Short Communication

Pharmacology

Pharmacology 2006;78:198–201
DOI: 10.1159/000096598

Received: August 29, 2006
Accepted: September 12, 2006
Published online: October 27, 2006

Rapid Endocytosis of Copper-Zinc Superoxide Dismutase into Human Endothelial Cells: Role for Its Vascular Activity

Ernst R. Waelti Matthias Barton

Department of Medicine, Internal Medicine I, Medical Policlinic, University Hospital Zürich, Zürich, and Department of Pathology, University Hospital Bern, Bern, Switzerland

Key Words
SOD1 · Atherosclerosis · Aging · Cytosol · Endothelium · FITC · NO · Receptor · Superoxide uptake · Vasodilation · Receptor

Abstract
Cytosolic CuZn-SOD (SOD1) is a dimeric, carbohydrate-free enzyme with a molecular weight of about 32 kDa and also circulates in human blood plasma. Due to its molecular mass it has been believed that the enzyme cannot penetrate the cell membrane. Here we report that rapid endocytosis of FITC-CuZn-SOD into human endothelial cells occurs within 5 min. Moreover, relaxation of rat aortic rings in response to CuZn-SOD is associated with a lag time of 45–60 s and only observed in the presence of intact endothelial cells. The results indicate acute and rapid endothelial cell endocytosis of CuZn-SOD, possibly via activation of a receptor-mediated pathway. Intracellular uptake via endocytosis may contribute to the vascular effects of CuZn-SOD, including vasodilation, and is likely to play a role in regulation of vascular tone and diseases such as atherosclerosis.

Copyright © 2006 S. Karger AG, Basel

Introduction

Cytosolic CuZn-SOD (SOD1) is a dimeric, carbohydrate-free enyzme with a molecular weight of about 32 kDa [1] whereas EC-SOD (SOD3) is a tetrameric extracellular enzyme which also contains copper and zinc [2]. CuZn-SOD is not only found intracellularly but also circulates in blood plasma where its levels have been associated with different disease states [3, 4]. Due to the molecular mass of the enzyme it has been believed that the enzyme cannot cross the cell membrane, and that encapsulation of CuZn-SOD protein into liposomes or other carrier molecules may be required to induce effects [5–7]. In a most recent report, Chu et al. [8] described endocytosis of native extracellular superoxide dismutase (EC-SOD) into mouse endothelial cells via a clathrin-dependent pathway. In the early 1990s, CuZn-SOD was reported to cause relaxation of precontracted rabbit arteries with a threshold concentration of about 1 U/ml [9], and similar effects were observed in rat aorta and femoral artery [10]. However, although rapid endocytosis of proteins into endothelial cells has been reported [11, 12], it is still unknown whether endothelial cell endocytosis of CuZn-SOD occurs.

Materials and Methods

Vascular Function Experiments

Vascular function experiments were performed as described [13]. Adult male Wistar rats were anesthetized, sacrificed by exsanguination, and the aorta removed. The thoracic aorta placed in cold Krebs-Ringer bicarbonate solution (in mmol/l: NaCl 118.6, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.1, EDTA calcium disodium 0.026, and glucose 11.1), cleaned from perivascular tissue, and cut into rings. Rings were suspended in organ

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

© 2006 S. Karger AG, Basel
0031–7012/06/0784–0198$23.50/0

Accessible online at:
www.karger.com/pha

Matthias Barton, MD
Department of Medicine, Internal Medicine I
Medical Policlinic, University Hospital Zurich
Ramistrasse 100, CH–8091 Zürich (Switzerland)
Tel. +41 44 255 5663, Fax +41 44 255 8747, E-Mail barton@usz.ch

source: https://doi.org/10.48350/20351 | downloaded: 1.5.2024

Downloaded from http://karger.com/pha/article-pdf/78/4/198/3420079/000096598.pdf by Universitätsbibliothek Bern user on 21 September 2023

chambers containing 25 ml of Krebs-bicarbonate solution (37°C, pH 7.4, 95% O_2 and 5% CO_2), and allowed to equilibrate for 1 h. Resting tension was gradually increased, and rings were repeatedly exposed to 100 mmol/l KCl until the optimal tension for generating force during isometric contraction was reached and equilibrated for 30 min. Vessels were left at resting tension throughout the study. Rings were precontracted with norepinephrine (Sigma Chemical Co., St. Louis, Mo., USA) to approximately 80% of the response obtained with KCl 100 mmol/l and exposed to cumulative concentrations of CuZn-SOD (0.075–75 U/ml, from bovine erythrocyes, Sigma Chemical Co.). Procedures and experimental protocols were performed in accordance with the local authorities for animal research and the American Heart Association guidelines on research animal use.

Cell Culture Experiments

Human umbilical vein endothelial cells were grown on glass slides to subconfluency in phenol-red free medium. Experiments using FITC-SOD were performed at 4 or at 37°C as described [14, 15]. Cells were then incubated with 40 U/ml of FITC-labeled SOD (40 U/ml, Sigma Chemical Co.) and experiments were terminated after 5 or 30 min. Cells were washed several times with phosphate-buffered saline to remove residual SOD protein from the cell surface and then viewed under a fluorescence microscope (Leitz AG). Fluorometry was performed using a PerkinElmer 3000 fluorometer (PerkinElmer, Boston, Mass., USA) as described [15], and samples were measured three times for each experiment and time point, and averaged values were used for calculation of SOD concentrations per 500,000 cells.

Statistical Analysis

Data are given as means ± SEM. The unpaired Student t test was used to compare the relaxant response at individual time points in rings with to those without endothelial cells [16]. Statistical significance was accepted at p values <0.05.

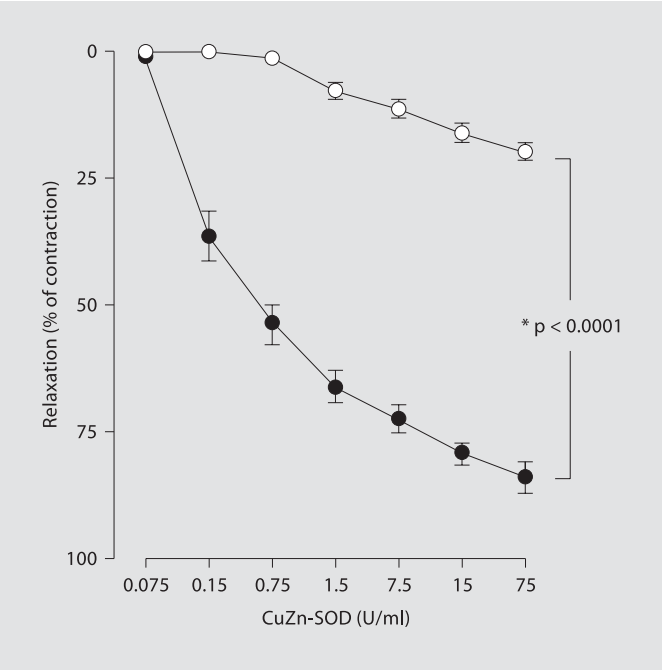

Fig. 1. Concentration-dependent relaxation responses to exogenous CuZn-SOD in precontracted rat aorta rings with (+, ●) and without (–, ○) endothelial cells. Note threshold concentration of about 0.1 U/ml, submaximal effects were observed at concentrations of 7.5 U/ml and above. n = 6–10/group; (+) endothelium vs. (–) endothelium.

Results

CuZn-SOD elicited a concentration-dependent dilator response only in the presence of intact endothelial cells (fig. 1). To test whether H_2O_2, a potent endothelium-derived vasodilator [17], contributes in the dilator effect of CuZn-SOD, experiments were also performed after preincubation with catalase (1,000 U/ml) with no effect on the relaxant response (data not shown). The threshold concentration of CuZn-SOD to cause a relaxant effect was at around 0.1 U/ml. We further observed that the relaxant effect in response to CuZn-SOD – unlike that in response to acetylcholine – does not occur instantaneously after adding the drug to the bath, but that relaxation shows a slow onset with a lag time of 45–60 s after application to the organ bath. The relaxant effect was initially interpreted as increased NO bioactivity by intracellular scavenging of superoxide anion thereby prolonging the intracellular half-life of NO [18], this, however, would require transport of the protein into the cytoplasm. In view of the observed slow-onset relaxant response, we hypothesized that other mechanisms such as binding of CuZn-SOD to endothelial cells or even uptake into the cells might be involved. Therefore, we next studied the effects of FITC-labeled SOD on cultured human endothelial cells. At 4°C, only binding but no uptake of SOD into the cells was observed. Binding to the cells' surface was 5.5×10^{11} molecules of SOD per 500,000 cells, equaling a SOD concentration of ca. 0.12 U/ml. This concentration is close to the threshold concentration (ca. 0.1 U/ml) found to cause relaxant responses in precontracted arterial rings in organ chamber experiments in our present (fig. 1) and in previous studies [9, 10]. By contrast and compared with experiments performed at 4°C, SOD uptake was observed at 37°C and cell SOD content increased by 31-fold to 1.73×10^{13} molecules of SOD per 500,000 cells, equaling a SOD concentration of ca. 4 U/ml. A SOD concentration of 4 U was

Downloaded from http://karger.com/pha/article-pdf/78/4/198/3420079/000096598.pdf by Universitätsbibliothek Bern user on 21 September 2023

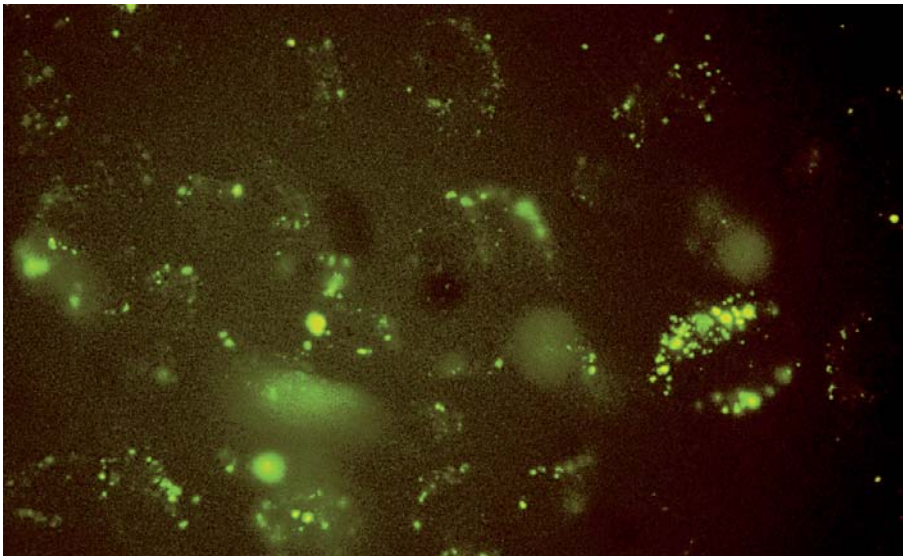

Fig. 2. Representative fluorescent image of human endothelial cells after 5 min of exposure to 40 U/ml of FITC-labeled CuZn-SOD. Internalized FITC-CuZn SOD is seen as fluorescent bright spots in the cytoplasm representing endocytosed vesicles. A part of FITC-CuZn-SOD is adsorbed and bound at the cell membrane.

found to be sufficient to cause about 80% of the maximal relaxant effect of the enzyme in organ chamber experiments at 37°C (fig. 1). Analysis of FITC-SOD-treated human endothelial cells showed uptake of the enzyme into the cell cytoplasm with the greatest fluorescence signal found in proximity to the plasma membrane. Numerous vesicles of different sizes were visible (fig. 2), compatible with a rapid endocytotic process. Fluorescent vesicles were observed after 5 min of incubation, and incubation for 30 min did further increase the fluorescent signal.

Discussion

The findings of the present study support the notion that CuZn-SOD rapidly binds to and crosses endothelial cell membranes, possibly via receptor-mediated endocytosis as has been most recently described for the extracellular isoform of this enzyme [8]. Rapid endocytosis of exogenously added CuZn-SOD into rat hepatocytes was first described almost 20 years ago [19–21] and endocytosis was associated with a 4-fold increase in intracellular enzyme activity [19]. The uptake and effects of SOD in hepatocytes were blocked by cytochalasin B, which typically blocks receptor-mediated uptake of proteins [19]. To our knowledge, until now a receptor mediating cellular binding and uptake of CuZn-SOD has not been identified. Similarly to our study, binding of FITC-labeled CuZn-SOD was observed after only 5 min of incubation in human monocytes [22], and uptake of SOD showed different patterns between monocytes and neutrophils, including perinuclear accumulation in the cytoplasm after 3 h of incubation [22, 23]. Again, CuZn-SOD endocytosis was associated with increased intracellular enzyme activity, leading to a reduction of PMA-induced monocyte superoxide production [23]. Finally, it has been reported that in certain cells not only endocytotic but also exocytotic pathways exist that control bioactivity of CuZn-SOD and involve ATP-sensitive, Golgi-apparatus-mediated mechanisms [24].

Based on findings of the present study we propose that endothelial cell endocytosis via activation of a receptor-mediated pathway – and possibly exocytosis of the protein – contribute to acute and chronic vascular effects of CuZn-SOD which are likely to play a role for vascular tone and atherogenesis [25]. Although CuZn-SOD is present in plasma in humans [3, 4], the physiological and pathophysiological relevance of circulating CuZn-SOD protein is still unclear. Cardiovascular risk factors affect the protective endothelial effects of CuZn-SOD as aging reduces cell binding and the rate of internalization of CuZn-SOD [26] and also attenuates endothelial cell-dependent vasodilator effects of the enzyme [10]. As CuZn-SOD transcriptionally downregulates HMG-CoA reductase [27] it likely also plays a role in lipid metabolism. Thus, the potential role of circulating levels and endothelial cell endocytosis of CuZn-SOD

Downloaded from http://karger.com/pha/article-pdf/78/4/198/3420079/000096598.pdf by Universitätsbibliothek Bern user on 21 September 2023

protein [3] in the context of atherosclerotic vascular disease requires further study and possibly may extend the understanding of this antioxidant enzyme beyond being simply a 'determinant of the bioactivity of nitric oxide' [18, 28, 29].

Acknowledgments

Supported by the Swiss National Foundation (32-58421.99, 32-58426.99/1 and 32-108258/1). The authors are indebted to the staff of Obstetrics and Gynecology, University Hospital Bern, for providing umbilical cords for endothelial cell culture experiments and thank Dr. Elvira Haas for stimulating scientific discussion and her suggestions.

References

1 McCord JM, Fridovich I: Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). J Biol Chem 1969;244:6049–6055.
2 Hjalmarsson K, Marklund SL, Engstrom A, Edlund T: Isolation and sequence of complementary DNA encoding human extracellular superoxide dismutase. Proc Natl Acad Sci USA 1987;84:6340–6344.
3 Marklund SL, Holme E, Hellner L: Superoxide dismutase in extracellular fluids. Clin Chim Acta 1982;126:41–51.
4 Andersen PM, Nilsson P, Forsgren L, Marklund SL: CuZn-superoxide dismutase, extracellular superoxide dismutase, and glutathione peroxidase in blood from individuals homozygous for Asp90Ala CuZu-superoxide dismutase mutation. J Neurochem 1998;70:715–720.
5 Michelson AM, Puget K: Cell penetration by exogenous superoxide dismutase. Acta Physiol Scand 1980;492(suppl):67–80.
6 Mügge A, Elwell JH, Peterson TE, Hofmeyer TG, Heistad DD, Harrison DG: Chronic treatment with polyethylene-glycolated superoxide dismutase partially restores endothelium-dependent vascular relaxations in cholesterol-fed rabbits. Circ Res 1991;69:1293–1300.
7 Freeman BA, Turrens JF, Mirza Z, Crapo JD, Young SL: Modulation of oxidant lung injury by using liposome-entrapped superoxide dismutase and catalase. Fed Proc 1985;44:2591–2595.
8 Chu Y, Piper R, Richardson S, Watanabe Y, Patel P, Heistad DD: Endocytosis of extracellular superoxide dismutase into endothelial cells. Role of the heparin-binding domain. Arterioscler Thromb Vasc Biol 2006;26:1985–1990.
9 Hayashi T, Fukuto JM, Ignarro LJ, Chaudhuri G: Basal release of nitric oxide from aortic rings is greater in female rabbits than in male rabbits: implications for atherosclerosis. Proc Natl Acad Sci USA 1992;89:11259–11263.
10 Barton M, Cosentino F, Brandes RP, Moreau P, Shaw S, Luscher TF: Anatomic heterogeneity of vascular aging: role of nitric oxide and endothelin. Hypertension 1997;30:817–824.
11 Williams SK, Greener DA, Solenski NJ: Endocytosis and exocytosis of protein in capillary endothelium. J Cell Physiol 1984;120:157–162.
12 Rybak ME, Gimbrone MA Jr, Davies PF, Handin RI: Interaction of platelet factor four with cultured vascular endothelial cells. Blood 1989;73:1534–1539.
13 Arnet UA, Novosel D, Barton M, Noll G, Ganten D, Luscher TF: Endothelial dysfunction in the aorta of transgenic rats harboring the mouse Ren-2 gene. Endothelium 1999;6:175–184.
14 Waelti ER, Gluck R: Delivery to cancer cells of antisense L-myc oligonucleotides incorporated in fusogenic, cationic-lipid-reconstituted influenza-virus envelopes (cationic virosomes). Int J Cancer 1998;77:728–733.
15 Tuffin G, Waelti E, Huwyler J, Hammer C, Marti HP: Immunoliposome targeting to mesangial cells: a promising strategy for specific drug delivery to the kidney. J Am Soc Nephrol 2005;16:3295–3305.
16 Mügge A, Riedel M, Barton M, Kuhn M, Lichtlen PR: Endothelium independent relaxation of human coronary arteries by 17β-oestradiol in vitro. Cardiovasc Res 1993;27:1939–1942.
17 Burke-Wolin T, Abate CJ, Wolin MS, Gurtner GH: Hydrogen peroxide-induced pulmonary vasodilation: role of guanosine 3′,5′-cyclic monophosphate. Am J Physiol 1991;261:L393–L398.
18 Ignarro LJ, Buga GM, Wood KS, Byrns RE, Chaudhuri G: Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. Proc Natl Acad Sci USA 1987;84:9265–9269.
19 Kyle ME, Nakae D, Sakaida I, Miccadei S, Farber JL: Endocytosis of superoxide dismutase is required in order for the enzyme to protect hepatocytes from the cytotoxicity of hydrogen peroxide. J Biol Chem 1988;263:3784–3789.
20 Dini L, Rotilio G: Electron microscopic evidence for endocytosis of superoxide dismutase by hepatocytes using protein-gold adducts. Biochem Biophys Res Commun 1989;162:940–944.
21 Dini L, Falasca L, Rossi L, Rotilio G: In vivo uptake of Cu, Zn superoxide dismutase. Morphological evidence for preferential endocytosis and accumulation by sinusoidal liver cells. Cell Mol Biol 1996;42:269–277.
22 Filipe P, Emerit I, Vassy J, Levy A, Huang V, Freitas J: Cellular penetration of fluorescently labeled superoxide dismutases of various origins. Mol Med 1999;5:517–525.
23 Emerit I, Garban F, Vassy J, Levy A, Filipe P, Freitas J: Superoxide-mediated clastogenesis and anticlastogenic effects of exogenous superoxide dismutase. Proc Natl Acad Sci USA 1996;93:12799–12804.
24 Mondola P, Ruggiero G, Seru R, Damiano S, Grimaldi S, Garbi C, Monda M, Greco D, Santillo M: The Cu, Zn superoxide dismutase in neuroblastoma SK-N-BE cells is exported by a microvesicles dependent pathway. Brain Res Mol Brain Res 2003;110:45–51.
25 Faraci FM, Didion SP: Vascular protection: superoxide dismutase isoforms in the vessel wall. Arterioscler Thromb Vasc Biol 2004;24:1367–1373.
26 Dini L, Rossi L, Marchese E, Ruzittu MT, Rotilio G: Age-related changes in the binding and uptake of Cu, Zn superoxide dismutase in rat liver cells. Mech Ageing Dev 1996;90:21–33.
27 De Felice B, Santillo M, Seru R, Damiano S, Matrone G, Wilson RR, Mondola P: Modulation of 3-hydroxy-3-methylglutaryl-CoA reductase gene expression by CuZn superoxide dismutase in human fibroblasts and HepG2 cells. Gene Expr 2004;12:29–38.
28 Mügge A, Elwell JH, Peterson TE, Harrison DG: Release of intact endothelium-derived relaxing factor depends on endothelial superoxide dismutase activity. Am J Physiol 1991;260:C219–C225.
29 Gryglewski RJ, Palmer RM, Moncada S: Superoxide anion is involved in the breakdown of endothelium-derived vascular relaxing factor. Nature 1986;320:454–456.

Downloaded from http://karger.com/pha/article-pdf/78/4/198/3420079/000096598.pdf by Universitätsbibliothek Bern user on 21 September 2023