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Original Contribution

The effects of hypochlorous acid and neutrophil proteases on the structure and function of extracellular superoxide dismutase

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ABSTRACT

Extracellular superoxide dismutase (EC-SOD) is expressed by both macrophages and neutrophils and is known to influence the inflammatory response. Upon activation, neutrophils generate hypochlorous acid (HOCl) and secrete proteases to combat invading microorganisms. This produces a hostile environment in which enzymatic activity in general is challenged. In this study, we show that EC-SOD exposed to physiologically relevant concentrations of HOCl remains enzymatically active and retains the heparin-binding capacity, although HOCl exposure established oxidative modification of the N-terminal region (Met32) and the formation of an intermolecular cross-link in a fraction of the molecules. The cross-linking was also induced by activated neutrophils. Moreover, we show that the neutrophil-derived proteases human neutrophil elastase and cathepsin G cleaved the N-terminal region of EC-SOD irrespective of HOCl oxidation. Although the cleavage by elastase did not affect the quaternary structure, the cleavage by cathepsin G dissociated the molecule to produce EC-SOD monomers. The present data suggest that EC-SOD is stable and active at the site of inflammation and that neutrophils have the capacity to modulate the biodistribution of the protein by generating EC-SOD monomers that can diffuse into tissue.

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Human extracellular superoxide dismutase (EC-SOD)[1] is a homotetrameric metalloenzyme present in the extracellular space. The protein subunit is composed of an N-terminal region involved in the formation of the tetramer, a central region facilitating the dismutation of the superoxide radical ($O_2^{\bullet -}$) into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) via a two-step mechanism involving sequential one-electron reduction and oxidation of a central copper atom, and a C-terminal region known to mediate the binding to ligands in the extracellular space (ECM-binding region) [1]. EC-SOD recovered from tissue is generally found to encompass both the intact subunit and a subunit lacking the ECM-binding region [2,3]. This finding is likely to reflect the presence of native EC-SOD tetramers displaying variable affinity for ECM-binding partners [4] and suggests that removal of the ECM-binding region is used to modulate the distribution of the protein in the extracellular space. Although not described in full detail, the proteolytic removal of the ECM-binding region has been shown to be an intracellular event and possibly regulated by redox conditions [3,5,6].

EC-SOD is the only extracellular enzyme with the ability to remove superoxide. The reaction kinetics are diffusion limited and greatly accelerate the spontaneous dismutation of superoxide, hence maintaining a low level of superoxide under physiological conditions [7]. Under conditions of oxidative stress, EC-SOD has been shown to inhibit the fragmentation of extracellular constituents including syndecan-1, hyaluronan, collagen, and heparan sulfate [8–11]. Moreover, as superoxide reacts very quickly with nitric oxide (NO) to produce the highly reactive peroxynitrite radical ($ONOO^-$), the presence of EC-SOD greatly enhances the bioavailability of nitric oxide [12,13]. Although the enzymatic activity of EC-SOD removes superoxide, it concomitantly generates another oxidant, i.e., hydrogen peroxide, which likewise (if not removed) may oxidize and potentially destroy other biomolecules. Indeed, we have recently shown that EC-SOD itself can be inhibited and fragmented by oxidations introduced by hydrogen peroxide [14]. Moreover, it is evident that hydrogen peroxide is used in diverse biological processes to define a biological response, e.g., by tuning the synthesis of

Abbreviations: CatG, cathepsin G; ECM, extracellular matrix; EC-SOD, extracellular superoxide dismutase; HNE, human neutrophil elastase; PMA, phorbol 12-myristate 13-acetate

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cytokines and growth factors [15]. In addition to protecting biomolecules against superoxide-mediated oxidation, it is therefore plausible that EC-SOD partakes in the orchestration of cellular responses by providing hydrogen peroxide as a source of oxidative capacity. From the reaction stoichiometry, it is clear that the enzymatic or spontaneous dismutation of superoxide will provide less hydrogen peroxide compared to superoxide reacting as an oxidant. However, the binding characteristics of EC-SOD and consequently the biodistribution of the protein may provide a relatively high local concentration of hydrogen peroxide, e.g., on the cell surface. Indeed, EC-SOD associated with the cell surface of endothelial cells has been shown to regulate angiogenesis by providing hydrogen peroxide to oxidize and reversibly inactivate phosphatases involved in cell proliferation and migration [16].

Macrophages and neutrophils are known to express EC-SOD [17,18], and we have recently shown that EC-SOD expressed by macrophages is associated with the cell surface but secreted only upon cellular activation induced by lipopolysaccharide [19]. However, the cellular activity of EC-SOD expressed by inflammatory cells is currently not well described. Several studies using models of bacterial infection [20,21], skin inflammation [22,23], and ischemia [24] provide evidence that EC-SOD modulates the expression and distribution of receptors and cytokines central to the inflammatory response. Hence, in models of pulmonary inflammation it has been established that overexpression of EC-SOD attenuates the inflammatory response by reducing the influx of neutrophils into the lung [9,25,26]. However, if inflammatory conditions are established in the lung, cleaved EC-SOD can be recovered from the bronchoalveolar space [27–30]. The basis for this observation was suggested to reflect limited proteolysis removing the ECM-binding region and hence allowing the protein to diffuse into this compartment. It is not clear whether this observation is supported by increased intracellular processing established in inflammatory cells producing EC-SOD or whether it resides under inflammatory conditions in the extracellular space facilitating proteolysis.

Because EC-SOD is involved in the inflammatory response, it is important to know how inflammation affects the properties of the protein. The major extracellular products generated by activated neutrophils are hypochlorous acid (HOCl) and proteases including cathepsin G (CatG) and human neutrophil elastase (HNE). Here we show that although EC-SOD is oxidized by HOCl the enzymatic activity is unaffected even at high concentrations ($<300\ \mu M$), suggesting that the enzyme is functional under inflammatory conditions. In addition, we find that both CatG and HNE cleave the N-terminal region of EC-SOD with no discernible effect of prior HOCl-mediated oxidation. Interestingly, although cleavage by HNE did not affect the quaternary structure of EC-SOD, the cleavage by CatG produced monomers, as evaluated by size-exclusion chromatography. Collectively, the present data show that EC-SOD has the structural and functional capacity to be an active player under inflammatory conditions established to combat invading microorganisms.

Materials and methods

Materials

Human EC-SOD was purified from aortic tissue by heparin-affinity chromatography and anion-exchange chromatography as previously described [31]. Bovine Cu/Zn-SOD was purchased from Sigma. Isolated HNE and CatG were generously provided by Professor Jan Potempa, Jagiellonian University, Krakow, Poland. Antibodies directed against human EC-SOD were purchased from Abcam (murine MAb 4G11G6) or produced in-house (rabbit anti-EC-SOD). GammaBind G Sepharose was obtained from GE Healthcare. Reagent hypochlorous acid was obtained from VWR. The concentration of diluted HOCl was verified by using the extension coefficient for OCl^- of $\varepsilon_{292}=350\ M^{-1}\ cm^{-1}$ under alkaline conditions.

Oxidation of purified EC-SOD

Purified EC-SOD (2 μM) was diluted 1:4 in phosphate-buffered saline (PBS) containing increasing amounts of HOCl prepared immediately before the reaction at concentrations as indicated. The oxidation was allowed to proceed for 30 min at 37 °C and quenched by the addition of 5 mM methionine.

SOD activity assay

The activity of EC-SOD exposed to HOCl was evaluated using the cytochrome *c* assay [32] modified for use in a 96-well plate format as described before [14]. In brief, samples containing 0.5 μM Cu/Zn-SOD or EC-SOD exposed to HOCl at the indicated concentrations were diluted 10-fold in 50 mM $NaHCO_3$, 0.1 mM EDTA, pH 10, containing 0.1 mM xanthine and cytochrome *c* and 100 μl was added to the wells of a microtiter plate. As a negative control, wells received 100 μl of reaction mixtures without SOD protein. To initiate the reaction, 100 μl of 50 mM $NaHCO_3$, 0.1 mM EDTA, pH 10, containing cytochrome *c* and xanthine oxidase was added and the increase in absorbance at 550 nm measured between 0 and 2 min at intervals of 20 s using an EnSpire 2300 multimode plate reader (PerkinElmer). The SOD activity was evaluated as $\Delta Abs\ min^{-1}$ and the relative activity determined by defining the activity of the sample containing no HOCl as 100%.

SDS–PAGE and protein visualization

Reaction products were separated using uniform 10% polyacrylamide gels and the glycine/2-amino-2-methyl-1,3-propanediol–HCl buffer system [33]. For analysis of proteins under reducing conditions, the samples were boiled in the presence of 0.5% (w/v) SDS and 50 mM dithiothreitol before electrophoresis. For Western blotting, separated proteins were electrophoretically transferred to a polyvinylidene difluoride membrane for 1 h at 150 mA in 10 mM 3-(cyclohexylamino)-1-propane sulfonic acid/10% ethanol, pH 11 [34]. The membranes were blocked with 5% (w/v) skimmed milk in 20 mM Tris–HCl, 137 mM NaCl, pH 7.4 (TBS) supplemented with 0.1% Tween 20, and EC-SOD protein was detected by using a rabbit anti-EC-SOD antiserum. Blots were developed by enhanced chemiluminescence using peroxidase-conjugated goat anti-rabbit Ig (DAKO) and data acquired using an ImageQuant LAS 4000 instrument (GE Healthcare). For activity staining, samples were prepared in sample buffer omitting both reducing agent and boiling before electrophoresis. Electrophoresis was conducted using buffer containing no SDS. After electrophoresis the gel was rinsed in water and equilibrated in nitroblue tetrazolium, riboflavin, and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine and the SOD activity detected by exposure to light as previously described [31].

Heparin enzyme-linked immunosorbent assay (ELISA)

To evaluate the heparin binding capacity of HOCl-oxidized EC-SOD, we used an ELISA essentially as described before [35]. Briefly, microtiter wells (MaxiSorp, Nunc) were coated with 50 ng heparin–bovine serum albumin (BSA) in 100 μl of 50 mM $NaHCO_3$, pH 9.6, overnight at room temperature. The heparin–BSA was prepared by conjugating heparin to BSA using sodium cyanoborohydride [36]. The wells were emptied and residual binding sites blocked by the addition of 200 μl 0.1% (w/v) BSA in TBS. The wells were incubated at 23 °C, washed three times using TBS containing 0.05% Tween 20 (TBST), and incubated with a twofold dilution series of controls and EC-SOD (3 μM) oxidized by 0.2 mM HOCl in TBST as

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