Free Rad. Res., Vol. 29, pp. 53–66
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Antioxidant Activity of 5-Aminosalicylic Acid Against Peroxidation of Phosphatidylcholine Liposomes in the Presence of α-Tocopherol: A Synergistic Interaction?

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Accepted by Prof. H. Sies

(Received 9 January 1998; In revised form 19 March 1998)

Oxidative damage has been implicated in the pathogenesis of inflammatory bowel diseases. 5-Aminosalicylic acid (5-ASA), the anti-inflammatory drug commonly used in the treatment of this condition, has been shown to possess antioxidant properties considered to be of particular importance in the pathologic context of these diseases. However, its action mechanisms are far from being completely elucidated, especially regarding its antioxidant properties in the presence of endogenous antioxidants such as α-tocopherol (α-T), the major defence system of biomembranes against lipid peroxidation. In this study we investigated the scavenging activity of 5-ASA toward peroxyl radicals generated at different sites of soybean PC liposomes, used as model membranes, either alone or in combination with α-T. 5-ASA, separately, shows strong scavenging activity toward peroxyl radicals generated in the aqueous phase by thermal decomposition of 2,2′-azobis(2-amidinopropane hydrochloride) (AAPH), inducing a clear concentration-dependent inhibition period, either of oxygen consumption or of conjugated diene hydroperoxides production. HPLC analysis indicates that 5-ASA is consumed, at a constant rate, throughout the reaction, and when the inhibition period is over, the oxidation rate is resumed. On the other hand, apart from a slight decrease in the rate of oxidation, 5-ASA is unable to suppress efficiently lipid peroxidation, when the reaction starts inside the lipid membranes, by thermal decomposition of 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN).

When 5-ASA is combined with α-T, and the oxidation starts in the aqueous phase, an additive inhibitory effect occurs between both compounds. 5-ASA protects efficiently α-T against initial attack from AAPH-peroxyl radicals, delaying its consumption. On the other hand, if the reaction starts inside the lipid bilayer, 5-ASA prolongs significantly the inhibitory period produced by α-T on the initial rate of oxidation, as measured by oxygen consumption and conjugated diene hydroperoxides. This inhibitory effect points to a synergistic interaction between 5-ASA and α-T, since 5-ASA, by itself, is unable to suppress the oxidation reaction. Therefore, 5-ASA reveals an important cooperative effect with α-T, either affording an efficient protection to this antioxidant compound, when free radicals are generated in the aqueous site, or potentiating its activity when oxidation is initiated inside the lipid bilayer. Taking into account that the ascorbic acid content decreases

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significantly in the inflamed mucosa of patients with inflammatory bowel diseases, our data are, certainly, a very important contribution to the knowledge of the anti-inflammatory action of 5-ASA.

Keywords: Antioxidant activity, 5-aminosalicylic acid, inflammatory bowel diseases

INTRODUCTION

5-Aminosalicylic acid (5-ASA) is a non-steroidal anti-inflammatory drug, widely used in the treatment of inflammatory bowel diseases (IBD). Active episodes of these diseases are characterized by inflammation of the intestinal mucosa, with accumulation of dense infiltrates of phagocytic leukocytes.[1,2] These leukocytes retain the ability to produce reactive oxygen species such as superoxide, hydroxyl radical, hydrogen peroxide and hypochlorous acid that have been related with the intestinal tissue injury developed in IBD.[3,4] Consequently, the tissue damage observed in these diseases may be related with the oxidative stress to which inflamed mucosa is subject.[4–6]

Furthermore, recently, it has been shown that the antioxidant defences, in particular the antioxidant protection assigned to ascorbic acid, in the inflamed mucosa of IBD patients is compromised.[7] Studies performed with paired mucosal tissues (inflamed and non-inflamed portions of the mucosa) obtained from IBD patients revealed that the contents of reduced and total ascorbic acid were markedly decreased in the inflamed mucosa. As a consequence, other antioxidant defences may also be compromised, weakening the overall antioxidant protection of the mucosa. In fact, not only the direct scavenging activity of ascorbic acid toward hydrophilic radicals will be decreased, but also its indirect antioxidant activity, by cooperative interaction with other important antioxidants, will be undermined in such situation.[7] In particular, the recovery of α-tocopherol (α-T) by ascorbic acid, a very important event in the cellular antioxidant defence system,[8,9] may be affected in these diseases.[7,10] Therefore, it is becoming increasingly evident that an imbalance between prooxidant species and antioxidants defences exists in the intestinal mucosa of IBD patients.

Among the multiple action mechanisms of 5-ASA, particular interest has been put on its antioxidant properties. Actually, there are experimental evidences that 5-ASA possesses antioxidant ability to scavenge a variety of free radicals and capacity to inhibit lipid peroxidation. Several groups have demonstrated that 5-ASA reacts with various oxygen-derived species, such as hydrogen peroxide,[1] superoxide radicals,[11] hydroxyl radicals,[12,13] hypochlorous acid,[12] and peroxyl radicals,[14,15] which are some of the mediators produced during inflammation and that have been found in the inflamed colon mucosa of IBD patients. Moreover, 5-ASA, a hydrophilic compound, could be an excellent potential substitute of ascorbic acid in the intestinal mucosa of IBD patients. Such possibility, if confirmed, would be an important advance to the knowledge of the anti-inflammatory action of 5-ASA.

These considerations prompted us to study 5-ASA antioxidant properties and to explore the putative interaction between 5-ASA and α-T. Thus, the goal of the present work was to study the antioxidant properties of 5-ASA, either alone or in combination with α-T, against lipid peroxidation using soybean phosphatidylcholine liposomes as a membrane model system. Liposomes oxidation was initiated by peroxyl radicals generated either in the aqueous phase or in the lipid phase, at a defined and constant rate, by thermal decomposition of azo initiators. The extent of lipid oxidation was followed by the measurements of oxygen consumption as well of conjugated diene hydroperoxides formation and the antioxidant activity was evaluated by the effects on the rates of oxygen consumption or conjugated dienes production. Additionally, 5-ASA and α-T consumptions throughout the oxidation reactions were

determined by HPLC to better understand the antioxidative effects of 5-ASA and its interaction with α-T.

MATERIALS AND METHODS

Chemicals

Commercial soybean phosphatidylcholine (PC), 5-ASA and α-T were purchased from Sigma Chemical Co. (St. Louis, MO). Ascorbic acid (ASC) was obtained from Fluka (Switzerland). The azo compounds 2,2′-azobis(2-amidinopropane hydrochloride) (AAPH) and 2,2′-azobis-(2,4-dimethylvaleronitrile) (AMVN) were purchased from Polysciences, Inc. (Warrington, PA). AAPH was used as supplied and AMVN was previously recrystallized from methanol. All the other reagents and chemicals were of the highest purity or HPLC grade. Solutions were prepared in ultra pure MilliQ water to minimize metal contamination and protected from light when necessary. 5-ASA and ASC solutions were prepared just before use.

Liposomes Preparation

Multilamellar Liposomes

Multilamellar liposomes (MLVs) were prepared by adding aliquots of soybean PC chloroform solution as well as the convenient amounts of lipid-soluble initiator (AMVN) and of lipid-soluble antioxidant (α-T), when required, in a pear-shaped flask. Solvents were evaporated in a rotary evaporator to obtain a thin film on the flask wall. This was hydrated in a phosphate-buffered saline solution (20 mM Na_2HPO_4, 100 mM NaCl, pH 7.4) containing 100 μM EDTA by shaking, in vortex, to obtain a milky suspension of MLVs.[16]

Unilamellar Liposomes

Unilamellar liposomes (LUVs) were prepared by extrusion of the MLVs suspension, using an Avestin Liposofast (Avestin, Inc., Ottawa, Canada) small-volume extrusion device. After preparing MLVs, the suspension was introduced into the extruder and then passed seven times back and forth through a policarbonate filter (200 nm pore diameter), by simply applying manual pressure. The resulting LUVs suspension proved to be a homogeneous population of large unilamellar liposomes as measured by photon correlation spectroscopy.[17,18]

Lipid Peroxidation Assays and Measurement of Antioxidant Activities

Lipid peroxidation was initiated by peroxyl radicals generated at a constant rate by thermal decomposition of azo compounds.[19] In studies with the water-soluble initiator, AAPH, oxidation reactions were started by injecting aliquots of an AAPH stock solution, in phosphate buffer, into the liposomes suspensions and the reactions were carried out at 37°C. When the lipophilic initiator AMVN was used, it was previously incorporated into the multilamellar vesicles before hydration, as referred above, and the reactions were carried out at 56°C. 5-ASA, a water-soluble compound, was added when required, as a phosphate buffer solution containing 100 μM EDTA, before the start of oxidation reaction.

In both cases, the oxidation reactions were measured following the oxygen consumption and the conjugated diene hydroperoxides production at 233 nm after second derivative spectroscopy.

The oxygen consumption was monitored in a closed glass vessel protected from light, thermostatted and provided with a magnetic stirring device, using a Clark-type electrode (YSI Model 5331, Yellow Springs Inst.). When AAPH was used as initiator, reactions were started by addition of AAPH (10 mM final concentration) to 1 ml of LUVs suspension (800 μM) in a small volume of phosphate buffer.[15] The hydrophilic antioxidant 5-ASA was added 1 min before the addition of AAPH and the vessel was kept at 37°C. When AMVN (500 μM final concentration)

was used as initiator, the reactions were started as soon as 1 ml of MLVs suspension (1.5 mM in PC) attained the reaction temperature in the vessel thermostatted at 56°C. 5-ASA was added immediately before the start of the reaction (at time zero). The changes in oxygen concentration were recorded continuously in a potentiometric chart record.

For conjugated diene hydroperoxides studies, soybean PC liposomes (5 mM final concentration) were incubated in a water bath, under air, at 37°C or 56°C, depending on the azo initiator used, AAPH (20 mM) or AMVN (1.67 mM), respectively. Aliquots of liposomes (20 µl) were taken at 10 min intervals and dissolved in 2 ml of absolute ethanol directly in 1 cm (light path) quartz cuvettes.[20] Conjugated diene hydroperoxides formation was evaluated by second derivative spectrophotometry[21] in a Perkin Elmer Lambda 6 spectrophotometer. After running a background correction without liposome suspension, but with an identical volume of phosphate buffer, spectra from 300 to 210 nm were scanned at the oxidation time intervals. Conjugated dienes were expressed as the height of the minimum peak at 233 nm (in arbitrary units) in the second derivative spectra, after subtracting the spectra obtained at "time 0" from that obtained at different times. The addition of 5-ASA to the liposomal system was carried out in a way similar to that described above for oxygen consumption experiments. The inhibition periods were determined graphically from the profiles of oxygen consumption and of conjugated dienes formation, taken from the intersections of tangents to the inhibited and uninhibited rates of oxidation.

5-ASA and α-T HPLC Analysis

The consumption of either α-T or 5-ASA during peroxidation of liposomal suspension was followed by HPLC quantitation (Beckman System Gold, UV detector model 166), on a Lichrospher 100 RP-18 (5 µm) column (Merck, Darmstadt, Germany). For α-T analysis, the elution was carried out with methanol (100%), at a flow rate of 2 ml/min and UV detection at 292 nm.[22] Sample aliquots of 2 ml were withdrawn along the time, chilled in ice and immediately extracted into hexane according to the SDS method.[23]

For 5-ASA analysis, sample preparation was performed by removing aliquots (2 ml) of liposomes suspensions, at different times along the oxidation reaction, which were further submitted to ultracentrifugation, in a Beckman TL-100 ultracentrifuge (Beckman Instruments, Inc., Palo Alto, CA), at 90 000 rpm, during 1 h, at 4°C. In these conditions, no lipid was detected in the supernatant, as revealed by spectroscopic analysis (data not shown). The elution was carried out with a solvent mixture consisting of 95% 0.1 M KH_2PO_4 (pH 4.4) and 5% methanol,[24] at a flow rate of 1 ml/min and UV detection at 240 nm.

RESULTS

The antioxidant properties and scavenger activity of 5-ASA toward peroxyl radicals, generated either in the aqueous phase by AAPH or in the lipid region by AMVN, alone or combined with α-T, were evaluated. Since free-radical-mediated chain reactions of lipids are accompanied by oxygen uptake and conjugated diene hydroperoxides formation, we followed the peroxyl-radicals-induced lipid peroxidation by measurement of oxygen consumption and conjugated dienes production. Additionally, the consumption of either 5-ASA or α-T was evaluated throughout the oxidation reaction by HPLC analysis.

Antioxidant Effects of 5-ASA against AAPH-induced Oxidation of Soybean PC Liposomes

Antioxidant Action of 5-ASA

We first examined the effects of 5-ASA on oxidation of soybean phosphatidylcholine liposomes, initiated in the aqueous phase by AAPH

thermal decomposition, in the absence of α-T. To achieve a more realistic model of biological membranes, unilamellar liposomes, with a homogeneous size distribution were used.

As shown in Figure 1, the generation of peroxyl radicals from AAPH leads to a high rate of oxygen uptake in liposomes without 5-ASA, pointing to a significant oxidation of phosphatidylcholine. When increasing concentrations of 5-ASA are added to the reaction mixture, the oxidation is suppressed and a clear initial concentration-dependent inhibition period occurs in the rate of oxygen consumption. Furthermore, when the inhibition period is over, the propagation rate is resumed, i.e., the oxidation proceeds at a rate similar to that observed without drug. This biphasic response is consistent with a chain-breaking antioxidant activity. A linear correlation between 5-ASA concentrations and the inhibition time of oxygen consumption is also observed (Figure 1, inset). Control assays were performed in similar experimental conditions without liposomes in the absence and presence of 5-ASA. In the absence of lipid, the rate of oxygen consumption is not significant as compared to that in its presence (Figure 1, dashed line) and 5 μM 5-ASA practically abolishes the oxygen consumption (data not shown).

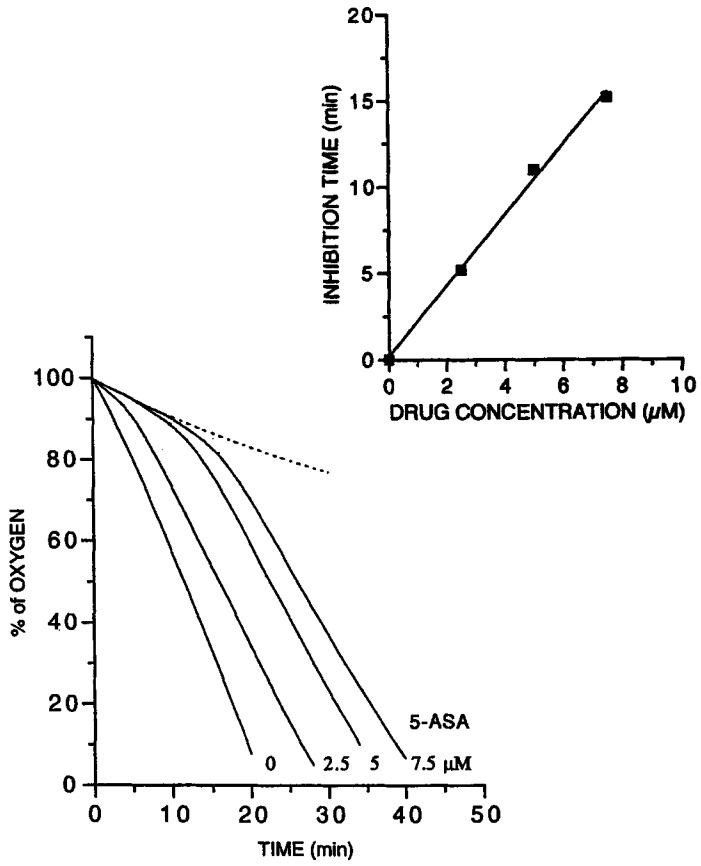

FIGURE 1 Rates of oxygen consumption during oxidation of soybean PC liposomes (800 μM), induced by AAPH (10 mM), added at time zero to lipid suspensions, in the absence (0) and presence of increasing concentrations of 5-ASA, as indicated besides the traces. The dashed line represents a control experiment in the absence of liposomes. Recordings are representative assays of at least three independent determinations. The inset depicts the linear correlation between 5-ASA concentrations and the inhibition periods in the rates of oxygen consumption.

Inhibition of Oxidation by a Combination of 5-ASA and α-T

In order to examine a potential interaction between 5-ASA in the aqueous phase, and α-T incorporated into the lipid membrane, we studied the inhibition of oxidation by a combination of 5-ASA and α-T in the same liposomal system. As observed in Figure 2, the presence of 2.5 μM α-T, previously incorporated in PC unilamellar vesicles, leads to a typical lag phase of oxygen consumption during oxidation induced by AAPH, followed by a constant propagation rate similar to that of control without α-T. Addition of 5-ASA to the lipid system, just before AAPH, produces an increase in this lag phase in a concentration dependent manner, as illustrated in Figure 2. However, the extent of such lag time proves to be only the sum of the inhibition periods observed with individual antioxidants, tested separately (Figure 3(A)). A similar additive effect is also observed when lipid peroxidation is evaluated by conjugated diene hydroperoxides formation (Figure 3(B)). Thus, these results show that 5-ASA and α-T, if combined, inhibit in an additive manner the membrane lipid peroxidation, when the reaction starts in the aqueous phase.

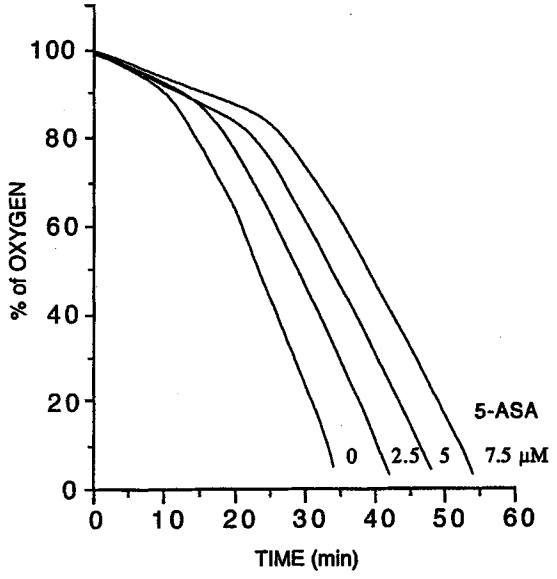

FIGURE 2 Effect of 5-ASA on AAPH-induced oxidation of PC liposomes, containing 2.5 μM α-T previously incorporated. Rates of oxygen consumption during lipid oxidation in the absence (0) and presence of increasing concentrations of 5-ASA, as indicated besides the traces. Recordings are representative assays of at least three independent determinations.

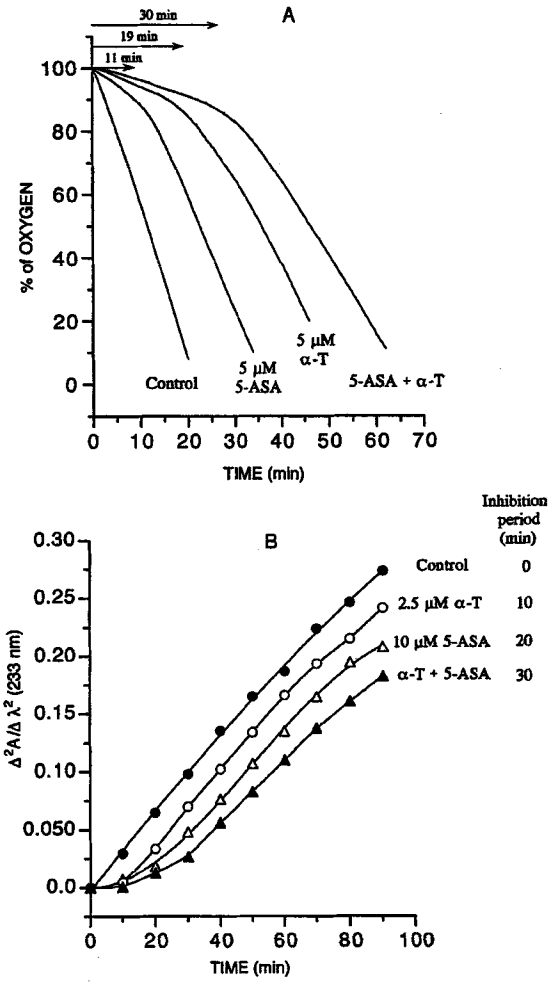

FIGURE 3 Inhibiting effects of 5-ASA and α-T, either alone or combined, on AAPH-induced peroxidation of soybean PC liposomes, as measured by oxygen consumption (A) and conjugated dienes formation (B). (A) Rates of oxygen consumption during PC oxidation in the absence (control) and presence of 5 μM 5-ASA or 5 μM α-T separately or together (5-ASA + α-T). The arrows indicate the inhibition times of oxygen consumption. (B) Conjugated diene hydroperoxides as evaluated by second derivative spectrophotometry in the absence (control ●) or presence of 2.5 μM α-T (○), 10 μM 5-ASA (△) and 2.5 μM α-T + 10 μM 5-ASA (▲). The inhibition periods of conjugated dienes formation are also indicated for all the curves. Notice the additive inhibiting effects of 5-ASA and α-T when combined. Experimental conditions are described in Materials and Methods.

Consumption of 5-ASA and α-T during the Oxidation Reaction

Measurements of 5-ASA and α-T were performed by HPLC analysis to follow their consumptions throughout the oxidation of soybean PC liposomes induced by AAPH. In the experiments with each antioxidant separately, either 5-ASA or α-T are consumed at a constant rate during the inhibition period of oxidation reaction, as shown in Figures 4(A) and (B), respectively. Actually, at the end of the inhibition period of oxygen consumption, during lipid oxidation, about 50% of 5-ASA (Figure 4(A)) or α-T (Figure 4(B)) have been already consumed.

On the other hand, when 5-ASA and α-T are combined in the same liposomal system it is observed that 5-ASA protects α-T from consumption. Figure 5 shows that in liposomes containing α-T, 5-ASA induces a clear inhibition period of α-T consumption, after addition of AAPH, which increases with increasing 5-ASA concentrations. During this inhibition period, 5-ASA concentration decreases significantly, being depleted at about 70% of the initial concentration in 30 min (Figure 6). Only at this time, the rate of α-T consumption increases drastically, in parallel with a fast oxidation reaction, as measured by the rate of oxygen consumption (Figure 6, inset). These results point to an efficient ability of 5-ASA to scavenge hydrophilic radicals, protecting the membrane lipids and α-T from their attack.

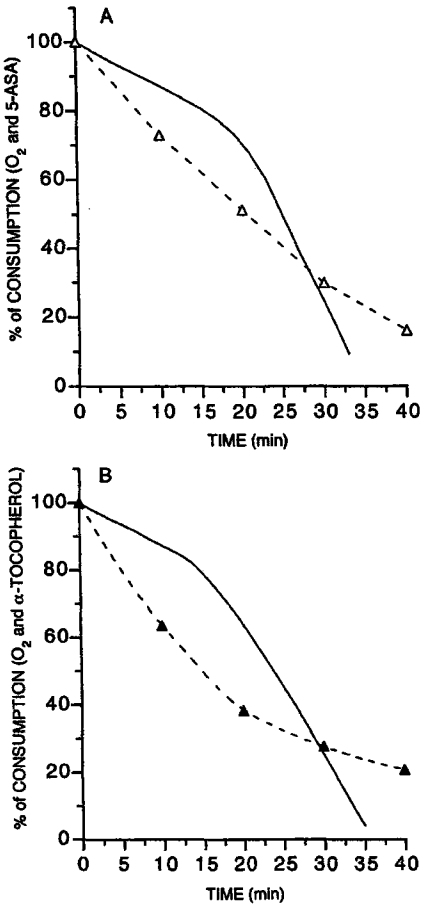

FIGURE 4 Time courses of oxygen, 5-ASA and α-T consumptions during AAPH-induced oxidation of soybean PC liposomes, at 37°C. (A) 5-ASA consumption (dashed line) or (B) α-T depletion (dashed line) were evaluated in parallel with the oxygen consumption rate (solid lines). 5-ASA (10 μM) was added just before addition of 10 mM AAPH to the lipid suspension and α-T (2.5 μM) was previously incorporated into the liposomes (800 μM PC). At indicated times, after challenging with AAPH, aliquots were withdrawn for 5-ASA and α-T measurements by HPLC analysis, as reported in Materials and Methods. Each point represents the mean of experiments performed in duplicate. Oxygen consumption record is a representative assay of three independent determinations.

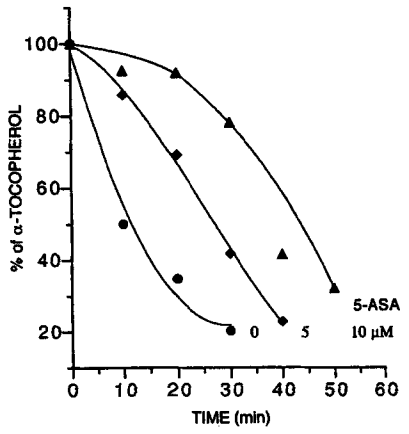

FIGURE 5 Effect of 5-ASA on α-T consumption rate in soybean PC liposomes during AAPH-induced peroxidation. 5-ASA was added to lipid suspension just before AAPH addition at time zero. Liposomes (800 μM PC, containing 2.5 μM α-T) were exposed to 10 mM AAPH and, at indicated times, aliquots were withdrawn for α-T extraction and measurement by HPLC. The concentrations of 5-ASA are indicated besides the curves. Each point represents the mean of experiments performed in duplicate.

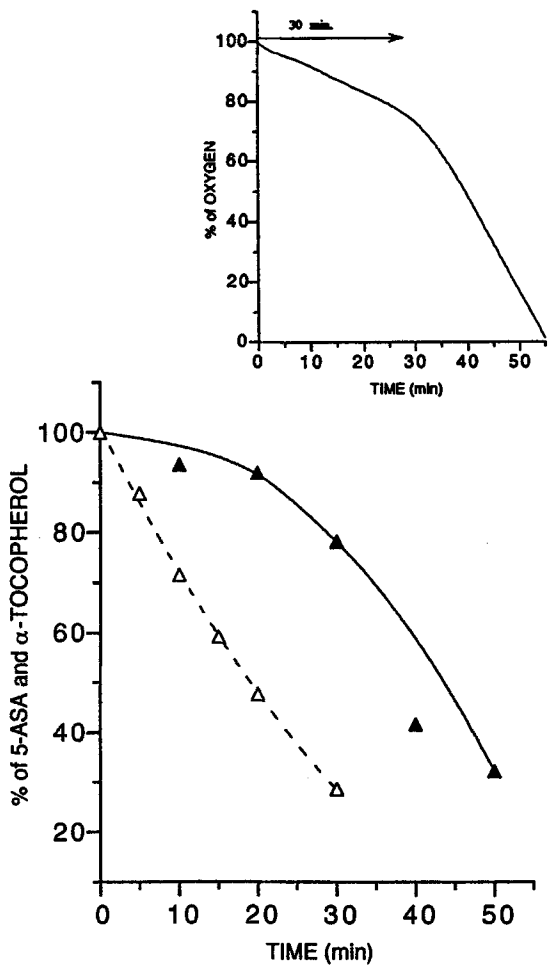

FIGURE 6 Parallel time courses of the consumptions of α-T (full line) and of 5-ASA (dashed line) in soybean PC liposomes (800 µM PC, 2.5 µM α-T) during AAPH-induced peroxidation, in the presence of 10 µM 5-ASA, added before starting the oxidation reaction. Inset depicts the oxygen consumption rate after addition of AAPH, where an inhibition period of 30 min is evident. Notice that during this period, the rapid consumption of 5-ASA occurs but α-T is almost spared. Only when 5-ASA is almost depleted the rates of α-T consumption and oxygen consumption increase significantly. Each point in both curves represents the mean of experiments performed in duplicate.

Antioxidant Effects of 5-ASA against AMVN-induced Oxidation of Soybean PC Liposomes

Independent Effect of 5-ASA

We examined also the effects of 5-ASA on soybean phosphatidylcholine liposomes oxidation initiated by hydrophobic peroxyl radicals derived from AMVN, in the absence of α-T. Considering the difficulties, in our assay conditions, to incorporate AMVN into the unilamellar structure, these studies were performed with multilamellar vesicles. Thus, Figure 7 shows the effects of 5-ASA on lipid peroxidation when the reaction was initiated within the membranes, in PC multilamellar vesicles, as evaluated by the oxygen uptake and conjugated diene hydroperoxides formation. In such conditions, 5-ASA does not suppress appreciably the oxidation reaction, suggesting that this drug is not an efficient scavenger of peroxyl radicals in the lipid region, being unable to induce a clear inhibition period, either in the rate of oxygen consumption or in the rate of conjugated diene hydroperoxides formation.

Actually, in both assays, the presence of 5-ASA only induces a slight decrease in the propagation rate of lipid peroxidation but no initial lag phase is detected.

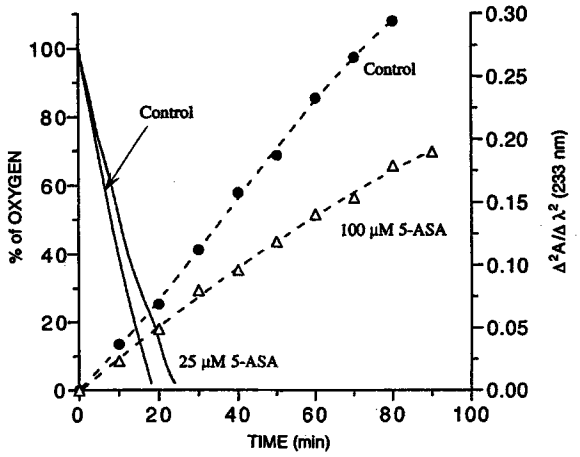

FIGURE 7 Effect of 5-ASA alone on AMVN-induced lipid peroxidation in soybean PC liposomes, as measured by the oxygen consumption (solid lines) and conjugated diene hydroperoxides formation as evaluated by second derivative spectrophotometry (dotted lines). The oxygen uptake was followed during the oxidation of PC liposomes (1.5 mM) challenged with AMVN (500 µM), at 56°C, in the absence (control) and presence of 25 µM 5-ASA. Conjugated dienes formation was evaluated during AMVN (1.67 mM) induced lipid oxidation of soybean PC (5 mM), at 56°C, in the absence (control) and presence of 100 µM 5-ASA. Recordings are representative assays of at least three independent experiments.

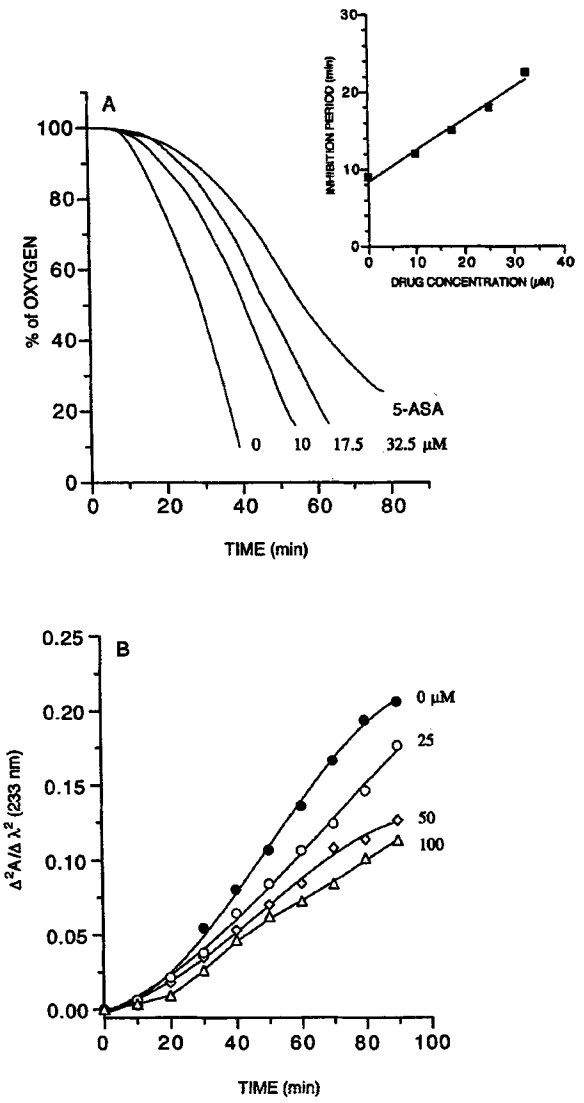

FIGURE 8 Effect of 5-ASA on AMVN-induced oxidation of PC liposomes containing α-T (2.5 μM), as evaluated by oxygen consumption and by conjugated diene hydroperoxides formation. (A) Rates of oxygen consumption during lipid oxidation in the absence (0) and presence of increasing concentrations of 5-ASA as indicated besides the traces. Recordings are representative assays of at least three independent determinations. The inset depicts the linear correlation between 5-ASA concentrations and the inhibition times observed in the rates of oxygen consumption. (B) Rates of conjugated dienes formation in the absence (●) and presence of 25 μM (○), 50 μM (◇) and 100 μM (△) 5-ASA. Experimental conditions are described in Materials and Methods.

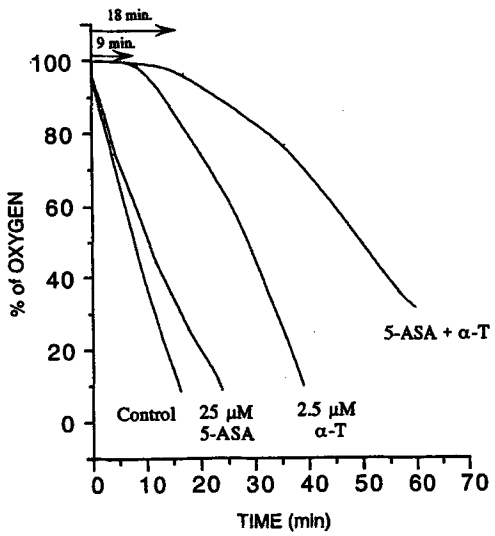

FIGURE 9 Effects of 5-ASA and α-T separately or combined on the inhibition of AMVN-induced peroxidation of soybean PC liposomes, pointing to a synergistic interaction between both compounds. Rates of oxygen consumption during PC oxidation in the absence of either 5-ASA or α-T (control) and presence of 25 μM 5-ASA and 2.5 μM α-T individually or combined (5-ASA + α-T). The arrows indicate the inhibition periods of oxygen consumption in the presence of α-T alone (9 min) or combined with 5-ASA (18 min).

Inhibition of Oxidation by a Combination of 5-ASA and α-T

Figure 8 shows the effects of 5-ASA on the oxidation of multilamellar PC liposomes when α-T and AMVN were incorporated previously into the lipid bilayer. In the absence of 5-ASA, an initial clear lag phase in the oxidation reaction initiated by AMVN is detected, when evaluated either by the oxygen uptake (Figure 8(A)) or by conjugated dienes formation (Figure 8(B)), showing that α-T efficiently suppresses the oxidation reaction. The presence of 5-ASA, added to the reaction mixture, extends significantly the inhibition period due to α-T, in a concentration dependent manner. In fact, a good linear correlation ($r^2 = 0.99$) is obtained between the induced lag period of oxygen consumption and 5-ASA concentration (Figure 8(A), inset). Thus, as evidenced in Figure 9, 5-ASA (25 μM) alone does not scavenge efficiently the radicals generated inside

membranes, not inducing an inhibition period of oxygen consumption, but together with α-T (2.5 μM) extends significantly the lag phase induced by α-T (it is elongated from 9 to 18 min). This behaviour suggests a synergistic interaction between 5-ASA and α-T.

Consumption of α-T throughout the Oxidation Reaction

The consumption of α-T was evaluated during the course of soybean PC oxidation reaction induced by AMVN, at 56°C. As shown in Figure 10, in the absence of 5-ASA, α-T slows down linearly with time until a residual concentration of about 20%.

Although 25 μM 5-ASA does not protect efficiently α-T from lipophilic radicals, it reduces the rate of α-T consumption. Thus, while in the absence of 5-ASA, the liposomes become completely depleted of α-T at 30 min of reaction, in the presence of 5-ASA this occurs at 40 min of reaction, evidencing some protection of α-T afforded by 5-ASA.

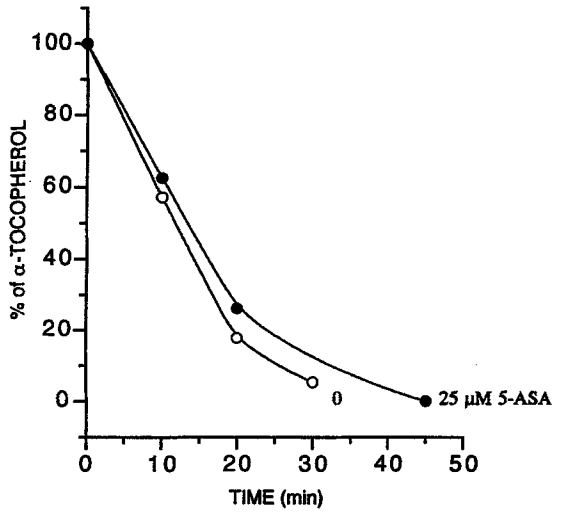

FIGURE 10 Rates of α-T consumption during the oxidation of soybean PC liposomes (1.5 mM PC, 2.5 μM α-T) initiated by AMVN (500 μM), at 56°C in the absence (○) and presence (●) of 25 μM 5-ASA. Each point represents the mean of experiments performed in duplicate. Deviations are generally encompassed by the size of the symbols.

DISCUSSION

Earlier studies have already shown that 5-ASA, an anti-inflammatory drug commonly used in the treatment of inflammatory bowel diseases,[25,26] possesses antioxidant properties, namely capacity to scavenge oxidants produced by the phagocytic cells[5] and to inhibit membrane lipid peroxidation.[15] Peroxyl radicals represent the main mediators in lipid peroxidation propagation, a free radical chain-reaction developed during inflammation.[27–29]

In this work, some advances were achieved to the knowledge of the antioxidant activity of 5-ASA toward peroxyl radicals, generated at different sites in a liposomal membrane system. Using the water-soluble azo initiator AAPH, or the lipid-soluble azo initiator AMVN, we could control exactly the site of free radicals generation, either in the aqueous phase, or inside the lipid region, respectively[19] and thus, develop a more detailed study, regarding the protection afforded by 5-ASA against lipid peroxidation.

Actually, depending on the azo compound used as initiator of peroxyl radicals, completely different inhibitory effects of 5-ASA have been observed against soybean PC induced peroxidation. When peroxyl radicals are generated initially in the aqueous phase, 5-ASA acts as a potent free radical scavenger, inducing a clear inhibition period either of oxygen consumption or of conjugated diene hydroperoxides formation (Figures 1 and 3). On the other hand, when the reaction starts inside the membranes, 5-ASA is unable to suppress the oxidation, only decreasing slightly the propagation rate of lipid oxidation (Figure 7). These results indicate that the antioxidant potency is determined by the site of peroxyl radicals generation as well as the location of the antioxidant. Thus, when free radicals are generated in the aqueous phase, at the site where the hydrophilic drug 5-ASA is located, a potent inhibitory effect on lipid peroxidation occurs. Under these conditions, 5-ASA is an accessible target to peroxyl radicals, reacting with them

rapidly, preventing the initiation of lipid peroxidation through hydrogen abstraction. The antioxidant radical species formed, apparently, are devoid of activity to initiate lipid peroxidation reaction. These considerations explain its potent chain-breaking antioxidant activity (Figure 1). Therefore, as long as 5-ASA is present in enough concentration to trap peroxyl radicals efficiently, inhibition of lipid peroxidation occurs, as evidenced by the lag phases of oxygen consumption. When 5-ASA is consumed over a certain level, the protective effect of 5-ASA against free radical attack to lipid membranes is undermined and lipid peroxidation propagates in a chain-reaction, at a rate similar to that in the absence of 5-ASA.

The antioxidant activity of 5-ASA has been detected for concentrations as small as 2.5 μM, which falls well below the concentration found by Grisham and Granger in the cat colonic mucosa (~164 μM) when 10 mM 5-ASA was perfused into the intestinal lumen.[30] Also, clinical studies with oral 5-ASA microgranules report human intraluminal concentrations of 5-ASA in the range of 589–1112 μM, from duodenal to ileal lumen.[31] Therefore, our results indicate that 5-ASA scavenges efficiently peroxyl radicals generated in the aqueous phase, at much lower concentrations than the therapeutic concentrations achieved locally in the human intestinal mucosa. At these higher concentrations, a much more potent inhibitory effect will be expected, as in our studies 5-ASA has been found to act in a concentration dependent manner.

A completely different antioxidant effect has been detected when peroxyl radicals are generated initially in the lipid region of multilamellar vesicles by AMVN. In such conditions, 5-ASA located outside the liposomal membranes shows an inefficient ability to scavenge free radicals, not inducing clear inhibition periods, neither of oxygen consumption nor of conjugated dienes formation (Figure 7). Being located at different sites, 5-ASA and peroxyl radicals are unable to react efficiently with each other, which explains the preferential attack of peroxyl radicals to lipid membranes and the propagation of the lipid chain reaction only at a little lower rate than that in the absence of 5-ASA. Therefore, as also demonstrated for other hydrophilic compounds, such as cysteine and ascorbic acid,[8,32] the accessibility of 5-ASA toward peroxyl radicals should be the key factor to consider in the analysis of these results and consequently in the discussion of the antioxidant potency of 5-ASA.

To assess the potential interaction between 5-ASA and the endogenous antioxidant α-T, the inhibition of lipid peroxidation by 5-ASA was evaluated in liposomes containing α-T, which is known to be located well inside the lipid bilayer with its phenolic hydroxyl group, the active site for radical scavenging, oriented to the polar region and the phytyl side chain embedded into the bilayer interior.[33] When AAPH has been used as the peroxyl radical initiator, the combination of 5-ASA and α-T in the same liposomal system leads to an additive antioxidant effect. In fact, the inhibition periods detected in the presence of both compounds, either of oxygen consumption or of conjugated diene hydroperoxides production, are similar to the sum of the individual inhibition periods observed for each antioxidant, when used separately (Figure 3). Under these conditions, when α-T and 5-ASA are allowed to act separately, most of these compounds are consumed, while the oxidation is suppressed to cause the inhibition periods of oxygen consumption (Figure 4). On the other hand, when α-T and 5-ASA have been combined, 5-ASA protects α-T from the attack of peroxyl radicals initially generated outside the lipid membranes, as evidenced by the very low rates of α-T consumption in the presence of 5-ASA (Figure 5) and the parallel high rate of 5-ASA consumption (Figure 6), during the oxidation reaction. Indeed, although both 5-ASA and α-T can act as efficient free radical scavengers of peroxyl radicals generated in the aqueous phase (Figure 3(A)), 5-ASA is expected to have a better access to these initiator radicals than α-T, because it has the same location, i.e., in the aqueous phase.

Therefore, as also reported for other hydrophilic compounds,[32] 5-ASA shows the ability to react efficiently with AAPH-peroxyl radicals readily before they can reach the lipid membrane and react with α-T or the soybean PC. The sparing of α-T by 5-ASA in these conditions is remarkable and concentration dependent, as evidenced in Figure 5.

Finally, to assess a potential synergistic interaction between 5-ASA and α-T we investigated the combined effect of 5-ASA with α-T against lipid peroxidation initiated inside the membrane by thermal decomposition of the lipid-soluble compound AMVN. The detection of such interaction, similar to that reported for ascorbic acid and α-T,[34] would be a major advance to the understanding of the action mechanism of this drug. Considering that 5-ASA, by itself, shows low ability to suppress lipid oxidation initiated in the lipid region of the membranes (Figure 7), the induced significant prolongation of inhibition periods of oxygen consumption, due to the presence of α-T, suggests a synergistic interaction between these two antioxidants (Figure 9). This synergism could be explained by a recovery mechanism of α-T, due to a 5-ASA-induced reduction of tocopheroxyl radical back to α-T, sustaining its antioxidant activity. However, HPLC determinations of α-T throughout the oxidation reaction reveal that 5-ASA does not inhibit but only retards the consumption of α-T, when they have been combined (Figure 10). Thus, although 5-ASA does not protect efficiently α-T from the attack of peroxyl radicals generated inside the membrane, it is able to delay its consumption. The specific locations of peroxyl radicals and of 5-ASA and α-T may explain such results. When the reaction starts inside the membranes, α-T has a better location to trap AMVN-peroxyl radicals more efficiently than 5-ASA, which is located outside the membranes, completely apart from these radicals. Moreover, the model membranes used in these assays are multilamellar vesicles, which certainly makes it difficult the accessibility of 5-ASA toward the α-tocopheroxyl radical. In fact, although this radical has the chromanoxyl head located at the interface between the lipid and the water phase, 5-ASA is not encapsulated within the liposomes but certainly is located only around the outer monolayer which may also account for the failure in detecting the α-T recovery. Therefore, the rapid consumption of α-T observed during the oxidation reaction only reflects its high capacity to scavenge peroxyl radicals. This does not exclude that 5-ASA may react with α-T radical in the lipid layer but that the α-T radical reacts with another radical much faster than with 5-ASA. As also reported in other works,[32] no significant protection will be to expect on α-T initial consumption, because this is the first target of free radicals when the reaction is initiated inside the membrane, in our experimental conditions. Motoyama *et al.*[32] have shown exactly the same results in a similar experiment to study the synergistic interaction between cysteine and α-T. In this study, the regeneration process of α-T by cysteine was only assessed successfully by ESR studies. Thus, although we could not prove the regeneration of α-T from its tocoferoxyl radical, our data suggest a synergistic interaction between α-T and 5-ASA against lipid peroxidation when the reaction is initiated inside the membranes, similarly to that reported between α-T and ascorbic acid or cysteine.[32]

Our results do not agree exactly with those reported by Pearson *et al.*[35] which suggest that 5-ASA acts as a chain-breaking antioxidant, independently of α-T regeneration, when AMVN initiates the oxidation reaction in membranes. However, it should be stressed that such conclusions were based only on data obtained with *cis*-parinaric as a fluorescent probe to evaluate the lipid oxidation.

In conclusion, the sparing effect on α-T consumption by 5-ASA, when the lipid oxidation is initiated by a flux of peroxyl radicals generated outside the membrane, together with the prolongation of the lag phase of lipid peroxidation due to α-T in the presence of 5-ASA, when the lipid

oxidation starts inside the membrane, play an important role in the pathologic context of inflammatory bowel diseases. Considering that the contents of total and reduced ascorbic acid have been shown to decrease in the inflamed intestinal mucosa of those patients, the regeneration of α-T by ascorbic acid may be compromised and this could account for the higher membrane susceptibility to oxidative stress. Consequently, the protection afforded by 5-ASA against α-T consumption has necessarily to be considered when analyzing the mechanisms by which 5-ASA exerts its anti-inflammatory action in the inflammatory bowel diseases.

Acknowledgements

We are greatly indebted to Prof. Vítor M.C. Madeira for his advice and many helpful suggestions. This work was supported by PRAXIS XXI. Elisabete Gonçalves was a recipient of a grant from PRAXIS XXI (BM/6568/95).

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