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Original Communication

Vasoprotective effect of the supramolecular superoxide dismutase-chondroitin sulfate-catalase nanoconjugate

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ABSTRACT

The bienzyme conjugate (SOD-CHS-CAT) of superoxide dismutase and catalase (covalently coupled with each other through chondroitin sulfate, a glycosaminoglycan of the endothelial glycocalyx) was assayed with respect to its vasoprotective activity in interaction with platelets and the rat arterial ring, and in normalization of the hemodynamic parameters in rats and rabbits pathologically altered by exposure to hydrogen peroxide to simulate the development of oxidative stress. The SOD-CHS-CAT conjugate has anti-platelet potential owing to the antiaggregatory effect of the combined enzymatic activities and the supramolecular structure acquired. The effect of SOD and CAT on arterial ring tonus was equivalent for both their native and conjugated forms. The normalizing effect of the SOD-CHS-CAT conjugate on blood arterial pressure and heart rate (after their perturbation by hydrogen peroxide) in rats and rabbits was significantly more effective than the control values. The study demonstrates the possibility of using the SOD-CHS-CAT conjugate in chronic prophylactic therapy and the feasibility of developing oral forms of the conjugate. These features of the SOD-CHS-CAT conjugate, its good tolerability and satisfactory acute toxicity, qualify this product as promising drug candidate, suggesting a versatile approach for developing enzyme conjugates for medical needs.

KEYWORDS: antioxidant therapy, superoxide dismutase, catalase, chondroitin sulfate, bienzyme conjugate, vascular wall

INTRODUCTION

The oxidative stress is now well known to accompany the development of many disorders [1]. Reactive oxygen species (ROS) participate in cell signaling processes under normal conditions, but if the oxidant-antioxidant balance has been disturbed (toward excessive production of ROS), they contribute to oxidative stress. The non-specific effect of ROS in the body results in damage to macromolecules, disruption of metabolic pathways, and progression of pathological processes [2]. Antioxidants are used preventively to arrest or slow down these processes [1, 2]. Among the antioxidants the enzymes stand out for the high efficiency of their protective action, exceedingly high specificity, and well-known mechanisms of catalyzed reactions [2]. A great majority of cardiovascular disorders develop through a phase of oxidative stress, which explains the highly productive results achieved by studying antioxidants for the needs of cardiology.

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Superoxide dismutase, catalase, and glutathione peroxidase are enzymes known to have antioxidant effects [1, 2]. The autonomous functioning (without co-factors) of the first two enzymes makes them promising candidates for developing an antioxidant agent designed to protect the cardiovascular system from oxidative stress. In view of the above-mentioned advantages of these enzymes, we have chosen to develop a combined antioxidant enzyme derivative using Cu, Zn-superoxide dismutase (SOD) and catalase (CAT).

Our approach was based on the use of the biochemically coupled action of SOD and CAT, in which the product of the first enzymatic (SOD) conversion (H_2O_2) becomes the substrate for the second conversion (CAT), which in turn generates water and molecular oxygen (end products that are relatively safe under these conditions) [1, 2]. Furthermore, because chondroitin sulfate (CHS), a glycosaminoglycan of the endothelial glycocalyx, accumulates in areas of incipient atherosclerotic changes in the blood vessels (i.e., areas of potential lesions in the vascular wall) [3], it was selected as a cross-linking modifier agent for enzymatic subunits [4]. The SOD-CHS [5] and CAT-CHS [6] conjugates were obtained by covalent coupling of these enzymes with CHS. The antithrombotic activity of these conjugates was significantly higher than those of their components used individually or as a mixture. On the rat model of arterial thrombosis induced by the treatment of the vessel with saturated solution of ferrous chloride was shown that the SOD-CHS conjugate reduced markedly the obtained thrombus mass [5], while CAT-CHS conjugate prolonged predominantly the time of occlusion emergence [6]. These results emphasized the importance of covalent conjugation of antioxidant enzymes with CHS. The antithrombotic efficacy of combined single-bolus injection of SOD-CHS/CAT-CHS mixture or other compositions of those native or CHS modified enzymes and CHS (free or coupled) was much lower than that of the bienzyme SOD-CHS-CAT conjugate [7]. This was probably due to different intravascular distribution of the enzyme derivatives, coupling of SOD and CAT activities in bienzyme conjugate via CHS and its large molecular size [2]. These data suggested the bienzyme SOD-CHS-CAT conjugate for sequential biopharmacological investigation. The water-soluble form of the resulting exogenous bienzyme SOD-CHS-CAT conjugate could potentially be administered both intravenously and orally. It should be noted that the linear dimensions of the CAT molecule are 10.5-10.5-5.0 nm [8] and those of SOD are 6.7-3.6-3.3 nm [9]. The CHS polymer chain (molecular weight 25-50 kDa) “entangles” the surfaces of the enzymatic subunits, thereby linking them to form a covalent conjugate [10]. The nature of the bond between the enzymatic subunits was confirmed by denaturing gel electrophoresis [4, 10] and by the very highest antithrombotic activity of the bienzyme conjugate obtained *in vivo* using various combinations of its constituents, which resulted in the optimal intravascular distribution of the conjugate and the greatest possible efficacy [2, 7]. The molecular size of the conjugate places it in the lower range of the nanoscale, which makes it a nanoparticle (with an estimated size of /17-20/ - /14-18/ - /8-12/ nm). It is believed that the physical, chemical, and biological properties of nano-size molecular objects can acquire a unique, and sometimes even surprising character (compared to the properties of their constituents), due to the quantum-mechanical effects induced by the resulting structures.

Since the supramolecular SOD-CHS-CAT conjugate was interpreted as a bienzyme device with nanoparticle dimensions, the purpose of the present study was to examine the interactions between the conjugate, on one hand, and platelets (as interactions in the bloodstream), ring arterial fragments (as interactions on the vascular surface), and the intact body of experimental animals (both under conditions of oxidative stress, simulated by the introduction of hydrogen peroxide, and without such stress), on the other hand.

MATERIALS AND METHODS

All studies on experimental animals have been conducted under protocols reviewed and approved by the author’s institutional animal care and use committee (Ethics Committee of Russian Cardiology Research-and-Production Complex) as adhering to generally accepted international guidelines for animal experimentation.

Reagents

The study was conducted using Cu, Zn-superoxide dismutase (SOD) extracted from bovine erythrocytes, with a specific activity of 3,000 U/mg protein; catalase (CAT) from bovine liver, with a specific activity of 11,000 U/mg protein; chondroitin-4-sulphate A (molecular weight 25-50 kDa) from bovine trachea; and benzoquinone, dimethyl formamide, beta-galactosidase (from *E. coli*), xanthine, hydrogen peroxide, noradrenaline (NA), N-nitro-L-arginine (L-NNA), acetylcholine, and sodium nitroprusside (SNP) produced by Sigma (USA). Xanthine oxidase was supplied by Calbiochem (USA), nitrotetrazolium blue by Reanal (Hungary), sephadex G-25 and sephacryl S-300 by Pharmacia (Sweden). The remaining reagents were analytically pure substances produced in Russia.

The bienzyme SOD-CHS-CAT derivative was produced using a method previously employed [10]. The protein content (by weight) of the SOD-CHS-CAT product was 4-6%; SOD and CAT had a specific activity of 60 and 140 U/mg of product, respectively. The SOD-CHS-CAT conjugate of irreversibly inactivated enzyme forms was produced with preliminarily inactivated SOD and CAT, following their incubation in 0.3 M hydrogen peroxide solution (pH 7.0, 0.02 M phosphate buffer, room temperature, 3 hours) and at pH 11.8-12.0 (0.05 M NaOH, room temperature, 2 hours), respectively [10].

Biochemical analysis

The protein content was determined by the Bradford method. SOD enzymatic activity was measured based on inhibition of nitrotetrazolium blue reduction in the xanthine/xanthine oxidase system, pH 7.8 [4]; CAT enzymatic activity was determined spectrophotometrically by measuring the decrease in absorption (hydrogen peroxide consumption) at 240 nm (pH 7.0, room temperature) [10].

***In vivo* experiments**

The tolerability and protective action of the SOD-CHS-CAT derivative against oxidative stress induced by intravenous injection of hydrogen peroxide were evaluated in male rabbits (n = 29) weighing 3.65 ± 0.10 kg and on male Wistar rats (n = 13) weighing 427 ± 7 g. All experiments were conducted on ketamine-anesthetized animals.

Under ketamine anesthesia induction (50-60 mg/kg body weight), the central artery in one of the rabbits' ears and the marginal veins of the rabbits' left and right ears were catheterized with needles glued into a catheter (PE-50 diameter). After induction of anesthesia (55 mg/kg), the administration of 5% ketamine was continued by infusion using a syringe pump (SAGE Instruments, USA) at 36-54 μL/h per kg of rabbit live weight. Physiological solution or the solution of SOD-CHS-CAT derivative were injected by bolus into the vein of the other ear; 0.8% hydrogen peroxide solution was also injected by infusion twice for 3 minutes at 0.4 mL/min with a 20-minute interval between injections; the interval was necessary for complete restoration of the parameters after the first dose of hydrogen peroxide and for distribution of the intravenously injected conjugate in the body. The sequence of injections in the acute experiment was as follows: hydrogen peroxide - physiological solution (control group) or SOD-CHS-CAT (experimental or experimental prophylactic group) - hydrogen peroxide. Mean arterial blood pressure (BP, mm Hg), heart rate (HR, beats/min), and the second-lead electrocardiogram (ECG) were recorded with a BIOGRAF-4 apparatus (Saint-Petersburg State University of Aerospace Instrumentation) using an ADC card (NI 6210 National Instruments, USA) for computerized data recording; the data were subsequently processed using a software package for physiological signal analysis developed by Dr. E. V. Lukoshkova. When the baseline parameters of BP, HR, and ECG were recorded (for 15 minutes), the animals were injected with hydrogen peroxide for 3 minutes and then BP, HR, and ECG were recorded again for 10 minutes. The total dose of hydrogen peroxide received by the animals throughout the experimental period was 0.31 μM/kg. In the control group n = 12, in the experimental group n = 15.

The catheters (diameter PE-50) were implanted into the carotid artery and jugular vein of the Wistar rats under ketamine anesthesia (100 mg/kg). The experimental settings were the same as those described above, except that hydrogen peroxide was administered 2 minutes longer to achieve

comparable effects on the hemodynamic parameters. The total dose of hydrogen peroxide received by rats throughout the experimental period was 4.5 μM/kg. All animals survived the experiments.

The tolerability of the SOD-CHS-CAT derivative, based on the effect of different doses (therapeutic dose of 1.5 mg product per kg of live weight [2, 7], as well as 7.5 and 15 mg/kg) on mean BP, HR, and ECG, was studied in a separate set of experiments on rabbits (n = 8). After a 15-minute control recording of BP, HR (taken as 100%), and ECG, the animals were injected with the first dose of the SOD-CHS-CAT derivative (1.5 mg/kg) and BP, HR, and ECG were then recorded for 15 minutes. After that, the next dose of SOD-CHS-CAT (7.5 mg/kg) was injected, and then after a 20-minute interval the animals were injected with a dose (15 mg/kg) ten times the therapeutic dose, with simultaneous recording of the hemodynamic parameters and ECG. The animals, having received cumulatively a 16-fold therapeutic dose of the SOD-CHS-CAT derivative, were evaluated 3 days later for the prophylactic effect of the bienzyme conjugate in the presence of oxidative stress (as described above) compared to the effects of its acute administration and of the control experiment in treatment-naïve intact rabbits.

Study of changes in the tonus of the rat arterial ring

After decapitation of the Wistar rats (males weighing 350-400 g), their abdominal cavity was dissected and the abdominal aorta was removed. The extracted segment of the aorta was carefully cleansed of connective tissue and was cut into ring fragments 3 mm in length. The aorta fragments were fitted onto the needles connected to a tensiometer (in μN) and then immersed in Krebs-Henseleit solution and gassed with carbogene at 37ºC (pH 7.4). Oxidative stress was simulated by adding hydrogen peroxide to the incubation solution under NA preconstriction. Change in vascular tonus was estimated relative to the magnitude of the constriction mediated by 0.1 μM NA, which was taken as 100%. The antioxidant enzyme derivatives were introduced 10 minutes prior to addition of hydrogen peroxide, using various doses of reagents. The effect of SOD (10 U/mL) on the tonus of the arterial ring fragment was determined by addition of native SOD or SOD-CHS-CAT under NA preconstriction. The production of endogenous NO and its effect on the tonus of the arterial ring was evaluated by introduction of an exogenous NO-synthase inhibitor, L-NNA (0.1 μM).

Platelet aggregation

The effect of hydrogen peroxide and SOD-CHS-CAT on platelet aggregation was studied using healthy fasting volunteers' blood drawn by gravity from the cubital vein, and collected in plastic test tubes containing 0.13 M sodium citrate (pH 7.3). Platelet-rich plasma (PRP) was extracted by centrifuging blood at 180 g for 15 minutes. Platelet aggregation was evaluated using a BIOLA dual-channel laser aggregation analyzer. Besides the conventional method of registering optical transmission (Born method), platelet aggregation was evaluated by fluctuation analysis of the light passing through the sample. The relative variance of these fluctuations is proportional to the average aggregate radius and, therefore, provides a tool for studying the formation of microaggregates that contain less than 100 platelets. It also helps eliminate the effect that the light-absorption activity of the plasma and changes in platelet shape have on precision of measurement; this is especially important for studying spontaneous aggregation.

The capacity to form small-sized aggregates (3-100 platelets) was evaluated by measuring spontaneous aggregation and the aggregation induced by 0.5 μM adenosine diphosphate (ADP), 0.5 μM serotonin, and 1 μM TRAP (thrombin receptor agonist peptide) using the method of registering aggregates of medium size (in relative units). The formation of large-sized aggregates (over 100 platelets) in response to 5 μM ADP and 6 μM TRAP was evaluated using the Born method and expressed as % optical transmission. The study was conducted no more than 2 hours after the blood draw.

For research of platelet morphology, ten μL of PRP were fixed in 2.5% glutaraldehyde for 1.5 hours at room temperature. Then the samples were placed on polycarbonate membranes with pores (diameter 0.22-0.40 μm), dehydrated, dried and prepared for microscopy. Platelets of various

shapes were counted in 25 scanning fields at x2500 magnification using a PHILLIPS PSEM 550x scanning electron microscope; the results were expressed as percentage of the total number of cells.

Determination of statistically significant differences

The results (expressed as the mean ± standard error (M ± m), n - number of animals) were statistically analyzed by comparing data from two groups using Student's paired t-test (statistically significant difference at $p<0.01$). If more than two groups were compared, the statistical analysis was performed using ANOVA ($p<0.01$) of Statistica 6.0 program.

RESULTS

Vasoactivity of the SOD-CHS-CAT conjugate *in vivo*

Intravenous bolus administration of various doses (10-fold range) of the SOD-CHS-CAT conjugate to the rabbits showed that BP and HR values deviated by no more than 4% from the mean values in intact anaesthetized animals. In rabbits, there were no changes in ST segment, no abnormalities in the rhythm, conduction, or other parameters even at 10-fold effective dosages of the conjugate. Testing of a single intraperitoneal administration of SOD-CHS-CAT to the BALB/c mice and to the hybrid F1(CBAxC57B16) mice revealed low acute toxicity of the conjugate in addition to the absence of mutagenic potential in the Ames test. These results, together with the above-mentioned tolerability of the SOD-CHS-CAT conjugate in the experimental animals and its marked antithrombotic activity [2, 7], confirm the appropriateness of its further biomedical study.

The first dose of hydrogen peroxide administered to the intact rabbits (curve 1, Fig. 1, A) caused a rapid drop in BP (to 60% of baseline), which returned to 90% of baseline in the control group within ten minutes. Similar changes in BP were observed in the experimental group (curve 2, Fig. 1, A). It is of interest to note that prophylactic administration of the SOD-CHS-CAT derivative three days prior to the experiment with hydrogen peroxide (Fig. 1) consistently prevented the drop in BP, causing at first only small fluctuations in it, smoothing these out and then maintaining the BP values close to baseline (curve 3, Fig. 1, A). Preventive administration of SOD-CHS-CAT also afforded its prophylactic effect in relation to HR in rabbits (curve 3, Fig. 1, B) compared to the results in the control and experimental (curve 1 and 2, Fig. 1, B, respectively) groups.

After a 10-minute distribution of the bienzyme conjugate in the rabbit body, which did not alter the central hemodynamic parameters, the rabbits were administered hydrogen peroxide for the second time (in 20 minutes) at the same dose and rate. The second infusion caused more substantial changes in BP and HR (Fig. 1). During BP recovery, two phases were observed: rapid (within first five minutes) and slow.

In the experimental group, the response of BP and HR to the second damaging dose of hydrogen peroxide after the administration of the SOD-CHS-CAT derivative was significantly lower and the recovery faster than those in the control group (Fig. 1).

In the rabbits, the ECG remained essentially unchanged after the first administration of hydrogen peroxide, although there was some ST-segment depression in the control group. After the second administration of hydrogen peroxide there were the greatest ST-segment elevations, especially in rabbits with labored breathing. Ventricular arrhythmia with single or grouped extrasystoles was observed after 5-8 min. If dyspnea was already apparent in rabbits after the first administration of hydrogen peroxide, then after the second administration there was labored breathing and bronchial spasms, more pronounced in the control group.

The first administration of hydrogen peroxide to the rats caused an initial short-term (no more than 1 minute) increase in BP by 3-5%, with a subsequent decrease by 15% and then full recovery within 10 minutes (Fig. 2, A). Under these conditions, HR decreased by 3-5% and recovered to 96-98% in 10 minutes (curves 1 and 2, Fig. 2, B). The administration of the SOD-CHS-CAT derivative did not alter the hemodynamic parameters and ECG in rats. After the bienzyme conjugate had been allowed to

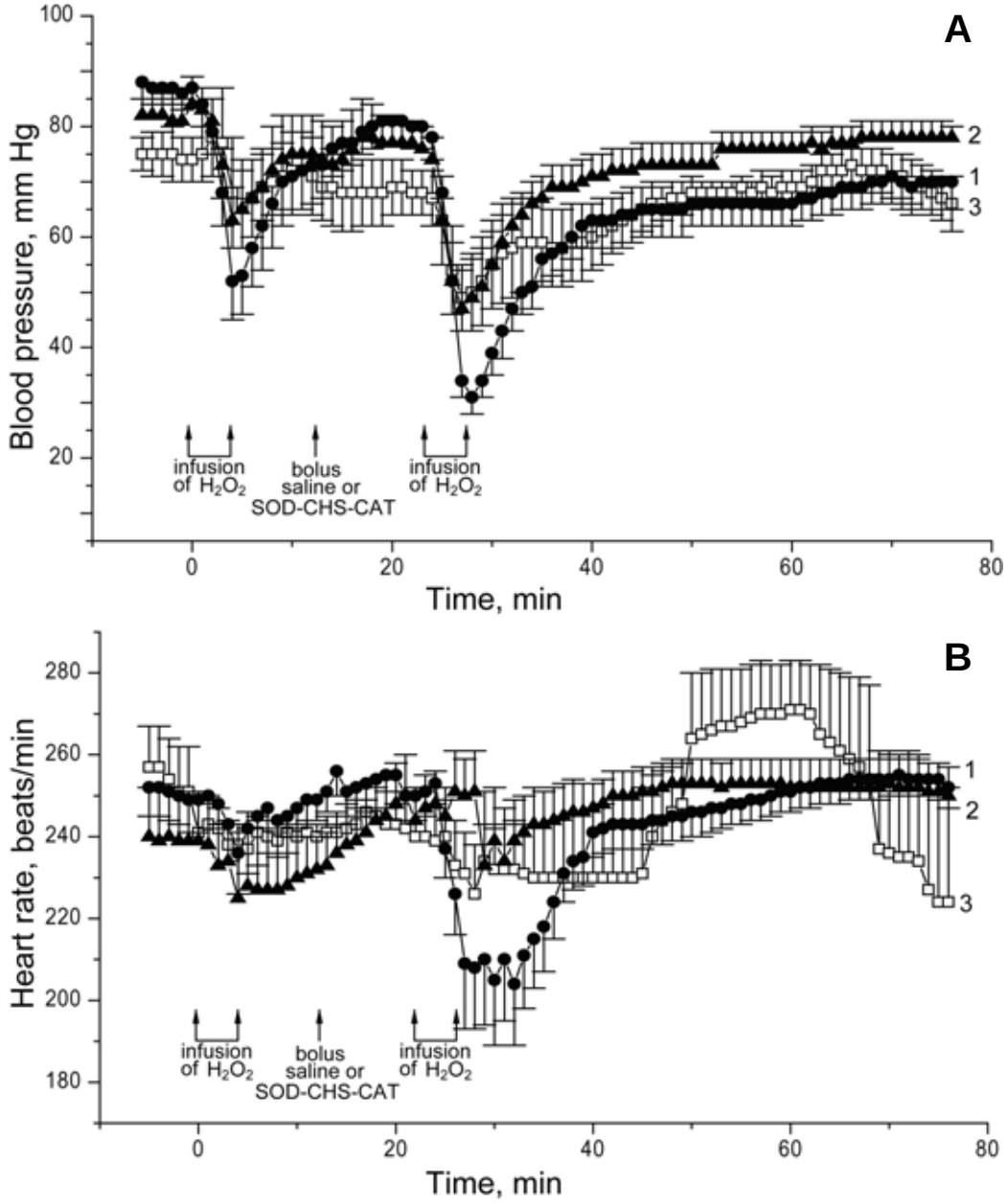

Fig. 1. Change in BP (**A,** mm Hg) and HR (**B,** beats/min) in anesthetized rabbits under oxidative stress (infusion with 0.8% hydrogen peroxide) in the control (n = 12, bolus injection of physiological solution (saline), curve 1) and experimental (n = 9, bolus injection of the SOD-CHS-CAT conjugate, curve 2) groups. Curve 3 shows results for the experimental prophylactic group (n = 6) of rabbits that received the SOD-CHS-CAT conjugate three days prior to the experiment with hydrogen peroxide and then after the first administration of hydrogen peroxide. Arrows indicate the time points for the start and end of the infusion of hydrogen peroxide and of the bolus injection of physiological solution or SOD-CHS-CAT conjugate (1.5 mg/kg).

distribute in the body for 10 minutes, the rats were given hydrogen peroxide for the second time. There were more pronounced (compared to the first infusion) changes in BP and HR. BP values dropped to 52% in the control group and to 73% in the experimental group (curves 1 and 2, respectively, $p<0.05$, Fig. 2, A). The BP recovery was much slower in the control group.

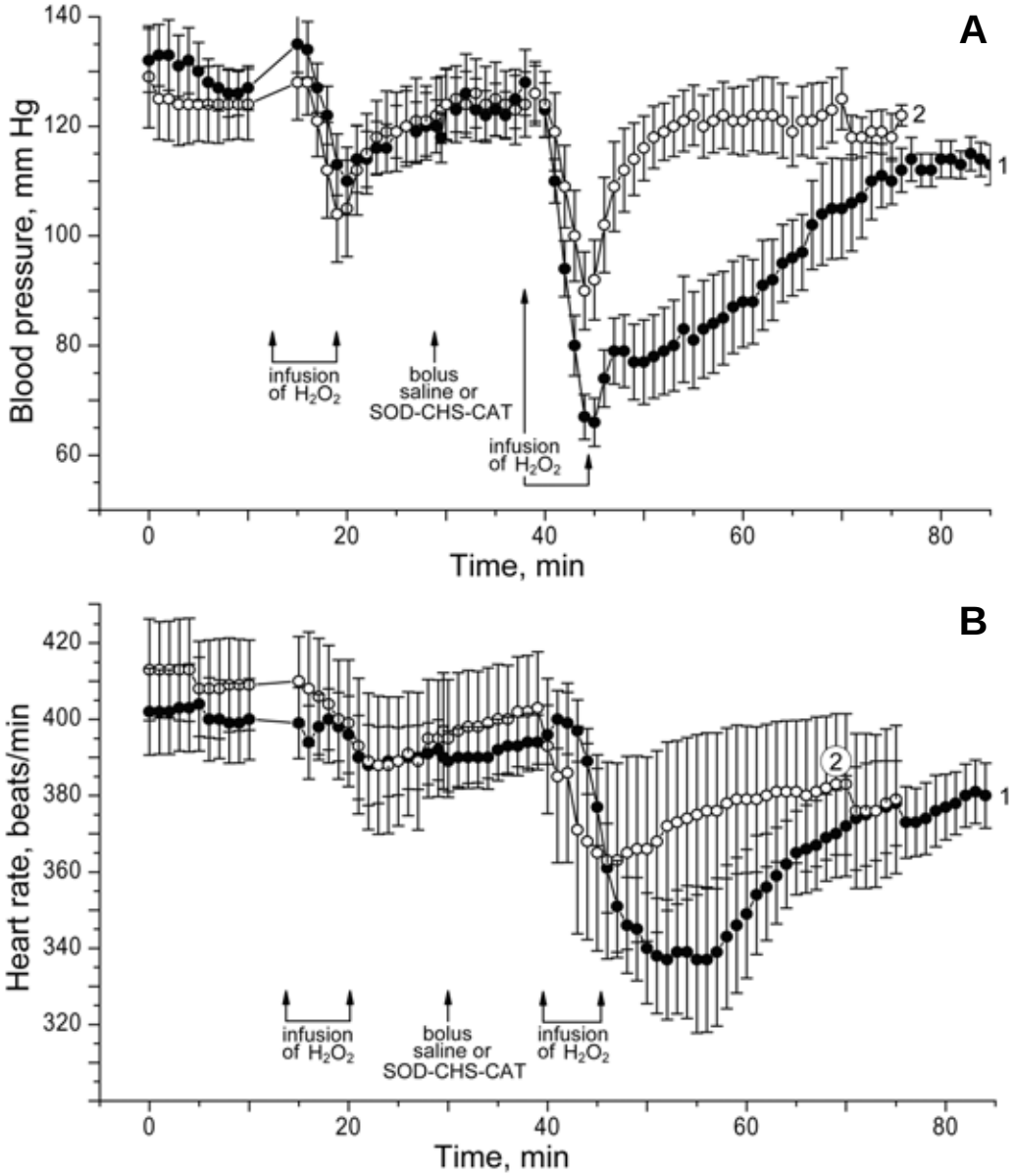

Fig. 2. Changes in BP (**A,** mm Hg) and HR (**B**, beats/min) in anesthetized rats. Arrows indicate the time points for the start and end of the intravenous infusion with 0.8% hydrogen peroxide (oxidative stress) and of the bolus injection of physiological solution (n = 6, control group, curve 1) or SOD-CHS-CAT conjugate (n = 7, experimental group, curve 2).

A statistically significant difference in recuperation of BP in the experimental and control groups was already observed during the first few minutes.

The form of the ECG signal in rats did not change significantly during the experiment, although the number of individual ventricular extrasystoles increased in both groups of animals, especially after the second administration of hydrogen peroxide.

Study of the changes in the tonus of the rat arterial ring

Oxidative stress of the tonus of the arterial ring fragment (of rat abdominal aorta) was simulated under NA preconstriction by introducing hydrogen peroxide into the incubation solution. NA preconstriction was 50-60% of the maximum possible constriction of the arterial ring fragment,

which permitted recording of the contraction as well as relaxation. Changes in vascular tonus were estimated relative to the magnitude of the constriction mediated by 0.1 μM NA, which was taken as 100% for the interval length, and as 0 for the baseline of the experiment. Hydrogen peroxide induced dose-dependent constriction of the vascular fragment. A fairly small (10-12%) rise in tonus was observed at a concentration of 0.01 mM H_2O_2; the tonus further increased at 0.1 mM H_2O_2 (48-50%) with a subsequent return to the baseline of NA preconstriction (relaxation 0-3%). There was a rapid constriction of the arterial fragment (88-90%) at 1.0 mM H_2O_2, which was followed by a relaxation phase (68-70%). The proposed model was, therefore, suitable for studying the effect that ROS has on the tonus of a vascular fragment, and it has provided experimental evidence of the dose-dependent effect of hydrogen peroxide.

After three rinses and a 15-minute recuperation period, the vascular ring was again exposed to NA and hydrogen peroxide was added at the same concentrations as before (0.01 - 1.0 mM H_2O_2). During the second response, the functional activity of the vascular fragment decreased with the escalation of hydrogen peroxide concentrations, both in relation to the second administration of NA and to the magnitude of constriction after the second administration of H_2O_2. The significant decrease in the magnitude of the second constriction of the arterial fragment in response to the second administration of high hydrogen peroxide concentrations is an indication of adequate maintenance of the vascular reactions.

Because the effect of antioxidant agents is preventive in nature, their protective action should be apparent even when physiological levels of ROS have just begun to rise. For this reason, we used 0.1 mM H_2O_2 to estimate the comparative effectiveness of the protective effect of CAT and the SOD-CHS-CAT conjugate in our experimental model in the test for maintenance of vascular function (Fig. 3, A). The CAT derivatives were

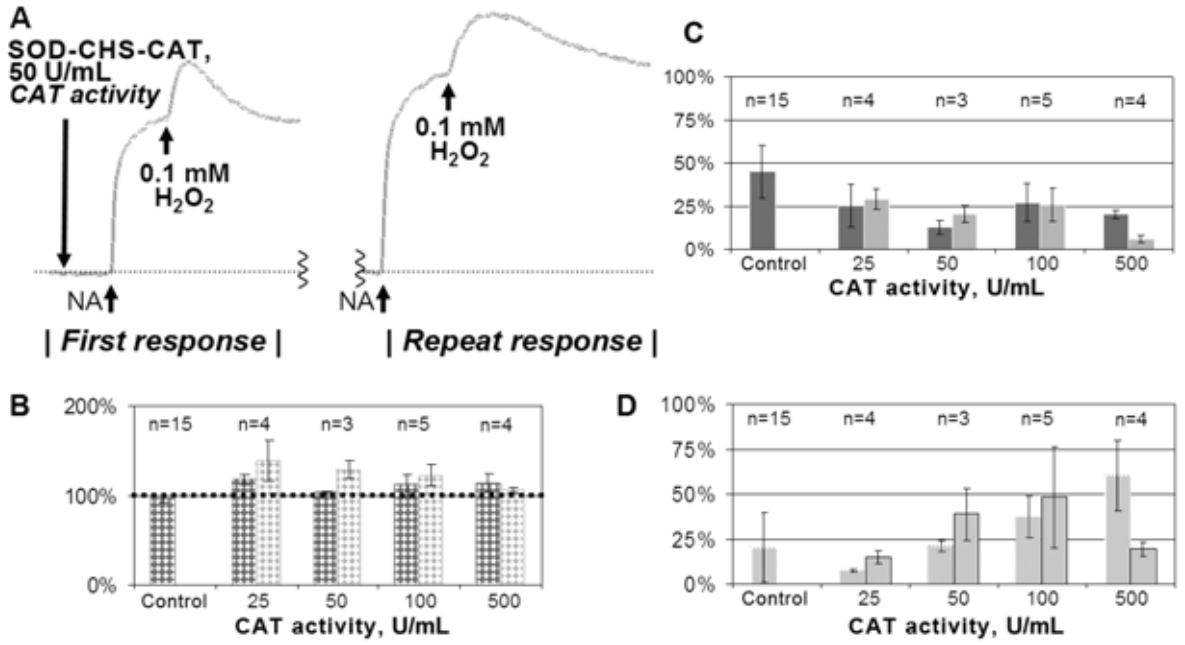

Fig. 3. The comparative effects of the protective action of CAT and SOD-CHS-CAT against 0.1 mM hydrogen peroxide. **A**: originals tracks. **B**: the magnitude of the second response to NA after exposure to various concentrations of CAT (dark columns) and SOD-CHS-CAT (grey columns), equal (within pair) by catalase activity. **C** and **D**: constriction of the aortic fragment during the first (**C**) and second (**D**) responses to 0.1 mM H_2O_2 during exposure (for the first response) to the CAT concentrations shown (on the left in the pair) and to SOD-CHS-CAT (on the right in the pair) by catalase activity (U/mL).

used at equal concentrations as measured by catalase activity (U/mL). Native CAT and SOD-CHS-CAT conjugate maintained the response to the second administration of NA (Fig. 3, B) comparable to the control values. In the catalase activity concentration range of 25-100 U/mL, the CAT and SOD-CHS-CAT derivatives manifested a similar protective effect against exposure to 0.1 mM H_2O_2 (Fig. 3, C, D). At a concentration of 500 U/mL, the SOD-CHS-CAT conjugate decreased the response to H_2O_2 more efficiently than CAT. The pronounced protective action of SOD-CHS-CAT can be associated with its affinity for the vascular wall (due to the conjugation of SOD and CAT through CHS, a glycosaminoglycan of the endothelial glycocalyx [2, 7]) and/or with the presence of SOD and endogenous NO in the model system. It is difficult to estimate sorption of enzyme derivatives in the system employed, because vascular reactions can proceed both from inside outwards and from outside inwards [11]; evaluation of the protective effect of SOD with respect to NO, on the other hand, is entirely possible.

This effect, measured against NA preconstriction of an arterial ring fragment, proved to be quite pronounced for native SOD and SOD-CHS-CAT (Fig. 4, A, B). Both derivatives caused significant vascular relaxation. This dilatation presumably resulted from retention of endogenous NO, because SOD neutralizes the superoxide radical, which is able to convert NO to peroxynitrite that lacks vasodilatative properties. This assumption was experimentally supported by administering an NO-synthase inhibitor, N^{ω}-nitro-L-arginine (L-NNA), which elicited an increase in vascular tonus (Fig. 4, C). These results show that the significant inhibition of NO-synthase activity and development of a contractile response is due to a deficiency of dilatation induction from endogenous NO. Administration of acetylcholine (as an inducer of vascular relaxation acting through endothelial receptors that trigger NO-synthase activity) did not lead to any apparent response, suggesting effective inhibition of NO-synthase. The injection of 10 U/mL SOD activity during exposure to L-NNA did not have any effect, which emphasizes the role of endogenous NO in the previously observed relaxation of the arterial fragment. The administration of SNP, an NO donor, caused dilatation (Fig. 4, C), which proves that the vascular fragment does not lose its ability to relax when exposed to NO. The increase in NO bioavailability (Fig. 4, B) observed after the administration of the SOD-CHS-CAT conjugate was similar in its magnitude to the effect of native SOD (Fig. 4, A); this demonstrates the high effectiveness of vasoprotective activity of the bienzyme conjugate as a result of the activity of its SOD constituent also.

Platelet study

Under normal conditions, hydrogen peroxide acts as a molecule of intra- and intercellular signal

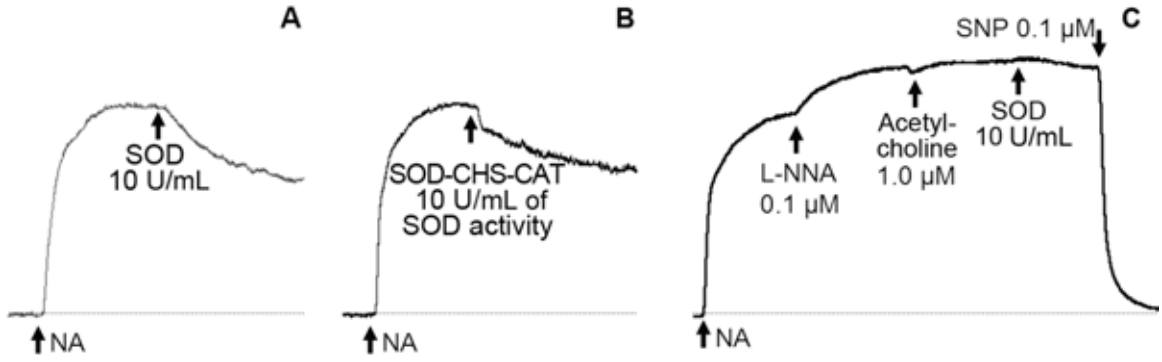

Fig. 4. Effect of SOD activity on the tonus of the arterial ring fragment. Original curves for 10 U/mL of native SOD (**A**) or SOD-CHS-CAT (**B**) administered under NA preconstriction. (**C**) change in arterial tonus during exposure to NA, N^{ω}-nitro-L-arginine (L-NNA), acetylcholine, native SOD and sodium nitroprusside (SNP). Similar curves were obtained in 3-4 experiments.

transmission; it shows limited toxicity against many cell types over the 20-50 μM concentration range, and under physiological conditions hydrogen peroxide levels over 50 μM are regarded as a high concentration [12]. In *in vitro* experiments, the addition of 50 μM - 2 mM hydrogen peroxide resulted in platelet aggregation (Fig. 5). Scanning electron microscopy showed that at the maximum aggregation response, the aggregates consist of densely associated platelets in their central part and of loosely associated platelets at the periphery (Fig. 5, B). After five minutes of aggregation, the size of aggregates decreases and their structure becomes so compact that individual cells are no longer discernible (Fig. 5, C). The decrease in aggregate size is caused both by dissociation of loosely associated platelets from aggregation centers and by consolidation of the central part of aggregates. 300 μM H_2O_2 was selected for experiments with CAT derivatives. Such concentration is quite physiological [12] and the aggregation peak was 1.8 ± 0.1 (in rel. u) in this case (Table 1). Simultaneous addition of 300 μM H_2O_2 and 0.5 μM ADP to the PRP induced the fast growth of platelet aggregate size. It was higher (3.3 ± 0.3 rel. u on first minute) than that for ADP-induced platelet aggregation (2.2 ± 0.1 rel. u, $p<0.001$, Table 1). After first minute, the primary aggregation of platelets was replaced by the fast desaggregation. A similar picture was observed after simultaneous addition of H_2O_2 and 0.5 μM serotonin or 1 μM TRAP (Table 1). The amount of three platelet forms (discoid shape, sphere, spread platelets) was evaluated with help

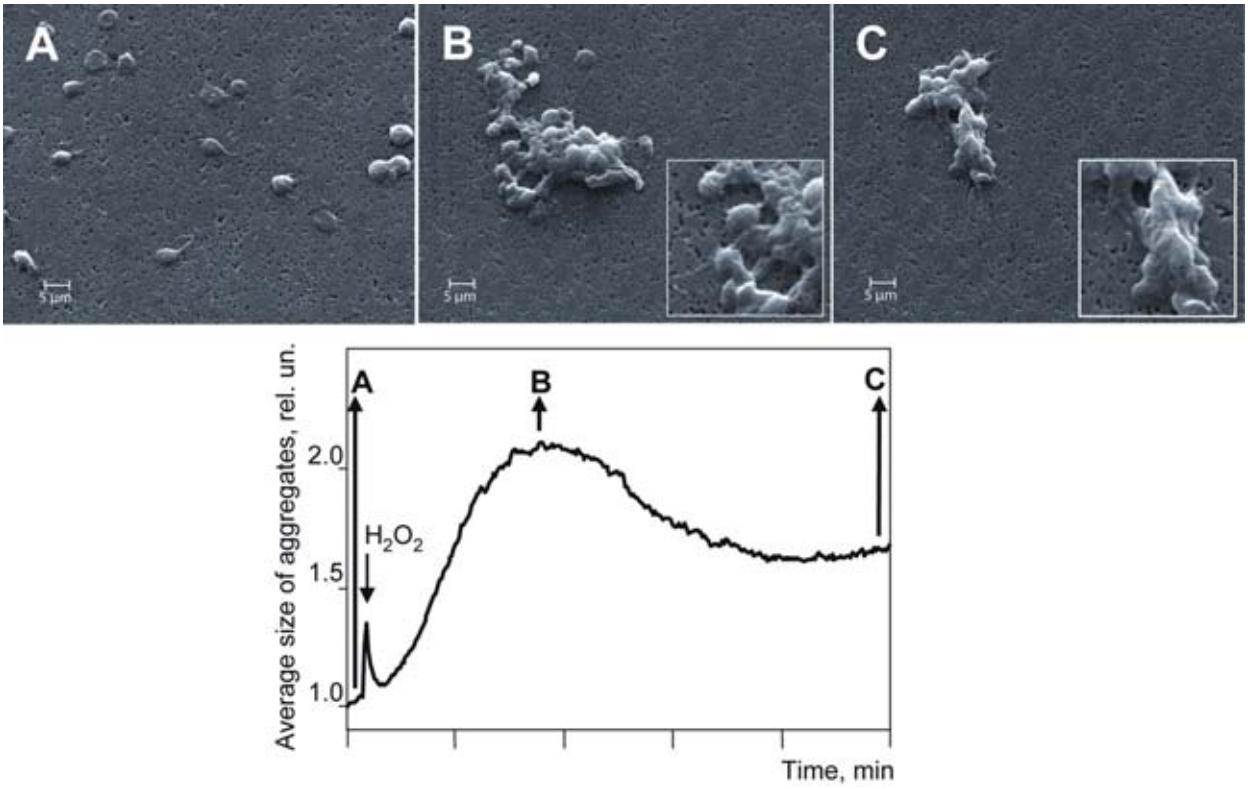

Fig. 5. A typical curve for the platelet aggregation induced by 50 μM - 2 mM H_2O_2, and the composition of the platelet aggregates at different stages of their formation. **A**: most individual platelets prior to aggregation have a discoidal shape; **B**: the sample collected during the peak aggregation: an aggregate with a dense core and loosely adherent platelets at the periphery (insert shows a magnified view of the peripheral region; note the looseness of the structure); **C**: the sample collected after 5 minutes of aggregation: the aggregate size decreased; there are no loosely adherent platelets at the periphery; insert shows strong platelet fusion in the aggregate at higher magnification. Magnification: x2500; inserts: x5000.

Table 1. The medium size (in relative units) of platelet aggregates on first minute of measurement (the peak of platelet aggregation induced by different inducers with or without CAT /3,000 U/ or SOD-CHS-CAT conjugate /400 U CAT activity/).

Type of platelet aggregation	Without hydrogen peroxide			With hydrogen peroxide (300 μM)		
	Control	CAT	SOD-CHS-CAT	Control	CAT	SOD-CHS-CAT
Without spontaneous aggregation	1.1 ± 0.03	-	-	1.8 ± 0.1	1.4 ± 0.1	1.6 ± 0.1
Spontaneous aggregation	1.6 ± 0.2	1.5 ± 0.1	1.4 ± 0.1	1.8 ± 0.2	1.5 ± 0.1	1.6 ± 0.1
Induced by 0.5 μM ADP	2.2 ± 0.1	2.1 ± 0.2	1.7 ± 0.1	3.3 ± 0.3	2.1 ± 0.3	2.1 ± 0.2
Induced by 0.5 μM serotonin	2.3 ± 0.1	2.3 ± 0.2	1.6 ± 0.2	3.2 ± 0.4	2.9 ± 0.2	2.4 ± 0.3
Induced by 1 μM TRAP	2.3 ± 0.3	2.3 ± 0.4	1.9 ± 0.1	3.0 ± 0.4	3.0 ± 0.3	2.4 ± 0.3

M ± SEM, n = 8 – 10.

of scanning electron microscopy (as counted their content /%/ from total amount of platelets). Adhesion and spreading of platelets are one of the critical phases of homeostasis that induce a cascade of reactions, leading to the formation of thrombus. The part of spread platelets was 21% for control and 24% for sample with hydrogen peroxide (data not shown).

Preventive addition of native CAT (3,000 U) substantially decreases the aggregation effect of 300 μM hydrogen peroxide (Table 1). The SOD-CHS-CAT conjugate neutralizes it at a dose as low as 400 CAT activity units, demonstrating an increased dose-dependent antioxidant efficacy compared to CAT. The platelet aggregate size was decreased from 1.8 ± 0.1 to 1.4 ± 0.1 and 1.6 ± 0.1 (rel. u), respectively (Table 1). CAT and CHS applied separately have no effect on platelet aggregation.

The presence of 300 μM H_2O_2 enhances platelet activation (assessed by platelet aggregation) triggered by inducers with different mechanisms of action: ADP, serotonin, and TRAP (Table 1). Thus, hydrogen peroxide had effect on platelet aggregation (inducing it in determined interval of concentration and increasing the peak of ADP-, serotonin-, TRAP-induced aggregation) and was reliable for a model of oxidative stress in platelet system for research of antioxidant action. Preventive addition of 3,000 U CAT or 400 SOD-CHS-CAT CAT activity units to PRP prevents activation by H_2O_2 (Table 1). The use of the SOD-CHS-CAT conjugate confirms its higher antioxidant activity, exceeding that of CAT. The effect proved to be dose-dependent, reaching its highest values (which remain essentially unchanged with further dose escalation) for CAT and SOD-CHS-CAT at 3,000 U and 400 CAT activity units, respectively. The SOD-CHS-CAT conjugate decreased the platelet aggregate size in spontaneous aggregation by 12-13% and in ADP-induced aggregation by 22-24%. Similar results were obtained with other assayed inducers (Table 1).

Due to their catalase activity, the CAT and SOD-CHS-CAT derivatives neutralize the H_2O_2 effect on platelets (Table 1). At the same time, it has been shown that platelets themselves can generate reactive oxygen species [13]. This is consistent with the inhibiting effect of SOD-CHS-CAT derivatives on ADP-induced platelet aggregation (Fig. 6), while the effect of the CAT-CHS derivative was very moderate, and CAT had no effect at all (data not shown). The anti-aggregation effect of the bienzyme conjugate was mediated both by enzymatic activity (compare

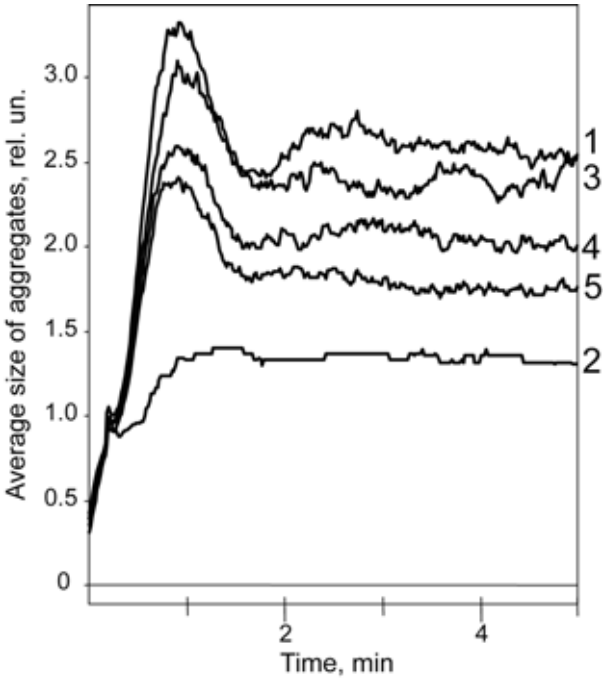

Fig. 6. Effect of the SOD-CHS-CAT derivatives on platelet aggregation induced by 0.5 μM ADP. Curves of aggregation in response to 0.5 μM ADP (1), and to preventive administration at equimolar protein concentrations, corresponding to 400 CAT activity units of SOD-CHS-CAT conjugate: SOD-CHS-CAT (2), SOD_{inact}-CHS-CAT_{inact} (3), SOD-CHS-CAT_{inact} (4), SOD_{inact}-CHS-CAT (5). Only typical aggregatograms, selected from 3-5 experiments, are shown. The X-axis represents time (min); the Y-axis represents the mean aggregate size (relative units).

curves 1 and 2, Fig. 6) and by its unique supramolecular structure generated with the participation of CHS (compare curves 1 and 3, Fig. 6) [2, 7]. Indeed, when β-galactosidase, which is comparable in size to SOD-CHS-CAT, was used at equimolecular protein concentrations under identical experimental settings, it did not inhibit ADP-induced platelet aggregation at all.

When SOD-CHS-CAT inhibits platelet aggregation induced by ADP, serotonin, or TRAP (inducers with different mechanisms of action added at different concentrations), the resulting effect is generalized in nature (Fig. 7, Table 1). This is a new property of SOD-CHS-CAT, not characteristic of its individual constituents under the same conditions.

DISCUSSION

In the *in vivo* experiments, the bienzyme SOD-CHS-CAT conjugate was consistently able to prevent the marked hemodynamic changes caused by hydrogen peroxide in the two animal species used in experiments. The SOD-CHS-CAT conjugate successfully counteracted the direct effect of hydrogen peroxide on myocardial cells, probably due to the neutralization of both the superoxide radical and hydrogen peroxide. The effect of SOD-CHS-CAT was consistently manifested prior to the second administration of hydrogen peroxide, when the antioxidant protection of the body was already attenuated by the first administration of H_2O_2.

The prophylactic effect of the SOD-CHS-CAT conjugate was much more pronounced in those rabbits that had received high doses of the conjugate 3 days prior to the acute experiment. This completely prevented the decrease in BP and HR occurring in response to the first administration of hydrogen peroxide. This was the best antioxidant effect as compared to the other cases examined in our study. It is likely that the conjugate, administered preventively, can successfully counteract, directly and/or indirectly, the acute effect of hydrogen peroxide. The importance of catalase activity was also underscored by the necessity of its presence in the experimental therapy with lecithinized SOD in mice with bleomycin-induced pulmonary fibrosis [14]. On the bell-shaped dose-response curve, the therapeutic effect of lecithinized SOD (in the high-dose area) was restored by additional administration of CAT, which eliminated the accumulation of hydrogen peroxide. The presence of coupled superoxide dismutase and catalase activities in the bienzyme conjugate optimizes its antithrombotic effect [2, 7] and provides safe recuperation of BP and HR under the perturbing effect of hydrogen peroxide (Figs. 1 and 2). The effectiveness of oral [15] and inhalatory [14] administration of the modified SOD form reported in other studies points to the urgency of developing oral forms of SOD-CHS-CAT for the prophylactic needs of antioxidant therapy [16].

Overall, the bienzyme SOD-CHS-CAT conjugate consistently manifests its vasoprotective properties

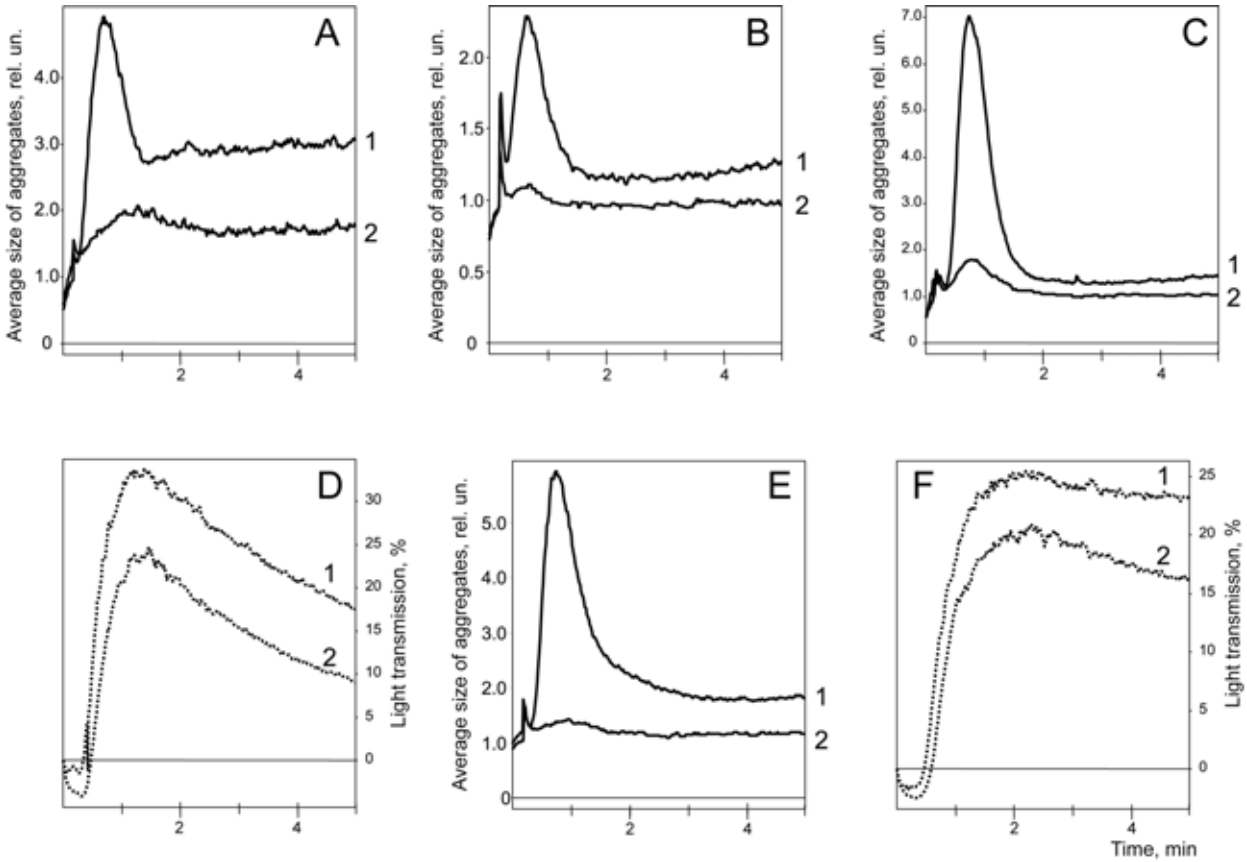

Fig. 7. Effect of the bienzymic SOD-CHS-CAT conjugate (400 U CAT activity, curve 2) on platelet aggregation, induced by (curve 1): 0.5 μM (**A**) and 5.0 μM (**D**) ADP; 0.5 μM (**B**) and 5.0 μM (**E**) serotonin; 1 μM (**C**) and 6 μM (**F**) TRAP. Only typical aggregatograms, selected from 3-6 experiments, are shown. The X-axis represents time (min); the Y-axis represents the mean aggregate size (relative units, **A-C**, **E**) or optical transmission (%, **D** and **F**).

when applied to the rat arterial ring, due to the catalytic activity of both enzymatic constituents (SOD and CAT), and is no less efficient than the native forms of biocatalysts. The latter strongly suggests the importance of biopharmaceutical development of the SOD-CHS-CAT conjugate as a drug candidate.

The results of the platelet study demonstrate a marked dose-dependent antioxidant effect of the bienzyme SOD-CHS-CAT conjugate in induced platelet aggregation in the presence of hydrogen peroxide. Antiaggregatory activity of the SOD-CHS-CAT conjugate is significantly higher than that of other CAT derivatives and is manifested within a wide range of conditions, when platelet aggregation was stimulated with inducers of various mechanisms of action: ADP, serotonin, and TRAP, both with and without hydrogen peroxide. The latter demonstrates a new feature of the antithrombocytic potential of SOD-CHS-CAT, which is absent in free CHS and in the native enzymes composing the derivative, and which is determined by its molecular composition and dimensions. This set of properties of the bienzyme SOD-CHS-CAT conjugate underscores the promising outlook for its biomedical study for the purposes of antioxidant therapy and the productiveness of developing medications based on enzyme conjugates.

CONCLUSIONS

The results of this study demonstrate the real possibility of developing potentially effective and safe supramolecular enzyme conjugates for the purposes of practical medicine. Covalent coupling of enzymatic subunits is fairly efficient and

provides mutual stabilization of the activity of biocatalysts. The higher molecular weight of the resulting conjugate (compared to the initial protein constituents) and the inclusion of chondroitin sulfate, a glycosaminoglycan of the endothelial glycocalyx, contribute to the vasoprotective activity of the SOD-CHS-CAT derivative at the luminal surface of the vascular wall. The beneficial effect of the experimental therapy with the SOD-CHS-CAT conjugate is determined by covalent linkage of the superoxide dismutase and catalase activities of the biocatalysts, which enhances their combined catalytic effect with the formation of safe end products of enzymatic conversion. Furthermore, the resulting supramolecular structure of the bienzyme nanoconjugate gives rise to new features (antiaggregatory activity upon induced platelet aggregation) and adds new activities to its components at a high level (the effect of the superoxide dismutase and catalase activities of the conjugate on the tonus of the vascular fragment similar to that of native enzymes; the contribution of chondroitin sulfate to the antiaggregatory effect of the conjugate), which determine its highest antioxidant effect among various combinations of components. The good tolerability of the SOD-CHS-CAT derivative, its satisfactory acute toxicity, the normalizing effect on the hemodynamic parameters under oxidative stress, and the pronounced prophylactic effect qualify this product as a promising drug candidate. The technique presented in this paper offers a highly versatile path to the development of medical treatments based on enzyme conjugates.

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