Vascular Bound Recombinant Extracellular Superoxide Dismutase Type C Protects Against the Detrimental Effects of Superoxide Radicals on Endothelium-Dependent Arterial Relaxation

T. Abrahamsson, U. Brandt, S.L. Marklund, and P.-O. Sjöqvist

Extracellular superoxide dismutase type C (EC-SOD C) is a secretory SOD isoenzyme that, in vivo, is bound to heparan sulfate proteoglycans in the glyocalyx of various cell types (e.g., endothelial cells) and in the connective tissue matrix. The aim of this study was to investigate the efficacy of vascular bound EC-SOD C in protecting arterial relaxation mediated by endothelium-derived relaxing factor (EDRF) against the inhibitory effects of superoxide radicals. For comparison, the effect of CuZn SOD was also studied. This SOD isoenzyme lacks affinity toward heparan sulfate and does not bind to cell surfaces. Rings from rabbit aorta were mounted in an organ bath and acetylcholine-induced endothelium-dependent relaxation was then studied in preparations precontracted with phenylephrine. Pyrogallol (10^-4 M), used to generate superoxide radicals, reduced the maximal relaxant effect of acetylcholine from about 65% to 25%. When present in the buffer throughout the experiment, CuZn SOD and EC-SOD C caused a concentration-dependent prevention of the pyrogallol effect on EDRF-mediated relaxation, with a half-maximal effect at about 100 units/ml (KO_2 assay). In a second set of experiments, the arterial rings were preincubated with 8,000 units/ml CuZn SOD (50 μg/ml) or EC-SOD C (69 μg/ml) during 30 minutes, followed by washing, before the effect of pyrogallol on EDRF-mediated relaxation was studied in SOD-free buffer. The maximal relaxant effects of acetylcholine, expressed as the percent decrease in tension, were (mean±SEM, n=6) 61±4% (control), 22±4% (pyrogallol), 27±5% (pyrogallol+CuZn SOD), and 46±6% (pyrogallol+EC-SOD C; p<0.05 versus CuZn SOD+pyrogallol). The preincubation resulted in considerable binding of EC-SOD C to the arterial tissue, whereas negligible amounts of enzyme were present in the medium after the washing procedure. The protection by EC-SOD C was lost when the preincubation was performed in the presence of heparin (125 IU/ml), which prevents the binding of the enzyme to the arterial rings. In this case, the maximal relaxant effects of acetylcholine were (n=6) 60±4% (control), 21±4% (pyrogallol), 43±4% (pyrogallol+EC-SOD C), and 27±4% (pyrogallol+EC-SOD C+heparin; p<0.05 versus EC-SOD C+pyrogallol). It is concluded that EC-SOD C, associated with heparan sulfate proteoglycans on endothelial cell surfaces and internal structures in the aortic rings, protects against the detrimental effects of superoxide radicals on endothelium-dependent arterial relaxation. (Circulation Research 1992;70:264–271)
heparan sulfate can be competitively antagonized by heparin.5-7

A number of substances (e.g., acetylcholine) may induce release of a humoral agent, endothelium-derived relaxing factor (EDRF), from the vascular endothelium.8,9 EDRF is a potent vasodilator and inhibitor of platelet and leukocyte aggregation10-12 and has recently been suggested to be identical to nitric oxide or a closely related nitric oxide-containing compound.13-15 Superoxide radicals have been shown to inhibit EDRF-mediated arterial relaxation,16,17 probably via direct reaction with EDRF. It was recently demonstrated that pyrogallol, added to the oxygenated buffer, inhibited acetylcholine-elicited arterial relaxation in vitro.18,19 Because CuZn SOD attenuated this inhibition, it was suggested that the inactivation of EDRF was caused by superoxide radicals, formed during pyrogallol auto-oxidation.18,19 Moreover, oxygen radicals generated at reperfusion of an ischemic tissue may cause attenuated endothelium-dependent vascular relaxation, probably via inactivation of EDRF.20,21

In this study the protective action of EC-SOD C on EDRF-mediated vascular relaxation was studied in isolated rings of rabbit thoracic aorta exposed to free radicals formed during pyrogallol auto-oxidation. Specifically, the importance of the association of EC-SOD C with cell surfaces and other structures was evaluated.

Materials and Methods

Experimental Preparation

Male New Zealand White rabbits were used in the experiments. The animals were anesthetized with pentobarbital sodium (30 mg/kg i.v.) and killed by exsanguination. The thoracic aorta was carefully removed and placed in oxygenated (3% CO2-97% O2) modified Krebs-Henseleit buffer of the following composition (in mmol/l): NaCl 122, KCl 4.7, CaCl2 2.5, NaHCO3 15.5, MgCl2 1.2, KH2PO4 1.2, and glucose 11.5. Transverse rings of 2 mm width were cut and mounted between fine stainless steel bars for isometric recording of force in an organ bath filled with 50 ml oxygenated Krebs buffer. The temperature of the buffer was kept at 37°C, and the pH was 7.4. Six to eight rings were taken from the aorta of each rabbit, and two rings were mounted in each organ bath. The arterial ring was attached to a force-displacement transducer (model FT03, Grass Instrument Co., Quincy, Mass.), and changes in isometric force were recorded on a Grass polygraph. The length–tension relation for the aortic rings was studied in initial experiments. The rings were progressively stretched, and the contractile response to KCl (120 mM) was found to be maximal when the preparations were prestretched to 20 mN. This level of passive force was then applied in all the subsequent experiments. The potassium concentration was increased to 120 mM by substituting NaCl with equimolar amounts of KCl.

Experimental Protocol

After the preparations were mounted, they were allowed to stabilize for 1 hour. All preparations were then depolarized by Krebs solution containing 120 mM KCl (see above). After the rings were washed with normal Krebs solution, they were allowed to equilibrate for 1 hour. The arterial preparations were contracted with phenylephrine (3×10⁻⁷ M), a concentration of the α-agonist producing about 70% of maximal contractile response. An inhibitor concentration–response curve for acetylcholine was obtained, starting at 10⁻⁹ M and continuing in increments of 0.5 log units until the maximal relaxing effect was reached. This first concentration–response curve for acetylcholine was performed in all the arterial rings studied. After the preparations were washed, they were allowed to equilibrate for 1 hour before a second concentration–response curve was constructed for acetylcholine on phenylephrine-contracted arterial rings.

In control experiments, two concentration–response curves were constructed for acetylcholine with a 1-hour interval. In several experimental groups (see below), the second acetylcholine curve was carried out in arterial rings exposed to pyrogallol, which was added to the oxygenated buffer to generate superoxide radicals.18,19,22 In preliminary experiments, increasing pyrogallol concentrations (10⁻⁶ to 10⁻⁴ M) were tested, and a concentration of 10⁻⁴ M was chosen for the subsequent experiments. This concentration caused a reduction in the maximal relaxant effect of acetylcholine from 60–70% to 20–30%.

Three different experimental protocols were used when the second acetylcholine curve was carried out in the presence of pyrogallol (Figure 1). In study 1 (13 rabbits), we investigated the effects of CuZn SOD and EC-SOD C on pyrogallol inhibition of EDRF-mediated relaxation. Increasing concentrations of the SOD isoenzymes were studied, and the enzymes were present in the Krebs buffer throughout the second set of acetylcholine concentration–response measurements. In study 2 (six rabbits), the preparations were preincubated with 8,000 units/ml EC-SOD C or CuZn SOD during 30 minutes. After the incubation period, the arterial rings were washed twice with SOD-free Krebs buffer. The effect of pyrogallol on the relaxant effect of acetylcholine in precontracted preparations was then studied in SOD-free buffer. In study 3 (six rabbits), the arterial rings were preincubated with EC-SOD C (8,000 units/ml) as in study 2 but with heparin (125 IU/ml) added to the incubation and washing media. Moreover, the possible effects of heparin (125 IU/ml) on the acetylcholine-elicited relaxation were investigated in precontracted arterial rings not exposed to pyrogallol.

To investigate whether the inhibitory effects of pyrogallol were specific for endothelium-dependent relaxation, we studied the effects of pyrogallol on the
arterial relaxant effect of nitroprusside and nitroglycerin. These experiments were performed in a similar way to that described above for the second acetylcholine concentration-response curve. Moreover, in a separate set of experiments we investigated whether the effect of pyrogallol on acetylcholine relaxation was reversible. After the second acetylcholine concentration-response curve, in the presence of pyrogallol (see above), the arterial rings were washed three times with Krebs buffer (over a period of 1 hour) before a third acetylcholine concentration-response curve was performed.

**Assay of Superoxide Dismutase in Buffer and Tissue**

Eight arterial rings, two in each organ bath, were incubated with 8,000 units/ml EC-SOD C in oxygenated Krebs buffer for 30 minutes followed by washing (see above, study 2). Aliquots of the buffer were taken 3 minutes after the first wash and 1, 3, 10, and 15 minutes after the second wash. After the addition of bovine serum albumin (final concentration, 0.5%), the samples were stored at -70°C until assayed. Fifteen minutes after the second wash the arterial rings were removed, blotted, weighed, and then kept at -70°C until assayed for SOD activity. In corresponding control experiments, eight arterial rings not exposed to EC-SOD C were taken for analysis of SOD activity.

For assay of SOD activity the frozen arterial rings (about 15 mg wet weight) were pulverized in a Braun Microdismembrator II (B. Braun Biotech, Inc., Allentown, Pa.). The powder was added to 0.5 ml ice-cold 50 mM sodium phosphate (pH 7.4) containing 0.3 M KBr, 10 mM diethylenetriamine-pentaacetic acid, 0.5 mM phenylmethylsulfonyl fluoride, and aprotinin (100 kallikrein inhibitor units/ml). After sonication, the extracts were centrifuged (20,000g, 10 minutes), and the supernatant was analyzed for SOD activity. The SOD enzymic activity was determined with a direct spectrophotometric method by using KO₂. Cyanide (3 mM) was used for the distinction between the cyanide-sensitive isoenzymes CuZn SOD and EC-SOD and the cyanide-resistant isoenzyme Mn SOD. The method is very sensitive, and one unit corresponds to 6.3 ng bovine CuZn SOD, 8.6 ng human EC-SOD, and 65 ng Mn SOD. One unit in the more commonly used xanthine oxidase–cytochrome c assay corresponds to 40 units in the KO₂ assay. The amount of EC-SOD C protein in the buffer samples was determined by enzyme-linked immunosorbent assay.

**Chemicals**

Acetylcholine chloride, phenylephrine hydrochloride, and pyrogallol were purchased from Sigma Chemical Co., St. Louis, Mo., and heparin from KabiVitrum, Stockholm, Sweden. Nitroprusside was obtained from Merck, Darmstadt, FRG, and nitroglycerin (Karo Bio Medica, Stockholm, Sweden) was purchased from a local pharmacy. Human recombinant EC-SOD C was prepared as previously described and was provided by SYMBICOM AB, Umeå, Sweden. The specific activity of EC-SOD C in the KO₂ assay was 116,000 units/mg. Bovine CuZn SOD from erythrocytes was purchased from Boeh-

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**Figure 1.** Schematic illustration of three different experimental protocols used for the isolated thoracic rings (for details, see "Materials and Methods"). One enzyme unit (KO₂ assay) corresponds to 6.3 ng CuZn superoxide dismutase (SOD) and 8.6 ng extracellular SOD type C (EC-SOD C).

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<td>Vehicle</td>
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<td>EC-SOD C (8000 U/ml) + Heparin (125 IU/ml)</td>
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Phenylephrine (3x10⁻⁷M)  
Pyrogallol (10⁻⁴ M)  
Acetylcholine (10⁻⁹-10⁻⁸M)  

Vehicle  
EC-SOD C (24-4800 U/ml)  
CuZn-SOD (24-4800 U/ml)
ringer Mannheim GmbH, FRG, and had a specific activity of 160,000 units/mg. The drug solutions were prepared on the day of the experiment, and all the compounds were dissolved in Krebs buffer.

Calculations and Statistics

The relaxation caused by acetylcholine was expressed as the percent decrease in tension of the precontracted arterial ring. The concentration–response curves for acetylcholine were determined by applying the experimental points to a hyperbolic function by the method of least-squares fit. For each concentration–response curve, the maximal effect and the negative log (pD₂) of the agonist concentration that elicited 50% of the maximal effect were determined. In this study, two rings from the thoracic aorta were mounted together in the organ bath. The means for the maximal effects and the pD₂ values from the two acetylcholine curves were then calculated, and this mean value was regarded as one experimental observation. The differences in pD₂ value and maximal response to acetylcholine were usually less than 10% when comparing the two arterial rings in the same organ bath.

Results are expressed as mean±SEM. Differences between groups were tested by one-way analysis of variance followed by Duncan’s multiple range test. A value of p<0.05 was considered to be statistically significant.

Results

Acetylcholine caused a marked concentration-dependent relaxation of precontracted arterial rings (Figure 2, top panel). The maximal relaxant responses and the pD₂ values for acetylcholine were similar when two concentration–response curves were performed with a 1-hour interval (n=12). The percent relaxation was 61±3 (first curve) and 60±3 (second curve), and the pD₂ values for both the first and second curves were 7.3±0.1. Addition of pyrogallol to the buffer markedly attenuated the maximal response to acetylcholine (Figure 2, middle panel).
This effect of pyrogallol on the acetylcholine response was reversible since the acetylcholine-elicited relaxation could be repeated after the preparations were washed. The maximal relaxant effects to acetylcholine in these experiments were \( n=5 \) 73±2% (first curve, control), 32±3% (second curve, pyrogallol \( 10^{-4} \) M), and 72±2% (third curve, 1 hour after pyrogallol). Pyrogallol did not affect the vascular relaxant effects of nitroprusside and nitroglycerin. In arterial rings contracted with phenylephrine, the maximal relaxant response was 89±6% (nitroprusside, \( n=3 \)) and 104±2% (nitroglycerin, \( n=2 \)). In the presence of pyrogallol \( (10^{-4} \) M) the corresponding relaxant response was 85±9% (nitroprusside, \( n=3 \)) and 103±2% (nitroglycerin, \( n=2 \)).

The inhibitory effect of pyrogallol on acetylcholine-elicited vascular relaxation was attenuated by SOD (Figure 2, bottom panel). When CuZn SOD and EC-SOD C were present in the buffer throughout the experiment (study 1; see Figure 1), the isoenzymes caused a concentration-dependent prevention of the inhibitory effect of pyrogallol on the acetylcholine response (Figure 3). The acetylcholine-induced vascular relaxation was almost totally restored at an SOD concentration of about 1,000 units/ml, with a half-maximal effect at about 100 units/ml. In arterial ring preparations not exposed to pyrogallol it was demonstrated that CuZn SOD and EC-SOD C did not affect the relaxant effect of acetylcholine (data not shown).

In study 2 (see Figure 1), we investigated whether preincubation with CuZn SOD and EC-SOD C followed by washing could protect against the inhibitory effect of pyrogallol on the relaxant response to acetylcholine. The acetylcholine concentration–response curves in these experiments were thus determined in SOD-free buffer. The results shown in Figure 4 demonstrate that preincubation with EC-SOD C \( (8,000 \) units/ml) followed by washing significantly attenuated the effect of pyrogallol, while preincubation with an equal activity concentration of CuZn SOD was ineffective. Moreover, in corresponding experiments with a lower concentration \( (2,400 \) units/ml) of EC-SOD C, there was also an
improvement in the maximal relaxant effect of acetylcholine (n=3): 76±3% (control), 28±3% (pyrogallol), and 49±6% (pyrogallol+EC-SOD C; p<0.05 versus pyrogallol).

These results suggested that EC-SOD C, bound to heparan sulfate proteoglycans in the arterial rings, was responsible for the protective action observed after washing. To examine this possibility further, corresponding preincubation experiments were performed with heparin (125 IU/ml) present in the medium to prevent binding of EC-SOD C (study 3; see Figure 1). Heparin was found to markedly attenuate the preventive effect of EC-SOD C (8,000 units/ml) on the inhibition by pyrogallol of acetylcholine-elicited relaxation (Figure 5). In separate control experiments, it was demonstrated that heparin alone did not affect the maximal relaxant effect of acetylcholine (n=4): 66±7% (control) and 61±7% (heparin 125 IU/ml).

To further characterize the preincubation experiments, we determined the basal SOD activity of arterial rings, the SOD activity of rings after preincubation with EC-SOD C and subsequent washing, and also the amount of EC-SOD C present in the buffer after the incubation and washing procedures. The basal cyanide-sensitive SOD activity of the arterial rings was 2,260±300 units/g wet wt (n=8). This activity is mainly caused by intracellular CuZn SOD with some minor contribution by endogenous rabbit EC-SOD. The Mn SOD activity of the rabbit arterial rings was very low, 19±9 units/g wet wt. The aortic rings preincubated with human recombinant EC-SOD C contained considerably more cyanide-sensitive SOD activity, 7,650±1,030 units/g wet wt (n=8). Thus, the EC-SOD C activity was about 5,000 units/g wet wt in these arterial preparations. After the preincubation period (8,000 units/ml EC-SOD C) the concentration of EC-SOD C in the buffer was already markedly reduced after the first wash (145±10 units/ml, n=4), and the levels at various times after the second wash were 4.3±1.0 (1 minute), 3.3±0.9 (3 minutes), 2.8±0.5 (10 minutes), and 2.6±0.2 units/ml (15 minutes).

Discussion

It is established that the arterial relaxant effect of acetylcholine is mediated via EDRF, suggested to be nitric oxide or a closely related nitrosothiol derivative. The effect of pyrogallol in inactivating EDRF and the capacity of CuZn SOD to attenuate this inhibition have previously been demonstrated in experiments with isolated arterial rings. These findings were confirmed in our first set of experiments (study 1; see Figure 1), in which CuZn SOD and EC-SOD C prevented the inhibitory effect of pyrogallol on acetylcholine-elicited relaxation. The effect of pyrogallol on EDRF is presumed to be due to superoxide radicals formed during auto-oxidation. However, the mechanism of action is not entirely clear. The superoxide radical is a chain-propagating species in the auto-oxidation of pyrogallol, but free radical species are also formed from the pyrogallol molecule in the process. These may also react with and inactivate EDRF. In this context,
the SOD isoenzymes are not specific probes for the involvement of superoxide radicals in the inactivation of EDRF, since by removing superoxide, they also inhibit the auto-oxidation and hence reduce the steady-state concentration of “pyrogallol radicals.” They will also reduce the concentration of hydrogen peroxide, the stable product of oxygen in the auto-oxidation.22 However, in this study we shall discuss the effects of the SOD isoenzymes as being due to reductions in the concentration of superoxide radicals.

When present in the Krebs buffer during the exposure to pyrogallol, the SOD isoenzymes were equally capable of protecting EDRF against the effects of this agent. However, there was a marked difference in efficacy between the enzymes in the experiments in which the SOD isoenzymes were present in the buffer only during a preincubation period, which was followed by washing with SOD-free buffer. In contrast to CuZn SOD, EC-SOD C also exerted a marked protective effect in these preincubation experiments. The effect of EC-SOD C was lost when the preincubation was performed in the presence of heparin, which would prevent the binding of the enzyme to heparan sulfate proteoglycans.5–7

Experiments with cultured cells have shown that an incubation period of 30–60 minutes is required to reach equilibrium in the binding of EC-SOD C to the culture and that half-maximal binding is obtained at about 1,000 units/ml.6 In this study, the 30-minute preincubation with 8,000 units/ml should thus result in very extensive binding of EC-SOD C to the arterial rings. Indeed, determination of the tissue content of EC-SOD C demonstrated large quantities of the enzyme in the arterial rings, about 5,000 units/g wet wt after preincubation and subsequent washing. This EC-SOD C activity represents enzyme bound to heparan sulfate proteoglycans in the surface of endothelial cells and other cell types and, furthermore, in the connective tissue matrix. It has previously been shown that EC-SOD C bound to heparan sulfate retains essentially all enzymic activity.27 The effective SOD activity in the extracellular space of the arterial tissue may thus be very high and it should, in this location, completely prevent inactivation of EDRF by superoxide radicals (see Figure 4). The binding of EC-SOD C to heparan sulfate is reversible,5,6 and the enzyme will be continuously lost to the medium. However, after preincubation and washing the EC-SOD activity in the medium was very low and would not contribute to the protection of EDRF to any significant extent.

The degree of EDRF protection was lower in EC-SOD C–preincubated arterial rings than when EC-SOD C and CuZn SOD were present in the medium throughout the experiment. It is possible that part of the EDRF acting on the arterial smooth muscles was released to the medium and exposed to superoxide radicals before entering the arterial tissue. The acetylcholine relaxing effect seen in the EC-SOD C preincubation experiments may thus represent EDRF diffusing directly abuminally from the endothelial cells to the smooth muscle cells. This would mimic the in vivo situation, in which EDRF in the vessel lumen would rapidly be sequestered by hemoglobin. It is likely that some endogenous rabbit EC-SOD C is present in the arterial rings during the experiments, but much of the initial content may be lost because of the long incubations in the organ bath and because rabbit EC-SOD C has an unusually low affinity for heparan sulfate.4 Arterial tissue in mammals may contain several hundred units of EC-SOD per gram wet weight,3 and the concentration per milliliter in the interstitial space may be an order of magnitude larger. Such concentrations would probably exert significant protection of EDRF against superoxide radicals produced physiologically and during pathological conditions. In vitro, superoxide radicals formed during auto-oxidation of pyrogallol are mainly produced extracellularly.19 The inactivation of EDRF in vivo may be caused by free radicals produced in the extracellular space by activated neutrophils and intracellularly by xanthine oxidase, for example. However, the relative contribution of these free radical sources may vary during different pathological conditions.

After intravenous injection and when added to isolated perfused organs, EC-SOD C rapidly associates with endothelial cell surfaces7 and may also bind to some extent to heparan sulfate proteoglycans deeper in the vessels. Compared with other nonbinding SOD isoenzymes, this property of EC-SOD C may enhance its protective action against superoxide radicals formed under pathophysiological conditions. Thus, EC-SOD C significantly reduced the burst of free radicals observed at reperfusion of the rat isolated heart subjected to global ischemia.28 Moreover, in studies of isolated hearts subjected to regional ischemia and reperfusion, EC-SOD C reduced the myocardial cellular injury and caused an improved recovery of coronary flow and cardiac contractile function at reperfusion.29 Furthermore, EC-SOD C inhibited the increase in posts ischemic microvascular permeability in the hamster cheek pouch.30 In all three studies, EC-SOD C tended to be more effective than CuZn SOD in decreasing ischemia/reperfusion injury. However, the experimental protocols in these studies were not particularly designed to investigate the role of endothelium-bound SOD enzyme. At present, an important role for endothelium-bound EC-SOD C in reducing ischemia/reperfusion injury is suggested only from preliminary findings in the isolated perfused heart subjected to ischemia and reperfusion31 and, furthermore, from studies on the reduction of infarct size in the conscious rat.32

In conclusion, the present results show that exogenous EC-SOD C, associated with heparan sulfate proteoglycans on endothelial cell surfaces and probably internal structures in the aortic rings, efficiently protects EDRF against inactivation by superoxide radicals. The findings further suggest that endogenous EC-SOD C may be an important physiological enhancer of EDRF stability and that increased
EDRF stability may contribute to the therapeutic efficacy of exogenous EC-SOD C.

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Key Words • endothelium-derived relaxing factor • free radicals • superoxide dismutase • aorta
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