Platelet-Endothelial Cell Adhesion Molecule-1-Directed Endothelial Targeting of Superoxide Dismutase Alleviates Oxidative Stress Caused by Either Extracellular or Intracellular Superoxide

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ABSTRACT

Targeting of the antioxidant enzyme catalase to endothelial cells protects against vascular oxidative stress induced by hydrogen peroxide (H$_2$O$_2$) (Am J Physiol 285:L283–L292, 2003; Nat Biotechnol 21:392–398, 2003; Am J Physiol 293:L162–L169, 2007). However, another reactive oxygen species, superoxide anion, is also involved in many forms of vascular oxidative stress, including ischemia/reperfusion, hypertension, and inflammation. To protect endothelium against superoxide attack, we designed and tested antibody-directed targeting of superoxide dismutase (SOD) to the endothelial surface determinant, platelet-endothelial cell adhesion molecule (PECAM)-1. We synthesized anti-PECAM/SOD conjugates that retained 70% of enzymatic activity (superoxide anion dismutation) and specifically bound to endothelial cells, but not PECAM-negative cells. The effect of anti-PECAM/SOD delivery to cells was tested in two distinct models of oxidative stress induced by either extracellular or intracellular generation of superoxide anion. In the first model, anti-PECAM/SOD, but not unconjugated SOD, protected endothelial cells against injury caused by superoxide produced in the medium by hypoxanthine-xanthine oxidase. At the optimal dose, anti-PECAM/SOD provided up to 40 to 50% protection against cell death in this model. In the second model, anti-PECAM/SOD at the optimal dose provided complete protection against necrosis caused by paraquat-induced intracellular superoxide generation. Endothelial targeting of SOD represents a new molecular antioxidant approach that could be used for the management of vascular oxidative stress.

Oxidative stress induced by a surplus of reactive oxygen species (ROS) plays an important role in human vascular pathology (Cai et al., 2003). Great efforts have been dedicated to the development of antioxidant molecular interventions capable of detoxifying ROS and thus alleviating vascular oxidative stress (for review, see Greenwald, 1990; Muzykantov, 2001). Nonenzymatic antioxidants, including scavengers of ROS and oxidized molecules or donors of reducing equivalents (e.g., glutathione precursors), may provide some degree of protection in chronic settings, but these agents are consumed in the protective reactions and not sufficiently potent to offset damage in acute severe oxidative stress (Greenwald, 1990; Christofidou-Solomidou and Muzykantov, 2006).

Enzymes, such as superoxide dismutase (SOD), which converts superoxide anion into H$_2$O$_2$ and catalase, which decomposes H$_2$O$_2$ into water and oxygen, represent more potent antioxidants capable of detoxifying unlimited number of ROS molecules (Mccord and Fridovich, 1969; Mccord, 2002). Unfortunately, these antioxidant enzymes have very limited medical utility due to inadequate vascular delivery, in part due to lack of targeting to the cells suffering oxidative stress (Greenwald, 1990; Muzykantov, 2001; Christofidou-Solomidou and Muzykantov, 2006). In particular, endothelial cells lining the luminal surface of blood vessels are susceptible to ROS-induced injury and represent an important therapeutic target in all forms of...

ABBREVIATIONS: ROS, reactive oxygen species; SOD, superoxide dismutase; PECAM, platelet-endothelial adhesion molecule; HX, hypoxanthine; NHS-LC-biotin, succinimidyl-6-[biotinamido]hexanoate; HABA, 4'-hydroxyazobenzene-2-carboxylic acid; HUVEC, human umbilical vein endothelial cell; PBS, phosphate-buffered saline; BXT-01050, 5,6,6a,11b-tetrahydro-3,9,10-trihydroxybenzo[c]fluorine; XO, xanthine oxidase; PAGE, polyacrylamide gel electrophoresis; LDH, lactate dehydrogenase; SA, streptavidin.
vascular oxidative stress (Terada et al., 1992; Houston et al., 1999; Muzykantov, 2001; Cai et al., 2003; Guo et al., 2007). Thus endothelial targeting of antioxidant enzymes may provide more potent antioxidant interventions. Recent studies showed that conjugating catalase with antibodies directed to endothelial surface determinants (i.e., vascular immunotargeting) provides targeted delivery of an active catalase into endothelium, resulting in protection against \( \text{H}_2\text{O}_2 \)-induced injury in diverse models of oxidative stress (Christofidou-Solomidou et al., 2003; Kozo et al., 2003; Nowak et al., 2007).

A particularly good EC molecule for antibody-targeting is platelet-endothelial cell adhesion molecule (PECAM)-1 (CD31), a molecule that is constitutively and stably expressed on the endothelial lumen at the level of approximately a million copies per cell and is involved in transcytosis of activated leukocytes via endothelium in inflammation (Scherpereel et al., 2002). Conjugation of diverse therapeutic cargoes and carriers with anti-PECAM antibodies provides robust intracellular drug delivery into endothelium (Li et al., 2000; Muro et al., 2003b). Inhibition of leukocyte transmigration by PECAM blocking may provide a secondary benefit in the context of vascular oxidative stress (Muzykantov, 2005). Therefore, PECAM-1 is a good target determinant for endothelial delivery of antioxidants. In fact, anti-PECAM/catalase conjugates provide effective protection against endothelial injury caused by \( \text{H}_2\text{O}_2 \) (Christofidou-Solomidou et al., 2003; Kozo et al., 2003).

However, an additional ROS, superoxide anion, is also involved in many types of vascular oxidative stress including ischemia/reperfusion, hypertension, stroke, infarction, and inflammation (Jia and Furchgott, 1993; Bonaventura and Gow, 2004; Loomis et al., 2005). In particular, superoxide anion, but not hydrogen peroxide, is the key culprit in vascular disorders associated with inactivation of NO by superoxide anion produced by endothelial NADPH-oxidase abnormally activated in response to angiotensin II and other proconstrictive mediators (Cai et al., 2003). To protect endothelium against superoxide attack in this and other relevant settings, we designed and tested targeting of Cu,Zn-SOD to PECAM-1. We characterized salient features of anti-PECAM/SOD conjugates (activity, size, and binding to endothelial cells) and tested the protective effect of conjugates against oxidative stress caused by extracellular and intracellular production of superoxide anion.

Materials and Methods

Materials and Cells. Cytochrome c, xanthine oxidase, hypoxanthine (3,7-dihydropurin-6-one; HX), xanthine (3,7-dihydro-purine-2,6-dione), allopurinol (4-hydroxy-2-pyrazolo[3,4-d]pyrimidine), dimethylformamide, and fetal bovine serum were purchased from Sigma (St. Louis, MO). Cu,Zn-SOD from bovine liver, streptavidin, and SOD-525 assay kit were from Calbiochem (San Diego, CA). Radiosotope-containing sodium iodide (Na\(^{125}\text{I}\)) and sodium chromate (Na\(^{51}\text{Cr} \text{O}_4 \)) were obtained from PerkinElmer (Wellesley, MA). Succinimidyl-6-[biotinamido]hexanoate (NHS-LC-biotin), 4'-hydroxyazobenzene-2-carboxylic acid (HABA) assay, and iodogen were from Pierce Biotechnology (Rockford, IL). CytoTox 96 NonRadioactive Cytoxicity Assay was purchased from Promega (Madison, WI). Anti-PECAM mAb 62, a monoclonal mouse IgG\(_1\)-\(\gamma\) antibody directed against human platelet-endothelial cell adhesion molecule-1, has been characterized in our previous studies (Muzykantov et al., 1999; Scherpereel et al., 2002). All cell culture medium components were from Life Technologies (Gaithersburg, MD) unless otherwise noted. Rat IgG was from Jackson ImmunoResearch Laboratory, Inc. (West Grove, PA). Human umbilical vein endothelial cells (HUVECs) were purchased from Clonetics (San Diego, CA). REN cells, a human mesothelioma cell line previously isolated in our laboratories, were transfected with full-length mouse PECAM (REN/PECAM cells) as described previously (Muzykantov et al., 1999). These two cell lines, PECAM-positive REN/PECAM and PECAM-1 negative REN, forming an endothelioid-like monolayer provide an ideal cell-type-matched control for the specificity of targeting and effects of anti-PECAM conjugates without potential confounding effects of different responses of various cell types to ROS, as described in our previous studies (Muzykantov et al., 1999).

Biotinylation of Proteins and Biotin Measurement. Proteins were biotinylated with NHS-LC-biotin freshly dissolved in dimethylformamide to a concentration of 0.1 M. SOD was biotinylated at 15:1 NHS-LC-biotin/protein molar excess. After 2 h of incubation on ice, free biotin was removed by dialysis against PBS. Protein-attached biotin was measured by HABA assay as we described previously (Shuvaev et al., 2004).

Measurements of SOD Activity. Activity of SOD was measured using two assays: ferricytochrome c assay (McCord and Fridovich, 1969) and SOD-525 assay (Nebot et al., 1993). Cytochrome c assay uses xanthine and xanthine oxidase as a source of superoxide anion and cytochrome c as the indicating scavenger of the radical competing with SOD. Working solution (0.6 ml) contained 50 mM phosphate buffer, pH 7.8, 0.1 mM EDTA, 50 \(\mu\)M xanthine, 20 \(\mu\)M cytochrome c, and 10 \(\mu\)l of sample. Reaction was initiated by the addition of 10 \(\mu\)l of 0.2 U/ml xanthine oxidase, and the absorbance was monitored at 550 nm using a Cary 50 spectrophotometer (Varian, Palo Alto, CA). One unit of SOD is defined as the amount of enzyme that inhibits the rate of cytochrome c reduction by 50\% at pH 7.8 and 25°C. Another assay, SOD-525, utilizes BXT-01050, which undergoes alkaline autooxidation and is accelerated by SOD (Nebot et al., 1993). Autoxidation yields a chromophore that absorbs at 525 nm. One SOD-525 activity unit is defined as the amount of enzyme that doubles the autoxidation rate of the control blank. Each measurement was performed at least three times, and results were expressed as mean ± S.D.

SOD Radiolabeling. Biotinylated SOD was iodinated with Na\(^{125}\text{I}\) (PerkinElmer) using Iodogen (Pierce) as recommended by the manufacturer. An excess of free iodine was removed by gel-filtration chromatography using Bio-Spin 6 Chromatography columns (Bio-Rad Labs, Hercules, CA), whereas iodinated b-SOD was transferred to PBS.

Preparation of Immunocoujugates. Biotinylated SOD was conjugated with biotinylated anti-PECAM or nonimmune IgG via streptavidin as described in detail for other cargo enzymes including glucose oxidase, catalase, and \(\beta\)-galactosidase (Muzykantov et al., 1999; Scherpereel et al., 2001; Shuvaev et al., 2004). Molar ratios of enzyme to antibody were kept 1:1. The effective diameter of the obtained conjugates was measured by a Dynamic light-scattering apparatus 90Plus Particle Sizer (Brookhaven Instruments Corporation, Holtsville, NY). Throughout the study, we used conjugates with a mean diameter of 300 nm. Radiolabeled anti-PECAM/SOD conjugates were prepared by introducing from 5 to 10 mol% \(^{125}\text{I}\)-SOD to unlabeled SOD before its conjugation.

Cell Culture. HUVECs were maintained in M199 medium (GIBCO, Grand Island, NY) with 15% fetal bovine serum supplemented with 100 \(\mu\)g/ml heparin (Sigma), 2 mM L-glutamine (GIBCO), 15 \(\mu\)g/ml endothelial cell growth supplement (Upstate, Lake Placid, NY), 100 U/ml penicillin, and 100 \(\mu\)g/ml streptomycin (GIBCO). REN human mesothelioma cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (Sigma), 2 mM L-glutamine, 100 U/ml penicillin, and 100 \(\mu\)g/ml streptomycin. Cell sensitivity to ROS depends on their confluence. To equalize for this factor, we used confluent cells throughout the study.

Binding of Anti-PECAM/SOD Conjugate to Endothelial Cells. HUVECs and REN cells were plated in 24-well culture dishes and grown to confluent culture. Radiolabeled conjugates were added...
to confluent cells at serial dilutions. Cells were incubated at 37°C for 1 h, unbound conjugates were washed out, and cells were lysed with 1% Triton X-100 and 1.0 M NaOH. Bound radioactivity was measured using a Wallac 1470 Wizard gamma counter (Gaithersburg, MD). Bound material was expressed as nanograms of SOD per well. Each point was performed in quadruplicates, and results were expressed as mean ± S.E.M.

A Model of Endothelial Oxidative Stress Induced by HX-Xanthine Oxidase. HUVECs were plated at 7 × 10⁴ cells per well in 24-well culture dishes and grown to confluent culture. Superoxide anion production was initiated by adding xanthine oxidase (25 mU/ml) to the wells with confluent cells containing medium supplemented with HX. In one setting, the medium was Hank’s balanced salt solution supplemented with 20 mM HEPES, pH 7.2, to maintain pH. Cells were incubated in the presence of HX-xanthine oxidase (XO) for 20 min, washed, and incubated with fresh Hank’s balanced salt solution for 5 h at 37°C. In another setting, the complete HUVEC medium was used, and cells were incubated with HX-XO at 37°C for 24 h without removal of xanthine oxidase. Xanthine oxidase is known to be able to bind to cellular glycosaminoglycans of endothelial cells, which decreases its sensitivity to inhibitors (Kelley et al., 2004). To test whether xanthine oxidase anchored to cells is a source of toxic oxidants in our model, cells were pretreated with xanthine oxidase, washed out, and exposed to HX. No cell injury or apoptosis was detected in these conditions (data not shown). Therefore, in this model, cells are affected predominantly by oxidants produced by HX-XO in the medium.

Western Blot Analysis of Apoptotic Activation of Procaspase in Endothelial Cells. Cells in 24-well culture dishes (approximately 100,000 cells per well) were washed twice with PBS and lysed in 100 µl of sample buffer for SDS-PAGE. Cell culture medium was centrifuged at 1000 rpm (Eppendorf centrifuge; Eppendorf AG, Hamburg, Germany), and the cell pellet was pooled with total cell lysate. Cells proteins were subjected to SDS-PAGE. Gels were transferred to a polyvinylidene difluoride membrane (Millipore Corporation, Billerica, MA) using a semidry transfer unit (GE Healthcare, Little Chalfont, Buckinghamshire, UK). After protein transfer, polyvinylidene difluoride membrane was blocked with 10% nonfat dry milk in Tris-buffered saline/Tween 20 (100 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween 20) for 1 h, and the membrane was treated with primary antibody overnight and corresponding horseradish peroxidase-labeled secondary antibody. The blot was washed three times with Tris-buffered saline/Tween 20, and bound horseradish peroxidase was detected using ECL Plus reagents (GE Healthcare). Procaspase-3 cleavage was assessed by degradation of 32-kDa procaspase-3 after 15% SDS-PAGE using anti-caspase-3 antibody (clone E-8; Santa Cruz Biotechnology, Santa Cruz, CA) followed by reaction with horseradish peroxidase-conjugated anti-mouse antibody. In some cases, active caspase 3 was visualized with biotin-labeled anti-caspase3 antibody (clone 31A1067; Imgenex, San Diego, CA), followed by reaction with horseradish peroxidase-conjugated streptavidin.

Analysis of Endothelial Cell Death. We have assayed cell death using three methods optimally suited for specific models. In a relatively long-term (24-h incubation) apoptosis study, we tested cell survival/death using a fluorescent microscopy kit (see next paragraph). Results of this semiquantitative analysis correlated well with the data using Western blotting analysis. To analyze cell death in short-term (5 h) necrosis studies, we tested release of ⁵¹Cr from the cells prelabeled with Na⁺⁵¹CrO₄ (see below). This simple high-throughput assay provides objective and accurate quantitative analysis of cell death. However, this analysis is suboptimal for analysis of cell death in longer experiments due to a high background of spontaneous leakage of isotope from undamaged cells. To obtain objective and accurate quantitative analysis of cell death, while avoiding this shortcoming in the studies of the paracquat toxicity involving 24-h incubation, we measured lactate dehydrogenase (LDH) release since there is a low background of spontaneous LDH release from undamaged cells (see next section).

Cell survival was visualized using the Live/Dead Viability/Cytotoxicity Kit “for mammalian cells” (Molecular Probes, Eugene, OR) in accordance with the manufacturer’s recommendations. After the treatment, cells were washed and incubated with 0.1 µM calcein-AM and 1 µM ethidium homodimer for 30 min at 37°C.

Endothelial cell death was also determined by the specific release of ⁵¹Cr from the cells prelabeled with Na⁺⁵¹CrO₄ (200,000 cpm/well, added 24 h before the experiment) as described elsewhere (Muzikantov et al., 1999). After exposure of cells to 200 µM HX and 25 mM/ml xanthine oxidase for 20 min at 37°C, they were washed and incubated for an additional 5 h, the time necessary to reveal irreversible damage to the plasmalemma, manifested as leakage of ⁵¹Cr-labeled intracellular components. The 200-µl aliquots of the supernatant medium were collected. Total radioactivity remaining in the wells was determined by cell lysis with 0.5 ml of 1% Triton X-100 and 1.0 M NaOH and measurement of extracted radioactivity in a Wallac 1470 Wizard gamma counter. Cellular injury was expressed as the percentage of the total ⁵¹Cr radioactivity released into the supernatant medium. Each point was performed in quadruplicate, and results were expressed as mean ± S.E.M.

Paraquat Cytotoxicity. REN and REN-hPECAM cells were plated on 96-well culture dishes. Confluent cells were treated with 5 mM paraquat for 24 h, and cell viability was measured by LDH release using CytoTox 96 NonRadioactive Cytotoxicity Assay (Promega). Protective effects of immunoconjugates were studied by incubation of cells with the formulation for 1 h, washing out the unbound fraction following exposure of cells to paraquat.

Cell Protection by Anti-PECAM/SOD. In protection experiments, confluent cells plated in 24-well culture dishes were pretreated with anti-PECAM/SOD for 1 h at 37°C in regular cell medium. Unbound material was washed out, and cells were subjected to oxidative stress as described above. The protective effect of conjugates was expressed as percentage of protection compared with untreated cells. All present values are mean ± S.E.M. of four repeats.

Results

Chemical Modification of SOD. We conjugated CuZn-SOD to anti-PECAM antibodies using a streptavidin (SA) cross-linker, as described previously for catalase conjugation (Shuvaev et al., 2004). Anti-PECAM mAb 62 was biotinylated at the level of coupling of approximately four to five biotin residues per molecule, as in previous studies (Christofidou-Solomidou et al., 2003; Shuvaev et al., 2004). To define the optimal extent of SOD biotinylation for conjugation, we modified SOD with NHS-LC-biotin at diverse molar ratios and tested the enzymatic activity of the resultant biotinylated SOD, h-SOD, and anti-PECAM/SA/SOD conjugates (Fig. 1A). HABA assay showed a linear correlation between biotin ester input and the extent of biotinylation of SOD primary amino groups (Fig. 1A). Denaturing electrophoresis analysis showed no change in mobility of biotinylated SOD (Fig. 1A, insets). This compares well with the rather trivial reduction of enzymatic activity of biotinylated SOD measured using cytochrome c assay (Fig. 1A). At a relatively high extent of biotinylation (≥11 biotin residues per SOD molecule), SOD activity decreased by 20%. The yield of the reaction, calculated as a fraction of coupled biotin to total added biotin linker (NHS-LC-biotin), was estimated to be 21 ± 4% in the experimental conditions. In the subsequent studies, we used SOD biotinylated to the extent of six to eight biotin residues per SOD.

Synthesis and Characterization of Anti-PECAM/SOD Conjugate. To form conjugates of controlled size, we varied the molar ratios between streptavidin and biotinylated proteins and used dynamic light-scattering analysis to measure...
the effective diameter of resultant conjugates, as described in our previous studies of catalase conjugates (Shuvaev et al., 2004). This study showed that molar ratio anti-PECAM/SA/SOD equal to 1:2:1 yielded b-anti-PECAM/SA/b-SOD conjugates (indicated thereafter as anti-PECAM/SOD) with mean diameters of ~300 nm. Previous studies showed that this size is optimal for PECAM-directed drug targeting to endothelial cells (Christofidou-Solomidou et al., 2003; Muro et al., 2003a; Shuvaev et al., 2004).

Measurement of the enzymatic activity of the prepared anti-PECAM/SOD conjugates using the SOD-525 assay (detecting the oxidation of synthetic substrate) showed that SOD lost 65% of its activity after conjugation with anti-PECAM (Fig. 1B, left bars). In contrast, the cytochrome c assay (detecting decomposition of superoxide) showed only ~25% reduction of SOD activity in the conjugate (Fig. 1B, right bars).

### Binding of Anti-PECAM/SOD to Endothelial Cells.

We compared anti-PECAM/125I-SOD binding with HUVECs versus PECA-negative REN cells by incubating the conjugates with confluent cells for 1 h at 37°C, followed by elimination of nonbound materials. Anti-PECAM/SOD bound to HUVECs but not to control REN cells (Fig. 1C). Scatchard transformation showed that the maximal binding capacity of HUVECs was ~4.3 × 10^7 SOD molecules per cell, i.e., anti-PECAM/SOD conjugate thus delivered approximately 2.25 pg of SOD per cell (Fig. 1C, inset). In this model, effectiveness of anti-PECAM/SOD binding to endothelial cells versus REN cells approached 5 versus <0.1% of added SOD, respectively. Thus, anti-PECAM/SOD binds to endothelial cells specifically and with high capacity.

### Anti-PECAM/SOD Alleviates Endothelial Toxicity Induced by Extracellular Oxidative Stress.

To test whether anti-PECAM/SOD targeting protects cells against oxidative stress caused by extracellular superoxide, we exposed endothelial cells to xanthine oxidase, the enzyme that oxidizes HX and xanthine and produces superoxide anion. To detect endothelial apoptosis, we measured the levels of procaspase-3, poly(ADP-ribose) polymerase, and Rh-associated kinase (ROCK)-I in the cell lysates by Western blotting. Incubation of endothelial cells for 24 h with 25 mU/ml xanthine oxidase in the presence of 10 to 100 μM HX caused apoptosis in a dose-dependent manner; thus, the procaspase-3 level was markedly diminished 24 h after exposure to an HX level above 20 μM (Fig. 2A). The pattern and dose dependence on HX of poly(ADP-ribose) polymerase and ROCK I cleavage were similar to that of procaspase-3 (data not shown). Xanthine oxidase alone did not cause detectable apoptosis. To test endothelial protection by anti-PECAM/SOD against apoptosis, we used 100 μM HX. Cells treated with anti-PECAM/SOD, but not with free SOD, showed a significant protection against HX-XO-induced apoptotic conversion of procaspase-3, achieving a maximal level of 45% protection by this parameter at optimal dose (Fig. 2B). This amplitude of the anti-PECAM/SOD protective effect (~50% protection) has been confirmed by analysis of cell survival by staining cells with the Live/Dead Viability/Cytotoxicity Kit (Fig. 2C). Xanthine oxidase inhibitor allopurinol at 100 μM completely protected cells, confirming that the injury is due to enzymatic activity of xanthine oxidase (Fig. 2C).

In the next series of experiments, we tested the protection by anti-PECAM/SOD against xanthine oxidase-induced necrosis, caused by more acute superoxide influx. In this study, cells were exposed to xanthine oxidase with higher concentrations of HX substrate for 20 min followed by replacement with regular FCS-containing HUVEC medium. Necrotic cell death was detected 5 h later by ^51^Cr release, reflecting irreversible membrane damage. Under these conditions, xanthine oxidase caused ~40% necrosis at high (200 μM) HX levels (Fig. 3A). Data from ^51^Cr release from prelabeled cells
were confirmed by examination of the cells in a phase-contrast microscope, revealing characteristic morphological changes including disruption of the monolayer, rounding, and detachment of the cells (Fig. 3A, inset). Specificity of the xanthine oxidase-mediated damage was verified by nearly complete cell protection by allopurinol (Fig. 3B, left bar). Treatment of cells with anti-PECAM/SOD, but not free SOD, protected cells against necrotic injury induced by extracellularly produced superoxide by 40% (Fig. 3B). This effect was not due to engagement of and signaling via endothelial PECAM-1 because control anti-PECAM/streptavidin conjugate provided no protective effect (Fig. 3B). Incomplete protection by anti-PECAM/SOD can be explained by the accumulation of H2O2 in the medium, which may result in the hydroxyl radical attacking the plasma membrane or diffusing into the cells and giving rise to injurious oxidants. Consistent with this notion, a detoxification of both superoxide anion and hydrogen peroxide by tandem anti-PECAM/SOD/catalase conjugate afforded complete protection against oxidative stress induced by extracellular xanthine oxidase (Fig. 3B).

Anti-PECAM/SOD Alleviates Endothelial Toxicity Induced by Intracellular Oxidative Stress. To induce intracellular production of reactive oxygen species, we treated endothelial cells with paraquat. Paraquat permeates the plasma membrane, enters cells, and produces superoxide anions inside the cell via redox cycling using NADH-dependent diaphorases (such as nitric oxide synthase or xanthine oxidase) and subsequent oxygen reduction (Day et al., 1995). To test specific protection by anti-PECAM/SOD in PECAM-1-expressing cells versus control cells, we utilized stably PECAM-1 transfected human endothelium-like mesothelioma REN cells, which naturally do not express PECAM-1 (i.e., REN/PECAM versus REN cells) and tested release of LDH, which reflects irreversible plasma membrane damage. LDH analysis was used in this series since high level of spontaneous release of 51Cr at 24 h incubation convolutes analysis of
cell death under conditions requiring relatively prolonged treatment, such as in the case of paraquat (see below).

Incubation of REN cells with paraquat for 24 h caused dose-dependent cellular damage as shown by LDH release (Fig. 4A). REN and REN/PECAM cells displayed similar sensitivity to paraquat-induced injury (data not shown). Paraquat injury was inhibited by N^r-nitro-L-arginine methyl ester and allopurinol by 20 and 14%, respectively (Fig. 4A, inset). This effect was probably due to inhibition of the NADH-dependent intracellular enzymes nitric oxide synthase and endogenous intracellular xanthine oxidase, respectively. However, treatment of REN/PECAM cells with anti-PECAM/SOD conjugates provided effective protection against the toxic effect of an otherwise lethal dose of 5 mM paraquat, reaching 100% protection, at the optimal dose of anti-PECAM/SOD (Fig. 4B), whereas anti-PECAM/SOD did not protect PECAM-negative control REN cells.

Discussion

Abnormal overproduction of ROS (including superoxide anion and hydrogen peroxide) in the vasculature is involved in pathogenesis of many human disease conditions (Greenwald, 1990; Cai et al., 2003; Bonaventura and Gow, 2004). In particular, both acute (e.g., acute lung injury, hyperoxia, ischemia/reperfusion injury, inflammation, and complications of organ transplantation) and chronic (e.g., hypertension, diabetes, and atherosclerosis) vascular disorders are associated with abnormally high level of superoxide anion produced by activated leukocytes and endothelial and smooth muscle cells by pathways including NADPH-oxidase, xanthine oxidase, and mitochondrial respiratory chain (Terada et al., 1992; Mc Cord, 2002; Christofidou-Solomidou and Muzykantov, 2006).

Superoxide anion is not a strong oxidant, but reacting with \(H_2O_2\), the product of superoxide dismutation, it forms the highly reactive and damaging hydroxyl radical, whereas superoxide reaction with NO gives rise to a strong nitrating agent, peroxinitrate, and results in NO depletion, leading to the hypertensive and prothrombotic state characteristic of vascular oxidative stress (Cai et al., 2003; Bonaventura and Gow, 2004). SODs are the family of metal-containing enzymes that catalyze superoxide dismutation into \(H_2O_2\) and \(O_2\), thus alleviating these aspects of oxidative stress (Mc Cord, 2002). There are three types of SOD in humans, dimeric cytosolic CuZnSOD (SOD1, 32 kDa), tetrameric mitochondrial MnSOD (SOD2, 86–88 kDa), and tetrameric extracellular SOD (SOD3, 135 kDa), that bind to negatively charged components of cellular glycocalyx (McCord, 2002; Kinnula and Crapo, 2003).

Administration of SOD for augmentation of antioxidant defense was proposed almost 4 decades ago and has been explored in numerous studies (Beckman et al., 1988; Muzykantov, 2001). Unfortunately, the rapid clearance of native SOD, due to fast renal uptake, does not favor its use in most settings (Giri and Misra, 1984). Diverse chemical modifications of SOD have been designed to prolong the circulation time and improve its delivery, including coupling with polyethylene glycol, mannose, succinylate, and putrescine encapsulation into liposomes (Freeman et al., 1983; Fujita et al., 1992; Corvo et al., 1999). Some of these derivatives (e.g., polyethylene glycol -SOD, and some SOD mimetics) showed enhanced potency in animal models of systemic (e.g., sepsis) and focal oxidative stress (Tamura et al., 1988; Day et al., 1995; Corvo et al., 1999; Duann et al., 2006).

Considerable efforts have also been devoted to optimize SOD delivery to endothelial cells, arguably one of the most important therapeutic targets in vascular oxidative stress. For example, lectinized SOD was shown to have increased affinity to endothelial cells (Igarashi et al., 1994; Koo et al., 2001). Chimeric SOD2/3 was synthesized as a fusion gene product of mitochondrial MnSOD (SOD2) and C-terminal heparin-binding tail of EC-SOD (SOD3) that delivered SOD activity to heparan sulfates of cellular surface (Gao et al., 2003; Bonder et al., 2004). Recently, a fusion protein that consists of bacterial Fe-SOD and single-chain variable fragment LC-1 anti-lung adenocarcinoma antibody was shown to keep the activities of both enzymatic and affinity moieties (Lu et al., 2006). However, these SOD derivatives represent monomolecular compounds. This factor favors fast clearance from the circulation, restricts the drug load, and does not favor intracellular delivery. Therefore, delivery of SOD to endothelial cells, which may be necessary for effective interception of intracellular superoxide (Jiang et al., 2007), remains rather suboptimal. Modern drug delivery strategies based on targeting conjugated drugs to cellular receptors providing specific delivery and endocytosis of cargoes repre-
sent a promising avenue for this goal (Muzykantov et al., 1999; Muro et al., 2003b).

Earlier, we have reported specific, efficient, and safe targeting of catalase and other therapeutic and reporter enzymes conjugated with PECAM antibodies to the interior of endothelial cells (Muzykantov et al., 1999; Scherpereel et al., 2002; Muro et al., 2003a, 2006). Synthesis of multimolecular, multivalent anti-PECAM conjugates with a diameter of 200 to 400 nm endows conjugated enzymes with high affinity to endothelium and enables their uptake via a unique endocytic pathway, cell adhesion molecule-mediated endocytosis induced by clustering of endothelial PECAM-1 (Muro et al., 2003a). Anti-PECAM/catalase conjugates target endothelium and provide protection against oxidant injury caused by H₂O₂ in vitro and in vivo (Muzykantov, 2001; Christofidou-Solomidou et al., 2003; Kozower et al., 2003).

In the present study, we used our immunotargeting approach to obtain antioxidant protection against superoxide anion-mediated injury by using SOD as a cargo. The anti-PECAM/SOD conjugate showed high enzymatic activity and affinity to endothelium (Fig. 1). The SOD-525 assay employs a relatively large molecule, BXT-0150 (molecular mass, 270 Da), as an artificial substrate. Therefore, a more profound reduction of SOD activity in this assay versus cytochrome c assay utilizing small natural substrate of SOD, i.e., superoxide anion, probably reflects more stringent limitations imposed on diffusion of a large substrate molecule to the active site of conjugated SOD. This result suggests that a major fraction of SOD molecules is masked from the milieu by other components of the conjugates (SA and antibody). Because catalytic activity of the SOD conjugate toward to superoxide anion is well preserved (Fig. 1A), partial masking of the cargo enzyme may, conceivably, be viewed as a benefit in terms of its protection against proteolysis. In theory, even partial reduction of SOD activity in the conjugate (i.e., ~25% reduction revealed by cytochrome c assay) may become a liability in in vivo applications. Interestingly, our previous studies revealed a similar extent of inactivation (~20%) of catalase by conjugating to anti-PECAM (Shuvaev et al., 2004). Furthermore, results of our recent in vivo studies indicate that both anti-PECAM/SOD and anti-PECAM/catalase conjugates afford significant protective effects against distinct types of vascular oxidative stress. Anti-PECAM/catalase protects against mouse lung ischemia-reperfusion more effectively than anti-PECAM/SOD, whereas anti-PECAM/SOD but not anti-PECAM/catalase is protective against vasocstriction induced by angiotensin II-induced overproduction of superoxide anion in arteries in mice (V. V. Shuvaev and K. Lande, unpublished data).

However, data presented in this article show that the anti-PECAM/SOD conjugate demonstrated high protective efficacy in two distinct models of oxidative stress caused by either extracellular or intracellular generation of superoxide anion. In the first model, we exposed target cells to xanthine oxidase that produces superoxide from hypoxanthine, a well-characterized experimental model of extracellular oxidative stress caused by superoxide. Furthermore, xanthine oxidase is one of the sources of vascular superoxide in ischemia (Terada et al., 1992). Xanthine oxidase released into the bloodstream due to tissue injury may lead to endothelial attack by extracellular superoxide, hence direct (patho-)physiological relevance of this model. Other scenarios in which endothelial cells are exposed to external superoxide attack include ROS generation by activated leukocytes via NADPH-oxidase pathway (Cai et al., 2003; Bonder et al., 2004). Anti-PECAM/SOD, but not unconjugated SOD, provided significant and marked protection against both apoptosis and necrosis caused by xanthine oxidase exposure (Figs. 2 and 3).

The protection by anti-PECAM/SOD against intracellular superoxide production induced by metabolism of paraquat was practically complete (Fig. 4). This may be explained by the more optimal localization of ROS and antioxidants and/or different kinetics and amplitude of superoxide flux in paraquat versus HX-XO models. The highly effective protection against intracellular ROS by anti-PECAM/SOD fits well with the fact that endothelial cells and REN/PECAM cells internalize diverse anti-PECAM conjugates, verified by confocal multilabel fluorescent and transmission electron microscopy (Muzykantov et al., 1999; Scherpereel et al., 2002; Muro et al., 2003a, 2006). Clinical cases of vascular toxicity of paraquat (used as a pesticide) and other xenobiotics, such as doxorubicin and other drugs causing intracellular generation of superoxide, emphasize the pathophysiological relevance of our findings.

Taken together, the effects of anti-PECAM/SOD reported in this study indicate that SOD immunotargeting to endothelium offers marked advantages over SOD in alleviation of endothelial oxidative stress caused by either extracellular or intracellular superoxide. This result warrants studies to examine the endothelial targeting and efficacy of anti-PECAM/SOD conjugates in animal models of oxidant stress, which are in progress. This approach may improve containment of ischemia/reperfusion, inflammation, hyperoxia, and other conditions characterized by acute superoxide surplus in the vasculature.

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References