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Superoxide dismutase entrapped-liposomes restore the impaired endothelium-dependent relaxation of resistance arteries in experimental diabetes

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Abstract

Diabetes is associated with impaired endothelium-dependent relaxation. We questioned whether administration of superoxide dismutase (superoxide: superoxide oxidoreductase, EC 1.15.1.1) entrapped in long-circulating liposomes improves the vascular reactivity of the resistance arteries. Using the myograph technique, the vasodilation in response to acetylcholine was measured in mesenteric resistance arteries isolated from diabetic or normal hamsters treated for 3 days with superoxide dismutase entrapped in liposomes, with the same concentrations of free superoxide dismutase and plain liposomes, or untreated. Superoxide dismutase activity and nitric oxide (NO) levels were assayed by spectrophotometry, superoxide dismutase levels by Western blot and the role of N^{ω}-nitro-L-arginine ethylester (L-NAME) on vasodilation by the myograph technique. Our data revealed that: (i) superoxide dismutase entrapped in liposomes restored to a great extent the endothelium-dependent relaxation of diabetic hamster resistance arteries; (ii) in superoxide dismutase entrapped in liposomes-treated diabetic animals, the activity and the level of superoxide dismutase in arterial homogenates as well as the serum nitrite levels were significantly higher than those in untreated hamsters or hamsters treated with free superoxide dismutase and plain liposomes: (iii) L-NAME inhibited the response of arteries to acetylcholine in superoxide dismutase entrapped in liposomes-treated diabetic hamsters. These results suggest that superoxide dismutase entrapped in liposomes is effective in scavenging superoxide anions, increases nitric oxide bioactivity and improves the vasorelaxation of resistance arteries in diabetic hamsters.

Keywords: Artery; Diabetes; Liposome; Superoxide dismutase; Vasodilation

1. Introduction

Notable alterations in endothelium-dependent relaxation have been observed in the vessels of diabetic patients (Johnstone et al., 1993) as well as in laboratory animals with experimental diabetes (Diederich et al., 1994). Although the nature of this feature is not fully understood, a major role is attributed to the oxidant stress induced by hyperglycemia (Tesfamariam, 1994; Cai and Harrison, 2000). Produced in excess, reactive oxygen species, especially superoxide anion (O_2^-), seem to be involved in the impaired ability of endothelium to mediate vasodilation either by decreasing nitric oxide (NO) production and/or its bioactivity (for review see Pieper, 1998). The O_2^- and NO react rapidly and generate peroxynitrite ($ONOO^-$), a potent oxidant and potential mediator of vascular tissue injury (Beckman et al., 1990; Mazzon et al., 2002). Moreover, it has been reported that hyperglycemia promotes the glycation of superoxide dismutase, leading to its inactivation and reduction in antioxidant defense (Arai et al., 1987).

An approach to decrease oxidative stress is to use antioxidants as therapeutic agents (Ting et al., 1996; Mayhan and Patel, 1998). Given the important role of O_2^-, superoxide dismutase, a scavenger enzyme, may have the ability to restore, at least in part, endothelial function. However, due to its rapid elimination from the circulation (half-life < 10 min) the therapeutic use of superoxide dismutase is limited.

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Consequently, attempts to increase its therapeutic index by designing other delivery strategies have been made. Among these, coupling of polyethylene glycol to superoxide dismutase (Mugge et al., 1991) or the use of lecithinized Cu^{2+}/Zn^{2+}-superoxide dismutase (Yunoki et al., 1997) has been shown to improve superoxide dismutase efficiency by extending its time in the circulation. Another alternative is to trap superoxide dismutase in liposomes, which has the advantage that superoxide dismutase is protected from inactivation and its life-span in the circulation is extended. There are studies that show that liposome-incorporated superoxide dismutase is internalized by cultured endothelial cells and that the cells are protected against extracellular oxidative stress (Beckman et al., 1986; Nakae et al., 1990a). Data from in vivo experiments indicate that encapsulation of superoxide dismutase in liposomes may extend their circulation in the blood stream. Moreover, in animal models parenteral administration of superoxide dismutase-entrapped liposomes provides protection against toxic hepatic necrosis (Nakae et al., 1990b), adjuvant-induced arthritis (Corvo et al., 1999, 2002), oxidative injury of the brain (Yusa et al., 1984), and cerebral ischemia/reperfusion injury (Imaizumi et al., 1990). Also, in a clinical trial, the administration of Cu^{2+}/Zn^{2+}-superoxide dismutase encapsulated in liposomes was effective in reducing late radiation-induced tissue fibrosis (Delanian et al., 1994).

In the present study, we questioned whether superoxide dismutase encapsulated in sterically stabilized liposomes improves the diminished vascular response of resistance arteries, a common feature of diabetes. We provide evidence that in vivo administration of superoxide dismutase entrapped in liposomes restores the endothelium-dependent relaxation of small arteries from diabetic hamsters, functioning as a superoxide anion scavenger and thus contributing to an increase in NO bioactivity.

2. Materials and methods

2.1. Reagents

Reagents were obtained from the following sources: egg phosphatidylcholine, cholesterol, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[poly(ethylene glycol)-2000] (PEG_{2000}-PE) from Avanti Polar Lipids (Alabaster, AL, USA); mouse anti-bovine superoxide dismutase from Biotrend Khemikalien, (Koln, Germany); the enzyme kit for glucose assay from Dialab (Vienna, Austria); the enhanced chemiluminiscence system (ECL), Super Signal West pico chemiluminiscent substrate from Pierce (Rockford, USA); streptozotocin, bovine Cu^{2+}/Zn^{2+} superoxide dismutase (superoxide:superoxide oxidoreductase; EC 1.15.1.1), acetylcholine, noradrenaline (bitartrate), sodium nitroprusside, N^{ω}-nitro-L-arginine ethylester (L-NAME), Aspergillus nitrate reductase, as well as all other chemicals from Sigma-Aldrich Chemie (Germany).

2.2. Preparation of liposome-entrapped superoxide dismutase

Unilamellar sterically stabilized liposomes were prepared essentially as previously described after extruding multilamellar vesicles through a polycarbonate membrane (Paternostre et al., 1996). Briefly, a mixture of phospholipids in chloroform was dried in a rotary evaporator under reduced pressure; the ratio of lipids was egg phosphatidylcholine: cholesterol: PEG_{2000}-PE (65: 30: 5 mol%). The superoxide dismutase was added in phosphate-buffered saline (PBS) so as to achieve a final lipid concentration of 25 μmol/ml. After a brief vortexing, the preparation was hydrated overnight in a rotating wheel. The resulting multilamellar vesicles were extruded 20 times through a 100-nm polycarbonate membrane, using a Mini-Extruder (Avanti Polar Lipids, Alabaster, AL, USA). The final liposome vesicles had an average diameter of 115 nm, as determined on electron micrographs after negative staining and transmission electron microscopy. The efficiency of superoxide dismutase encapsulation was ~ 25% of the initial superoxide dismutase added; free superoxide dismutase was separated from liposome-encapsulated superoxide dismutase using a Sepharose 4B column. The final superoxide dismutase concentration in liposomes was 750U/25 μmol/ml.

2.3. Experimental protocol

Forty male Golden Syrian hamsters, 115–135 g in body weight, were divided into two groups: (i) in 30 animals, diabetes was induced by one i.p. injection of 50 mg streptozotocin/kg body weight; (ii) 10 age-matched were used as control animals. After 8 weeks from the induction of diabetes, both the diabetic and control hamsters were subdivided into three groups: (a) hamsters (under slight ether anesthesia) injected daily with superoxide dismutase entrapped in liposomes at the concentration of 1500U SOD/50 μmol liposome/kg body weight, via venous retro-orbital plexus for 3 days; (b) hamsters injected with free SOD and plain liposomes at the same dose and time as above and (c) control hamsters received no treatment; all hamsters were killed after 3 days. The experiments were approved by the Ethics Committee from our institute and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no 85-23, revised 1996).

2.4. Biochemical assays

At the time of death, animals had been fasted for 18 hours. Blood was collected from the venous plexus of the eye and used for biochemical assays.

2.4.1. Glucose assay

At the beginning of the experiment and at 8 weeks after the induction of diabetes, plasma glucose levels were

determined by an enzymatic method according to the manufacturer's protocol (Dialab, Viena, Austria).

2.4.2. Nitric oxide determination

Detection of inorganic nitrite (the stable end metabolite of NO) was performed in animal sera using NADPH oxidation by Aspergillus nitrate reductase, followed by Griess reaction (Smárason et al., 1997).

2.4.3. Superoxide dismutase activity assay

For each experimental condition used, the mesenteric bed was isolated and stored at − 70 °C. Frozen fragments were homogenized on ice and resuspended in lysis buffer containing 20 mM Tris, 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 0.5% sodium deoxycholate, 1 mM sodium orthovanadate, 0.25 mM phenylmethyl sulfonylfluoride and 1 μg/μl proteases inhibitors (aprotinin, leupeptin, pepstatin). After centrifugation at $10\,000 \times g$ for 10 min at 4 °C, the supernatant was collected and superoxide dismutase activity was assayed by inhibition of NADH oxidation mediated by $MnCl_2$, monitored by spectrophotometry at 340 nm as described (Paoletti and Mocali, 1990). The reaction was initiated by addition of 1 mM β-mercaptoethanol in a mixture consisting of 100 mM triethanolamine-diethanolamine-HCl buffer, pH: 7.4, 0.3 mM NADH, 100 mM/50 mM EDTA-$MnCl_2$ and 10 μl of each sample. The SOD activity was expressed as units/mg total protein, where one unit is defined as the amount of enzyme able to inhibit by 50% the rate of NADH oxidation of the control (reaction mixture without sample).

2.4.4. Determination of superoxide dismutase levels by Western blot

Homogenates of mesenteric arteries were prepared as described above. Total protein concentration was determined with the Amido Black technique, using bovine serum albumin as standard (Schaffuer and Weissmann, 1973). Each sample was resuspended in sample buffer (20 mM dithiothreitol, 6% sodium dodecyl sulfate, 0.25 M Tris pH: 6.8, 10% glycerol, 5% β mercaptoethanol, 0.001% bromphenol blue) and after boiling was subjected to sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) electrophoresis (15%) and then transferred to nitrocellulose membranes. After incubation with the blocking buffer (supplied by Pierce) supplemented with 0.05% polyoxyethyenesorbitan monolaurate (Tween-20), the blot was incubated with anti-bovine SOD (1:1000). Immunodetection was performed with an enhanced chemiluminescence (ECL) system using horseradish peroxidase-conjugated secondary antibody according to the manufacturer's protocol (Pierce, Rockford, USA).

2.5. Vascular reactivity assay

The hamsters were killed by cervical dislocation, and after laparotomy the mesenteric vascular bed was carefully dissected, and small segments (1–2 mm length) of resistance arteries were removed and mounted in a small vessel myograph chamber (model 410 A, JP Trading, Denmark) filled with HEPES salt solution containing HEPES (5 mM); NaCl (140 mM); KCl (4.6 mM); Mg_2SO_4 (1.17 mM); $CaCl_2$ (2.5 mM), and glucose (10 mM), pH 7.4. Throughout the experiment the buffer was maintained at 37 °C with continuous oxygen supply. Each artery was set to a "normalized lumen diameter" estimated to be 90% of that it would have when relaxed (in situ) and subjected to a transmural pressure of 100 mm Hg (Mulvany and Halpern, 1977). To this purpose, the artery was stepwise distended, and the force developed in the wall was continuously monitored (in mN) on the interface of the myograph. The values corresponding to the internal circumference-active tension relation were introduced into a computer BASIC program (a gift from Prof. Mulvany, Aarhus University, Denmark) that gives the values of the "normalized" lumen diameter of the artery. The mesenteric resistance arteries used in the experiments had "normalized" lumen diameters of $\sim 225 \pm 7$ μm. Then, a "standard start procedure" was carried out, which consisted of three contractions (3 min each) in response to noradrenaline (10 μM) and KCl (123.7 mM) and two contractions in response to noradrenaline (10 μM) and NaCl (140 mM), followed by 5 min rest in HEPES salt solution, until a stable, reproducible response was obtained. After the "standard start procedure" the arteries produced a maximum active force equivalent to a pressure of 100 mm Hg and were ready to use for the experiments. In preliminary experiments, we established the concentration of noradrenaline that gives a similar state of precontraction for diabetic and control groups. The data showed that at low noradrenaline concentrations (10^{-8} to 10^{-6} M), the contraction was increased in diabetic hamsters in comparison with control hamsters while in the range of 3×10^{-6}–10^{-4} M (the plateau zone) no statistically significant modifications of the contractile tension were recorded for the two groups. Thus, 10^{-5} M noradrenaline was used for precontraction, a concentration that did not interfere with the magnitude of the relaxation elicited by acetylcholine or sodium nitroprusside. After precontraction with noradrenaline, the arteries were exposed to a cumulative concentration of acetylcholine (10^{-8}–10^{-4} M) to evaluate the endothelium-dependent vasorelaxation or to sodium nitroprusside (10^{-8}–10^{-4} M) to determine the endothelium-independent relaxation. The vascular response was measured at 2-min intervals. To estimate the role of NO in acetylcholine relaxation, similar experiments were performed in the presence of 10^{-4} M L-NAME (a nitric oxide synthase inhibitor) for 15 min.

2.6. Expression of results and statistics

The vasodilator responses to acetylcholine and sodium nitroprusside were calculated as percentage of the force (in

mN) developed during the precontraction in response to 10^{-5} M noradrenaline. The IC_{50} was determined from the concentration–response curves for agonist-induced relaxation and represents the molar concentration of agonist that produced 50% of the maximum response (Graves and Poston, 1993). The sensitivity to acetylcholine and sodium nitroprusside is expressed as pD_2 ($pD_2 = -\log EC_{50}$). All other results are given as means ± S.E.M. The statistical analysis was performed by One-Way ANalysis Of VAriance (ANOVA) and differences were considered significant when $P < 0.05$.

3. Results

3.1. Plasma glucose levels

Eight weeks after the induction of diabetes, the plasma glucose concentration increased ~3.4 fold in diabetic hamsters above the values determined in the aged-matched control hamsters (241 ± 16.52 mg/dl versus 72.16 ± 3.84 mg/dl). The mortality was 6% in diabetic hamsters and 0% in the control animals.

3.2. Effect of SOD/Lipo administration on endothelial-dependent relaxation of resistance arteries of diabetic hamsters

Small resistance arteries dissected from the mesenteric vascular bed of normal and diabetic hamsters that received for 3 days superoxide dismutase entrapped in liposomes or the same quantities of separately injected superoxide dismutase and plain liposomes were tested by the myograph technique; for comparison similar vessels collected from non-treated, diabetic and control animals were used (Figs. 1, 2). The experiments showed that the endothelium-dependent relaxation in response to acetylcholine (determined in noradrenaline-precontracted resistance arteries) differed widely between the experimental groups. As compared to control hamsters, which exhibited a dose-dependent relaxation in response to acetylcholine, in the diabetic group the relaxation was concentration dependent but greatly reduced. The sensitivity of the response to acetylcholine (expressed as pD2) was significantly diminished from 6.5 ± 0.03 in control group to 5.83 ± 0.07 in diabetic group ($P < 0.05$). The maximum effect on relaxation was found at 10^{-4} M acetylcholine, when the values obtained for the arterial relaxation were 45.32 ± 4.65% for diabetic animals versus 81.58 ± 3.2% for control animals ($P < 0.05$).

Treatment of hamsters with superoxide dismutase entrapped in liposomes partially restored the endothelium-dependent relaxation of resistance arteries of diabetic animals; the sensitivity to acetylcholine was significantly increased ($P < 0.05$) to 6.15 ± 0.05, compared to that of untreated diabetic animals, and the maximal vessel relaxation averaged 71.42 ± 4.65%. By comparison, treatment of diabetic

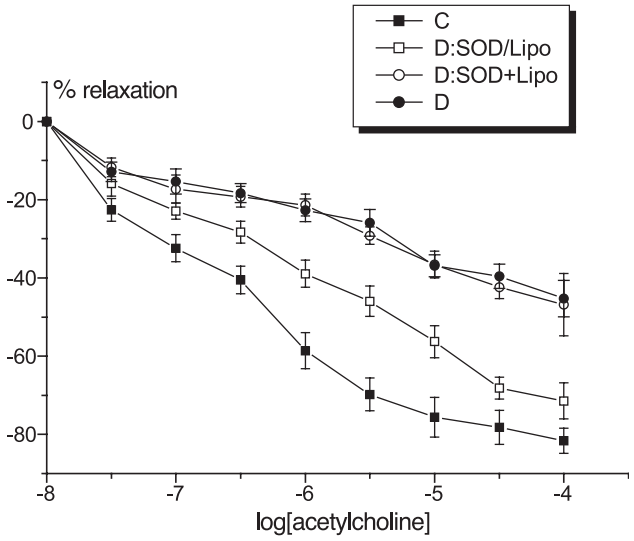

Fig. 1. Comparative effect of in vivo administration of superoxide dismutase (SOD) entrapped in liposomes (SOD/Lipo) and of free SOD and plain liposomes (SOD + Lipo) on endothelium-dependent relaxation in response to cumulative doses (10^{-8}–10^{-4} M) of acetylcholine, determined in noradrenaline-precontracted mesenteric resistance arteries from diabetic (D) and control hamsters (C) determined with the myograph technique. Compared to C group (■, $n = 4$), the vessels explanted from D hamsters (●, $n = 8$) exhibit an impaired vasodilator response to acetylcholine. Treatment (3 days) of diabetic hamsters with SOD/Lipo (□, $n = 10$) led to almost complete restoration of the response of arteries to acetylcholine. By contrast, administration of SOD + Lipo (○, $n = 10$) has no effect on artery relaxation to acetylcholine, which remained at the same level as that of untreated diabetic hamsters. Data are means ± S.E.M. *$P < 0.05$ versus D and D:SOD + Lipo; n = number of animals used.

hamsters with free superoxide dismutase and plain liposomes had no effect on vessel relaxation; the values obtained for sensitivity and maximal relaxation were 5.89 ± 0.05 and 46.8 ± 7.98%, respectively. Also, in the experiments performed on resistance arteries isolated from the control group, superoxide dismutase entrapped in liposomes administration did not increase the response to acetylcholine over that obtained in animals untreated or treated with free superoxide dismutase and plain liposomes (data not shown).

Another series of experiments were performed to assess the endothelium-independent relaxation elicited by cumulative concentrations of the NO donor, sodium nitroprusside. The myograph determinations indicated that the noradrenaline-precontracted arteries exhibited a dose-dependent relaxation in response to sodium nitroprusside; however, similar curves were obtained irrespective of the experimental group used. Thus, at 10^{-4} M sodium nitroprusside the maximal vasorelaxation attained was 85.24 ± 4.53% in the control hamsters and 82.5 ± 6.32% in the diabetic hamsters. The superoxide dismutase entrapped in liposomes and free superoxide dismutase and plain liposomes treatment had no effect on the response of vessels to sodium nitroprusside: the resulting values were 86.71 ± 2.96% and 87.98 ± 4.94%, respectively (Fig. 2).

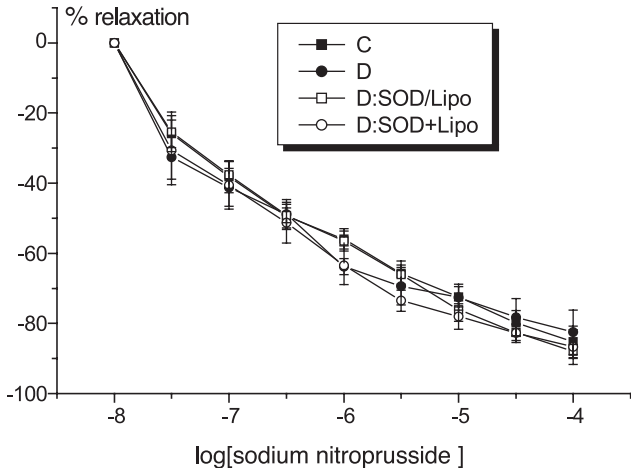

Fig. 2. The endothelium-independent relaxation of resistance arteries from normal (C, $n=4$), diabetic hamsters (D, $n=8$) or diabetic hamsters treated with superoxide dismutase (SOD) entrapped in liposomes (D: SOD/Lipo, $n=10$) or free SOD and plain liposomes (D: SOD + Lipo, $n=10$) in response to cumulative concentrations of sodium nitroprusside. In all experimental groups, sodium nitroprusside induced a similar vasorelaxation of the noradrenaline-precontracted vessels. Data are means ± S.E.M. n, number of animals.

No significant differences in sensitivity of the response to sodium nitroprusside were detected: the pD_2 values were 6.8 ± 0.09 in the control group, 6.93 ± 0.12 in the diabetic group, 6.76 ± 0.17 in diabetic animals treated with superoxide dismutase entrapped in liposomes and 6.85 ± 0.15 in diabetic animals treated with free superoxide dismutase and plain liposomes.

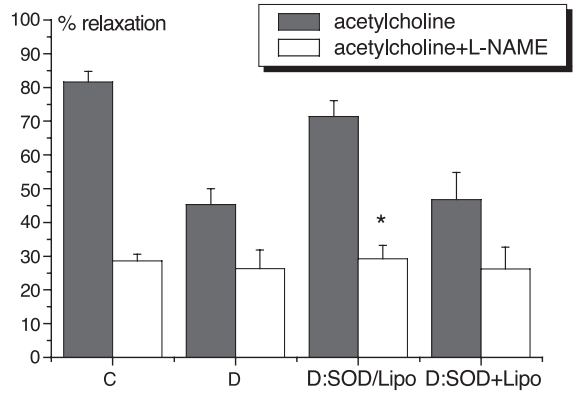

Fig. 3. Effects of pre-incubation of mesenteric artery segments with 10^{-4} M N (ω)-nitro-L-arginine methyl ester (L-NAME), on the relaxation elicited by 10^{-4} M acetylcholine in control (C, $n=4$) and diabetic (D, $n=8$) groups, and in D animals treated with superoxide dismutase (SOD)-encapsulated liposomes (SOD/Lipo, $n=10$) or with similar amounts of free SOD and plain liposomes (SOD + Lipo, $n=10$). For all experimental groups, incubation with L-NAME diminished the vasodilator response of the resistance arteries to acetylcholine, indicating a NO-dependent mechanism of vessel relaxation. Data are means ± S.E.M. * % inhibition of acetylcholine relaxation by L-NAME: $P<0.05$ versus D and D: SOD + Lipo. n, number of animals used.

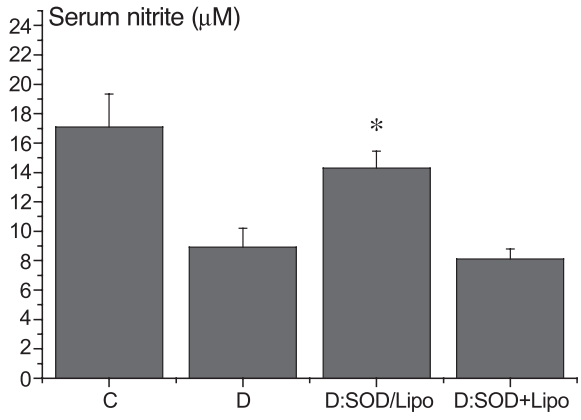

Fig. 4. The inorganic nitrite levels determined in the sera of control (C), untreated diabetic (D) hamsters or diabetics treated with SOD entrapped in liposomes, (D: SOD/Lipo) or with free SOD and plain liposomes (D: SOD + Lipo). The level of nitrites was significantly increased (*$P<0.05$) in D: SOD/Lipo versus D or D: SOD + Lipo. Number of animals used: 4 for C; 8 for D; 10 for D: SOD/Lipo; 10 for D: SOD + Lipo.

3.3. Nitric oxide involvement

For all experimental groups, the vasodilator response of the resistance arteries to acetylcholine was diminished by incubation with 10^{-4} M L-NAME, an NO synthase inhibitor. The percent inhibition of the acetylcholine-induced vasorelaxation by L-NAME was significantly higher ($P<0.05$) in controls (~ 65%) as compared with diabetic animals (~ 42%), suggesting that the impaired relaxation of arteries from the diabetic group is mainly due to the reduced production of NO.

The effect of superoxide dismutase entrapped in liposomes administration to diabetic hamsters was suppressed in the presence of L-NAME, which diminished the vasodi-

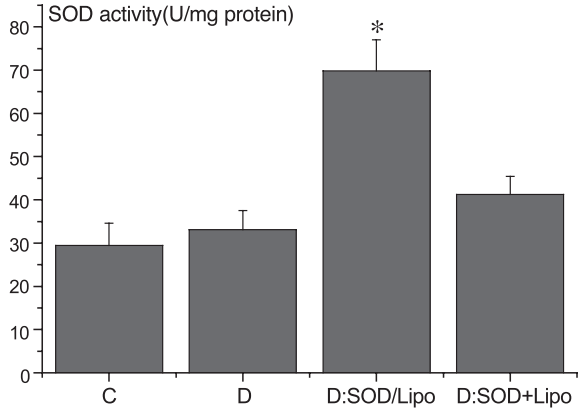

Fig. 5. Superoxide dismutase activity determined in homogenates of resistance arteries from control (C, $n=4$), diabetic (D, $n=8$) and D hamsters treated with superoxide dismutase (SOD)-encapsulated liposomes (D: SOD/Lipo, $n=10$) or with separately administered SOD and liposomes (D: SOD + Lipo, $n=10$). SOD/Lipo administration led to a significant increase in SOD activity in comparison with that in all experimental groups (*$P<0.05$).

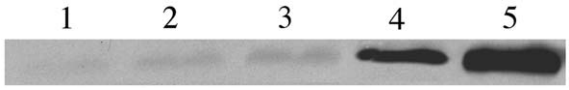

Fig. 6. Western blot of homogenates of resistance arteries probed with anti-bovine SOD. Samples were isolated from: 1—control hamsters, 2—diabetic hamsters, 3—diabetic hamsters treated with free superoxide dismutase and plain liposomes, 4—diabetic hamsters treated with superoxide dismutase encapsulated in liposomes, 5—positive control (Cu^{2+}/Zn^{2+}-superoxide dismutase from bovine erythrocytes). The results are representative for three animals from each experimental group. Note that there was only a small cross-reaction between endogenous superoxide dismutase and anti-bovine superoxide dismutase (lane 1).

lator response by 59%, suggesting a NO-dependent mechanism ($P < 0.05$ versus untreated diabetic hamsters and diabetic animals treated with free superoxide dismutase and plain liposomes). In the case of administration of free SOD and plain liposomes to diabetic animals, L-NAME blocked the acetylcholine-induced relaxation by ~ 44%, a figure similar to that obtained for untreated diabetic animals (Fig. 3). These data correlate well with the determination of serum nitrite levels, which increased significantly in diabetic animals treated with superoxide dismutase entrapped in liposomes in comparison with untreated diabetic animals (Fig. 4).

3.4. The activity and the level of superoxide dismutase in mesenteric artery homogenates

The superoxide dismutase activity was assayed using the NADH oxidation method. As shown in Fig. 5, comparatively, the superoxide dismutase activity was not significantly different in diabetic and control hamsters (33.1 ± 4.41 U/mg versus 29.47 ± 5.14 U/mg). The treatment of diabetic animals with superoxide dismutase entrapped in liposomes led to an increase in superoxide dismutase activity in vessel homogenates as compared with that of animals not treated or treated with free superoxide dismutase and plain liposomes (69.82 ± 7.2, 33.1 ± 4.41 and 41.3 ± 4.16 U/mg, respectively). Moreover, analysis of homogenates of arteries by Western blot probed with anti-bovine superoxide dismutase indicated that the level of retained superoxide dismutase in the vessels in the case of administration of SOD/Lipo was significantly higher than in the case of separately administered SOD + Lipo (Fig. 6).

4. Discussion

Diabetes has been reported to be associated with impaired endothelium-dependent relaxation in vivo (Ting et al., 1996) and in vitro (Tesfamariam et al., 1991). High glucose concentrations may increase oxidant stress by several mechanisms: glucose auto-oxidation, activation of the diacylglycerol-protein Kinase C pathway (Ishii et al., 1998), activation of the aldose-reductase pathway, abnormal arachidonic acid metabolism or non-enzymatic glycation by depleting tetrahydrobiopterin, leading to uncoupled nitric oxide synthase (Hink et al., 2001). All these processes contribute to a marked augmentation of superoxide anion production, which increases the degradation of NO, thus leading to an impaired vasorelaxation.

Our data strengthen and extend the concept that, in diabetes, oxidative stress is responsible for endothelial dysfunction and that administration of the antioxidant enzyme superoxide dismutase encapsulated in small sterically stabilized liposomes restores the endothelium-dependent relaxation, elicited by acetylcholine, of resistance arteries of diabetic animals.

The effect of diabetes on the level of superoxide dismutase in the vascular beds of diabetic animals is controversial. Superoxide dismutase activity has been reported to be increased (Kakkar et al., 1996), decreased (Kamata and Kobayashi, 1996) or unaltered (Pieper et al., 1995). Our study is in line with the latter report, revealing that the superoxide dismutase level in the diabetic group was not significantly different from that of the control group (33.1 ± 4.41 versus 29.47 ± 5.14 U/mg). However, the results suggest that the normal levels of superoxide dismutase may be insufficient to scavenge the excess of superoxide anion due to the hyperglycemic condition. Thus, we postulated that administration of superoxide dismutase, a superoxide anion-scavenging enzyme, could restore endothelial function. Our data demonstrate that free superoxide dismutase had no effect on relaxation. However, administration of superoxide dismutase encapsulated in small long-circulating liposomes restored the endothelium-dependent relaxation in resistance arteries of diabetic hamsters. These result are consistent with data that show that the administration of superoxide dismutase encapsulated in pH-sensitive liposomes re-establishes the relaxation of aortic segments from hyperlipemic rabbits (White et al., 1994). The data indicate the advantage of using superoxide dismutase-encapsulated liposomes, since the free enzyme, due to its rapid elimination, cannot prevent the decomposition of NO (by scavenging superoxide anion) and thus cannot maintain normal resistance artery vasodilation.

Mesenteric arteries from diabetic hamsters did not differ from arteries from normal animals in their response to the endothelial-independent vasodilator (sodium nitroprusside), suggesting that the vasorelaxation dependent on smooth muscle cells is not affected by hyperglycemia. Also, the administration of superoxide dismutase entrapped in liposomes or free superoxide dismutase and plain liposomes had no effect on the sodium nitroprusside-induced relaxation, indicating a role for endothelial cells rather than for vascular smooth muscle cells.

In our experiments, the liposomes proved to be effective vectors for the delivery of superoxide dismutase to the vessel wall. The superoxide dismutase activity of the mesenteric resistance artery homogenates was significantly increased in diabetic animals treated with superoxide dis-

mutase entrapped in liposomes in comparison with that of diabetic animals untreated or treated with superoxide dismutase and plain liposomes, separately. Also, there was a significantly increased level of retained exogenous administered superoxide dismutase in arteries (as revealed by Western blot probed with anti-bovine superoxide dismutase) from superoxide dismutase entrapped in liposomes-treated animals compared with superoxide dismutase and plain liposomes-treated animals. We can safely assume that superoxide dismutase encapsulated and delivered by liposomes to the arterial wall is effective in superoxide anion scavenging, thus contributing to an increase in NO bioactivity, which consequently leads to an improved vasodilation elicited by acetylcholine in diabetic animals. This assumption is based on the fact that L-NAME, the NO synthase inhibitor, inhibited the response of arteries from superoxide dismutase entrapped in liposomes-treated animals and increased the serum nitrite levels in diabetic hamsters. In addition, superoxide dismutase enhances the conversion of superoxide to H_2O_2, which has been reported to act as an endothelium-derived hyperpolarizing factor (EDHF) in mouse mesenteric arteries, leading to an increased vasorelaxation (Matoba et al., 2000).

One possible mechanism for the favorable action of superoxide dismutase entrapped in liposomes is that encapsulation of the enzyme in liposomes prolongs the circulation time of superoxide dismutase, thus increasing either the likelihood of cellular uptake or the extravasation of superoxide dismutase entrapped in liposomes. The permeability of the mesenteric vessels is reportedly profoundly altered in diabetes: the vessels are characterized by an increased number of endothelial plasmalemmal vesicles (Arshi et al., 2000; Costache et al., 2000) Moreover, it has been shown that the advanced glycation end-products disrupt the endothelial cadherin complex and this disruption was correlated with increases in vascular permeability (Otero et al., 2001).

Previous studies have shown that long-circulating liposomes (of small dimensions) may escape from the circulation at sites of increased permeability, as in inflammation or cancer (Oyen et al., 1996). In our experiments, it is likely that the liposomes (~ 115 nm diameter) were taken up by endothelial cells and/or were extravasated to the subendothelial space, in areas of increased intercellular transport (i.e. open junctions). In this way, liposomes may deliver superoxide dismutase to endothelial cells or within the subendothelial space, contributing to superoxide anion neutralization.

Taken together, our results indicate that (i) long-circulating liposomes (of small dimensions) are effective vectors for superoxide dismutase delivery to the arterial wall and (ii) in diabetes, the superoxide dismutase entrapped in liposomes is efficient in scavenging superoxide, thus contributing to an increase in NO bioactivity and to an improved endothelium-dependent vasorelaxation of resistance arteries.

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