Endocytosis of Extracellular Superoxide Dismutase Into Endothelial Cells
Role of the Heparin-Binding Domain

Yi Chu, Robert Piper, Simon Richardson, Yoshimasa Watanabe, Pragnesh Patel, Donald D. Heistad

Objective—Extracellular superoxide dismutase (EC-SOD) is a secreted antioxidant enzyme that binds to the outer plasma membrane and extracellular matrix through its heparin-binding domain (HBD). Carriers of a common genetic variant of EC-SOD (EC-SOD_{R213G}) within the HBD have higher plasma concentration of EC-SOD and increased risk for vascular disease. In the present study, we used confocal fluorescence microscopy to examine mechanisms of endocytosis of EC-SOD to determine whether EC-SOD translocates to the nucleus of endothelial cells, and to test the hypothesis that EC-SOD, but not EC-SOD_{R213G} is endocytosed into endothelial cells.

Methods and Results—Mouse endothelial cells (MS-1) were incubated with EC-SOD, EC-SOD_{R213G}, or HBD-deleted EC-SOD (EC-SOD_{ΔHBD}). Binding to MS-1 was observed only with EC-SOD, but not EC-SOD_{R213G} or EC-SOD_{ΔHBD}. Endocytosis of EC-SODs was monitored after coincubation of MS-1 cells with EC-SODs and BSA-Texas Red (BSA-TR), which marks endosomes and lysosomes. Only EC-SOD was endocytosed, colocalizing with BSA-TR. EC-SOD also colocalized with early endosome antigen 1 (EEA-1), a specific marker for endocytosis. Endocytosis of EC-SOD was inhibited by chlorpromazine, but not by methyl-β-cyclodextrin or nystatin, which suggests that endocytosis of EC-SOD is mediated by clathrin but not by caveolae. Minimal or no localization of EC-SOD in the nucleus of MS-1 cells was detected.

Conclusions—Our findings indicate that EC-SOD, but not EC-SOD_{R213G}, is endocytosed into endothelial cells through clathrin-mediated pathway, but does not translocate to the nucleus. We speculate that impairment of endocytosis may contribute to high plasma levels of EC-SOD_{R213G} in R213G carriers. (Arterioscler Thromb Vasc Biol. 2006;26:1985-1990.)

Key Words: antioxidant enzyme ■ superoxide dismutase ■ endothelium ■ endocytosis

Extracellular SOD (EC-SOD or SOD3) is a major extracellular antioxidant enzyme,1,2 distributed in extracellular matrix of many tissues and especially blood vessels.3-5 A fundamental property of EC-SOD is its affinity, through its heparin-binding domain (HBD), for heparan sulfate proteoglycans located on cell surfaces and in extracellular matrix.6-9 We have recently reported that the HBD is required for beneficial vascular effects of EC-SOD in several pathophysiologic conditions including hypertension, vasospasm after subarachnoid hemorrhage, heart failure, and endotoxemia.10-13

A gene variant within the HBD, with a substitution of arginine-213 by glycine (R213G) resulting from a C to G transversion at the first base of codon 213, is found in 2% to 5% of humans in whom blood concentration of EC-SOD is increased ≈10-fold.14-17 This alteration in the HBD reduces affinity for heparin but does not affect the enzymatic activity of EC-SOD.15,16 Binding to bovine aortic endothelial cells in vitro is 50-fold less by EC-SOD_{R213G} than normal EC-SOD.16 A recent large study suggested a 2.3-fold increase in risk of ischemic heart disease in heterozygotes carrying the variant or a 9-fold increase when corrected for blood levels of EC-SOD.18 Consistent with this population study, we have demonstrated recently that EC-SOD_{R213G} is deficient in beneficial vascular effects in a genetic animal model of spontaneous hypertension.19 Binding to blood vessels both in vitro and in vivo is much less by EC-SOD_{R213G} than EC-SOD.19

Internalization of human EC-SOD by bovine aortic endothelial cells was reported with the use of 125I-labeled EC-SOD.20 Based on our findings of deficient binding of EC-SOD_{R213G} to blood vessels, we hypothesized that EC-SOD_{R213G} also may be deficient in internalization into endothelial cells, which could contribute to higher blood concentrations of EC-SOD_{R213G} in carriers.14-17 In the present study, we used fluorescence confocal microscopy to determine whether EC-SOD_{R213G} versus EC-SOD, enters endothelial cells and whether endocytosis is mediated by clathrin or caveolae. In...
addition, based on 2 recent reports that suggested that the HBD mediates translocation of exogenous EC-SOD into the nucleus of 3T3-L1 mouse preadipocytes, we determined whether EC-SOD translocates into the nucleus of endothelial cells. As a control for the role of HBD, we also studied EC-SOD with deletion of the HBD (EC-SODΔHBD).

Methods

Cells

HeLa cells, 3T3-L1 cells, and a mouse endothelial cell line, MS-1, were purchased from American Type Culture Collection. Cells were grown in Dulbecco-modified Eagle medium (DMEM) with 10% fetal bovine serum, 10 U/mL penicillin, and 10 μg/mL streptomycin.

Recombinant Adenoviral Vectors

We studied replication-deficient adenoviruses that express normal human EC-SOD (AdEC-SOD), EC-SOD with deletion of the HBD (AdEC-SODΔHBD), or the R213G variant of EC-SOD (AdEC-SODR213G), constructed as described previously. The ratio of viral particles to plaque-forming units was ~50:1, for all viruses used in the study.

Binding of EC-SOD Proteins to MS-1 Cells

To obtain recombinant EC-SOD proteins, HeLa cells (70% confluent) were incubated with AdEC-SOD, AdEC-SODR213G, or AdEC-SODΔHBD at 20 PFU cell for 3 hours. After removal of unbound viruses by extensive washing, cells were incubated in DMEM with 10% fetal bovine serum for 3 days. Cell-free culture medium was then harvested by centrifugation followed by filtration through a 0.22-μm low-protein binding filter. The EC-SOD activity of the media, measured with nitroblue tetrazolium reduction, was ~70% for all 3 forms of EC-SOD.

Chamber slides containing subconfluent cells were equilibrated at 4°C and incubated with 0.4 mL of cold (4°C) EC-SOD protein in the absence or presence of 10 μg/mL heparin, which binds to EC-SOD to prevent binding of the protein to cells, for 30 minutes at 4°C. After EC-SOD protein was aspirated, cells were washed with cold phosphate-buffered saline (PBS) and fixed in 2% paraformaldehyde for 15 minutes. After blocking with 5% bovine serum albumin (BSA), cells were incubated with anti-human EC-SOD antibody (obtained from Dr James Crapo of National Jewish Research Center, Denver, CO), or with normal rabbit serum as a control, at 4°C for ~12 hours. Cells were washed with PBS, and then incubated with AlexaFluor-488 goat anti-rabbit IgG (1:200) at room temperature for 1 hour. Cells were washed with PBS, and covered with a coverslip. Confocal microscopy was performed as described previously.

Endocytosis of EC-SODs Into MS-1 Cells

Cells were seeded in chamber slides and incubated with EC-SOD protein containing 1.5 mg/mL Texas Red-conjugated BSA (made by co-author Simon Richardson) as a marker for endosomes and lysosomes in the absence or presence of 200 μmol/L leupeptin to inhibit lysosome-mediated proteolysis, at 37°C for 1 hour. Cells were washed with PBS containing 10 μg/mL heparin to remove extracellular EC-SOD, and then incubated in DMEM containing 10% fetal bovine serum, in the absence or presence of leupeptin for 0, 3, 16, or 24 hours. Cells were fixed, permeabilized with 0.2% Triton X-100 for 15 to 30 minutes, blocked with 5% BSA for 30 minutes, and then incubated with anti-human EC-SOD at 4°C for ~12 hours. Cells were washed and incubated with AlexaFluor-488 goat anti-rabbit IgG (1:200) for 1 hour, washed, and incubated with TO-PRO 3 (1:2000) for 10 minutes, before confocal microscopy.

Colocalization of EC-SOD and EEA-1 in MS-1 Cells

MS-1 cells were seeded in chamber slides, and incubated with EC-SOD protein at 37°C for 30 minutes. Cells were then washed with PBS containing 10 μg/mL heparin, fixed with paraformalde-
EC-SOD protein to cells and blood vessels, and the single amino acid substitution results in marked impairment in binding of EC-SODR213G.

Endocytosis of EC-SODs Into Endothelial Cells

To determine whether exogenous EC-SOD enters into endothelial cells, MS-1 endothelial cells were coincubated with the 3 types of EC-SOD protein and Texas Red-conjugated BSA for 1 hour, and examined for up to 24 hours. Only normal EC-SOD protein was endocytosed (Figure 2A). There was no detectable endocytosis of EC-SODR213G (Figure 2B) or EC-SOD/H9004/HBD (not shown).

After endocytosis, EC-SOD was present in cells for 3 hours in the absence of leupeptin, and for 16 hours in the presence of leupeptin (Figure 2A). Because leupeptin inhibits degradation of proteins in lysosomes, the findings suggest that EC-SOD may be degraded by lysosome-mediated proteolysis after uptake by endothelial cells.

A portion of EC-SOD was colocalized with EEA-1, a specific marker for early endosome, which suggests that EC-SOD indeed travels via the endocytic pathway (Figure 3). It is likely that EC-SOD that was not colocalized with EEA-1 had progressed to late endosomes.

Inhibitors of endocytosis were used to further delineate mechanisms of endocytosis of EC-SOD in MS-1 endothelial cells. Chlorpromazine (10 µg/mL, which inhibits clathrin-mediated endocytosis) greatly inhibited endocytosis of EC-SOD. In contrast, neither methyl-β-cyclodextrin (10 mmol/L) nor nystatin (25 µg/mL) (which inhibit caveolae-mediated endocytosis) affected endocytosis (Figure 4). The findings suggest that endocytosis of EC-SOD into MS-1 cells is mediated by the clathrin-dependent, but not the caveolae-dependent, pathway.

Evaluation of Nuclear Localization

A previous study using conventional immunocytochemistry and subcellular fractionation indicated nuclear localization of EC-SOD in 3T3-L1 cells after only 1 hour of incubation. In this study, little or no nuclear localization of exogenous EC-SOD was observed in MS-1 endothelial cells during the studies of endocytosis described above, even when incubation of cells with EC-SOD was extended to or beyond 4 hours (not shown), the time point used in another report.

We also examined nuclear localization when EC-SOD was produced inside cells. After adenovirus-mediated gene transfer of EC-SODs to MS-1 cells, no nuclear localization was observed with confocal microscopy with any of the 3 types of EC-SOD (Figure 5).

An important caveat in interpreting the findings with confocal microscopy is that the nucleus might not be accessible to the antibody against human EC-SOD and/or the secondary antibody, so that detection of nuclear localization of EC-SOD is not possible. To test this possibility, we...
processed MS-1 cells with Triton X-100 (0.2%) in the identical way as used for staining EC-SOD and stained for nucleolin, a nuclear protein. The nucleus was clearly stained for nucleolin in MS-1 cells (Figure 6). The finding suggests that the absence of staining of EC-SOD in the nucleus resulted from absence of translocation of EC-SOD to the nucleus of endothelial cells.

Discussion

In the present study, we determined whether exogenous EC-SOD, the R213G gene variant, and EC-SOD with deletion of the HBD endocytose into endothelial cells and translocate to the nucleus using fluorescence confocal microscopy. The major findings of this study are that: (1) EC-SOD can enter endothelial cells by endocytosis, but does not localize in the nucleus; (2) the HBD is required for endocytosis of EC-SOD, and there is little endocytosis of the R213G variant; and (3) endocytosis of EC-SOD into endothelial cells appears to be mediated by the clathrin, but not a caveolae-dependent, pathway.

Endocytosis of EC-SOD: Role of the HBD

Because EC-SOD is present in the circulation as well as bound to tissues, and the level of EC-SOD in plasma can be dramatically different based on the genotype of EC-SOD, it is important to know whether circulating EC-SOD (mimicked by exogenous EC-SOD in this study) enters endothelial cells and possibly exerts its antioxidant function intracellularly as well as extracellularly, or is degraded to maintain low blood level of EC-SOD. Although these 2 functions have not been resolved by the present study, this study, with the use of fluorescence confocal microscopy, provides the first direct evidence to our knowledge that exogenous EC-SOD is intracellular, as well as extracellular, in endothelial cell, and that EC-SOD enters endothelial cells through clathrin-mediated endocytosis.

In a previous study using centrifugal fractionation, radio-labeled human EC-SOD was reported to be internalized by bovine aortic endothelial cells, and internalization was inhibited by heparin. The method, because of intrinsic limitations of specificity of radiolabeling and centrifugation, however, is...
not appropriate for elucidation of particular cellular processes such as endocytosis. After internalization, EC-SOD was found to be degraded, and degradation was inhibited by chloroquine, which suggests that degradation is mediated by the lysosome.20 Our findings using leupeptin, an inhibitor of lysosome-mediated proteolysis, is consistent with this previous report. It is not known, however, whether endocytosed EC-SOD, before degradation, is enzymatically active.

Endocytosis of the gene variant R213G of EC-SOD was first examined in the present study. Interestingly, there is no detectable endocytosis of the variant, which is similar to the HBD-deleted EC-SOD. This finding suggests that binding, which is absent for the variant (Figure 1), is a prerequisite to endocytosis in MS-1 cells. Absence of binding of the R213G variant in MS-1 cells contrasts with small, but definite, binding to blood vessels.19 This difference may result from differences in cell types and/or sensitivity of detection. Nevertheless, these findings suggest that the HBD is necessary for EC-SOD to be endocytosed into endothelial cells. Consistent with the findings is the fact that heparan sulfate proteoglycan, the binding partner of the HBD, mediates binding and endocytosis of a diverse array of molecules, including fibroblast growth factors, HIV-1 TAT protein, and thrombospondin-1.30–33

Implications of endocytosis of exogenous EC-SOD into endothelial cells are not fully understood. The finding that the genetic variant R213G is not endocytosed may suggest that its high concentrations in plasma of carriers of the gene variant may be caused in part by impaired endocytosis.

Is There Nuclear Localization of EC-SOD?
EC-SOD is principally localized on cell surface and in extracellular matrix,1–5 with exceptions at some stages of fetal development, when EC-SOD is localized in cytoplasm but not nucleus, in placental cells and in fetal lung cells.34,35 Later in development, extracellular localization becomes the primary site of expression of EC-SOD. Although mechanisms responsible for intracellular expression and transition to extracellular localization during development are not understood, these findings indicate that endogenous expression of EC-SOD can be intracellular, and developmentally regulated. Intracellular expression was also observed by immunohistochemistry in some neurons of adult mice.36

In cells that produce EC-SOD endogenously, between 5% and 25% of EC-SOD protein is not secreted but is retained in the cell lysate.37 Subcellular localization of the cell-associated EC-SOD, however, has not been characterized.

Nuclear localization of mouse EC-SOD was reported in 3T3-L1, a mouse preadipocyte cell line.21,22 The major evidence, however, was based on conventional immunocytochemistry and subcellular fractionation, which may not be optimal for precise subcellular localization. Using fluorescence confocal microscopy, we found that EC-SOD, after incubation, does not localize in the nucleus of 3T3-L1 cells (data not presented). We also found (data not presented) that exogenous EC-SOD partitioned in the nuclear fraction after incubation at 4°C, at which temperature endocytosis does not occur. We suggest that the conclusion that nuclear localization of EC-SOD may occur21,22 may not be valid, based on the methods used in the studies.21,22

There are several reasons that we considered the possibility that EC-SOD is present inside the nucleus. First, based on a homology search, we have found that the RKKRRR basic amino acid sequence that constitutes the HBD of EC-SOD is present in 10 different nuclear proteins, which are not secreted. Second, secreted proteins such as fibroblast growth factors which contain the HBD, although not of the RKKRRR sequence, can translocate into the nucleus. Third, a recent article demonstrated that EC-SOD level in cells is positively associated with the length of telomeres in human fibroblast lines.38 Because telomeres are confined to the nucleus, this finding implies that EC-SOD might function in the nucleus.

As the possibility of nuclear localization of EC-SOD seems to be strong, it is surprising that no previous studies have used state-of-the-art approaches to demonstrate nuclear localization convincingly. Thus, the absence of EC-SOD in the nucleus in the present study, with the use of confocal microscopy, appears of interest.

Our finding that nuclear localization of EC-SOD does not occur in endothelial cells may have an explanation. Nuclear import of proteins containing a classical nuclear localization signal (NLS) is an energy-dependent process that requires the heterodimer importin alpha/beta. Three to 6 basic contiguous arginine/lysine residues characterize a classical NLS and are thought to form a basic patch on the surface of the import cargo.39 In many proteins which contain an NLS, the NLS is functional only when it is in an exposed configuration that allows binding of importins.40–43 Thus, it is possible that the HBD of human EC-SOD is in such a configuration that it does not function as an NLS.

In conclusion, we have presented evidence that EC-SOD can enter cells via endocytic vesicles through clathrin-mediated endocytosis but is not localized in the nucleus of endothelial cells. Endocytosis requires the heparin-binding domain of EC-SOD. The gene variant R213G is not endocytosed, which may explain in part higher plasma concentration in human carriers of R213G than wild-type EC-SOD.

Acknowledgments
We thank Jianqiang Shao for technical assistance, Teresa Ruggle for preparation of figures, Arlinda LaRose for typing the manuscript, and Drs Henry Keen and Curt Sigmund for assistance in homology search for the HBD-containing proteins.

Sources of Funding
We acknowledge the University of Iowa Gene Transfer Vector Core, supported in part by the NIH and Roy J. Carver Foundation, for viral vector preparations, and the UI Central Microscopy Facility for use of instruments. Studies were supported by NIH grants HL 16066, HL 62984, NS 24621, HL 14388, HL 38901, DK 54759, DK 52617, and HL 55006, funds provided by the Veterans Affairs Medical Service, and a Carver Trust Research Program of Excellence.

Disclosures
None.

References


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Arterioscler Thromb Vasc Biol. 2006;26:1985-1990; originally published online June 29, 2006; doi: 10.1161/01.ATV.0000234921.88489.5c

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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