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Effects of fumonisin B_1 on lipid peroxidation in membranes

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Abstract

Electron spin resonance (ESR)[1] spin-label oximetry and spin trapping techniques have been used to study the effect of fumonisin B_1 (FB_1), an amphipathic mycotoxin on lipid peroxidation in egg yolk phosphatidylcholine (EYPC) bilayers. In the study of the interaction between FB_1 and lipid bilayers our results show that fumonisin disturbs the ordering of membranes, enhances oxygen transport in membranes, and also increases membrane permeability. In our model system, lipid peroxidations were initiated by extended incubation of the liposomes, or by inducing Fe^{2+} ions, UV illumination of H_2O_2 or ultrasound irradiation. As an indication of the rates of lipid oxidation in EYPC, the consumption of molecular oxygen was studied by monitoring the oxygen concentration in the aqueous phases of the liposomes. Lipid-derived free radicals generated during the oxidation process were measured by a spin trapping method. The incorporation of FB_1 in the test samples made the membranes highly susceptible to oxidation. Our results provide the first evidence that the fumonisins appear to increase the rate of oxidation, promote the free radical intermediate production and accelerate the chain reactions associated with lipid peroxidation. The disruption of membrane structure, the enlargement of the relative oxygen diffusion–concentration products, as well as the enhancement effects on membrane permeability, thus provide additional insights into potential mechanisms by which the fumonisins could enhance oxidative stress and cell damage. © 1998 Elsevier Science B.V.

Keywords: Membrane permeability; Fumonisin; Lipid peroxidation; Electron spin resonance

1. Introduction

The involvement of lipid peroxidation in human pathology continues to be studied extensively. The induction of membrane damage has been postulated to play a possible role in many diseases, such as cancer, atherosclerosis, rheumatoid arthritis, as well as in aging processes [1–3]. In general, the methods used to study lipid peroxidation either monitor changes of the principal reactants (e.g., unsaturated fatty acids, hydroperoxides, or oxygen consumption) or one of a variety of reaction products (e.g., thiobarbituric acid, fluorescent compounds, liberated hydrocarbons, or other putative end products). For review, see Ref. [4]. For instance, highly sensitive ESR oximetry enables one to study the early stages of lipid peroxidation, e.g., by measuring oxygen uptake

Abbreviations: [1]ESR, electron spin resonance; FB_1, fumonisin B_1; DMPC, L-α-dimyristoylphosphatidylcholine; EYPC, egg yolk phosphatidylcholine; 5-SASL, 5-doxylstearic acid spin label; CTPO, 3-carbamoyl-2,2,5,5-tetramethyl-3-pyrroline-1-yloxyl; TEMPO-choline, 4-(*N*,*N*-dimethyl-*N*-(2-hydroxylethyl)ammonium-2,2,6,6-tetramethylpiperidine-1-oxyl, chloride; 4-POBN, α-(4-pyridyl-1-oxide)-*N*-*tert*-butyl-nitrone

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[5–7]. The extent of lipid peroxidation may also be estimated via ESR (in conjunction with spin trapping techniques) by directly detecting the free radicals [8,9].

The fumonisins are a relatively newly identified family of aminopolycarboxylic acids that possess a sphingoid-like lipophilic backbone reminiscent of the sphingolipid sphinganine. In light of epidemiological investigations that associate *Fusarium moniliforme*, which produces the fumonisins, with human esophageal cancer [10–12] as well as several animal feeding studies that demonstrate a causative role for these mycotoxins in leukoencephalomalacia in horses [13], pulmonary edema in swine [14], and hepatic cancer in rats [15], extensive efforts are directed toward elucidating their toxicological mode(s) of action [16,17]. The finding that the fumonisins are potent inhibitors of the enzyme sphinganine *N*-acyl transferase disclosed another likely aspect of their biological activity [18]. Cellular membranes are postulated to be a principal target for the fumonisins in vivo [17]. The composition and the physical state of membranes are important because they influence the conformations and the activities of many membrane-associated enzymes and receptors, some of which are involved in second messenger systems and cell signaling. Membrane damage caused by the free radical-mediated processes of lipid peroxidation may also contribute to the oxidative damage of DNA, which has often been implicated in carcinogenesis. In addition to a growing appreciation of the impact of the fumonisins on lipid metabolism, the affects of these mycotoxins on oxidative stress and on the overall integrity of cell membranes represent new avenues of investigation [19–22].

Lipid peroxidation is a free radical-mediated chain reaction that consists of initiation, propagation and termination [23–25]. However, a literature search failed to disclose any molecular mechanistic studies that specifically address the potential pro-oxidative effects of the fumonisins on lipids in membranes. An understanding of the basic physical and chemical aspects of oxidative stress and cell damage provides a basis for investigating molecular changes induced by the fumonisins. Our long-term objective is to understand how the fumonisins physically and chemically affect each of the discrete stages within the overall process of lipid peroxidation. An earlier paper [26] showed that the fumonisins affected the structural and dynamic properties of lipid bilayer membranes in unique ways, such as decreasing the order of alkyl chains (i.e., increasing motion) near the surface of fluid-phase membranes; sharpening the phase transition (enlarging the change in ordering) and restricting the alkyl chain motions in gel-phase membranes. Also, our work dealing with the measurement of oxygen transport within lipid bilayers indicated that the presence of fumonisin B_1 increased oxygen transport near the surface of model membranes [27]. The enhancement effects of FB_1 on oxygen transport in membranes suggests that in the hydrophobic region of the membrane, the fumonisins might accelerate oxidative reactions involving molecular oxygen and its reactive species and increase free radical intermediate production as well. In this work, we have focused on the relation between its structural and dynamic membrane properties and its pro-oxidation activity during lipid peroxidation. Multilamellar liposomes, consisting of egg yolk phosphatidylcholine (EYPC), were again used as a model system. In addition to the study of the effects of fumonisin on membrane structure and oxygen transport, the effect of FB_1 on membrane permeability was also investigated by using spin label methods. Lipid peroxidation was initiated either by incubation, inducing Fe^{2+} ions, ultraviolet (UV) or ultrasound irradiation. Oxygen consumption during the lipid peroxidation of EYPC was studied by monitoring oxygen concentrations in the aqueous phases of test samples. The lipid-derived free radicals generated during the oxidation process have also been measured by using a spin trapping method.

2. Materials and methods

The spin labels 3-carbamoyl-2-2-5,5-tetramethyl-3-pyrroline-L-yloxyl (CTPO), 5-doxyl stearic acid (5-SASL) and the spin traps 5,5-dimethyl-L-pyrroline-*N*-oxide (DMPO), α-(4-pyridyl-1-oxide)-*N*-*tert*-butyl-nitrone (4-POBN) and the lipids egg yolk phosphatidylcholine (EYPC), and L-α-dimyristoylphosphatidylcholine (DMPC) were purchased from Sigma (St. Louis, MO). 4-(*N*,*N*-dimethyl-*N*-(2-hydroxylethyl)ammonium-2,2,6,6-tetramethylpiperidine-1-oxyl, chloride (TEMPO-choline) was a product of Molecu-

lar Probes (Eugene, OR); Fumonisin B_1 was prepared as reported previously [26,28]. High purity (18-MΩ) water was used throughout the study. Trace metal impurities in buffers were removed by treatment with Chelex-100 chelating ion-exchange resin (100–200 mesh sodium from Bio-Rad, Melville, NY). Particular care was taken to prevent oxidation of Fe^{++} before reactions by holding the freshly prepared solutions of ferrous sulfate under argon.

The membranes used in this work were a multilamellar dispersion of investigated PC containing 1.4×10^{-4} M CTPO or 0.5 mol% of spin label (5-SASL), in the absence or presence of various concentrations of FB_1. Membranes were prepared according to the methods of Feix et al. [29]. All test samples were run in 50 μl capillaries.

The relative permeabilities of liposomes were studied by monitoring the net chemical reduction of 5-SASL spin labels by sodium ascorbate, which had subsequently been added to the aqueous phase [30]. At 37°C, ascorbate ions can diffuse through the lipid bilayer and abolish the ESR signals of the spin labels; the relative rate of reduction of the 5- and 16-SASL spin labels by ascorbate were determined by monitoring changes in the height of the center-field line from the ESR spectra, as a function of time. Another method to study the degree of membrane leakage is to monitor entrapped spin labels, such as TEMPO-choline, within the membrane liposomes [31]. In this work, TEMPO-choline solutions were added to liposomes containing various amounts of FB_1 and incubated for 10 min; the reaction mixture was cooled to 4°C for 2 min, prior to the addition of aqueous sodium ascorbate at 4°C. The sample was then transferred to a capillary for immediate measurements. Because TEMPO-choline and ascorbate are essentially impermeable to membranes at 4°C, the abolishment of ESR signals from TEMPO-choline molecules that had remained outside of the liposomes effectively enabled us to monitor changes in the signal intensities from TEMPO-choline molecules entrapped within the liposomes.

An oximetry method that uses electron spin resonance (ESR) spin labels and a closed chamber [5,6] was used to measure the oxygen concentrations during lipid peroxidation. ESR spectra of nitroxide spin labels exhibit three lines because of the hyperfine interaction of the unpaired electron with the nitrogen nucleus; each line consists of another group of lines because of proton superhyperfine interactions. Resolution of the proton superhyperfine lines depends on the concentration of oxygen—due to bimolecular collisions of the spin label with molecular oxygen—a paramagnetic molecule which broadens each line via Heisenberg exchange interactions. Resolution of the superhyperfine structure of the low field line of the ESR spectrum of CTPO depends on the oxygen concentration in the solution; oxygen consumption was thus obtained from the calibration curves relating the K parameter [Refs. [5,6]; see Fig. 1] to oxygen concentration.

Conventional ESR spectra were obtained with a Varian E-109 X-band spectrometer and a variable temperature controller accessory. ESR signals were obtained with a 1 or 10 mW incident microwave power and 100 kHz field modulation of 1 G (for order parameter and spin trapping studies) or 0.05 G (for oxygen transport and oxygen consumption studies). Spectra were recorded, stored, and manipulated on an IBM/PC computer. Ultrasound irradiation was carried out with VC 50 Ultrasonic Liquid Processor (Sonics and Materials) working at 25 Hz with 2 mm microtip inserted into the liposome suspensions in a glass tube cooled in an ice bath.

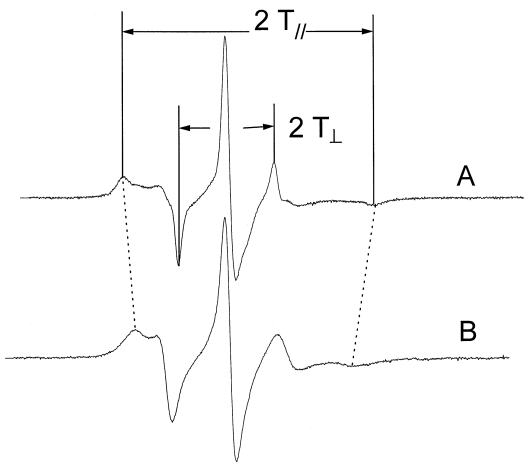

Fig. 1. Typical conventional ESR spectra of 5-SASL in EYPC (75 mg/ml) with 0 mM of FB_1 (A) and 10 mM of FB_1 (B) in 0.01 M PBS buffer, pH 7.4. The spectra were recorded at 37°C; microwave power, 1 mW; modulation amplitude, 1 G; scan range, 100 G. The order parameters were calculated from the ESR spectra by using the equation $S = 0.5407(T_{\|} - T_{\perp})/a$, where $a = (T_{\|} + 2T_{\perp})/3$.

UV photolyses were performed in situ at room temperature in liposome suspensions, in 50 μl quartz capillaries placed in the ESR cavity, using a Schoeffel 1000 W Xenon lamp coupled with a Schoeffel grating monochromator. The excitation light had a maximum centered at 270 nm. The oxygen consumption experiments were performed in duplicate and the spin trapping analyses were performed in triplicate.

3. Results and discussion

Typical ESR spectra obtained with 5-SASL in EYPC membranes at 37°C are displayed in Fig. 1. In the membrane, SASL undergoes anisotropic rotational motion. The ESR spectra of 5-doxylstearic acid spin labels in membranes were further characterized by calculating the values of the order parameter S [32,33]. By use of ESR oximetry, the effect of FB_1 on oxygen transport (the diffusion–concentration product) in membranes was also studied by measuring the line broadening on the center line of 5-SASL in the presence of 0 and 10 mM of FB_1, before or after incubation. After 40 h incubation at 37°C, the S values for 5-SASL in EYPC with and without 10 mM of FB_1 were 0.419 and 0.558, respectively. During the experiments, there were no significant time-dependent (before and after incubation) changes in the order parameters for either sample. The line width changes (ΔH) of 5-SASL in EYPC with and without 10 mM of FB_1 were 0.74 G and 0.56 G before incubation, and 0.78 G and 0.56 G after incubation, respectively. Reduction of the value of the order parameter for 5-SASL indicated that FB_1 perturbs the hydrocarbon chains near the surface of the membranes, and the increase in ΔH indicated the enhancement of oxygen transport in membranes by fumonisins.

The chemical reduction of 5- and 16-SASL spin labels by ascorbate ions were used to evaluate the effects of fumonisin B_1 on the permeabilities of lipid bilayers. The rate of ascorbate permeation into membranes is temperature-dependent [30,31]; at elevated temperatures, e.g., 37°C, ascorbate ions can penetrate membranes and (chemically) reduce spin labels located within the lipid bilayer, thereby decreasing the corresponding intensities of the ESR signal. Fig. 2 illustrates the kinetics of signal decay at different

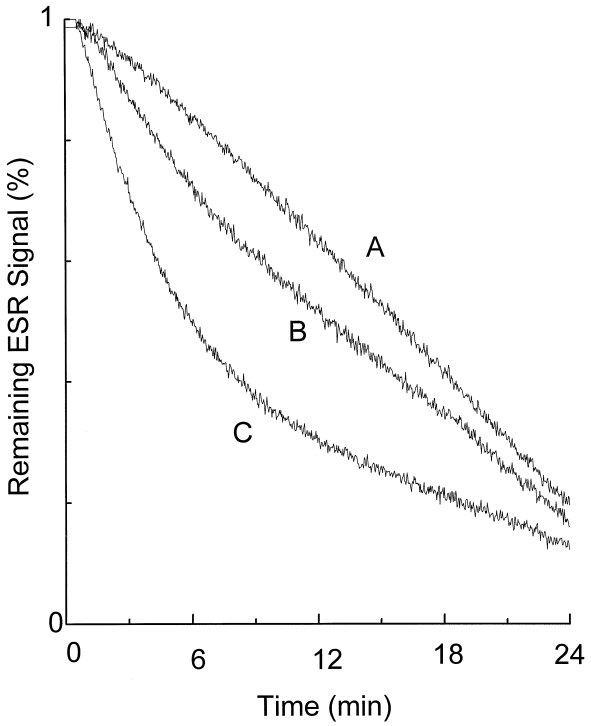

Fig. 2. Remaining ESR signal of 5-SASL as a function of time for FB_1 concentration (mM): (A) 0, (B) 1 and (C) 3 in EYPC membrane suspensions (25 mg/ml) in 25 mM HEPES buffer (pH 7.2) at 37°C. Scan range, off; time constant, 0.5 s; modulation amplitude, 1 G; microwave power, 1 mW; scan time 24 min.

fumonisin concentrations. The addition of FB_1 dramatically decreases the intensities of the ESR signals from 5-SASL spin labels, thereby indicating that fumonisin apparently increases the permeability of membranes to ascorbate ions at 37°C. We see smaller but similar effects of fumonisin on the reduction of the 16-SASL signal (data not shown). This finding corresponds to the smaller structure effects of FB_1 near the center of membranes as shown in our previous work [26].

Another experiment on membrane permeability was carried out. Lipid vesicles composed of EYPC were incubated with TEMPO-choline at 37°C for 10 min, followed by the addition of ascorbate (at 4°C) in order to abolish the ESR signal from TEMPO-choline remaining outside of the liposomes. Fig. 3A shows a typical 3-line TEMPO-choline signal overlapping with an ascorbyl radical signal (doublet) at the center field position. Because such membranes are largely impermeable to TEMPO-choline and ascorbate at 4°C [31],

the small ESR signal from TEMPO-choline that could not be abolished by ascorbate is attributable to TEMPO-choline molecules entrapped in the EYPC liposomes. In contrast, the presence of FB_1 at 1 mM to 3 mM concentrations increased the relative amounts of TEMPO-choline molecules entrapped in liposomes by factors of 6- to 20-fold, as depicted by the signal amplitudes of the corresponding ESR spectra of TEMPO-choline in Fig. 3B and C. As shown in Fig. 3D, the addition of a 10% solution of Triton X-100 emulsified (solubilized) the liposomes; the ESR signal from the previously entrapped TEMPO-choline was now completely abolished by ascorbate. These results collectively indicate that the fumonisins increase membrane leakage to small ions and hydrophillic solutes.

Loss of molecular oxygen in closed capillaries containing liposomes has been used as a diagnostic indicator of lipid peroxidation [5–7]. The rate of lipid peroxidation was estimated by monitoring oxygen consumption with a spin label, such as CTPO; Fig. 4 displays typical results when a spin-probe closed-chamber technique was used. The incubation of liposomes composed of EYPC—which contains a cis double bond in the alkyl chain—led to a depletion of oxygen from the medium. No oxygen depletion was observed in liposomes composed of DMPC, a fully saturated phospholipid. Lipid peroxidation was enhanced by incorporating 10 mM FB_1 into EYPC, as indicated by the dramatically increased rate of O_2 uptake. These results disclosed an apparent link (correlation) between the (physical) effects of FB_1 on the structural integrity of membranes and their enhancement of lipid peroxidation.

Fe^{2+} ions are known to strongly induce lipid peroxidations in cellular and reconstituted membranes [34–36]. In DMPC and EYPC membranes, in

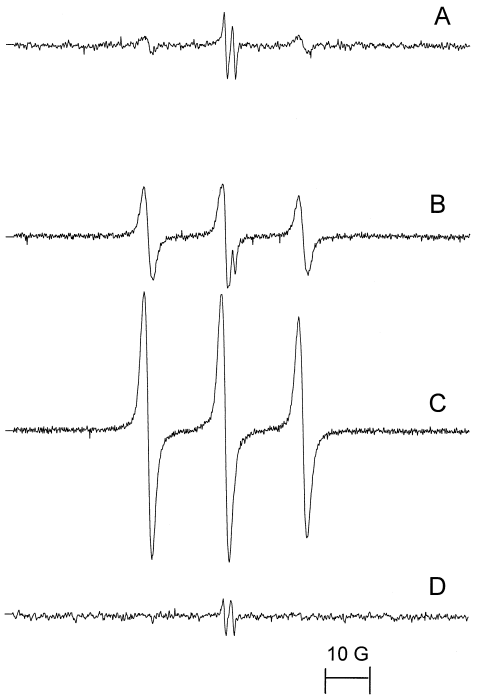

Fig. 3. Effect of FB_1 on the ESR spectrum of TEMPO-choline entrapped in the EYPC membrane suspensions (25 mg/ml) containing (A) no FB_1 or (B) 1 mM FB_1 or (C) 3 mM FB_1 prepared in 25 mM HEPES buffer (pH 7.2) were incubated with 3 mM TEMPO-choline at 37°C for 10 min. The liposome were cooled to 4°C and after the addition of cold ascorbate (90 mM), the ESR spectra were recorded at 4°C, (D) same as (C) except for the addition of 10% Triton X-100.

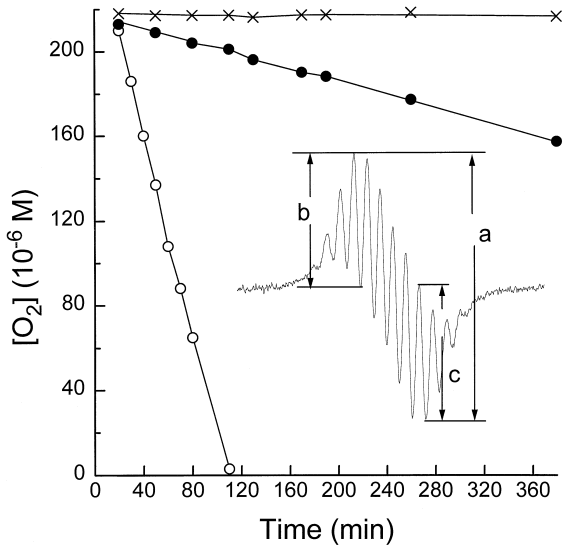

Fig. 4. Oxygen consumption measured in a closed chamber with samples of DMPC (×) or EYPC membrane suspensions (75 mg/ml) at 37°C in 3 mM NaN_3. CTPO (0.14 mM) was incorporated, together with 0 mM of FB_1 (●) or 10 mM FB_1 (○). Aliquots were taken from (aerated) stock samples that had been incubated at 37°C for 40 h. The inset depicts the superhyperfine structure of the low field line from ESR spectra of CTPO in nitrogen-saturated aqueous solutions. The definition of K, a parameter used in calibration charts to determine the oxygen concentration, is indicated. K parameters were calculated by using the equation $K=(b+C)/2a$. Spectra were recorded at 37°C; microwave power, 1 mW; modulation amplitude, 0.05 G; scan range, 5 G.

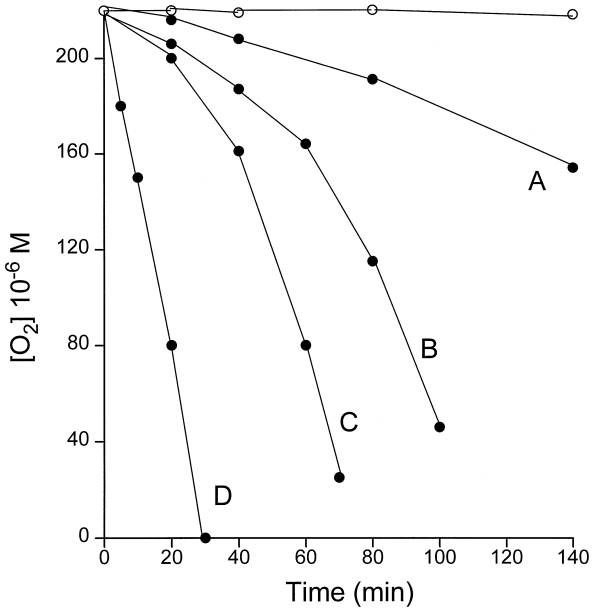

Fig. 5. Oxygen consumption measured in a closed chamber with samples of DMPC (○) or EYPC (●) membrane suspensions (75 mg/ml) at 37°C in 25 mM HEPES buffer (pH 7.2). CTPO (0.14 mM) was also incorporated, together with 0 mM (A), 1 mM (B), 2.5 mM (C) and 5 mM FB_1 (D). Aliquots were taken from stock samples that had been incubated at 37°C for 1 h after adding 10 μl of 1 mM Fe^{2+} into already prepared 100 μl liposomes.

the presence or absence of FB_1 and Fe^{2+}, the relative rates of lipid peroxidation were estimated from the corresponding rates of oxygen consumption. As shown in Fig. 5, there was no depletion of oxygen in DMPC. In contrast, lipid peroxidations occurred in EYPC membranes (alone), while successively higher concentrations of FB_1 led to enhanced rates. Several different mechanisms may be involved. For instance, the physical disruption of membranes by FB_1 could increase the mobility of reactive oxygen species in the propagation cycle of lipid peroxidations. Moreover, our preliminary results indicate that the fumonisins form metal chelates (unpublished data), and such metal complexes might enhance site-specific effects [37] favorable to free radical formation [36].

Two additional methods [38,39] to generate hydroxyl radicals were also used, i.e., UV illumination of H_2O_2 and ultrasound irradiation. Because these approaches do not rely on the (chemical) participation of the fumonisins during free radical generation, they could approximate certain conditions in vivo more closely. These methods also give rise to all of the free radical species relevant to biological reactions, such as inflammations. Moreover, in contrast to oxidations that are initiated by metal ions, these approaches can avoid complications due to the possible involvement of metal complexes. Several representative experiments in which hydroxyl radicals were generated by using UV illumination of 1% H_2O_2 are depicted in Fig. 6. Each test sample was exposed to UV irradiation for 15 min to initiate lipid peroxidation (by generating hydroxyl radicals). The depletion of oxygen was nonlinear during the irradiation of EYPC samples (data not shown). Based on the *K* parameters obtained, samples that contained successively higher concentrations of FB_1 exhibited correspondingly lower levels of residual oxygen. Likewise, the relative rates of oxygen consumption increased as the concentration of FB1 increased, as illustrated in Fig. 6 by the slopes of the respective reactions. Our results indicate that the relative rates

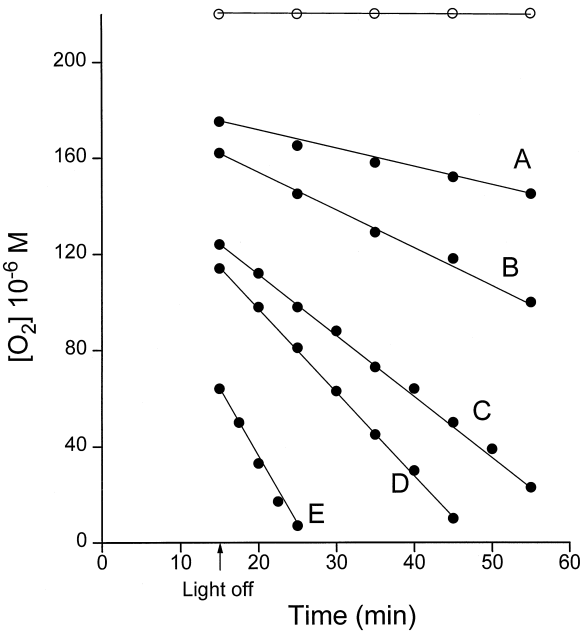

Fig. 6. Oxygen consumption measured in a closed chamber with samples of DMPC (○) or EYPC (●) membrane suspensions (75 mg/ml) at 37°C mixed with 1% H_2O_2, 10^{-4} M CTPO and 25 mM HEPES buffer (pH 7.2), incorporated with 0 mM (A), 0.25 mM (B), 0.5 mM (C), 1 mM (D) and 2.5 mM FB_1 (E). Samples were prepared at 37°C, transferred to the quartz capillary and placed in the ESR cavity. Each sample was UV-irradiated (270 nm, power 1 kW and distance 70 cm) for 15 min, and spectra were recorded afterwards.

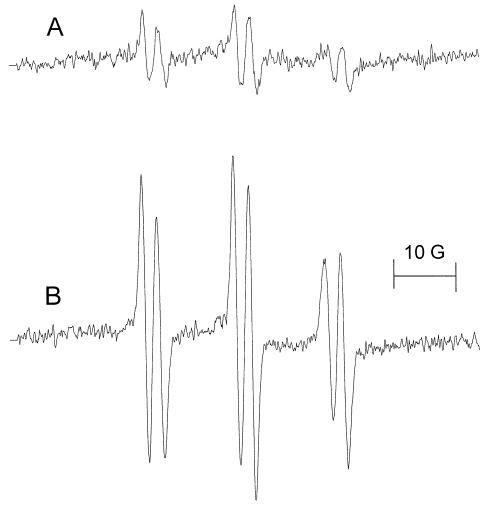

Fig. 7. ESR spectra of the 4-POBN spin adducts formed in the suspensions of 15 mg/ml EYPC membranes containing 0 mM (A) or 2 mM of FB_1 (B), in 100 mM 4-POBN and 25 mM PBS buffer (pH 7.4) at room temperature. Samples were irradiated with ultrasound at a frequency of 25 Hz with a 2-mm microtip for 20 min, transferred to the capillary, and placed in the ESR cavity. The spectra were recorded 2 min after irradiation.

of lipid peroxidation were enhanced by FB_1, both during and after the UV irradiation of EYPC samples. As expected, there was no depletion of oxygen in DMPC samples.

Fig. 7 shows the ESR spectra of POBN lipid radical adducts formed in the EYPC system after ultrasound irradiation. Sonication of EYPC liposome suspensions generated hydroxyl radicals through water sonolysis and induced the initiation of lipid peroxidation [39,40]; lipid-derived free radicals were detected with ESR spin trapping methods. ESR spectra of the POBN spin adducts displayed hyperfine splitting parameters ($a^N = 15.62$; $a^H = 2.64$ G) identical to those for carbon-centered, lipid-derived spin adducts (POBN/$L^{\cdot}$). These have previously been reported [7] in intact cells subjected to free radical-mediated oxidative stress. The pronounced difference in POBN/$L^{\cdot}$ ESR signal intensities demonstrated that incorporating of FB_1 into membranes enhanced the product formation during lipid peroxidation, thereby suggesting that such membranes were more susceptible to free radical-mediated cytotoxicity. The results of UV and ultrasound irradiation experiments not only offer additional evidence that FB_1 accelerates lipid peroxidation but also indicate that such enhancements evidently can occur without the direct involvement of FB_1 in free radical generation.

Our results indicate that the interaction of the fumonisins with lipid bilayers has multiple effects. Fig. 1 and our previous work [26] indicate that the fumonisins can disturb membrane structure and affect the oxygen transport properties of membranes [27]. The permeability studies (Figs. 2 and 3) indicate that the fumonisins also increase the rate of leakage of anions and cations solutes across lipid bilayers. In other words, the increases in permeability and oxygen transport induced by the fumonisins are correlated to corresponding decreases in the ordering of the membranes. As shown by Hubbell and McConnell [32], the disordering of lipid alkyl chains can be ascribed to an increase in the amplitude of motion of the long molecular axis and to an increase in the probability of gauche conformations, P_g, of carbon–carbon single bonds. The increase in P_g leads to an increase in the formation of alkyl chain 'kinks', which provide one molecular mechanism for the associated increase in membrane permeability.

The physiological significance of the effects of the fumonisins on membrane permeability observed in this study are not well-understood. Some additional insights into the mechanisms by which the fumonisins affect membrane transport might be obtained from their ability to enhance the permeabilities of membranes to (anionic) sodium ascorbate and (cationic) TEMPO-choline.

A correlation between the structural properties of membranes and the dependence of lipid peroxidation on the concentration of the fumonisins is also apparent. The results reported here and our earlier studies [26,27] collectively indicate that fumonisin B_1 perturbs a complex interrelationship between membrane structure (vis-à-vis *S*) and the susceptibility of such cellular components to oxidative damage; at physiological temperatures, i.e., in the fluid phase, the region near the surface of the membrane was primarily disrupted by FB_1, which also enhanced oxygen transport in this region of the membrane [27]. One effect of such mycotoxins may thus include making lipids more susceptible to the attack of free radicals (generated in the aqueous phase and/or at the membrane–water interface). The rate of chemical reactions (such as lipid peroxidation) involving molecular oxygen and/or reactive oxygen species (ROS) de-

pends on their collision frequency with target molecules. The combined effects of disrupting membrane structure, enhancing membrane permeability and increasing the oxygen diffusion–concentration product by the fumonisins imply that such molecules probably increase the bimolecular collision frequency of oxygen and/or ROS with target molecules, thereby accelerating the entire process of lipid peroxidation.

The effect of the fumonisins on oxidative stress is not well-understood either. As a first step in our mechanistic studies, we have focused on the relationship between its structural and dynamic effects on membranes and its pro-oxidative activities during lipid peroxidation. Although our studies have been primarily directed at physical aspects of oxidative stress and membrane damage, our long-term goal is to provide insights into the modes of actions of such compounds in vivo.

It has been shown that the efficiency of antioxidants (such as vitamin E and the carotenoids) depends on both chemical reactivity and physical factors, such as location and mobility of such molecules in a specific microenvironment [41–44]. To the best of our knowledge, our results provide the first evidence that the fumonisins apparently increase the rate of oxidation, promote free radical intermediate production and accelerate the chain reaction associated with lipid peroxidation. The disruption of membrane structure, the enlargement of the relative oxygen diffusion–concentration product, as well as the enhancement of membrane permeability by the fumonisins thus provide additional insights into potential mechanisms by which the fumonisins might enhance oxidative stress and cellular damage.

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