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Hydroxytamoxifen interaction with human erythrocyte membrane and induction of permeabilization and subsequent hemolysis

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Abstract

4-Hydroxytamoxifen (OHTAM) is the most active metabolite of the widely prescribed anticancer drug tamoxifen (TAM) used in breast cancer therapy. This work describes the effects of OHTAM on isolated human erythrocytes, using standardized test conditions, to check for a putative contribution to the TAM-induced hemolysis and to study basic mechanisms involved in the interaction of OHTAM with cell membranes. Incubation of isolated human erythrocytes with relatively high concentrations of OHTAM results in a concentration-dependent hemolysis, its hemolytic effect being about one-third of that induced by TAM. OHTAM-induced hemolysis is prevented by either α-tocopherol (α-T) or α-tocopherol acetate (α-TAc) and it occurs in the absence of oxygen consumption and hemoglobin oxidation, ruling out the oxidative damage of erythrocytes. However, OHTAM remarkably increases the osmotic fragility of erythrocytes, increasing the susceptibility of erythrocytes to hypotonic lysis. Additionally, the hemoglobin release induced by OHTAM is preceded by a rapid efflux of intracellular K^+. Therefore, our data suggest that OHTAM-induced hemolysis does not contribute to TAM-induced hemolytic anemia and it is a much weaker toxic drug as compared with TAM. Moreover, at variance with the membrane disrupting effects of TAM, OHTAM promotes perturbation of the membrane's backbone region due to its strong binding to proteins with consequent formation of membrane paths of permeability to small solutes and retention of large solutes like hemoglobin, followed by osmotic swelling and cell lysis. The prevention of OHTAM-induced hemolysis by α-T and α-TAc is probably committed to the permeability sealing resulting from structural stabilization of membrane.

Keywords: 4-Hydroxytamoxifen; Tamoxifen; Human erythrocytes; Hemolysis; Oxidative stress; Osmotic swelling

1. Introduction

Tamoxifen (TAM) is a triphenylethylenic anti-estrogen commonly prescribed in the breast cancer therapy (Jordan, 1990) and potentially useful in the prevention of this malignant process (Nayfield et al., 1991).

Most studies indicated that the anticancer action of TAM is not solely mediated by the antagonism at the estrogen receptors (ER). Among the several mechanisms proposed, the interaction with biomembranes has been favoured (Koedijk et al., 1994). In fact, TAM that binds to proteins in a unspecific way (Oosbree et al., 1984) partitions in a greater extent in native membranes relatively to the respective lipid dispersions (Custódio et al., 1991), where it interacts with lipids (Custódio et al., 1993a) and proteins (Lopes et al., 1990; Custódio et al., 1996), explaining the modifications in the morphology and structure of breast tumor cell membranes (Sica et al., 1984). Moreover, TAM disrupts model (Vogel et al., 1995; Custódio et al., 1996) and mitochondrial membranes (Custódio et al., 1998) and its hemolytic effect in isolated human erythrocytes has been assigned to a structural perturbation of the membrane integrity caused by its strong accumulation within membranes (Cruz-Silva et al., 2000). Therefore, the TAM-induced structural disruption of biomembranes, as observed in different membrane systems, may contribute for its ER-independent anticancer activity and adverse effects, including hemolytic anemia (Ching et al., 1992; Thangaraju et al., 1995).

The metabolites of TAM transformation, mostly 4-hydroxytamoxifen (OHTAM), have been reported to be

Abbreviations: AAPH, 2,2′-azobis(2-amidinopropane)dihydrochloride; α-T, α-tocopherol; α-TAc, α-tocopherol acetate; OHTAM, 4-hydroxytamoxifen; PBS, phosphate buffered saline; TAM, tamoxifen.

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pharmacologically active (Kemp et al., 1983), playing an important role in the therapeutic activity and side-effects of TAM (Jordan, 1990). Actually, the anti-estrogenic and antiproliferative activities of OHTAM in vitro are much stronger as compared with TAM (Borgna and Rochefort, 1981; Etienne et al., 1989), but the pharmacological relevance of OHTAM has been questioned (McCague et al., 1990) due to its low serum concentrations (Lien et al., 1987; MacCallum et al., 1996). However, the strong affinity of OHTAM for biomembrane proteins (Custódio et al., 1991) and its higher partitioning in the lipid bilayer as compared with TAM (Custódio et al., 1991), perturbing the membrane lipid-protein interface (Custódio et al., 1993b) suggest that OHTAM may accumulate in tissues, reaching there the required effective concentrations for its cytotoxic effects, as described by Lien et al. (1991).

Therefore, the reports of hemolytic anemia caused by TAM (Ching et al., 1992; Thangaraju et al., 1995), the strong incorporation of OHTAM into biomembranes (Custódio et al., 1991) and the putative role of OHTAM in the multiple cellular effects of TAM (Jordan, 1990) prompted us to search for the effects of OHTAM on isolated erythrocytes, attempting to clarify the contribution of OHTAM to the hemolytic anemia caused by TAM and the basic mechanisms underlying the interaction of this metabolite with biomembranes. The use of standardized methods is helpful to compare the effects of OHTAM with data previously published (Cruz-Silva et al., 2000).

2. Materials and methods

2.1. Chemicals

4-Hydroxytamoxifen, tamoxifen and α-tocopherol acetate (α-TAc) were purchased from Sigma Chemical Co (St Louis, MO, USA). 2,2′-Azobis(2-amidinopropane)dihydrochloride (AAPH) was obtained from Polysciences, Inc. (Warrington, PA, USA) and α-tocopherol (α-T) from Fluka BioChemika (Switzerland). All the other chemicals were of research grade. Solutions were prepared in deionized ultra-pure water.

2.2. Red blood cells preparation

Heparinised human blood samples were centrifuged at 3000 rpm for 10 min. The sediment of erythrocytes was washed three times with 6 volumes of isotonic phosphate buffered saline (PBS) (150 mM NaCl, 10 mM sodium phosphate, pH 7.4) and the buffy coat was removed with each wash, according to previous reports (Urano et al., 1992; Dwight and Hendry, 1996; Koga et al., 1998). The retrieved cells were resuspended in PBS and the hematocrit determined. All the erythrocyte suspensions used were prepared daily from freshly collected blood obtained from 12 healthy donors (25–50 years old and not submitted to any drug treatment) who are working in our laboratory, as described elsewhere (Cruz-Silva et al., 2000).

2.3. Hemolysis measurements

The hemolysis studies were performed in erythrocyte suspensions (4 ml) in PBS with the hematocrit at 0.33%. OHTAM or TAM were added from stock ethanolic solutions (2–4 μl) to the erythrocyte suspensions and incubated at 37 °C in a shaking water-bath with magnetic stirring. The effects of α-T and α-TAc were assessed by pre-incubating the erythrocyte suspensions with these compounds at 37 °C for 2 h, before the drug addition. A set of samples, after the pre-incubation period with the tocopherols, were washed three times with 10 ml of PBS before the addition of OHTAM.

After the incubation periods, the erythrocyte suspensions were centrifuged at 3000 rpm for 10 min and the hemolysis degree was estimated by visible spectroscopy at 540 nm from the hemoglobin released into the supernatant, as described elsewhere (Koga et al., 1998). The results were expressed as percent hemolysis. The absorbance of the supernatant in the absence of OHTAM was taken as zero hemolysis and the total hemolysis (100%) was assigned when PBS was replaced by water containing identical volume of ethanol as used in the assays with OHTAM.

The osmotic fragility experiments were carried out according to standardized procedures (Takeuchi et al., 1989; Jackson et al., 1996) in phosphate buffered solutions (10 mM sodium phosphate, pH 7.4) with increasing concentrations of NaCl and a hematocrit of 0.33%. After centrifugation at 3000 rpm for 10 min, the absorbance of supernatants was read at 540 nm.

The K^+ leakage from erythrocytes was monitored with a K^+ ion-selective electrode. The total amount of K^+ was measured after the addition of 0.5% Triton X-100 to the erythrocyte suspensions.

2.4. Erythrocytes oxidation measurements

The erythrocyte hemolysis induced by OHTAM were followed by oxygen consumption and changes in hemoglobin absorption spectrum, as described elsewhere (Cruz-Silva et al., 2000).

The rate of oxygen consumption was measured with a Clark-type oxygen electrode (YSI model 5331, Yellow Spring Inst.), as previously described (Custódio et al., 1994). The reactions were carried out at 37 °C in a closed glass vessel with magnetic stirring and the hemolysis of erythrocyte suspension (hematocrit of 0.33%) was induced by addition of 35 μM OHTAM. After 90 min of OHTAM incubation, the free radicals generator

AAPH (20 mM) was added and oxygen consumption was followed for another period of 60 min. The oxygen consumption was calculated assuming that the O_2 concentration in water was 177 nmol/ml at 37 °C.

In the course of incubation of erythrocyte suspensions with OHTAM and after addition of 20 mM AAPH, aliquots of the suspension were withdrawn, centrifuged at 3000 rpm for 5 min and the spectrum of the released hemoglobin was recorded. The withdrawn aliquots were chilled prior to centrifugation to stop the thermal decomposition reaction of the azoinitiator. Hemoglobin oxidation was followed by the decay of the absorption spectrum in the range 450–650 nm, using a Perkin–Elmer Lambda 6 UV/VIS spectrophotometer (Norwalk, USA).

The results are typical assays or represent the mean ±S.D. of three independent experiments.

3. Results

3.1. OHTAM-induced hemolysis

The effects of OHTAM in isolated human erythrocytes were assayed as a function of incubation time and drug concentration in erythrocyte suspensions from different human donors (Fig. 1, lines 1–4). This figure shows the time-dependent hemolysis induced by 30 μM OHTAM. Variations are observed in the susceptibility of samples to hemolysis depending on the blood donor, but an almost complete hemolysis is observed for all samples with 30 μM OHTAM after 90 min of incubation, whereas 35 μM OHTAM induces full hemolysis after this incubation time. As previously reported for TAM (Cruz-Silva et al., 2000), the sensitivity of erythrocytes to hemolysis induced by OHTAM is also lower for older subjects (Fig. 1, lines 3–4), according to earlier reports pointing out that the hemolysis extension decreases as a function of age as a consequence of increased membrane cholesterol (Araki and Rifkind, 1980). As OHTAM partitioning in biomembranes decreases as a function of cholesterol (Custodio et al., 1991), the different effects induced by OHTAM in diverse erythrocyte suspensions could be explained by the putative protection ascribed to membrane cholesterol. The hemolytic effect of OHTAM is also concentration dependent and total hemolysis is detected in all the erythrocyte samples when incubated with 35 μM OHTAM, at 37 °C, for 90 min (Fig. 2).

The hemolytic action of OHTAM was also compared with the effects of TAM in the same erythrocyte samples, as shown in Fig. 2. TAM (12.5 μM) induces complete hemolysis after 1 h of incubation, whereas the same concentration of OHTAM does not promote any significant effect and a total hemolysis is only achieved at 35 μM OHTAM after 1.5 h of incubation. Identical experiments carried out with both drugs at 37 °C for 1 h demonstrated that 12.5 μM TAM induces total hemolysis whereas 35 μM OHTAM promotes only about 70% hemolysis (data not shown). The combination of low concentrations of TAM (<7.5 μM) with OHTAM (<10 μM) does not change the effects observed with TAM alone, meaning that in vivo the interaction between these drugs will not cause increased hemolysis, in addition to the effect of TAM. Moreover, as described to TAM (Cruz-Silva et al., 2000), OHTAM induces the release of hemoglobin bound to the membrane, as evaluated by SDS–polyacrylamide gel electrophoresis, explaining the extra absorbance as compared to the total hemolysis induced by water (Fig. 2), also meaning that OHTAM changes the binding of cytosol and cytoskeleton proteins to band 3, the most abundant intrinsic protein of erythrocytes (Schuck and Schubert, 1991).

To clarify the potential mechanisms involved in the hemolytic effect of high concentrations of OHTAM, namely cellular oxidative stress or membrane structural perturbations, the protective effects of antioxidants and membrane stabilizers, for example α-T and α-TAc, were studied.

OHTAM-induced hemolysis is partially prevented by α-T (Fig. 3A) or α-TAc (Fig. 3B) previously incubated with erythrocyte suspensions. When erythrocytes are washed after the pre-incubation period to avoid the interference of non-incorporated tocopherol compounds,

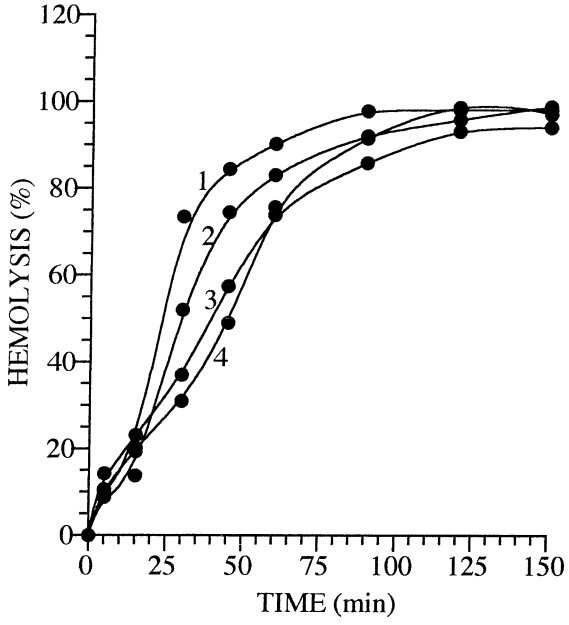

Fig. 1. OHTAM-induced hemolysis in human erythrocytes obtained from different donors (lines 1–4). Erythrocyte suspensions in phosphate buffer saline (PBS) (0.33% hematocrit) were incubated with 30 μM OHTAM at 37 °C, as a function of time. After incubation with the drug, erythrocyte suspensions were centrifuged at 3000 rpm for 10 min and hemolysis was estimated from the 540 nm absorbance of hemoglobin released into the supernatant. Data are expressed as percentage of total hemolysis induced by replacing the PBS by identical volume of water.

the protection against OHTAM-induced hemolysis is nearly maintained as in unwashed erythrocytes (Fig. 3A,B). Therefore, these protective effects putatively result from the interaction of tocopherols with the erythrocyte membrane core (Shinitzky and Barenholz, 1978), in agreement with the hydrophobicity, the high membrane partition and ordering effects of these compounds in lipid bilayer (Micol et al., 1990; Fukuzawa et al., 1992; Cruz-Silva et al., 2000), indicating that OHTAM-induced hemolysis is related with membrane interaction. Moreover, the fact that α-TAc (with an inactivated functional hydroxyl and deprived of antioxidant activity) (Urano et al., 1992) inhibits also the hemolytic process suggests that the free hydroxyl group of α-T is not critical for protection against OHTAM-induced damage and that the hemolysis will be not caused by oxidative disruption of erythrocyte.

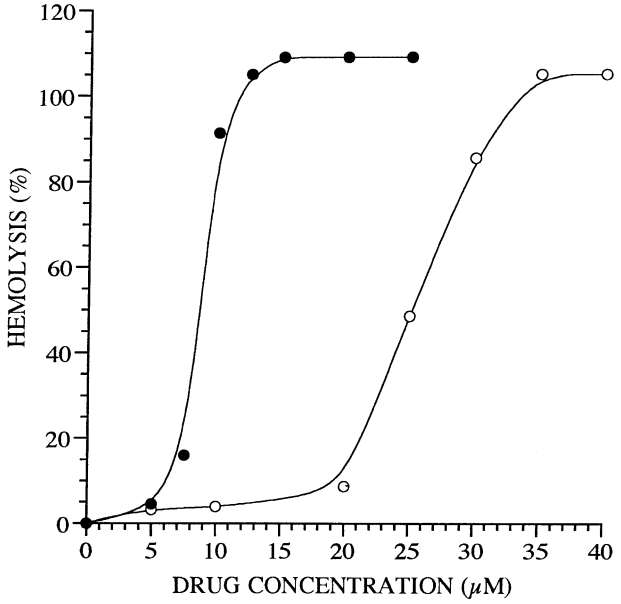

Fig. 2. Comparison of the hemolytic effects induced by TAM (●) and OHTAM (○) in the same erythrocyte suspension, as a function of drug concentration. Erythrocyte suspension in PBS (0.33% hematocrit) were incubated with different concentrations of either TAM at 37 °C for 1 h or OHTAM at 37 °C for 1.5 h, and the hemolysis was calculated from the absorbance of supernatants at 540 nm after centrifugation of erythrocyte suspension. The recordings obtained from the results expressed as percentage of total hemolysis are typical of three independent experiments.

3.2. *Effect of OHTAM on oxidative stress of erythrocytes*

To exclude the putative involvement of peroxidative degradation mechanisms on the hemolytic effect of OHTAM, the oxygen consumption and hemoglobin oxidation were evaluated simultaneously in the same erythrocyte suspension during the time course of the drug-induced hemolysis. The hemolytic effect promoted by 35 μM OHTAM, as followed by the increase in the hemoglobin released to the supernatant, is not accompanied by a significant decrease in O_2 concentration in the medium (Fig. 4A) or changes in the spectra of the released oxyhemoglobin (Fig. 4B), in contrast to the abrupt O_2 consumption (Fig. 4A) and conversion of the typical spectra of oxyhemoglobin into methemoglobin (Fig. 4C) induced by 20 mM AAPH, revealing an extensive peroxidation of lipids and proteins induced by the AAPH-derived radicals, as previously reported (Miki et al., 1987; Sato et al., 1995). Therefore, an oxidative

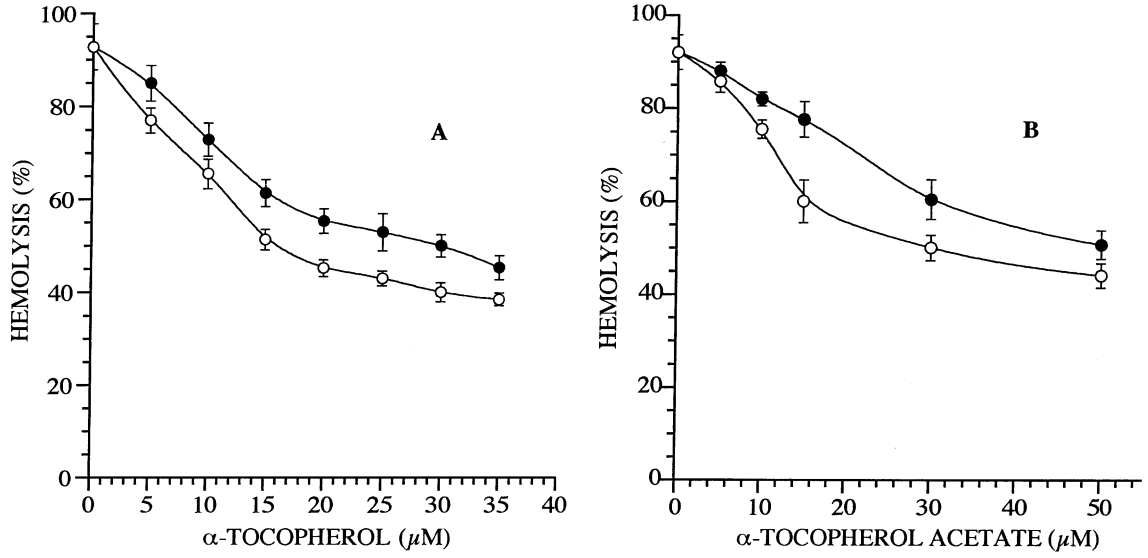

Fig. 3. Protection of α-T (A) and α-TAc (B) against OHTAM-induced hemolysis. The erythrocyte suspensions in PBS (0.33% hematocrit) were pre-incubated with the tocopherols at 37 °C for 2 h and afterwards the erythrocytes were washed (●) or not (○) prior to the addition of 35 μM OHTAM, followed by incubation at 37 °C for 90 min. The hemolysis extension was estimated by the absorbance of supernatant at 540 nm as described in the legend to Fig. 1. Each point represents the mean ±S.D. of three independent experiments.

challenge of erythrocytes by the presence of OHTAM is not observed as compared with AAPH, a well-known reference oxidant (Miki et al., 1987; Niki, 1990; Sato et al., 1995). Moreover, the peroxidation process induced by AAPH-derived peroxyl radicals in erythrocyte suspensions, previously lysed with hypotonic PBS, is significantly inhibited by OHTAM, as assessed by O_2 consumption (results not shown), pointing to the antioxidant activity of OHTAM in this biological system, as previously reported in other studies regarding the antioxidant mechanisms of OHTAM (Custódio et al., 1994; Wei et al., 1998).

3.3. Osmotic behavior of erythrocytes in the presence of OHTAM

The hemolytic action dependent on putative membrane perturbations was evaluated by the hemolytic fragility analysis as a function of NaCl concentration (Fig. 5). Incubation of the erythrocyte suspensions with 10 μM OHTAM does not change the osmotic fragility profile, as compared to the control conditions. However, 20 μM OHTAM, with no significant hemolytic effect in isotonic medium, considerably shifts the fragility profile. As already observed before, 35 μM OHTAM induces total hemolysis independently of the osmolarity (data not shown). In contrast to TAM (Cruz-Silva et al., 2000), OHTAM increases the susceptibility of erythrocytes to hypotonic lysis, through changes in membrane permeability.

To further explore this permeabilizing effect, the efflux of intracellular K^+ in parallel with hemoglobin release was searched, since K^+ efflux has revealed a reliable methodology for the assay of permeability changes induced by several hemolytic compounds (Cybulska et al., 1995; Jackson et al., 1996).

Fig. 6 displays the K^+ and hemoglobin leakage curves resulting from the incubation of erythrocyte suspensions with 35 μM OHTAM at 25 °C. Release of about 60% of intracellular K^+ occurs in a short period (2 min) and the remaining K^+ is gradually released after 20 min of incubation. In contrast, the release of hemoglobin to the

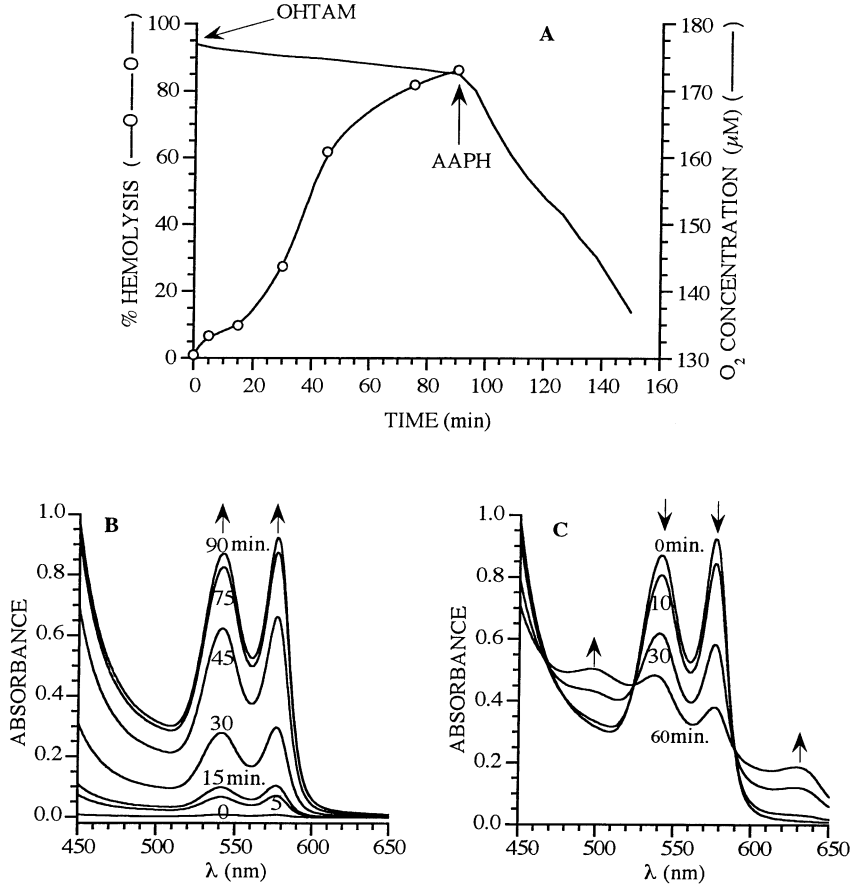

Fig. 4. Oxygen consumption rates (—) associated with hemolysis of erythrocytes (○) incubated with 35 μM OHTAM and afterwards with 20 mM AAPH (A) and spectra of hemoglobin after the addition of OHTAM (B) and AAPH (C). O_2 consumption (A) and hemoglobin spectra were monitored simultaneously during incubation of erythrocyte suspensions in PBS (0.33% hematocrit) with OHTAM at 37 °C for 1.5 h (B) and after AAPH addition (C), as described in Materials and Methods. Hemoglobin spectra were recorded at different reaction times indicated by the numbers adjacent to the traces. Upward and downward arrows indicate increase or decrease in absorbance due to hemoglobin release (B) and hemoglobin oxidation by AAPH added 1.5 h after OHTAM (C). The results are representative of three different assays.

extracellular medium is a late phenomenon and total hemolysis is observed only after 90 min (Fig. 6). Therefore, OHTAM induces the early formation of permeability paths in the erythrocyte membrane, allowing the rapid efflux of K^+ prior to a large scale disruption of the membrane, which permits the release of hemoglobin.

4. Discussion

OHTAM interacts with human erythrocytes in vitro in a different way from that recently described for TAM (Cruz-Silva et al., 2000). The extension of OHTAM hemolytic effects depends on blood donor, being lower for older subjects (Fig. 1) as observed for TAM (Cruz-Silva et al., 2000). However, a complete hemolysis occurs only at 35 μM OHTAM, incubated at 37 °C for 1.5 h, independently of the erythrocyte donor, whereas 12.5 μM TAM incubated at 37 °C for 1 h induces total hemolysis of erythrocytes (Fig. 2). Therefore, OHTAM requires a threefold higher concentration to induce the same hemolytic effect observed for TAM and the hemolysis takes longer, pointing out that OHTAM shows a much weaker hemolytic action than TAM. Considering that the tissue and serum concentrations of OHTAM are much lower as compared with TAM (Lien et al., 1987, 1991; MacCallum et al., 1996), OHTAM may not reach concentrations high enough to cause hemolysis, suggesting that this drug does not contribute to TAM-induced hemolytic anemia.

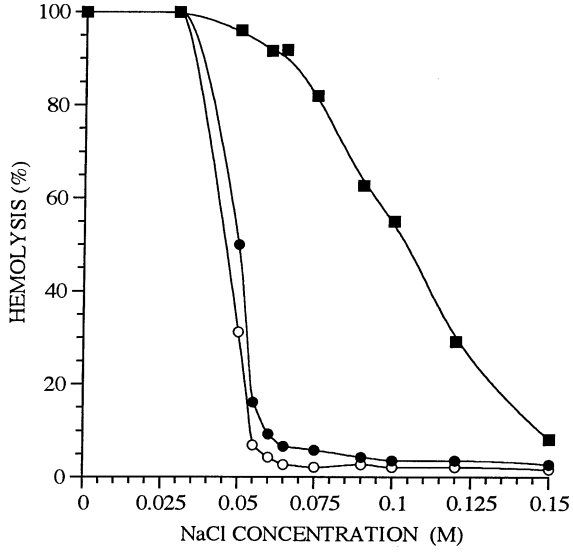

Fig. 5. Osmotic fragility curves of human erythrocytes as affected by the addition of OHTAM. The erythrocyte suspensions (0.33% hematocrit) in a 10 mM phosphate buffer, pH 7.4, containing increasing concentrations of NaCl were incubated at 37 °C for 1.5 h in the absence (○) and in the presence of 10 (●) and 20 μM OHTAM (■). Hemolysis was estimated and expressed as described in the legend to the Fig. 1.

The inhibition of OHTAM-induced hemolysis by α-T (Fig. 3A) could insinuate the oxidative damage of erythrocyte membrane as the mechanism involved in the hemolytic effect, since α-T has a remarkable antioxidant capacity, interrupting free radical chain reactions in several membrane systems (Yamamoto and Niki, 1988; Ernster, 1993), including the human erythrocyte membrane (Yamamoto et al., 1985; Miki et al., 1987). However, α-TAc, with its chromanol hydroxyl group acylated and thus deprived of antioxidant activity, also suppresses that hemolytic effect (Fig. 3B). Additionally, the hemolytic effect of OHTAM does not involve an increase in the membrane peroxidation or hemoglobin oxidative degradation (Fig. 4). On the other hand, OHTAM protects the peroxidative degradation of erythrocyte components induced by peroxyl radicals, according to its antioxidant properties in vitro (Custódio et al., 1994; Wei et al., 1998) and in vivo (Wei et al., 1998), acting as a potent intramembraneous scavenger of peroxyl radicals (Custódio et al., 1994). Therefore, OHTAM-induced hemolysis is not caused by the oxidative injury of the erythrocytes.

The effects of OHTAM on osmotic fragility and K^+ release from erythrocytes are considerably different from those described for TAM (Cruz-Silva et al., 2000), and suggest different mechanisms of interaction with the erythrocyte membrane. TAM does not change the osmotic fragility curves of erythrocytes and shows only a weak prelytic release of K^+ from the cells (Cruz-Silva et al., 2000), whereas OHTAM strongly shifts the osmotic fragility profile and makes the red blood cells leaky to K^+ preceding the slower hemolysis process (Fig. 6), supporting the conclusion that OHTAM-

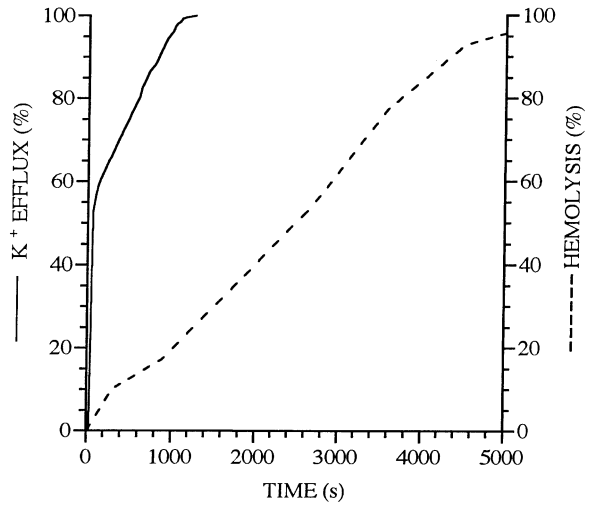

Fig. 6. Time courses of K^+ (—) and hemoglobin (---) release from human erythrocytes in 150 mM NaCl, 10 mM sodium phosphate, pH = 7.4 (0.33% hematocrit) incubated with 35 μM OHTAM at 25 °C. The release of K^+ was estimated with a K^+ ion-sensitive electrode and hemolysis was evaluated by spectrophotometry of the supernatant at 540 nm. The results are expressed as the percentage of total release in complete hemolysis and are typical of several independent assays.

induced hemolysis occurs via an osmotic swelling. In fact, the osmotic hemolysis described for several compounds (Lieber et al., 1984; Deuticke et al., 1989; Jackson et al., 1996) involves the formation of permeability paths for small ions, which allow water osmosis as consequence of the increased osmotic pressure inside the erythrocyte. The osmotic imbalance causes cells to swell, inducing physical rupture of the membrane followed by hemoglobin leakage. OHTAM strongly partitions in biomembranes (Custódio et al., 1991) and distributes in the outer region of the lipid bilayer, mainly in the lipid–protein interfaces (Custódio et al., 1993b), which may account for membrane structural perturbations reflected in the permeability status (Figs. 5 and 6). The inhibitory effects of α-T and α-TAc towards OHTAM-induced hemolysis (Fig. 3) reflect a decrease of the permeability (Urano et al., 1992) or the exclusion of OHTAM from the membrane; that is, withdrawing the drug from the interaction with membrane components involved in the control of permeability due to the membrane stabilization induced by both tocopherols (Micol et al., 1990; Fukuzawa et al., 1992; Cruz-Silva et al., 2000).

In conclusion, OHTAM induces hemolysis, but to a much smaller extent as compared with TAM, ruling out this hemolytic effect in TAM-induced hemolytic anemia. Moreover, our findings suggest that, differently from those described for TAM (Cruz-Silva et al., 2000), OHTAM induces permeabilization of the erythrocyte membrane and a subsequent time-dependent hemolytic process, via an osmotic mechanism, occurring at relatively high concentrations. It is difficult to calculate precisely the concentrations of OHTAM resulting from pharmacological doses of TAM due to its high hydrophobic character, partitioning in biological membranes (Custódio et al., 1991) and high affinity to receptors in various target tissues (Borgna and Rochefort, 1980, 1981), which in humans is 10–60-fold higher in tissues than in serum (Lien et al., 1991). However, the concentrations of OHTAM that induce hemolysis (>25 μM) are not easily reached in vivo in tissues and plasma, suggesting that little or no toxicity is expected with OHTAM doses as compared with TAM.

Acknowledgements

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