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Local Prevention of Oxidative Stress in the Intestinal Epithelium of the Rat by Adhesive Liposomes of Superoxide Dismutase and Tempamine

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Abstract: The purpose of the present study was to investigate whether the local prevention of luminal superoxide-mediated biological damage in the rat jejunal mucosa could be achieved by liposomal superoxide dismutase (SOD) and the SOD mimic tempamine (TMN). Cationic liposomes loaded with either SOD or TMN were perfused in the rat jejunum prior to the induction of oxidative insult. Reactive hydroxyl radicals were generated in situ in a closed circulating intestinal loop of the rat from the reaction between hypoxanthine and xanthine oxidase in the presence of chelated ferrous sulfate. Mucosal activity of lactate dehydrogenase and levels of potassium ions were used to quantify the tissue damage. Intracellular uptake and locality of SOD were examined in HT-29 cells. The intestinal uptake of SOD and TMN was further measured by using rat colon sacs. Entrapment in cationic liposomes was found to significantly enhance the antioxidant effect of SOD and TMN against the induced oxidative damage in the jejunal mucosa, compared with their free forms. The effect was found to be local and was caused by the increased mucosal adhesion of the liposomes. The cationic liposomes also triggered SOD uptake into the HT-29 cell line. It is concluded that the increased residence time of the cationic liposomes of SOD and TMN in the jejunal mucosa resulted in a local effect against oxidative injury. This local protection may be exploited for drug delivery purposes.

Keywords: Adhesive liposomes; mucosal oxidative injury; superoxide dismutase; tempamine; antioxidants; rat; perfusion; HT-29 cell line

Introduction

It has been shown that a variety of gastrointestinal (GI) diseases are associated with reactive oxygen species (ROS) and oxidative stress.[1−4] Although the mucus lining of the gastrointestinal tract possesses antioxidant properties,[5] it is exposed to a continuous oxidative damage by the efflux of ROS. One way to reduce this stress is to introduce antioxidants that scavenge $OH^{\bullet}$ radicals produced via the Haber−Wiess reaction. One such drug is the 5-aminosalicylic acid prodrug sulfasalazine, which was suggested to act as a scavenger of $OH^{\bullet}$ radicals and of the neutrophil-derived hypochlorous acid.[6]

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Natural antioxidant enzymes such as superoxide dismutase (SOD) or catalase are obvious candidates for the treatment of intestinal injuries. SOD has been tested, clinically, for the treatment of Crohn's disease.[7] We have postulated and later demonstrated that, by localizing the antioxidant activity of catalase and SOD, an improved therapeutic effect could be accomplished. In this context cationized catalase and cationized SOD were tested in both intestinal oxidative stress models and experimental colitis.[8−10] This approach, however, involves chemical modification of the enzymes. In this report we examine the possibility of localizing the native (unmodified) enzyme SOD with the aid of a sticky vehicle, namely, cationic liposomes, which have already been shown to possess good mucosal targeting properties.[11]

Tempamine (TMN) is a stable radical scavenger of the nitroxide family and was found to be effective at both the cellular and tissue levels, against a variety of reactive oxygen species (ROS) mediated insults. It is a cell-permeable, low molecular weight compound that mimics the activity of SOD by catalyzing the dismutation of the superoxide anion, producing molecular oxygen and H_2O_2. First oxidized by superoxide, nitroxides form the unstable intermediate, oxoammonium cation, which in turn is reduced by another superoxide anion to form the initial nitroxide molecule and molecular oxygen.[14] It has been therefore suggested as a complementary modulator against oxidative injury,[12,13] including intestinal injuries.[14]

The purpose of this study was to examine whether local administration of SOD or TMN to the small intestine of the rat by a delivery system capable of residing in the vicinity of the mucosa (cationized liposomes) can improve the mucosal protection against oxidative stress. More specifically, the study goals were to (a) induce a hydroxyl radical mediated oxidative stress in the rat jejunum and to characterize the resulting damage, (b) encapsulate (separately) SOD and TMN in cationic liposomes, (c) assess the ability of the antioxidant loaded liposomes to protect the mucosa of the rat jejunum, and (d) examine for the internalization of liposomes in an in vitro cell culture model.

Materials

Hydrogenated soybean phosphatidylcholine (HSPC) (iodine value 3) was obtained from Lipoid (Ludwigshafen, Germany). 1,2-Dioleolyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine Rhodamine B) sulfonyl ammonium salt (PEA-Rhod) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-carboxyfluorescein (CF-PE) were obtained from Avanti Polar Lipids (Alabaster, AL). Cholesterol, dioctadecyldimethylammonium bromide (DODAB), dinitrobenzenesulfonic acid (DNBS), xanthine oxidase, hypoxanthine, 4-amino tempol (tempamine, denoted as TMN), Triton X-100, fluoresceine isothiocyanate (FITC), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolinium bromide (MTT), 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI), and Cu-Zn-superoxide dismutase (SOD) were obtained from Sigma (Sigma Chemical Co., St. Louis, MO).

All other chemicals were of analytical grade unless otherwise stated in the text. Water was double distilled and freshly used.

Animals, Maintenance, Anesthesia, and Euthanasia

Male Sabra rats (220−250 g) were obtained from the Animal Farm of Hadassah Medical Center at The Hebrew University of Jerusalem. They were kept under controlled environmental conditions (22 °C, 12-h light/dark cycles) and fed with standard laboratory chow and tap water. All animal studies were conducted in accord with the Principles of Laboratory Animal Care (NIH Publication No. 85-23, revised 1985). The Mutual Committee of Hadassah University Hospital and the Faculty of Medicine for Animal Welfare approved the study protocol. Anesthesia was performed by an intraperitoneal injection of 100 mg/kg body weight of ketamine (Ketaset, Fort Dodge). Euthanasia of the anesthetized rats was carried out by chest wall puncturing.

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Methods

Liposome Preparation. All liposomal formulations used in the study were composed of 16 mM HSPC, 12 mM cholesterol, and 8 mM DODAB. The three components were weighed, dissolved in *tert*-butyl alcohol, and lyophilized overnight.

For cytotoxicity studies of the cationic liposomes, the lyophilized bed was then hydrated by L-histidine buffer (5 mM in normal saline), pH 6.5, at 60 °C, a temperature above the HSPC gel-to-liquid crystalline phase transition temperature (52 °C).

For cellular trafficking studies (see below) liposomes were stained with Rhodamine by addition of PEA-Rhod to the lipid mixture prior to lyophilization, at a molar ratio of 1:200 of the total lipids.

TMN Encapsulation. TMN was loaded into the liposomes by the pH-gradient method.[15] Briefly, lipids where lyophilized as above and water (70 °C) was added to the lipid film. The resulting liposomes were sonicated to form SUVs. Ammonium sulfate solution (0.25 M) was added, and the preparation was freeze−thawed 10 times, after which it was lyophilized overnight. L-Histidine buffer (70 °C) was then added, and the liposomes were extruded at 65 °C, 11 times through 400-nm pore-size polycarbonate filters using the LiposoFast syringe extruder (Avestin, Ottawa, ON, Canada) to prepare sized multilamellar liposomes (MLVs). The size distribution of the liposomes was analyzed by a submicrometer particle sizer (Coulter, Luton, U.K.). For each batch, a size-distribution curve was plotted and the average size of the liposomes was 400 ± 50 nm (mean value ± SD). Free ammonium sulfate was removed by dialysis against normal saline. Existence of the acidic gradient was verified by the acridine orange test. TMN (10 mM) was added at 70 °C and the system incubated for 15 min. Nonencapsulated TMN was separated by dialysis against normal saline, and the encapsulated TMN concentration was measured by electron paramagnetic resonance analysis (EPR) using an 8 point calibration curve of TMN.[12] The encapsulation yield averaged at 80%.

SOD Encapsulation. The lyophilized bed of lipids (see above) was hydrated in acetate buffer, pH = 5.5, and the SOD solution (0.75 mg/mL, see below) added. The mixture was freeze−thawed three times (60 °C).[11] The MLVs obtained were extruded as mentioned above to an average size distribution of 400 nm. Excess unloaded SOD was removed by ultracentrifugation and decantation (3 times). SOD content in the liposomes was determined by measuring its activity by the cytochrome *c* method.[16] The encapsulation yield averaged at 50 ± 5%.

Tagging SOD with FITC. SOD was tagged with FITC to enable the detection of its uptake into the cells. FITC was dissolved in DMSO, and SOD was dissolved in sodium bicarbonate buffer pH 9, 0.1 M. The two solutions were mixed (magnetic stirrer, l h, room temperature) to form a molar ratio of 1:3 SOD:FITC. Unreacted FITC was separated from the FITC-SOD by dialysis against saline. The FITC-SOD was then immediately loaded into the liposomes.

FITC-SOD Encapsulation. FITC-SOD was encapsulated in a similar way to native SOD. FITC-SOD content in the liposomes was determined by (a) activity measurements using the cytochrome *c* method,[16] (b) fluorescence measurements, and (c) quantification of the protein contents.[17] Measurement of the supernatant FITC-SOD activity verified the results of the liposomal FITC-SOD activity measurements. The encapsulation yield averaged at 50 ± 5%.

Liposomal charge was assessed by ζ potential analysis after dilution (1/100) in a 0.01 M NaCl (Zetasizer 3000 HS, Malvern, England).

SOD Locality within the Liposomes. To verify whether the liposomal FITC-SOD was inside the liposomes or adsorbed onto the surface of the lipid layers outside the liposome, FITC-SOD-loaded liposomes and free FITC-SOD were diluted in buffer solutions of the following pH values: 4.5 (citrate buffer, 0.1 M), 5.5, 6.5, 7.5 (bicarbonate buffer, 0.1 M), and 8.5 (borate buffer, 0.1M). The increase in fluorescence was recorded spectrofluorimetrically (Perkin-Elmer LS-SB, Bucks, England). λ emission = 525 nm, λ excitation = 495 nm. As FITC fluorescence is pH sensitive and increased pH is associated with increased fluorescence, an increase in fluorescence in the presence of more alkaline buffers can be interpreted to represent liposomal surface adsorbed FITC-SOD.

Cytotoxicity Studies. The cytotoxicity of the cationic liposomes was assessed in the human colon adenocarcinoma cell line HT-29 using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolinium bromide (MTT) assay.[18] Cells were maintained in DMEM containing 10% FCS and grown at 37 °C in 5% CO_2 (v/v) in air. Cells were seeded in 96-well microtiter plates at a density of 10000 cells per well. Twenty-four hours after plating, DODAB liposomes, diluted to final concentrations of 19, 56, 168, and 503 μM DODAB, were added to the wells. In addition, liposomes that did not contain DODAB (concentration 0) were added. The cells were then incubated for an additional 24, 48, or 72 h. Cellular metabolic activity was assayed by incubating with MTT (0.5 mg/mL per well) for the final 4 h of the designated exposure period, solubilizing the formed formazan crystals with 10% SDS in 0.01 N HCl, and monitoring the sample absorbance at 570 nm, with a background reference wavelength of 630 nm.

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Internalization of SOD-Loaded Liposome into HT-29 Cells. The ability of HT-29 cells to internalize SOD-loaded liposomes in culture was examined. HT-29 cells (2×10^4/200 μL) were seeded onto cover slips contained in 24-well trays. After 24 h the cells were exposed to SOD-loaded liposomes for 1, 2, and 4 h at 37 °C. To determine SOD internalization, FITC-SOD was introduced into nonlabeled liposomes. To verify cellular internalization of both SOD and liposomes, FITC-SOD was loaded in Rhodamine-labeled liposomes. At the end of incubation the cells were rinsed three times with cold PBS and fixed in 3% paraformaldehyde. Nuclear morphology visualization was enabled by soaking the fixed cells in 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI), 50 μg/mL in PBS, for 20−30 min, at room temperature. After PBS rinsing (three times) the cells were mounted in Mowiol-DABCO mounting media. Cell internalization and cytosol locality (in relation to the nucleus) were examined by confocal microscopy (Zeiss, Gottingen, Germany).

Colon Sac Preparation and Uptake Studies of SOD and TMN. The abdomens of anesthetized healthy Sabra rats were cut open, and 10 cm long segments of the distal colon were excised, separated, and rinsed with PBS. Colon sacs (5 cm each) were prepared by tying one end with 3/0 silk suture, filling with 0.4 mL of free or liposomal suspension of either TMN or FITC-SOD (diluted ×10 in PBS, final concentrations of 150 units/mL SOD and 0.5 mM TMN), and tying at the other end.

The sacs were incubated in 15 mL of PBS, containing 10 mM glucose, at 37 °C, in a glass vial in a shaking bath for 60 min. At the end of the incubation, the sacs were cut open, rinsed three times by PBS immersion, and weighed. For detection of FITC-SOD attachment and uptake (subsequently referred to generally as uptake, as internalization and attachment cannot be discriminated using these methods), the colon sac preparations were homogenized (Polytron, Kinematicas, Berlin, Germany) in a solution containing 2-propanol: pH 9.0 borate buffer 9:1. One milliliter of the homogenate was centrifuged at 14000 rpm for 15 min. The level of fluorescence in the supernatant was then measured by a spectrofluorimeter (Perkin-Elmer LS-SB, Bucks, England) at λ excitation = 495, λ emission = 525. For the detection of TMN uptake, tissues were homogenized in water containing 1% v/v TritonX-100. One milliliter of the homogenate was centrifuged (5000 rpm, 15 min), and TMN concentration in the supernatant was measured by EPR.

Local Induction of Oxidative Stress in Rat Jejunum. Hydroxyl radicals were induced in the jejunum (15 cm long segment) of each of four groups of anesthetized Sabra rats, by the perfusion of a mixture of 6 mM hypoxanthine, 3 units/mL xanthine oxidase, 10 mM $FeSO_4$ and 1 mM EDTA in saline over 30 min, using a closed rat jejunal loop system at a rate of 1 mL/min, as described elsewhere.[8]

In separate studies two groups of rats (4 rats in each group) were perfused with either 35 units/mL SOD or liposomal SOD (lipidic dose of 1.3 mg/mL) over 20 min. A 10-min saline rinse was then performed, followed by the hydroxyl radical induction as described above. Similarly, the other two groups (4 rats each) were perfused with 5 mM free TMN or liposomal TMN over 20 min. A 10-min saline rinse was then performed, followed by hydroxyl radical induction as described above. Control studies included (a) a group of rats perfused with normal saline over 30 min prior to the oxidative damage induction and (b) a group of rats perfused with normal saline for 20 min (naive control). At the end of each study, the perfused jejunal segment was separated from the anesthetized animal, and the mucosal epithelial layer was delicately scraped and homogenized in 5 mL of distilled water on ice.

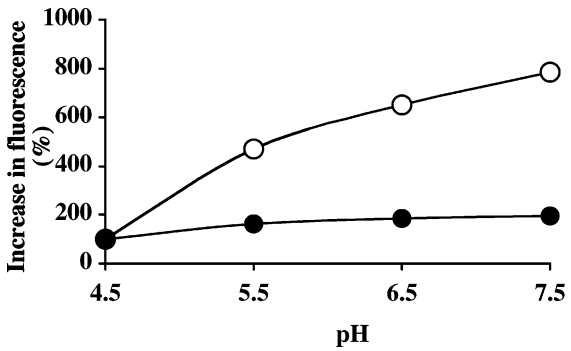

Figure 1. The change in fluorescence (expressed as a % of initial fluorescence at pH 4.5) of encapsulated (filled circles) or free (empty circles) FITC-SOD with pH. The pH resistance of the encapsulated FITC-SOD suggests that the enzyme was internalized and was not adsorbed on the liposomes' surface.

Injury Quantification. Assuming that tissue insult would cause leakage of enterocyte contents, the induced damage was characterized by measuring (a) tissue activity of lactate dehydrogenase (LDH) at 340 nm with pyruvic acid as the substrate and NADH as the electron donor (for this purpose, 1 mL of the tissue homogenate was centrifuged at 5000*g* for 15 min; LDH activity was measured in the supernatant) and (b) tissue potassium levels by atomic absorption. All measurements were repeated four times, and the results were normalized to tissue dry weight.

Statistical Analysis. Data were analyzed by the Kruskal−Wallis test. A difference was considered to be statistically significant when the p value was <0.05. When a difference between the groups was obtained, a Mann−Whitney test was used to analyze the significance of the difference between the individual group means ($p < 0.05$).

Results

To identify internalization of the FITC-SOD into the liposomal preparations, the loaded liposomes were incubated in buffer solutions of increasing pH. Figure 1 shows that while the fluorescence of free FITC-SOD intensified with pH increase, the fluorescence of the liposomal FITC-SOD changed very little with pH, indicating that the tagged liposomal enzyme was entrapped in the liposome construct (where the pH was 5.5, resulting in a typically weak fluorescence at that pH) and was not adsorbed to the liposomes surface.

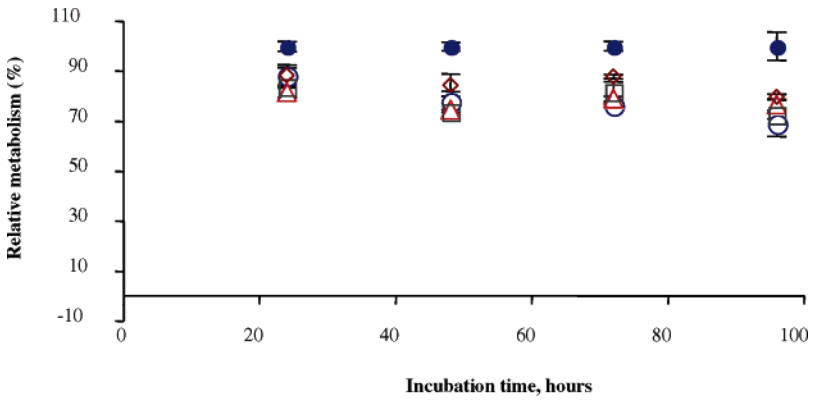

Figure 2. The toxicity of the cationic liposomes (400 nm), diluted to final concentrations of 19 (circles), 56 (triangles), 168 (squares), and 503 (hexagonals) μM DODAB, and liposomes without DODAB (concentration "0", filled circles) as analyzed by the MTT test in HT-29 colorectal adenocarcinoma cells. Shown are the means of triplicate data ± SD.

Characterization of the cytotoxicity and the cell permeability of the liposomes were undertaken on an immortalized colon cell line, as normal primary colon cells were not available for the assessment of these properties. The cationic liposomes were well tolerated by the colorectal adenocarcinoma cell line HT-29, for up to 96 h, as measured by the MTT assay, summarized in Figure 2. The same cell line was used to characterize cellular internalization of the liposomes. The liposomes were labeled with Rhodamine and loaded with FITC-SOD. Typical examples of visualization following 1, 2, and 4 h of incubation are presented in Figure 3B−D. Figure 3A shows the cells before incubation with liposomes. It was observed that FITC-SOD (yellow-green) was localized at discrete points within the cell cytoplasm. Rhodamine-labeled phospholipids (red) were also identified within the cytoplasm, indicating that the HT-29 cells engulfed both liposomal lipids and the FITC-labeled contents. DAPI staining showed that the location of the liposomal FITC-SOD was confined to the cytosol, excluding the nucleus (Figure 3E).

Tissue uptake studies as shown in Figure 4 revealed that the liposomal preparations of both SOD and TMN were efficiently uptaken by the epithelium of the rat colon compared with the native enzyme and free TMN (13.5 ± 3.9 and 15.4 ± 1.2 compared with 2.5 ± 1.6 and 6.8 ± 1.1% uptake of initial amount ± SEM, respectively).

The oxidative damage induced by hydroxyl radicals caused a 3-fold decrease in the activity of tissue LDH and almost 2-fold reduction in the tissue potassium levels in epithelium of the rat jejunum compared to the control (the untreated group, healthy jejunum). Pretreatment at the location of injury with native SOD or empty liposomes was unable to prevent this damage. However, perfusing the jejunum with liposomal SOD significantly protected the intestinal tissues against the noxious effects of the hydroxyl radicals. LDH activity was 22.4 ± 0.2, 8.7 ± 1.8, 11.8 ± 0.3, 10.4 ± 0.3 and 17.8 ± 1.9 units/mg tissue ± SEM for the untreated group, the oxidative damage treated group, the native SOD pretreated group, the empty liposome pretreated group, and the liposomal SOD pretreated group, respectively. Potassium levels were 0.7 ± 0.06, 0.33 ± 0.07, 0.37 ± 0.03, 0.27 ± 0.05, and 0.56 ±0.06 μmol of potassium/mg of tissue for the untreated group, the oxidative damage treated group, the native SOD pretreated group, the empty liposome pretreated group, and the liposomal SOD pretreated group, respectively (Figure 5).

Similarly, Figure 6 summarizes the advantages of liposomal TMN compared with free TMN in the local treatment of oxidative injury caused by hydroxyl radicals. While free TMN was not able to protect the epithelium against oxidative damage, liposomal TMN enhanced the protection effect as expressed by tissue activity of LDH and potassium levels. Mucosal LDH activity was 22.4 ± 0.2, 8.7 ± 1.8, 9.0 ± 1.3, 10.4 ± 0.3, and 17.9 ± 1.1 units/mg tissue ± SEM for the untreated group, the oxidative damage induced group, the free TMN pretreated group, the empty liposome pretreated group, and the liposomal TMN pretreated group, respectively. The corresponding mucosal potassium levels were found to be 0.6 ± 0.04, 0.35 ± 0.07, 0.33 ± 0.08, 0.27 ± 0.05, and 0.55 ± 0.05 μmol/mg tissue for the untreated group, the oxidative damage induced group, the free TMN pretreated group, the empty liposome pretreated group, and the liposomal TMN pretreated group, respectively.

Discussion

In this study we induced oxidative insult in the jejunal mucosa of the rat by perfusing the small intestine with a mixture of chelated ferrous sulfate, hypoxanthine, and xanthine oxidase. The metabolism of hypoxanthine by xanthine oxidase results in the release of superoxide anions.[19] The observed damage is understood to be caused by hydroxyl radicals, however, and not by superoxide radicals. The production of hydroxyl radicals in this system depends on the availability of superoxide anions and ferrous. In the absence of SOD, the ferrous ions that adhere to the lumen of the intestine during the perfusion interact with hydrogen peroxide (generated by the spontaneous dismutation of superoxide) resulting in the formation of hydroxyl radicals, while ferrous is oxidized to ferric. The continuous production

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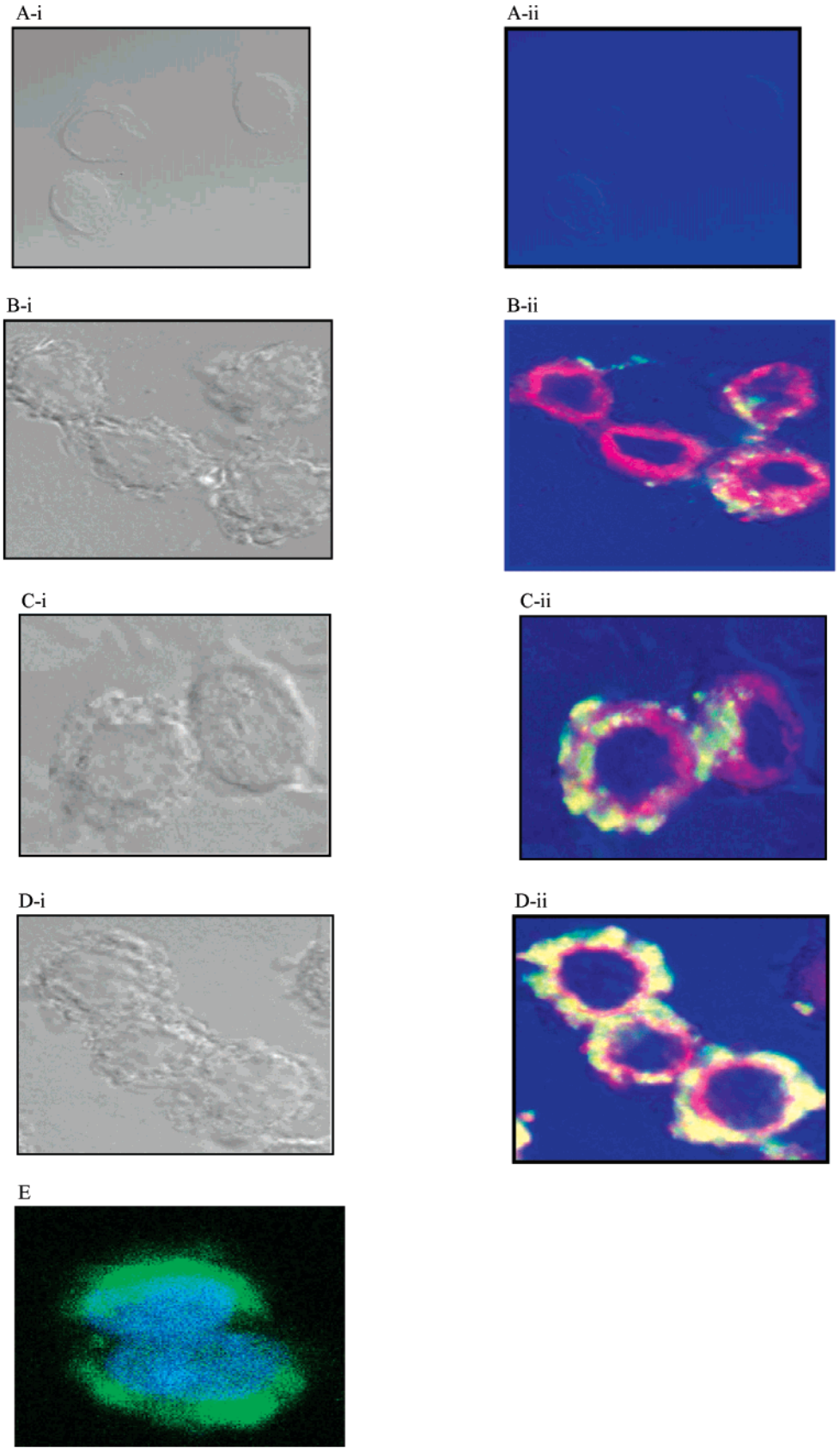

Figure 3. Images of HT-29 cells before (A) and after incubation with Rhodamine-labeled liposomes containing FITC-SOD for 1 h (B), 2 h (C), and 4 h (D). Panel i in each group depicts the phase-contrast images of the confocal fluorescence images of panel ii. (E) DAPI stain of HT-29 cells containing FITC-SOD cationic liposomes.

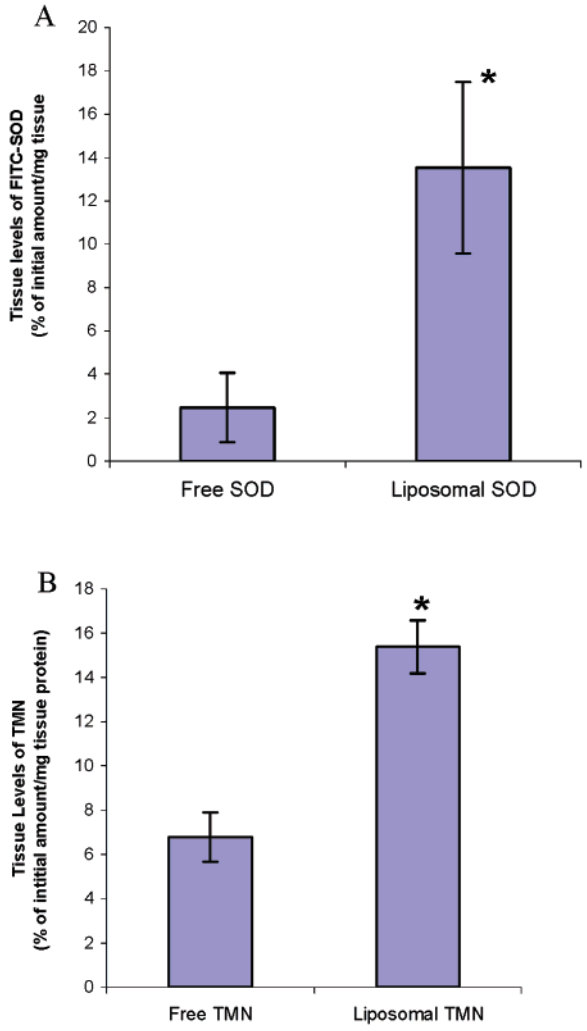

Figure 4. Uptake of free and liposomal FITC-labeled SOD (FITC-SOD) (A) and TMN (B) by the epithelium of gut sacs from the rat colon as expressed by % of initial amount (150 units of SOD or 0.5 mM TMN) in the bathing solution. Shown are the results of four different experiments ± SEM. (*) $p < 0.05$ compared with the unencapsulated group.

of superoxide in the system (by the reaction of xanthine oxidase with hypoxanthin) regenerates the ferrous ions, which in turn continue to react with hydrogen peroxide. This production of hydroxyl radicals in a site-specific manner[20,21] can cause biological damage either similarly to the way neutrophils do in inflammation or similarly to pathological situations such as anoxia caused by ischemic reperfusion, when xanthine dehydrogenase (which, under normal conditions, converts hypoxanthine to uric acid) is converted to xanthine oxidase. In the presence of superoxide dismutase, however, superoxide is dismutated at a faster rate, leading to insufficient superoxide to regenerate ferrous ions. In the oxidized state ferric does not interact with hydrogen peroxide, and the production of hydroxyl radicals is decreased.

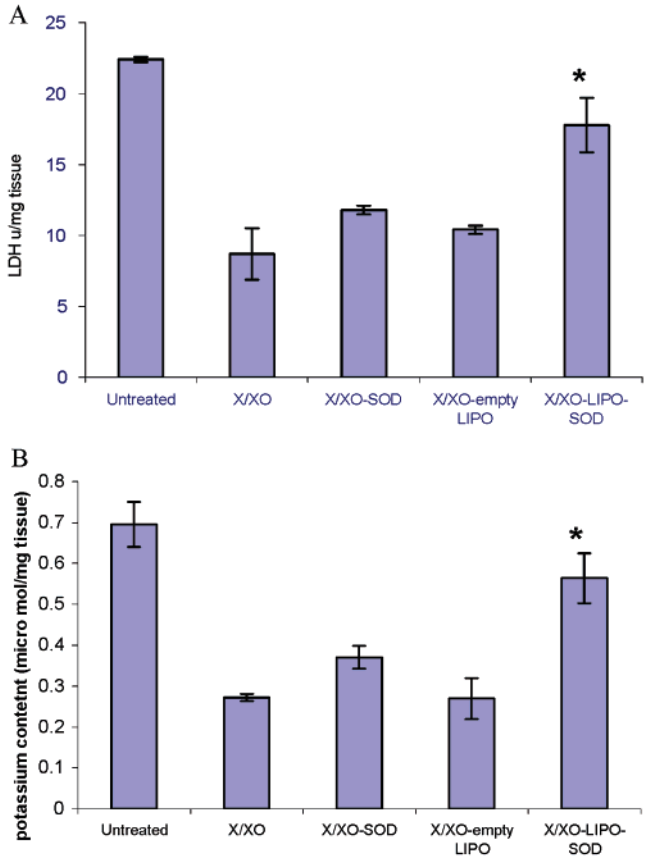

Figure 5. (A) Tissue LDH activity (expressed in units/mg tissue) of healthy jejunal mucosa (naive control, untreated) or mucosal tissues treated with a hypoxanthine (6 mM)/xanthine oxidase (3 units/mL)−$FeSO_4$ (10 mM) mixture followed by saline perfusion (X/XO), native SOD perfusion (35 units/mL) (X/XO-SOD), empty liposome perfusion (X/XO-empty LIPO), and SOD loaded cationic liposomes (35 units/mL) (X/XO-LIPO-SOD). Shown are the results of four experiments ± SEM. (*) $p < 0.05$ compared with the X/XO group. (B) Tissue content of potassium (expressed in μmol/mg tissue) of healthy jejunal mucosa (naive control, untreated) or mucosal tissues treated with hypoxanthine (6 mM)/xanthine oxidase (3 units/mL)−$FeSO_4$ (10 mM) mixture followed by saline perfusion (X/XO), native SOD perfusion (35 units/mL) (X/XO-SOD), empty liposome perfusion (X/XO-empty LIPO), and SOD loaded cationic liposomes (35 units/mL) (X/XO-LIPO-SOD). Shown are the results of four experiments ± SEM. (*) $p < 0.05$ compared with the X/XO group.

Consistent with this interpretation is our observation that when ferrous ions are removed from the system, biological damage is significantly reduced.

Localizing antioxidant treatment at the site of injury is relatively new and has been previously examined successfully with cationized catalase and cationized SOD in the rat jejunum and the rat colon.[10,21] To avoid the enzyme activity loss due to chemical modification, we adopted a different

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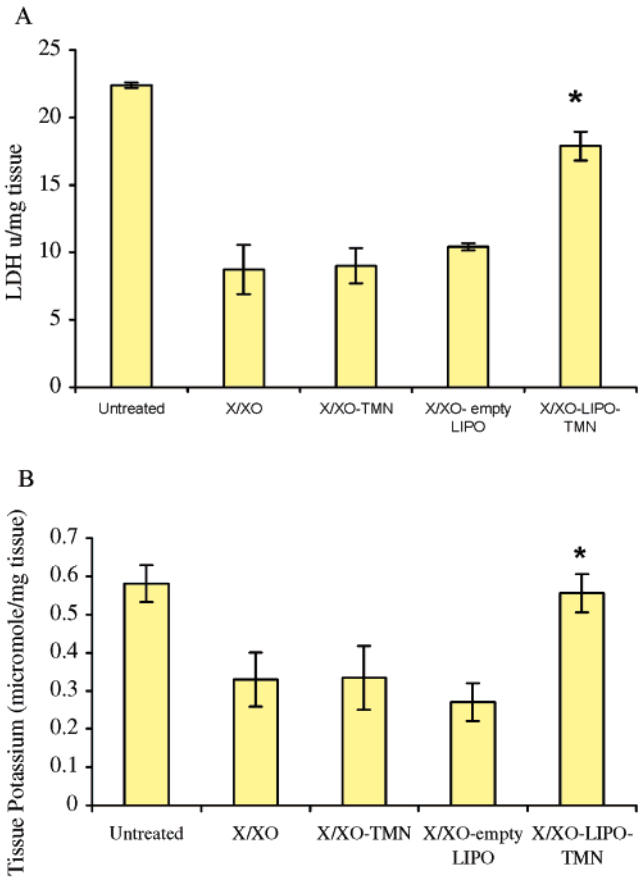

Figure 6. (A) Tissue LDH activity (expressed in units/mg tissue) of healthy jejunal mucosa (naive control, untreated) or mucosal tissues treated with a hypoxanthine (6 mM)/xanthine oxidase (3 units/mL)−$FeSO_4$ (10 mM) mixture followed by saline perfusion (X/XO), TMN perfusion (5 mM) (X/XO-TMN), empty liposome perfusion (X/XO-empty LIPO), and TMN loaded cationic liposomes (5 mM) (X/XO-LIPO-TMN). Shown are the results of four experiments ± SEM. (*) $p < 0.05$ compared with the X/XO group. (B) Tissue content of potassium (expressed in μmol/mg tissue) of healthy jejunal mucosa (naive control, untreated) or mucosal tissues treated with hypoxanthine (6 mM)/xanthine oxidase (3 units/mL)−$FeSO_4$ (10 mM) mixture followed by saline perfusion (X/XO), TMN perfusion (5 mM) (X/XO-TMN), empty liposome perfusion (X/XO-empty LIPO), and TMN loaded (5 mM) cationic liposomes (X/XO-LIPO-TMN). Shown are the results of four experiments ± SEM. (*) $p < 0.05$ compared with the X/XO group.

approach this time. Localization was accomplished by entrapping the enzyme in cationic liposomes, which were found to adhere more abundantly to the intestinal epithelium than neutral or anionic liposomes.[11] In this context the SOD mimic TMN was also tested in the same biological system.

DODAB-cationized liposomes have been reported to exhibit cytotoxicity in certain cellular contexts.[22] While cationization, in general, does not always involve cell damage,[23] the use of DODAB was found to be cytotoxic toward phagocytic cells (macrophages and U937 cells), but not toward nonphagocytic T lymphocytes.[24] In our study we found that DODAB containing liposomes were well tolerated by the HT-29 cell line, for 80 h (Figure 2). The different cell lines used as well as the different lipid combination could explain this discrepancy. The lipid combination of our liposomes was selected to ensure their stability and minimize leakage (data not shown). Thus, HPC, which contains saturated alkyl chains, provides rigid bilayers with low permeability.[25] Cholesterol was added to further stabilize the membrane and reduce premature release.[26] The use of charged compounds, such as DODAB, further increases the stability of the liposomes by decreasing potential aggregation and eventual fusion of the vesicles. In addition, entrapment of SOD inside the liposomes was verified (Figure 1) and the possibility that it was adsorbed to the liposome surface was excluded.

The use of liposomal TMN and SOD significantly increased the ability of the two antioxidants to protect the rat jejunum against the induced oxidative stress. To exclude the possibility that this protection was caused by a physical barrier of the liposomes' phospholipids (as was previously detected with calcitonin emulsions when tested in diffusion cells[27]), empty liposomes were perfused in the same experimental protocol and showed no preventive effect (Figure 5, Figure 6). When antioxidant-loaded liposomes adhered to the jejunal mucosa, an increase of tissue SOD activity or enrichment with TMN, respectively, was observed (Figure 4). The observed increased adhesion is important because, even with local administration, the antioxidants are still exposed to rapid removal processes. In addition, the liposomes potentially provide protection for the SOD against proteolysis and for both molecules against premature scavenging by mucin components. This speculation is currently being tested in our laboratory. As for TMN, an SOD mimic, which was shown to neutralize both intracellular and extracellular superoxide, with no preference to the time of administration (prior or after oxidative insult), delivery tools (e.g., liposomes) are highly important, especially because it is a small molecule with the capability of diffusing easily among

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cellular and tissue components.[14] Anchoring to the site of injury is important due to its mode of action which, unlike other antioxidants that act in a sacrificial mode, provides protection in a (constant) catalytic way.[14,28] Indeed, Figure 4 shows that free TMN tissue uptake was 3-fold higher than free SOD uptake, most probably because of its molecular dimensions (MW 171 compared to 33000) and hence better penetration through the mucus layer.

Internalization studies of liposomal FITC-SOD (Figure 3B−D) clearly show that the cationized liposomes entered the HT-29 cells within the first hour of incubation. Accumulation increased over 4 h. The precise mode of internalization and the fate of the SOD are still being explored in our laboratory. DAPI staining (Figure 3E) showed that the FITC-SOD (green) voyage ended at the cytosol and was not found in the cell nucleus (blue) within 4 h. While these studies indicate the capacity of liposomes to penetrate permissive cells and deliver their contents, we have yet to definitively demonstrate that this is the mechanism occurring in the rat intestine.

An interesting question is whether positioning SOD inside the cell is imperative for the antiinflammatory action. The paradigm is that, in inflammatory processes, ROS are generated in interstitial spaces by circulating neutrophils that consume higher amounts of oxygen when activated.[29,30] Due to the relatively short biological half-life of most radicals, and the nonspecific manner of their action, it is expected that they are active at neighboring cellular structures (cell membrane).[31] Indeed, Hata and co-workers reported that, after exposure to oxidative stress, the survival rate of colonic epithelial cells from the rabbit decreased significantly when intracellular SOD was deactivated by diethyldithiocarbamate.[32] However, internalization is not enough. It should be accompanied by a constant input into the site of injury. In this current study this was accomplished by using sticky liposomes (cationized). The ability of the liposomes to stay in the vicinity of the insulted mucosa clearly made them effective therapeutic tools. This is demonstrated by the TMN case. Nitroxide radicals readily cross cell membranes.[14] However, in our study, TMN solution was unable to prevent oxidative insult even after intimate contact with the injured jejunal mucosa (Figure 6). It was only after being incorporated into cationized liposomes that the TMN expressed therapeutic effect because of the much larger residence time at the site of action (Figure 4). Yet, it should be recognized that when cationic liposomes adhere to the mucosa of the intestine, they do so, most probably, by sticking to the mucus lining which covers the epithelium first. Indeed, reduction of whole mucins in the gut sac preparation with dithiothreitol (DTT), prior to incubation with the liposomes, decreased the amount of liposomes adhering to the intestine (data not shown).

The potential of microparticulate carriers in tissue deposition and, thus, extending the therapeutic effect was previously pointed out by Lamprecht and co-workers, who noticed a prolonged reduction in the activity of an inflammation marker (myeloperoxidase) in the colitis induced rat after oral administration of poly(lactic-coglycolic acid) spheres, loaded with the c-AMP selective inhibitor Rolipram.[33] In our study we measured directly the attachment of liposomal antioxidants and found a significant tissue accumulation, coupled with an improved therapeutic effect as described above.

Increasing the residence time of antioxidant enzymes such as SOD or catalase in the vicinity of a target organ, to enhance the local enzyme activity, has been suggested in the context of pulmonary injuries[34] and arthritis.[35] However, apart from studies associated with burn wounds,[36] all studies suggest systemic administration. The uniqueness of our hypothesis is the topical administration of the liposomal antioxidants to the mucosa of the injured epithelium. Supporting colonic delivery systems previously developed in our laboratory could be used for the local delivery of these liposomal borne remedies.[37]

Abbreviations Used

CF-PE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-carboxyfluorescein; DAPI, 4′,6-diamidine-2′-phenylindole dihydrochloride; DODAB, cholesterol, dioctadecyldimethylammonium bromide; DNBS, dinitrobenzenesulfonic acid; EPR, electron paramagnetic resonance analysis; FITC, fluoresceine isothiocyanate; FITC-SOD; FITC tagged SOD; HSPC, hydrogenated soybean phosphatidylcholine; IBD, inflammatory bowel diseases; LDH, lactate dehydrogenase;

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MLVs, multilamellar liposomes; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolinium bromide; PEA-Rhod, 1,2-dioleolyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine Rhodamine B) sulfonyl ammonium salt; ROS, reactive oxygen species; SOD, superoxide dismutase; TMN, 4-amino tempol (tempamine); XO, xanthine oxidase.

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