Superoxide Dismutase and Catalase Conjugated to Polyethylene Glycol Increases Endothelial Enzyme Activity and Oxidant Resistance*

(Received for publication, September 3, 1987)

Joseph S. Beckman†, Robert L. Minor, Jr.‡, Carl W. White, John E. Repine, Gerald M. Rosen‡, and Bruce A. Freeman†

From the †Departments of Anesthesiology and Biochemistry, University of Alabama at Birmingham, University Station, Birmingham, Alabama 35293, the ‡School of Medicine, University of Iowa, Iowa City, Iowa 52242, the †Webb-Waring Lung Institute, University of Colorado Health Sciences Center, Denver, Colorado 80262, and the †Department of Pharmacology, Duke University Medical Center, Durham, North Carolina 27710

Covalent conjugation of superoxide dismutase and catalase with polyethylene glycol (PEG) increases the circulatory half-lives of these enzymes from <10 min to 40 h, reduces immunogenicity, and decreases sensitivity to proteolysis. Because PEG has surface active properties and can induce cell fusion, we hypothesized that PEG conjugation could enhance cell binding and association of normally membrane-impermeable enzymes. Incubation of cultured aortic endothelial cells with 125I-PEG-catalase or 125I-PEG-superoxide dismutase produced a linear, concentration-dependent increase in cellular enzyme activity and radioactivity. Fluorescently labeled PEG-superoxide dismutase incubated with endothelial cells showed a vesicular localization. Mechanical injury to cell monolayers, which is known to stimulate endocytosis, further increased the uptake of fluorescent PEG-superoxide dismutase. Endothelial cell cultures incubated with PEG-superoxide dismutase and PEG-catalase for 24 h and then extensively washed were protected from the damaging effects of reactive oxygen species derived from exogenous xanthine oxidase as judged by two criteria: decreased release of intracellular 51Cr-labeled proteins and free radical-induced changes in membrane fluidity, measured by electron paramagnetic resonance spectroscopy of endothelial membrane proteins covalently labeled with 4-maleimido-2,2,6,6-tetramethylpiperidinooxyl. Addition of PEG and PEG-conjugated enzymes perturbed the spin-label binding environment, indicative of producing an increase in plasma membrane fluidity. Thus, PEG conjugation to superoxide dismutase and catalase enhances cell association of these enzymes in a manner which increases cellular enzyme activities and provides prolonged protection from partially reduced oxygen species.

Controlled manipulation of cellular superoxide dismutase and catalase-specific activities can help define mechanisms of tissue injury mediated by superoxide and hydrogen peroxide (1). The toxicity of reactive oxygen species, whose rate of production can be amplified by pathological events including neutrophil activation, hypoxia, metabolism of redox-active drugs, radiation exposure, and ischemia (2, 3), has also stimulated the use of antioxidant enzymes as therapeutic agents. However, the experimental and therapeutic potentials of superoxide dismutase and catalase are limited by two factors. First, both proteins are rapidly cleared by kidneys (4, 5), leading to circulatory half-lives of only 6–10 min following intravenous injection. Second, reactive oxygen species diffuse very short distances before reacting with cellular components (6), and neither superoxide dismutase nor catalase can penetrate across cell membranes. Thus, antioxidant enzymes cannot gain access to intracellular sites of free radical generation to achieve effective pharmacological value.

The renal clearance of superoxide dismutase and catalase can be prevented by increasing their molecular weight through covalent attachment of the inert linear polymer, monomethyloxy-polyethylene glycol (PEG; Refs. 7–9). The structure of PEG is H-O-(CH₂-O)n-CH₂ with n = 150 for PEG weighing 6000 Da. The free hydroxyl of PEG is conjugated to ε-amino groups of lysine with a bifunctional reagent such as cyanuric chloride. Typically, from 10 to 15 of the 20 available amino groups on superoxide dismutase are modified with PEG monomers of an average molecular mass of 5000 Da each, increasing the molecular mass of superoxide dismutase from 32 kDa to about 100 kDa for the modified protein. Similarly, the molecular weight of catalase may be increased by 300% following PEG conjugation. Modification by PEG blocks renal clearance and increases the circulating enzyme half-life from 6 min to 30–40 h in the rat (9). The inert nature of PEG also reduces the antigenicity of the native protein and inhibits the hydrolysis of protease-sensitive proteins such as catalase (8). Additionally, the long term stability of enzymes in aqueous solution is frequently increased by PEG conjugation.

Polyethylene glycol is a surface-active molecule that is used extensively to induce fusion for cell hybridization (10). Membranes can absorb substantial quantities of PEG, binding 1 PEG molecule/12 dipalmityloxy-phosphatidylcholine molecules in liposomes with a dissociation constant of 6 μM (11). Because PEG constitutes up to two-thirds of the total molecular weight of PEG-conjugated enzymes, we have investigated the possible membrane association of PEG-conjugated enzymes with cultured endothelial cells. Endothelial cells were used as target cells in this study, because the vascular endothelium is a significant site of oxidant injury (12) and because the anatomical localization of the vascular endothelium provides direct contact with circulating antioxidant enzymes. We re

* This work was supported by National Institutes of Health Grants NS-23760 and NS-24275 (to H. A. F.), HL-29182 (to J. E. R.), and HL-33550 (to G. M. R.) and by grants from the Health Effects Institute (to B. A. F.) and the Alabama Affiliate of the American Heart Association (to J. S. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1794 solely to indicate this fact.

† The abbreviations used are: PEG, polyethylene glycol; FITC, fluorescein isothiocyanate; HBSS, Hank's balanced salt solution; Mel-6-TEMPO, 3-maleimido-2,5,6,6-tetramethylpiperidinooxyl; W/S ratio, the weakly to strongly immobilized peak ratio.
port that PEG conjugation of superoxide dismutase and catalase enhances cell association of these antioxidant enzymes, alters membrane fluidity, and confers resistance to oxidant stress.

**EXPERIMENTAL PROCEDURES**

**Materials**—Pharmaceutical grade, bovine Cu,Zn superoxide dismutase was generously provided by Diagnostic Data Inc. (Mountain View, CA) and by Grunenthal GmbH (Aachen, Germany). The superoxide dismutase obtained from Diagnostic Data Inc. was dialyzed overnight against phosphate-buffered saline to remove sucrose added as a preservative. Bovine catalase was obtained from Cooper-Biomedical, Inc. Bovine milk xanthine oxidase was purchased from Behring Diagnostics. Tissue culture media were obtained from Gibco, and fetal calf serum was obtained from HyClone (Logan, UT). All other reagents were obtained from Sigma.

**Enzyme Assays**—Superoxide dismutase was assayed by inhibition of cytochrome c reduction, and units of activity are defined as given by McCord and Fridovich (13). Catalase activity was determined by the disappearance of 10 mM hydrogen peroxide in 50 mM K+ phosphate, pH 7.0, monitored at 240 nm (Δε240 = 43.6 M−1 cm−1; Ref. 14). Xanthine oxidase was assayed by monitoring the production of uric acid at 295 nm (Δε295 = 1.1 × 104 M−1 cm−1; Ref. 15) at 25°C. The reduction contained 50 mU xanthine, 100 μM EDTA, and 50 mM K+ phosphate, pH 7.5. Units of activity for catalase and xanthine oxidase are defined as 1 μmol produced per min.

**Preparation of PEG-Superoxide Dismutase and PEG-Catalase**—Cyanuric chloride (10 g) was dissolved in 100 ml of chloroform, filtered through Whatman No. 1 paper, evaporated to dryness immediately before use (16). Monomethoxy-polyethylene glycol (5000 Da) was activated with cyanuric chloride and coupled to superoxide dismutase and catalase by standard methods (7–9). The extent of conjugation, measured by the number of reactive amino groups, was determined by titration with trinitrobenzene sulfonic acid (17), and protein concentrations were measured by the Biuret method (8).

**Fluorescent and Radioactive Labeling of Proteins**—Proteins were labeled with 125I via a solid phase oxidative reaction using 1,3,4,6-tetrachloro-3-amin-6-diphenylglycoluril (Fierce Chemical Co.; Ref. 18) and then exhaustively dialyzed against phosphate-buffered saline to remove free 125I. Fluorescence labeling of Cu,Zn superoxide dismutase was accomplished by addition of 0.3 mg of fluorescein isothiocyanate (FITC) to 1.0 ml of 10 mg/ml protein in 50 mM sodium carbonate, pH 9.5, and stirred for 1 h at 4°C while maintaining pH at 9.5 by addition of Na2CO3. Unbound FITC was removed by gel filtration chromatography on Sephadex G-25 eluted with phosphate-buffered saline (150 mM NaCl, 10 mM K+ phosphate, pH 7.4).

**Cell Culture**—Porcine thoracic aortic endothelial cells were cultured as described previously (20). Third or fourth passage cells were grown in 2.2-cm 2 flask culture medium (Cambrex, Walkersville, MD) in M199 medium with 10% fetal calf serum at 37°C. Cells were passaged in a 1:3 split ratio with 0.05% trypsin and used by the fourth passage to avoid changes in endogenous antioxidant enzyme activities (21).

**Sample Preparation**—Cells were washed three times with Hank's balanced salt solution (HBSS) before being removed from culture flasks with a cell scraper (Costar), washed again in 5 ml of HBSS, and sonicated in 1 ml of 50 mM K+ phosphate, 0.1% Triton X-100, pH 7.8. The sample was centrifuged at 15,000 × g for 30 min and the supernatant assayed for either catalase or superoxide dismutase activity. The amount of 125I-label uptake was determined from 150-μl samples of cell homogenate by γ-counting. Protein concentrations of cell homogenates were measured by the method of Lowry et al. (22).

**125I Release Studies**—Endothelial cells were grown in 2.2-cm 2 well plates (Costar) and prelabeled with 3 μCi/ml 125I added to the culture medium for 4 h, washed 3 times, and then incubated for 6 h with either native or PEG-catalase. The cells were then washed five times and maintained in HBSS for the duration of the chase experiment. Xanthine oxidase (10 milliunits/ml) and xanthine (100 μM) were added to HBSS, and the release of 125I from cells into the medium was measured 4 h later. The percentage of 125I release for each treatment was calculated by subtracting the amount of 125I released by untreated cells (not exposed to xanthine oxidase) and dividing by the additional amount of 125I released when untreated cells were exposed to xanthine plus xanthine oxidase.

**Spin-labeling Measurements**—Endothelial cell monolayers were first washed free of serum-containing medium with HBSS containing 0.7 mM L-glutamine and 5 mM glucose, pH 7.4, and labeled for 3 h at 37°C in the same buffer with 100 μM 4-maleimido-2,6,6-tetramethylpiperidinoxyl (Mal-6-TEMPO) as reported previously (23). The labeled cells were washed free of unreacted labeling agent, removed from flasks by scraping, and resuspended to a density of 400,000 cells/ml, which corresponds to about 0.4 μg of protein. The labeling procedure did not affect cell viability or the rate of cell respiration. Immunoblot analysis using rabbit anti-Mal-6-TEMPO IgG following sodium dodecyl sulfate-polyacrylamide gel electrophoresis of solubilized endothelial cell membranes showed predominant labeling of two major membrane proteins (23). The EPR spectra were obtained from a Varian Associates E9 spectrometer (Palo Alto, CA) operated at 9.5 GHz with 100-kHz modulation. Spectra were recorded at room temperature in a flat quartz cuvette with a microwave power of 14 mW and a modulation amplitude of 0.63 gauss.

**Statistical Analysis**—The rate of PEG-conjugated enzyme uptake by endothelial cells was determined by multiple regression. The rate of change in the W/S ratio during exposure to xanthine oxidase was determined by linear regression with the difference between slopes from the regression lines determined by Tukey's post hoc procedure (24). Plate changes in the extent of 125I release from endothelial cells by catalase and PEG-catalase were determined by analysis of variance and Scheffe's post hoc test (25).

**RESULTS**

**Association of PEG-Catalase with Endothelium**—Incubation of cultured porcine aortic endothelial cells with 125I-labeled PEG-catalase produced a linear, dose-dependent increase of cell-associated enzyme activity with time (Fig. 1). There was an initial rapid cell association of PEG-catalase within the first 4 h which was followed by a slower, continuous uptake over the next 24 h. This pattern of PEG-catalase uptake was described by the following equation:

\[
    \text{Units} = a_1 + a_2 \text{[PEG-CAT]} + a_3 \text{[PEG-CAT]} + a_4
\]

where units equal the catalase activity/mg cell protein measured at time \( t \) following the addition of PEG-catalase. [PEG-CAT] is the concentration of PEG-catalase added to the medium, \( a_1 \) is the slow, continuous secondary rate of PEG-catalase uptake/h, \( a_2 \) is the initial rapid phase of PEG-catalase uptake, and \( a_3 \) is the endogenous catalase activity of control cells. Multiple regression analysis of the data shown in Fig. 1 yielded a correlation coefficient of 0.93, and the values determined for \( a_1 \), \( a_2 \), and \( a_3 \) are given in Table I. These values were used to calculate the fitted lines shown in Fig. 1 for each concentration of PEG-catalase. This was addition of 1 mg/ml PEG-catalase to cell monolayers doubled endogenous catalase activity within 4 h and resulted in an additional increase in activity of 11%/h over the next 20 h. This increase in both catalase activity and enzyme-associated radioactivity could not be removed after extensive washing of PEG-catalase-treated cells.

In the above experiment, the cell uptake of PEG-catalase measured by 125I radioactivity closely paralleled the increase in catalase specific activity. The specific activity of PEG-
Thus, significant augmentation of cellular catalase activity was 20 times greater than could be accounted for by the increase in catalase enzyme activity after 24 h of incubation (Table II). The most likely explanation for this is that native catalase was avidly bound to and degraded by endothelial cells and that radiolabeled amino acids or peptides remained associated with cell.

Association of PEG-Superoxide Dismutase with Endothelium—Incubation of cells with 0.5 mg/ml PEG-superoxide dismutase increased cellular superoxide dismutase-specific activity by about 17%/h (Fig. 5). During the first 4 h, incubation with 0.5 mg/ml native bovine Cu,Zn superoxide dismutase nearly doubled endothelial superoxide dismutase activities from 4.4 ± 0.8 to 8.2 ± 0.5 units/mg cell protein. Thereafter, the activity for native superoxide dismutase-treated cells did not increase significantly, rising only to 9.0 ± 0.6 units/mg cell protein after 24 h (Fig. 5). As with native catalase, Cu,Zn superoxide dismutase was predominantly incorporated as an inactive species, since there was a progressive increase in the ratio of cpm of 125I/unit superoxide dismutase in endothelial cells compared with medium counts/min of 125I/unit superoxide dismutase (Fig. 6). If cells incubated with native superoxide dismutase for 8 h were washed and placed in fresh medium, the counts/min of 125I/unit superoxide dismutase level remained constant over the next 16 h to near control values. In contrast, cells incubated with PEG-superoxide dismutase showed a constant ratio of counts/min of 125I/unit superoxide dismutase over endogenous superoxide dismutase activity that was the same as the specific activity of 125I-PEG-superoxide dismutase added to the medium. Furthermore, if medium containing PEG-superoxide dismutase was replaced with fresh medium after 8 h incubation, cellular superoxide dismutase activities remained elevated and the ratio of counts/min of 125I/unit superoxide dismutase remained constant.

When lysates of endothelial cells incubated for 24 h with 125I-labeled PEG-superoxide dismutase were chromatographed over Sephadex G-25, all of the radioactivity from the PEG-superoxide dismutase-treated cell lysates eluted in the void volume. These results show that PEG-superoxide dismutase is resistant to degradation by endothelial cells to smaller peptides or amino acids.

The uptake of FITC-PEG-superoxide dismutase could also be observed by fluorescence microscopy, demonstrating a punctate fluorescence distribution pattern (Fig. 7). Mechanical injury to the endothelium, produced by scratching the monolayer with the sealed tip of a glass Pasteur pipette, greatly stimulates pinocytosis in the endothelial layer that grows into the "injured" area (26). Forty-eight h after injuring endothelium in the presence of FITC-PEG-superoxide dismutase, the injured area showed numerous fluorescent patches which, upon focusing through the cell, were uniformly distributed at all depths. This was consistent with the uptake of fluorescent protein into discrete subcellular vesicles which might be endosomes and lysosomes.

Resistance to Oxidant Stress—The degree of protection afforded by superoxide dismutase or catalase against an oxidant stress imposed by xanthine plus xanthine oxidase depends upon the method chosen for assaying cellular injury. We have made use of two markers of cellular injury: the xanthine plus xanthine oxidase-induced release of 51Cr-labeled proteins from cells, and EPR detection of membrane organizational changes measured by changes in the peak amplitude of the two major binding environments representing weakly and strongly immobilized proteins labeled with Mal-6-TEMPO. Our previous studies have shown that xanthine plus xanthine oxidase-induced 51Cr release from endothelial cells is predominantly inhabitable by catalase (21),

---

**Augmenting Endothelial Antioxidant Enzymes**

**Table I**

*Analysis of the cell association of PEG-catalase by multiple regression*

The data shown in Fig. 1 were fitted to Equation 1 to determine the coefficients $a_1$, $a_2$, and $a_3$. Each incubation time and PEG-catalase concentration contained three replicates with a total of 29 observations used in the regression. The overall correlation coefficient was 0.93, and all coefficients were significant at the 0.0001 level.

<table>
<thead>
<tr>
<th>Coefficient</th>
<th>Estimate ± S.E.</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>$a_1$</td>
<td>2.5 ± 0.24</td>
<td>Units (mg PEG-catalase)$^{-1}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>h$^{-1}$ (mg cell protein)$^{-1}$</td>
</tr>
<tr>
<td>$a_2$</td>
<td>21 ± 4.2</td>
<td>Units (mg PEG-catalase)$^{-1}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(mg cell protein)$^{-1}$</td>
</tr>
<tr>
<td>$a_3$</td>
<td>22 ± 1.5</td>
<td>Units (mg cell protein)$^{-1}$</td>
</tr>
</tbody>
</table>

---

**Fig. 1.** Polyethylene glycol-catalase uptake by endothelium. The increase in catalase specific activity/mg cell protein is shown for endothelial cell monolayers incubated with the following concentrations of PEG-catalase: O, 1.0 mg/ml; Δ, 0.5 mg/ml; ●, 0.1 mg/ml; ◊, 0.05 mg/ml; A, untreated cells. Regression lines were calculated from Equation 1 using the coefficients given in Table I for each concentration of PEG-catalase. Each point represents the mean ± S.E. of three replicates.

---

**Mechanisms of action**

Catalase added to culture medium was 6.5 cpm of 125I/unit catalase. When the radioactivity uptake by cells was plotted against the increase in catalase activity (Fig. 2), it was found that 7.6 ± 0.6 cpm of 125I/unit catalase was taken up by the endothelial cells. From these data, we estimate that at least 85% of PEG-catalase taken up by endothelial cells retained its activity, probably by electrostatic membrane-protein interactions (29).

Incubation of cell monolayers with 0.5 mg/ml native catalase increased catalase activity from 11 units/mg cell protein to 30 units/mg cell protein within the first 4 h (Fig. 3). Unlike PEG-catalase-treated cells, longer incubation with native catalase did not lead to further increases of cellular catalase activity. This difference was not due to the presence of PEG alone, because the addition of 1% PEG to native catalase did not increase endothelial catalase specific activities (Fig. 3). Thus, significant augmentation of cellular catalase activity depended upon PEG being covalently bound to catalase. The uptake of radiolabel by cells incubated with 125I-catalase was far greater than that observed when cells were incubated with a similar isotope specific activity and protein concentration of 125I-PEG-catalase (Fig. 4). Radiolabel incorporation of 125I-catalase was 20 times greater than could be accounted for by the increase in catalase enzyme activity after 24 h of incubation (Table II). The most likely explanation for this is that native catalase was avidly bound to and degraded by endothelial cells and that radiolabeled amino acids or peptides remained associated with cell.
while xanthine oxidase-induced changes in endothelial membrane protein mobility are inhibitable by superoxide dismutase (23).

The release of $^{51}$Cr into culture medium from cells following injury is analogous to the release of endogenous lactate dehydrogenase (27, 28). Control experiments showed that incubation of PEG-superoxide dismutase or PEG-catalase (1 mg/ml) with endothelial cells for 24 h (without exposure to xanthine plus xanthine oxidase) did not enhance $^{51}$Cr release from endothelial cells. Under the experimental conditions we employed, exposure to 10 milliunits of xanthine oxidase/ml medium before xanthine oxidase exposure prevented 80% of the $^{51}$Cr release compared with untreated controls. Preincubation with 0.5 or 1.0 mg of native catalase/ml medium for 24 h and washed free of enzyme-containing medium reduced the amount of $^{51}$Cr release compared with untreated cells.

The protection provided by preincubation of cells with native catalase might be due to the apparent electrostatic...
attachment of native catalase to membranes (29). Addition of native catalase to endothelial cells followed by washing of the cells increased the catalase specific activity from 11 ± 1.2 to 20 ± 1.6 units/mg cell protein. We have found that addition of catalase to cationic liposomes leads to membrane aggregation (29). Modification of the surface change of catalase with succinic anhydride greatly diminished the aggregation of liposomes, suggesting that electrostatic charges on catalase mediate its association with membranes.

Preincubation of cells with PEG-superoxide dismutase did not protect endothelial cells from xanthine oxidase-induced 51Cr release, consistent with previous studies where that native superoxide dismutase added to culture medium at the time of cell exposure to xanthine oxidase was not protective (28). Thus, formation of hydroxyl radical by the superoxide-driven, iron-catalyzed Fenton reaction can be ruled out in the present study, although trace amounts of iron were undoubt-

**Membrane Fluidity**—Pretreatment of cells with PEG-superoxide dismutase confers endothelial oxidant resistance using another index of injury based upon oxidant-induced membrane fluidity changes. Membrane fluidity was measured by conjugating a maleimide derivative of the spin label TEMPO to sulfhydryl groups of endothelial membrane pro-

<table>
<thead>
<tr>
<th>Percentage uptake</th>
<th>Enzyme activity</th>
<th>Radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG-catalase</td>
<td>0.07</td>
<td>0.12</td>
</tr>
<tr>
<td>Catalase</td>
<td>0.01</td>
<td>0.20</td>
</tr>
<tr>
<td>PEG-superoxide dismutase</td>
<td>0.13</td>
<td>0.10</td>
</tr>
<tr>
<td>Superoxide dismutase</td>
<td>0.01</td>
<td>0.04</td>
</tr>
</tbody>
</table>

**TABLE II**

Percentages of enzyme added to the medium which was taken up by endothelial cell monolayers after 24-h incubation.

The catalase data were derived from the experiment shown in Figs. 3 and 4, while the superoxide dismutase data came from Fig. 5.

**Fig. 5.** The time course of increased cellular superoxide dismutase (SOD) specific activities after incubation with 0.5 mg/ml either native superoxide dismutase (●) or PEG-superoxide dismutase (■). Control cell activities are shown (▲).

**Fig. 6.** The ratio of cellular 125I counts/min versus units of superoxide dismutase (SOD) activity taken up by endothelial cells incubated with 0.5 mg/ml 125I-PEG-superoxide dismutase (●) or 125I-superoxide dismutase (♦). After 8 h of incubation with 125I-PEG-superoxide dismutase or 125I-superoxide dismutase, some flasks were washed three times and placed in fresh medium to observe the disappearance of cell associated enzyme activities (dashed line). The 125I/unit superoxide dismutase ratios shown at time 0 reflect the specific activities of the 125I-labeled superoxide dismutase and PEG-superoxide dismutase added to the media.

*P-20% of the rate of increase in the W/S ratio could be prevented by the addition of superoxide dismutase (100 units/ml) during exposure to...
Augmenting Endothelial Antioxidant Enzymes

FIG. 7. Cell uptake of FITC-labeled PEG-superoxide dismutase. Endothelial monolayers were incubated with 1 mg/ml FITC-PEG-superoxide dismutase in M199 medium plus 10% fetal calf serum for 48 h. Immediately before adding FITC-PEG-superoxide dismutase, the monolayer was scored with the sealed end of a sterile Pasteur pipette (upper panel). This removed a narrow strip of cells which was completely recolonized 48 h later (middle panel). The middle panel was photographed from a different location than the upper panel but is superimposable with the lower panel. After the 48-h incubation, the Petri plates were washed with HBSS three times. The lower panel shows the punctate distribution of fluorescence within the endothelial cells. The region with intense fluorescence dots in the lower panel corresponds to part of the area scraped 48 h before.

xanthine oxidase (23). In the present study, we found that pretreating endothelial cells with 1 mg/ml PEG-superoxide dismutase for 24 h, followed by washing, was as protective as that afforded by a much greater concentration of superoxide dismutase present during exposure to xanthine oxidase. Addition of superoxide dismutase to suspensions of PEG-superoxide dismutase-pretreated cells during xanthine oxidase exposure did not enhance protection against membrane fluidity changes provided by PEG-superoxide dismutase pretreatment alone (Fig. 10). Although the initial W/S ratio of labeled cells was increased by 1% PEG, preincubation with 1% PEG did not affect the rate of change in the W/S ratio caused by xanthine oxidase-derived free radicals or the protection afforded by native superoxide dismutase present during the xanthine oxidase exposure. Because catalase previously did not attenuate xanthine oxidase-induced membrane fluidity changes when added either alone or with superoxide dismutase (23), no EPR experiments were performed with PEG-catalase.

FIG. 8. Increased endothelial cell resistance to oxidant stress following treatment with PEG-catalase. Endothelial monolayers were prelabeled with %Cr and then incubated for 24 h with 0.5 mg/ml either native (○) or PEG-catalase (■). The cell monolayers were then washed five times and placed in HBSS. Xanthine oxidase (10 milliunits/ml) and xanthine (100 μM) were added to the medium and the release of %Cr by cells was measured 4 h later. Each point represents the mean ± S.E. of eight replicates. At concentrations at and above 0.4 mg/ml, the differences in %Cr release between PEG-catalase- and catalase-treated cells were significantly different at the 0.05 level.

DISCUSSION

We have shown that conjugation of superoxide dismutase and catalase to PEG enhanced uptake of the active form of these enzymes by cultured endothelial cells and that prior incubation of cells with these enzyme conjugates provided greater resistance to oxidant stress than that afforded by native enzymes. Three possibilities could account for the augmentation of endothelial cell enzyme activity following incubation with PEG-conjugated enzymes: (a) direct penetration of membranes by PEG-enzymes, (b) binding of PEG-enzymes to membrane surfaces, and (c) uptake of PEG-enzymes by endocytosis (Fig. 11). Polyethylene glycol conjugation of proteins probably does not mediate migration directly through cell membranes. Horseradish peroxidase becomes soluble in benzene when conjugated to PEG, suggesting
that enough hydrophilic residues are masked by PEG to prevent an otherwise hydrophilic protein to partition into hydrophobic solvents (30). While PEG itself is quite soluble in aromatic solvents such as benzene, it precipitates rapidly in alkane solvents, a fact made use of in the synthesis of activated PEG for coupling to proteins (7). Hence, PEG-derivatized enzymes would not be expected to be soluble within the aliphatic core of phospholipid membranes and therefore are unlikely to traverse the membrane core.

Polyethylene glycol avidly associates with membranes in the phospholipid headgroup region (11), which might allow anchoring of PEG-conjugated proteins to exofacial plasma membrane surfaces. Evidence in favor of this concept is the increased W/S ratio of Mal-6-TEMPO-treated endothelial membranes secondary to incubation with PEG-superoxide dismutase, suggesting some influence is exerted upon cell membranes by superoxide dismutase after PEG conjugation.

The binding of PEG-enzymes to cell membranes does not explain the continuous uptake of enzyme conjugates by endothelial cells over 24 h because the binding of PEG to membranes should be rapidly saturated (11).

The third explanation entails the uptake of PEG-conjugated enzymes by endocytosis from the medium. In confluent stationary phase bovine aortic endothelial cells, the rate of medium uptake by endocytosis is reported to be 50 nl h−1/106 cells (26). If we assume that this rate of endocytosis is similar in cultured porcine endothelium and take into account cell number and medium volume, between 0.06 and 0.09% of the medium would be taken up by cells by endocytosis in 24 h. The fraction of cellular uptake of both PEG-superoxide dismutase and PEG-catalase present in culture medium was 0.07–0.13% in 24 h (Table II), consistent with the calculated rate of uptake by endocytosis. The punctate distribution of fluorescence observed in endothelial cells incubated with FITC-labeled PEG-superoxide dismutase also suggests that a vesicular uptake process plays a major role in the uptake of PEG-conjugated proteins. Mechanical injury to the monolayer, known to stimulate endocytosis (26), considerably augmented the uptake of FITC-PEG-superoxide dismutase, supporting endocytosis as a contributing factor in the uptake of PEG-conjugated enzymes.

The difference in the rate of native and PEG-conjugated enzyme uptake by endocytosis can be explained by the increased resistance of PEG-conjugated enzymes to proteolysis. Because PEG-protein conjugates are resistant to proteases (7), they can survive within lysosomes, yielding higher cell-associated specific activities. Native catalase added to either crude endothelial cell lysates or delivered to endothelial cells via liposomes is degraded with a half-life of 2–3 h (31). On the other hand, superoxide dismutase is more resistant to proteolysis and liposome-delivered superoxide dismutase was far more stable in endothelial cells than catalase (20). However, Cu,Zn superoxide dismutase loses its zinc cofactor when exposed to an acidic pH and becomes susceptible to protease degradation (32). Thus, native superoxide dismutase present in acidic endosomal and lysosomal compartments should be degraded. In the present study, degradation of both cell-associated native superoxide dismutase and catalase was ob-

![Diagram](image-url)
FIG. 11. Three possible mechanisms for PEG-conjugated antioxidant enzyme uptake by endothelial cells. Direct membrane penetration of PEG-enzymes due to the amphiphilic nature of PEG is considered to be unlikely because PEG would be insoluble in the aliphatic core of the membrane. However, evidence suggests that PEG-conjugated proteins may bind to the polar head groups of phospholipid membranes as well as being taken up by endocytosis. The same mechanisms apply equally to PEG-catalase. SOD, superoxide dismutase.

served, as evidenced by the continuous increase in the ratio of counts/min of $^{125}$I/unit enzyme in cells incubated with $^{125}$I-labeled proteins (Figs. 4 and 6). This ratio for PEG-superoxide dismutase- and catalase-treated cells remained almost the same as the specific activity of the PEG-superoxide dismutase and PEG-catalase added to the medium, even after 24 h of incubation with endothelial cells.

On balance, there is evidence to support cell uptake of PEG-enzymes by both membrane binding and endocytosis. These two explanations are not mutually exclusive and could even complement each other. For example, the binding of PEG-enzymes to cell surfaces may enhance endocytosis, thereby increasing rates of endocytic uptake. Further studies are required to differentiate between PEG-enzyme uptake by endocytic bulk phase transport of protein conjugates in solution, or the internalization of PEG-enzymes bound to plasma membrane, which would then be distributed in endocytic vacuoles and throughout the cell by membrane flow (33).

Can PEG-conjugated antioxidant enzymes entrapped within endosomes or lysosomes also provide protection against intracellularly generated superoxide and hydrogen peroxide, since these enzymes are separated from the cytoplasm by a membrane barrier? Endosomes and lysosomes become strongly acidic by an active proton pumping mechanism that requires the presence of an anion channel which allows migration of chloride ions to counterbalance proton accumulation within the endosome (34). Because anion channels permit migration of superoxide across red blood cell membranes (35), it is possible that PEG-superoxide dismutase entrapped within lysosomes or endosomes could act as a sump for intracellular superoxide radicals which traverse the chloride anion channel. Recently, Saez et al. (36) have shown that native superoxide dismutase added to the medium of cultured superior cervical ganglion neurons is rapidly taken up by endocytosis after the cells are transiently depolarized with potassium chloride. These neurons, which had superoxide dismutase entrapped within an endocytic compartment, were more resistant to a 40-min starvation stress, whereas starved neurons that were depolarized but had superoxide dismutase added to the medium were not protected.

The increased activity of PEG-conjugated enzymes observed in cultured endothelial cells suggests that prolonged administration of PEG-conjugated enzymes may gradually augment vesicular-associated cell antioxidant enzyme activities in vivo, implying a more complex pharmacokinetics than reflected by plasma clearance. Although the uptake of PEG-superoxide dismutase and PEG-catalase by endothelium reported here is a slow process, the continual exposure of endothelium to PEG-conjugated enzymes having long circulatory half-lives and stability may lead to significant augmentation of cellular antioxidant activities. Furthermore, areas of reversibly injured endothelium in vivo may concentrate even greater levels of PEG-conjugated enzymes due to injury-enhanced endocytic processes (26).

Our results suggest that an additional consequence of PEG-conjugation to proteins is the enhancement of protein association with cells. Uptake of PEG-conjugated enzymes by other cell types almost certainly will occur in vivo as well. The prolonged treatment with PEG-conjugated adenosine deaminase in children afflicted with severe combined immunodeficiency disease due to adenosine deaminase deficiency led to restoration of immune function after 6 months (37). Conceivably, uptake of PEG-adenosine deaminase by immature lymphocytes may have contributed to the intracellular removal of inhibitory metabolites in addition to those secreted into plasma, enabling these cells to differentiate and restore immune function.

Acknowledgments—Both Diagnostic Data Inc. (Mountain View, CA) and Grunenthal GmbH (Aachen, Germany) generously provided Cu,Zn superoxide dismutase for these studies. We thank Zermeena Mirza and M. Kathy Cunningham for their expert assistance in the culture of endothelial cells.

REFERENCES