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Nitric oxide and its decomposed derivatives decrease the binding of extracellular-superoxide dismutase to the endothelial cell surface

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Abstract Extracellular-superoxide dismutase (EC-SOD) is bound to the vascular endothelial cell surface with an affinity for heparan sulfate proteoglycan. The binding of EC-SOD to the human umbilical vein endothelial cell (HUVEC) and bovine aortic endothelial cell surface proteoglycans was significantly decreased by the incubation with *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP) and (±)-*N*-[(*E*)-4-ethyl-2-[(*Z*)-hydroxyimino]-5-nitro-3-hexene-1-yl]-3-pyridine carboxamide (NOR4), potent nitric oxide (NO) donors. NO derived from lipopolysaccharide-stimulated J774 A-1 cells also decreased the binding of EC-SOD to HUVEC, and this decrease was blocked by N^G-nitro-L-arginine, a nitric oxide synthase inhibitor. SNAP and NOR4 also decreased the binding of EC-SOD to immobilized heparin. Furthermore, the decomposed derivatives of NO donors and sodium nitrite decreased the binding of EC-SOD. These observations suggest that excess NO produced in the inflammatory conditions decreases the binding of EC-SOD to the vascular endothelial cell surface, which results in a loss of the ability to protect the endothelial cell surface from oxidative stress.

Key words: Extracellular-superoxide dismutase; Nitric oxide; Nitrite; Endothelial cell; Heparan sulfate proteoglycan; Oxidative stress

1. Introduction

Extracellular-superoxide dismutase (EC-SOD) is a secretory glycoprotein [1,2] and is the principal enzymatic scavenger of superoxide in the extracellular space. EC-SOD has an affinity for heparin-like substances [3,4] and is present in the circulation in equilibrium between the plasma phase and the glycosaminoglycans on the endothelium [5]. In a previous study, we showed that the plasma EC-SOD level was associated with age [6,7], and was elevated in patients with renal diseases [8], and reduced in patients with a history of myocardial infarction [9]. Recently, we showed that the plasma EC-SOD level was elevated in patients with homocysteinuria [10] via decreasing the binding of EC-SOD to the endothelial cell surface by the treatment of homocysteine [11]. Therefore, physiological and pathological changes of heparan sulfate proteoglycans of the endothelial cell surface modulate the variation of the amount of EC-SOD binding to the endothelial cell surface, which widely affects the plasma EC-SOD levels and the oxidative conditions of the vascular system.

Nitric oxide (NO) is produced by three distinct isoforms of nitric oxide synthase (NOS) [12] and regulates physiological and pathological events in cellular systems. The induction of inducible NOS (iNOS) is involved in the inflammatory process evoked by endogenous signals such as infectious agents, lipopolysaccharide (LPS) and inflammatory cytokines, in phagocytes and other cells [13–17]. Excessive amounts of NO are associated with plasma leakage and microvascular injury [18] as well as cytotoxic effects against host cells in the inflammatory focus. NO reacts extremely rapidly with superoxide to produce peroxynitrite [19], a potent mediator of oxidant-induced cellular injury, and is finally degraded to nitrite and nitrate.

NO has been reported to degrade heparin and heparan sulfate on endothelial cells [20]. So NO or its derivatives might decrease the amount of EC-SOD bound on the endothelial cell surface. In the present study, we investigated the effects of NO and its decomposed derivatives on the binding of EC-SOD to heparin and the endothelial cell surface.

2. Materials and methods

2.1. Reagents

NO donors *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP) and (±)-*N*-[(*E*)-4-ethyl-2-[(*Z*)-hydroxyimino]-5-nitro-3-hexene-1-yl]-3-pyridine carboxamide (NOR4), peroxynitrite donor 3-(4-morpholinyl) sydnonimine hydrochloride (SIN-1), and NOS inhibitor N^G-nitro-L-arginine (L-NNA) were purchased from Dojindo Laboratories (Kumamoto, Japan). Human recombinant EC-SOD (r-EC-SOD), prepared as described previously [2], was kindly provided by Symbicom AB (Umeå, Sweden). Heparin–bovine serum albumin (BSA) complex and LPS (from *Escherichia coli* serotype O55:B5) were purchased from Sigma (St. Louis, MO, USA). Mouse anti-r-EC-SOD monoclonal antibody was prepared as described previously [21]. Alkaline phosphatase-coupled rabbit anti-mouse IgG antibody was purchased from Zymed Laboratories Inc. (San Francisco, CA, USA). Dulbecco's modified Eagle's medium (DMEM) was obtained from Nissui Pharmaceutical Co. (Tokyo, Japan). Human umbilical vein endothelial cells (HUVEC) and bovine aortic endothelial cells (BAEC) were purchased from Dainippon Pharmaceutical Co. Ltd. (Osaka, Japan). J774 A-1

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Abbreviations: BAEC, bovine aortic endothelial cell(s); BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; EC-SOD, extracellular-superoxide dismutase; FCS, fetal calf serum; HUVEC, human umbilical vein endothelial cell(s); iNOS, inducible nitric oxide synthase; L-NNA, N^G-nitro-L-arginine; LPS, lipopolysaccharide; NO, nitric oxide; NOS, nitric oxide synthase; NOR4, (±)-*N*-[(*E*)-4-ethyl-2-[(*Z*)-hydroxyimino]-5-nitro-3-hexene-1-yl]-3-pyridine carboxamide; PBS, phosphate-buffered saline; SIN-1, 3-(4-morpholinyl) sydnonimine hydrochloride; SNAP, *S*-nitroso-*N*-acetyl-DL-penicillamine

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murine macrophage cells (JCRB9108) were purchased from Health Science Research Resources Bank (Osaka, Japan). Immunoplates and TC insert Anopore membrane eight-well strips were purchased from Nunc (Roskilde, Denmark). Collagen Type I-coated 96-well microplates were purchased from Asahi Techno Glass Co. Ltd. (Tokyo, Japan).

2.2. Binding of EC-SOD to endothelial cells

Binding of r-EC-SOD to the endothelial cells was performed as described previously [11]. HUVEC or BAEC were suspended in DMEM containing 10% (v/v) fetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin and cultured in Collagen Type I-coated 96-well plates. When the cells were nearly confluent, the media were replaced with fresh FCS-free DMEM and cultured for 24 h. After the replacement of the medium with the fresh one, various concentrations of NO donors were added or J774 A-1 cell-plated TC inserts (as described below) were set on the wells, and followed by incubation in a CO_2 incubator for 24 h. Decomposed NO donors were prepared by incubation in phosphate-buffered saline (PBS) for 48 h at room temperature before use. The endothelial cell layers were rinsed with DMEM containing 0.5% FCS, and r-EC-SOD dissolved in the same medium was added followed by incubation at 4°C for 1 h. At the end of the incubation period, the unbound r-EC-SOD was collected and the cells were washed twice with PBS. The cells were then incubated at 4°C for 30 min with 10 mg/ml of heparin dissolved in the same medium to remove the cell surface-bound r-EC-SOD, which was then quantified by ELISA [22].

2.3. NO generation from macrophage cells

J774 A-1 murine macrophage cells were cultured in DMEM containing 10% (v/v) heat-inactivated FCS, 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were collected by scraping, mixed with or without 10 μg/ml LPS and/or 1 mM L-NNA, and then plated into TC inserts at densities of 5×10^4 or 1×10^5 cells/well. The TC inserts were set on the 96-well plate in which HUVEC was cultured confluently as described above. Production of NO was assayed by measuring the accumulation of nitrite in the conditioned media by the Griess reaction [23].

2.4. Binding of EC-SOD to immobilized heparin

Binding of r-EC-SOD to immobilized heparin was performed as described previously [11]. Briefly, immunoplate wells were coated with 100 μl of 50 mM Tris–HCl, pH 7.4, containing 12.7 mM EDTA and 0.4 μg/ml heparin–BSA complex and left to stand overnight at 4°C. Each well was washed three times with PBS containing 0.05% (v/v) Tween 20 (PBS-Tween), blocked with PBS-Tween containing 1% (w/v) BSA, and the plates were then left to stand overnight at 4°C.

A volume of 100 μl of the various concentrations of NO donors or the other reagents diluted in PBS was added to each well and the plates were kept for 24 h in a CO_2 incubator. The wells were then rinsed once with DMEM containing 0.5% (v/v) FCS and incubated for 3 h with r-EC-SOD diluted in the same medium. The wells were washed three times with PBS-Tween, and bound r-EC-SOD was then determined by the method mentioned above.

2.5. Statistical analysis

The data shown are the means ± S.D. of three or four separate experiments. Statistical significance was estimated using the Student's *t*-test, and a value of $P<0.05$ was considered to be statistically significant.

3. Results

3.1. Effects of NO on r-EC-SOD binding to the endothelial cells

It is known that EC-SOD can bind to cultured anchorage-dependent cell lines including endothelial cells [11,24]. We assessed whether NO affects the binding of EC-SOD to the endothelial cells. As shown in Fig. 1A, the amount of r-EC-SOD bound to the HUVEC surface was significantly decreased by pretreatment with SNAP or NOR4 in a concentration-dependent manner. The binding of r-EC-SOD to HUVEC surface was reduced to 74.0% ($P=0.005$) and 74.1% ($P=0.019$) with 200 μM SNAP or NOR4, respectively. The decrease of the binding of r-EC-SOD by NO donors was also observed in BAEC as well as HUVEC (Fig. 1B), which suggests that this phenomenon commonly occurs in the vascular wall in vivo.

3.2. Effects of NO released from macrophages on r-EC-SOD binding to the endothelial cells

In inflammatory conditions, LPS or cytokines have been shown to stimulate the expression of iNOS and NO production by several cells, especially by macrophages [13,14]. Therefore we studied the change of r-EC-SOD binding to HUVEC treated with macrophage-derived NO, using a TC insert culture system. As shown in Fig. 2A, the binding of r-EC-SOD to HUVEC was significantly decreased by co-culture of J774 A-1 cells in the presence of 10 μg/ml LPS. Addition of L-NNA, an NOS inhibitor, to the above reaction mixture reversed the binding of r-EC-SOD to HUVEC, whereas L-NNA alone did not affect it (Fig. 2B). The amounts of NO released from the J774 A-1 cells supported the above observations (Fig. 2C and D). These results indicate that NO released by LPS-stimulated J774 A-1 cells as well as NO donor decreases r-EC-SOD binding to the endothelial cells.

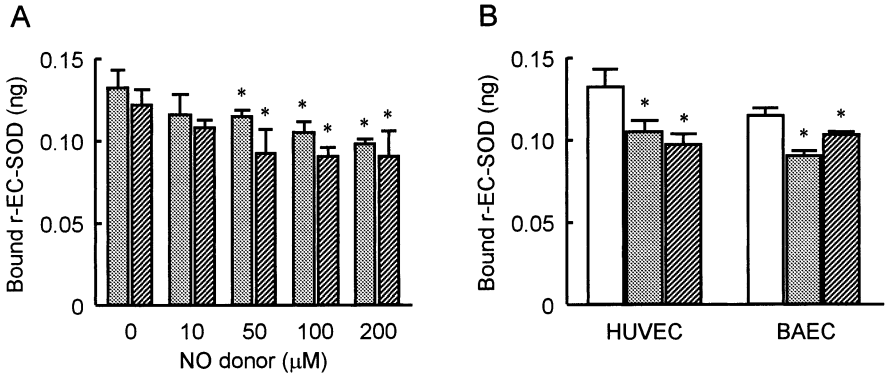

Fig. 1. Inhibition of EC-SOD binding to the endothelial cells by NO donors. A: NO donor concentration-dependent inhibition of r-EC-SOD binding to HUVEC. HUVEC was incubated with various concentrations of SNAP (filled columns) or NOR4 (hatched columns) for 24 h. B: Inhibition of r-EC-SOD binding to the endothelial cells. HUVEC and BAEC were incubated without (open columns) or with 100 μM of SNAP (filled columns) or NOR4 (hatched columns) for 24 h. The cells were followed by addition of r-EC-SOD as described in Section 2. The amounts of bound r-EC-SOD were determined by ELISA. Data represent the means ± S.D. of four experiments. *$P<0.05$ vs. control.

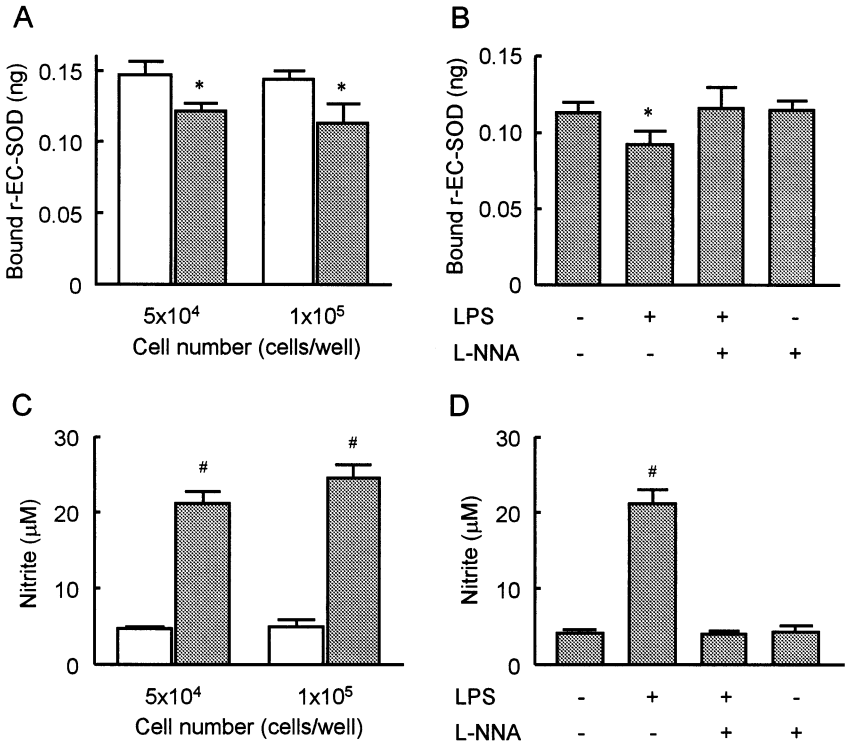

Fig. 2. Inhibition of EC-SOD binding to HUVEC by macrophage-derived NO. A: Inhibition of r-EC-SOD binding to HUVEC by J774 A-1 cells stimulated with LPS. HUVEC were incubated for 24 h with 5×10^4 or 1×10^5 cells/well of J774 A-1 cells mixed without (open columns) or with 10 μg/ml LPS (filled columns). B: Effect of NOS inhibitor on the decrease of the binding of r-EC-SOD to HUVEC by LPS-stimulated J774 A-1 cells. HUVEC and 5×10^4 cells/well of J774 A-1 cells mixed with or without 10 μg/ml LPS and/or 1 mM L-NNA were incubated for 24 h. The conditioned media of the above experiments were collected and the nitrite concentrations were measured by the Griess reaction (C and D). Data represent the means ± S.D. of three experiments. $^*P<0.05$, $^{\#}P<0.001$ vs. control.

3.3. *Effects of NO on r-EC-SOD binding to immobilized heparin*

Because EC-SOD binds to the vascular endothelial cells via, especially, heparin-like heparan sulfate proteoglycans on the endothelial cell surface, we next assessed the effects of NO on the binding of EC-SOD to heparin. The amount of r-EC-SOD binding to immobilized heparin was significantly decreased by pretreatment with NO donors in a concentration- and time-dependent manner (Fig. 3). The binding of r-EC-SOD was significantly reduced to 66.6% of the control ($P=0.017$) by 20 μM SNAP and to 73.6% ($P=0.012$) by 10 μM NOR4 for 24 h. The decrease of the binding of r-EC-SOD to immobilized heparin was observed after more than 3 h incubation with 100 μM SNAP or NOR4.

3.4. *Effects of decomposed derivatives of NO on r-EC-SOD binding*

We next studied whether the decomposed NO donors

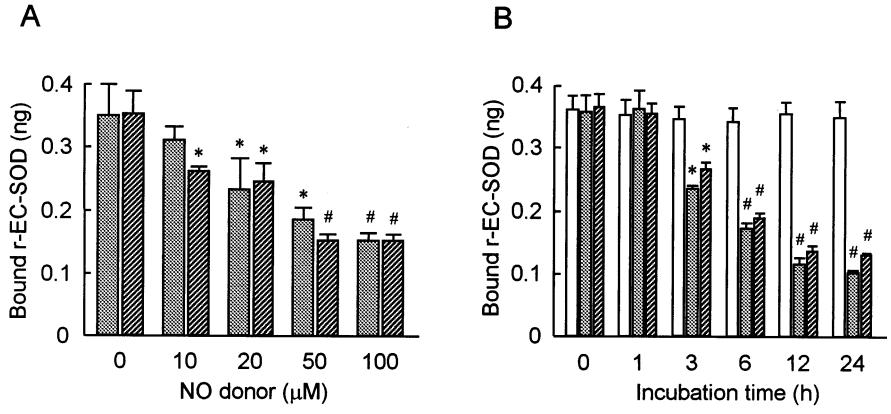

Fig. 3. Inhibition of EC-SOD binding to immobilized heparin by NO donors. A: NO donor concentration-dependent inhibition of r-EC-SOD binding to immobilized heparin. Heparin–BSA-coated plates were incubated with various concentrations of SNAP (filled columns) or NOR4 (hatched columns) for 24 h and then the binding of r-EC-SOD was assayed. B: Time-dependent inhibition of r-EC-SOD binding to immobilized heparin. Heparin–BSA-coated plates were incubated without (open columns) or with 100 μM SNAP (filled columns) or 100 μM NOR4 (hatched columns) for the indicated times and then the binding of r-EC-SOD was assayed. The amounts of bound r-EC-SOD were determined as described in Section 2. Data represent the means ± S.D. of four experiments. $^*P<0.05$, $^{\#}P<0.001$ vs. control.

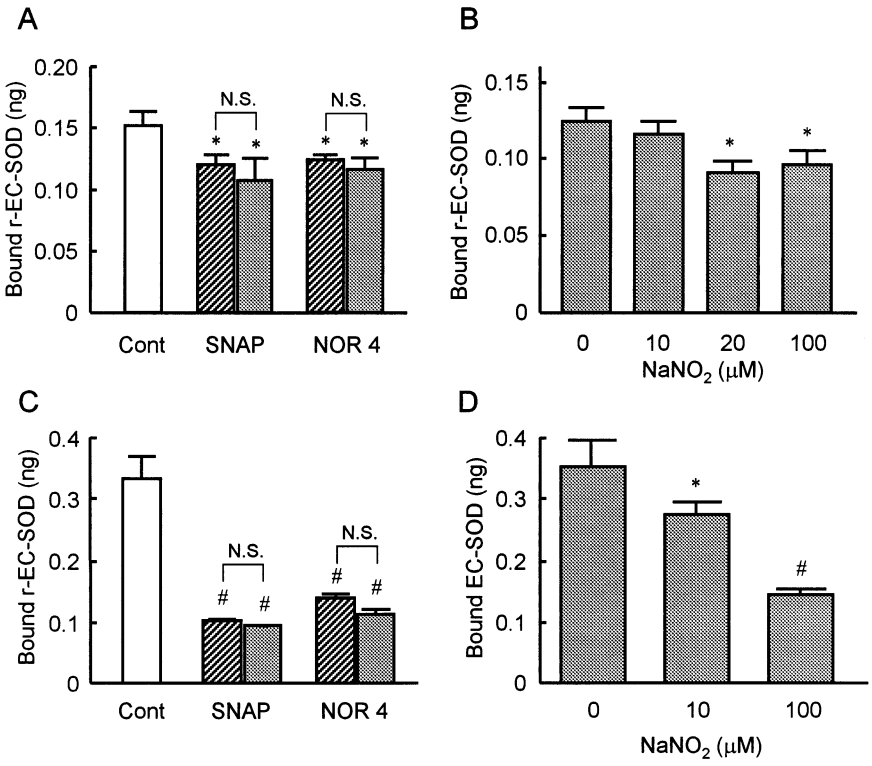

Fig. 4. Inhibition of EC-SOD binding by decomposed derivatives of NO. HUVEC (A and B) or heparin–BSA-coated plates (C and D) were incubated without (open columns) or with 100 μM active (hatched columns) or decomposed (filled columns) SNAP or NOR4 (A and C), various concentrations of sodium nitrite (B and D) for 24 h and then the binding of r-EC-SOD was assayed. Data represent the means ± S.D. of four experiments. $^{*}P < 0.05$, $^{\#}P < 0.001$ vs. control, N.S., no significant difference.

would decrease the binding of r-EC-SOD to the endothelial cells and immobilized heparin. As shown in Fig. 4A, the binding of r-EC-SOD to HUVEC was also significantly inhibited by decomposed NO donors as well as active NO donors. We observed no significant differences of the potentialities between active and decomposed NO donors. Moreover, the binding was also reduced to 73.5% ($P = 0.012$) by sodium nitrite, a stable product of NO in aqueous solution (Fig. 4B). Similar to the binding to HUVEC, decomposed NO donors and sodium nitrite markedly inhibited the binding of r-EC-SOD to immobilized heparin (Fig. 4C and D). In contrast, peroxynitrite donor SIN-1 or nitrate did not decrease the binding of EC-SOD (data not shown). NO donors and nitrite did not affect cellular protein contents at the concentration used (data not shown). These results demonstrate that NO derivatives, especially nitrite, also inhibited the r-EC-SOD binding to heparin and the endothelial cells.

4. Discussion

EC-SOD binds to the endothelial cells by the interaction of C-terminal basic amino acids with anionic charged residues in heparan sulfate proteoglycan or other proteoglycans on the endothelial cell surface [25]. In this study, we found that exogenous NO decreased r-EC-SOD binding to the endothelial cell surface. The NO productions from 100 μM SNAP and NOR4 for 24 h were 52.5 ± 0.9 and 64.2 ± 3.2 μM, respectively under the experimental conditions shown in Fig. 1, which are consistent with plasma concentration of nitrite in inflammatory conditions [26,27]. It is known that large amounts of NO were produced by several cells including macrophages, endothelial cells and smooth muscle cells, stimulated by inflammatory cytokines or LPS [15,28–30]. We found that the co-culture with LPS-stimulated J774 A-1 cells decreased the binding ability of HUVEC with r-EC-SOD, as shown in Fig. 2. The decrease of the binding of r-EC-SOD was accompanied with NO release measured by the Griess reaction and was blocked by NOS inhibitor. These results suggest that large amounts of NO released from activated macrophages would cause the decrease of EC-SOD binding to the endothelial cell surface, followed by the decrease of capacity to scavenge superoxide in the microenvironment of the vascular system, causing vascular injury.

As shown in Fig. 4, decomposed NO donors as well as active donors reduced r-EC-SOD bindings to HUVEC and to immobilized heparin. One of the stable end products of NO in aqueous solution is nitrite [31,32]. The question of nitrite as a new potent oxidant has recently been raised. Nitrite levels of plasma and other tissue fluids were elevated in several inflammatory diseases including chronic glomerular nephritis [26], cerebral systemic lupus erythematodes [33] and infective gastroenteritis [34], but the mechanism causing oxidative stress remains poorly understood. Recently, it has been suggested that nitrite can be oxidized by myeloperoxidase, an enzyme secreted from activated neutrophils, to form nitrogen dioxide or a related species, which can contribute to tyrosine nitration during inflammatory processes [35,36]. Moreover, nitrous acid (HNO_2) is known to cleave heparin and heparan sulfate at *N*-sulfate or free amino glucosamine residues by a deaminative mechanism [37–40]. The active species, the nitrosonium cation (NO^+), may be formed directly from NO or HNO_2 in the presence of an appropriate electron

acceptor. Along with cleavage of the glycosidic bond, the amino group and the sulfate group are known to be eliminated, yielding an anhydromannose at the new reducing end of each oligosaccharide [40]. Our observation that sodium nitrite decreased r-EC-SOD binding to the endothelial cell surface and immobilized heparin might be explained by these mechanisms. In contrast, the results that peroxynitrite did not decrease r-EC-SOD binding to heparin-like substances are consistent with a previous report that peroxynitrite did not degrade heparin and heparan sulfate [20]. Therefore, it is suggested that the main material that decreases the EC-SOD binding to heparin-like substances is NO or its decomposed derivatives, including nitrite.

The blood vessel walls were found to contain large amounts of EC-SOD, whereas the levels of other SOD isozymes were relative low, and EC-SOD was shown to be distributed throughout the wall with significant amounts in all layers [41]. It is possible that EC-SOD in the vascular system diffuses into the extracellular space and suppresses the oxidative stress by scavenging superoxide. We have reported that reduction of heparin affinity of EC-SOD contributes to endothelial dysfunction [42]. Elimination of EC-SOD from the endothelium by NO derivatives may result in the elevation of oxidative stress.

In conclusion, our study demonstrated that the binding of EC-SOD to glycosaminoglycans was significantly decreased by macrophage-derived NO, and NO donors and their decomposed derivatives, especially nitrite. These findings suggest that excess NO decreases the protection of the vascular endothelial cell surface from superoxide and results in the pathogenesis of inflammation and the other oxidative stress-derived diseases.

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