Immunotargeting of glucose oxidase: intracellular production of H$_2$O$_2$ and endothelial oxidative stress

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Gow, Andrew J., Frank Branco, Melpo Christofidou-Solomidou, Linda Black-Schultz, Steven M. Albelda, and Vladimir R. Muzykantov. Immunotargeting of glucose oxidase: intracellular production of H$_2$O$_2$ and endothelial oxidative stress. Am. J. Physiol. 277 (Lung Cell. Mol. Physiol. 21): L271–L281, 1999.—Extracellular and intracellular reactive oxygen species attack different targets and may, therefore, result in different forms of oxidative stress. To specifically study an oxidative stress induced by a regulated intracellular flux of a defined reactive oxygen species, we used immunotargeting of the H$_2$O$_2$-generating enzyme glucose oxidase (GOX) conjugated with an antibody to platelet-endothelial cell adhesion molecule (PECAM)-1, an endothelial surface antigen. Anti-PECAM-1-GOX conjugates specifically bind to both endothelial and PECAM-transfected cells. Approximately 70% of cell-bound anti-PECAM-GOX was internalized. The cell-bound conjugate was enzymatically active and generated H$_2$O$_2$ from glucose. Use of the fluorescent dye dihydroorhodamine 123 revealed that 70% of H$_2$O$_2$ was generated intracellularly, whereas 30% of H$_2$O$_2$ was detected in the cell medium. Catalase added to the cells eliminated H$_2$O$_2$ in the medium but had little effect on the intracellular generation of H$_2$O$_2$ by anti-PECAM-GOX. Both H$_2$O$_2$ added exogenously to the cell medium (extracellular H$_2$O$_2$) and that generated by anti-PECAM-GOX caused oxidative stress manifested by time- and dose-dependent irreversible plasma membrane damage. Inactivation of cellular catalase by aminotriazole treatment augmented damage caused by either extracellular H$_2$O$_2$ or anti-PECAM-GOX. Catalase added to the medium protected either normal or aminotriazole-treated cells against extracellular H$_2$O$_2$, yet failed to protect cells against injury induced by anti-PECAM-GOX. Therefore, treatment of PECAM-positive cells with anti-PECAM-GOX leads to conjugate internalization, predominantly intracellular H$_2$O$_2$ generation and intracellular oxidative stress. These results indicate that anti-PECAM-GOX 1) provides cell-specific intracellular delivery of an active enzyme and 2) causes intracellular oxidative stress in PECAM-positive cells.

hydrogen peroxide; drug delivery; bioconjugation; CD31; platelet-endothelial cell adhesion molecule-1

OXIDATIVE STRESS in endothelial cells plays an important role in cardiovascular and lung diseases (3, 10, 12, 21). Endothelium, particularly pulmonary endothelium, is predisposed to injury by reactive oxygen species (ROS) and their derivatives. The major factors involved in pulmonary oxidative endothelial injury include: 1) exposure to high levels of environmental oxygen, ROS (e.g., ozone), and compounds that induce ROS generation in the lung (e.g., smoke) (6); 2) a pulmonary vasculature that is a target for sequestration of activated leukocytes that release ROS (3, 9, 10); and 3) pulmonary endothelial cells as well as other endothelial cells that are capable of ROS generation (5, 7, 11, 26, 32).

The effect of ROS generation is critically dependent on the available targets for the reaction, which are determined by both the reaction rate and the local concentrations of these potential reactants. Presumably, the available targets for the reaction will vary between the intracellular and extracellular environments. Because they interact with and modify different targets, it is likely that ROS generated internally and externally have different effects in cells and cause different forms of oxidative stress.

The aim of the present study was to construct a model to study an oxidative stress induced by regulated intracellular flux of a defined ROS in a cultured cell system. We hypothesized that this could be achieved by the intracellular delivery of an ROS-generating enzyme. To deliver such an enzyme in a cell-specific manner (allowing for in vivo applications), we utilized an immunotargeting strategy. In this strategy, an active compound (e.g., an ROS-generating enzyme) is conjugated with an antibody directed against a specific surface antigen on the target cell. In the present study, we used the anti-platelet-endothelial cell adhesion molecule (PECAM), a monoclonal antibody directed against PECAM-1, PECAM-1, or CD31 antigen, is a 130-kDa glycoprotein constitutively expressed on the endothelial surface (1, 20). Platelets also possess PECAM-1 but at a much lower level. In a previous study, Muzykantov et al. (17) have documented that endothelial cells internalize biotinylated PECAM antibodies conjugated with streptavidin (SA) and that the anti-PECAM-SA carrier provides intracellular delivery of the conjugated proteins to endothelial cells. Importantly, the anti-PECAM-SA carrier accumulates in the pulmonary vasculature in perfused rat lungs and in intact animals in vivo (17).

We postulated that conjugation of an ROS-generating enzyme with anti-PECAM-SA would provide specific binding of the enzyme to endothelial cells, facilitate its intracellular delivery (internalization), and cause intracellular generation of a defined ROS. To test this hypothesis, we 1) conjugated the H$_2$O$_2$-generating enzyme glucose oxidase (GOX) to a monoclonal anti-PECAM (anti-PECAM-GOX), 2) studied the properties of the conjugate in cultures of human endothelial cells.
and cells transfected with PECAM-1 antigen, and 3) characterized intracellular ROS generation and oxidative stress caused by the anti-PECAM-GOX conjugate.

MATERIALS AND METHODS

Reagents. The following materials were used in the study: IODO-GEN from Pierce (Rockford, IL); Na\textsuperscript{125}I and \textsuperscript{51}Cr from Amersham (Arlington Heights, IL); fatty acid-free bovine serum albumin (BSA) from Boehringer Mannheim (Indianapolis, IN); dimethyl formamide, a 30% aqueous solution of \textsubscript{10}H\textsubscript{2}O\textsubscript{2}, mouse IgG, biotinylated GOX (b-GOX), 3-amino-1,2,4-triazole (ATZ), D-\textsubscript{(+)}-glucose, Triton X-100, and components of the buffer solutions from Sigma (St. Louis, MO); lyophilized bovine liver catalase (activity 20,000 U/mg) from Fluka (Ronkonkoma, NY); and SA, goat antibodies against mouse IgG, and 6-biotinylaminocaproic acid N-hydroxysuccinimide ester (BxNHS) from Calbiochem (San Diego, CA). Protein concentration was determined with a Bio-Rad (Hercules, CA) microassay kit. A monoclonal antibody to human PECAM-1, MAB 62 (provided by Dr. Marian Nakada, Centocor, Malvern, PA), is a mouse monoclonal IgG2a reacting with the first Ig-like loop of human and rat PECAM-1 (17). Soluble purified PECAM (a chimeric Ig-CD31 fusion protein) was generously provided by Dr. Peter Newman (Blood Center of Southeastern Wisconsin, Milwaukee).

Modification of proteins and conjugation of b-GOX with anti-PECAM. Immunoglobulins were biotinylated at a 10-fold molar excess of the biotinylating reagent BxNHS as previously described (14, 18). The biotinylated proteins will be designated as b-IgG, b-MAB, and b-GOX. b-GOX was labeled with \textsuperscript{125}I with IODO-GEN-coated tubes according to the manufacturer’s recommendation. To construct the trimolecular heteropolymer complex b-IgG-SA-b-GOX, we used a two-step procedure with other enzymes established in our laboratory (14, 15). Briefly, in the first step, SA and b-GOX were mixed at a molar ratio of SA to b-GOX of 5 and incubated for 1 h on ice to form a bimolecular complex, SA-b-GOX. In the second step, the SA-b-GOX complex was incubated with b-anti-PECAM or b-IgG to form the trimolecular complex b-anti-PECAM-SA-b-GOX or its nonimmune counterpart b-IgG-SA-GOX. As in the preliminary experiments, we determined that the SA-b-GOX molar ratio of 5 provides optimal conditions for the further conjugation with biotinylated murine immunoglobulins. The enzymatic activity of GOX conjugated with either the immune or nonimmune carrier did not differ from that of the initial preparation of b-GOX (\textasciitilde 100 U/mg). These conjugates will be designated as anti-PECAM-GOX and IgG-GOX, respectively.

Binding of anti-PECAM-GOX to immobilized PECAM-1 and detection of generation of H\textsubscript{2}O\textsubscript{2}. To confirm that the conjugation procedure yields a trimolecular complex (b-anti-PECAM-SA-b-GOX), we determined its binding in wells coated with a goat polyclonal antibody to murine IgG (anti-IgG). First, plastic wells of a 96-well plate were incubated overnight at 4°C with 100 µl of borate-buffered saline (BBS; pH 8.1) containing 0.1 mg/ml of anti-IgG. After being washed, the wells were incubated for 1 h at room temperature with BBS containing 2 mg/ml of BSA (BBS-BSA) to block the sites for nonspecific binding. Control wells were coated with albumin instead of anti-IgG. Anti-PECAM-GOX (10 µg/ml in BBS-BSA) was incubated in the wells for 1 h at room temperature. After elimination of nonbound material, 100 µl of a 10 mM glucose solution in PBS was incubated in the wells to provide bound GOX with a substrate for H\textsubscript{2}O\textsubscript{2} generation. At the indicated time, 100 µl of a substrate solution (10 µmol of peroxidase and 1 mg/ml of o-phenylenediamine in PBS) were added to the wells. After the 5-min reaction was terminated with sulfuric acid, absorbance at 495 nm was determined in an ELISA reader.

To determine the antigen-binding capacity of the conjugate, the chimeric Ig-CD31 protein was immobilized in the wells as described above for the anti-IgG. Incubation with the conjugates, elimination of nonbound material, incubation with glucose, and detection of H\textsubscript{2}O\textsubscript{2} generated in the wells were performed as described above.

Binding and internalization of anti-PECAM-\textsubscript{125}I-GOX in cell culture. We utilized two cell types to address the binding and uptake of anti-PECAM: 1) human umbilical vein endothelial cells (HUVECs) and 2) transformed human mesothelioma REN cells transfected with human PECAM-1 cDNA (REN/PECAM cells) (1). Nontransfected REN cells were used as PECAM-negative control cells (28). Binding and internalization of the radiolabeled anti-PECAM-GOX conjugate in cell culture were studied as previously described (16). Cells were subcultured in 24-well plates for 2–3 days to reach confluence. For an estimation of cellular binding, the conjugates containing \textsuperscript{125}I-GOX were added to washed cells (1 µg/well) in Hanks’ buffer solution containing 0.2% BSA and incubated for the indicated time at 37°C. After being washed with buffer, the cells were lysed with 0.1% Triton X-100, and radioactivity in the cell lysates was determined with a gamma counter. To determine the internalization of the conjugated GOX, the cells were incubated at 37°C with conjugates containing \textsuperscript{125}I-GOX for 90 min at 37°C. After being washed to remove unbound radioactivity, the cells were incubated with 50 mM glucose and 100 mM NaCl, pH 2.5 (15 min at room temperature) to release surface-associated antibody. There was no detectable cell detachment or visible morphological changes after treatment with glucose buffer as determined by light microscopy. After collection of the glucose eluates, the cells were detached by incubation with trypsin-EDTA. Surface-associated radioactivity (i.e., radioactivity of the glucose eluates) and cell-associated radioactivity (i.e., radioactivity of cell lysates) were determined with a gamma counter. The percentage of internalization was calculated as [(total radioactivity – glycine eluted) \times 100]/total radioactivity.

Detection of intracellular and extracellular H\textsubscript{2}O\textsubscript{2} generated by the cell-bound anti-PECAM-GOX in REN/PECAM cells. To detect and localize H\textsubscript{2}O\textsubscript{2} in cell culture, we used the oxidant-sensitive fluorescent dye dihydrorhodamine (DHR) 123. DHR is not readily oxidized by the superoxide anion, but it is rapidly oxidized by peroxynitrite and the hydroxyl radical as well as by H\textsubscript{2}O\textsubscript{2} in the presence of peroxidases (25). In our experiments, we incubated the cells with 15 µM DHR for 30 min before treatment with the conjugate. Nonbound extracellular dye was removed by washing. DHR-loaded cells were incubated with anti-PECAM-GOX (10 µg/ml in Hanks’ buffer containing 5 mg/ml of BSA, pH 7.4). After elimination of nonbound conjugate, the cells were further incubated for 30 min at 37°C with RPMI medium containing 10 mM glucose and inspected in a fluorescent microscope with a triple-band dichroic mirror (D/F/R-BS&M, Chroma Technology, Brattleboro, VT), with a wide-range rhodamine red filter providing good resolution from green (excitation 560 nm and emission 630 nm). To avoid potential artifacts associated with photoexposure (e.g., photobleaching), the cells were exposed to exciting light for time intervals of 10–15 s. The conditions were consistent when all images presented were taken.

Afterward, supernatants and cell lysates were collected, and rhodamine fluorescence (excitation at 510 nm and emission at 529 nm) was determined with a spectrofluorimeter. Background fluorescence in control wells containing DHR...
123-labeled cells was 220 ± 21 arbitrary fluorescence units (AFU). Bovine liver catalase (50 µg/well) was added in some wells to degrade H2O2 in the extracellular medium.

Determination of cytotoxicity of anti-PECAM-GOX or H2O2 added to the cells. Cell death in culture was determined by the specific release of 51Cr. Reports from our laboratory (19) and another group (4) document that this assay permits an accurate and quantitative assessment of oxidant-induced cellular death (more precisely, irreversible damage to the plasma membrane) comparable with the results obtained with other methods of cell death detection, for example, trypan blue staining. To label the cells, 51Cr isotope (200,000 counts·min⁻¹·well⁻¹) was added 24 h before the experiment. The cells were washed with Hanks’ buffer to eliminate nonbound isotope, incubated with the indicated amount of anti-PECAM-GOX or IgG-GOX conjugate for 1 h at 37°C in Hanks’ buffer containing 5 mg/ml of BSA, and washed again to eliminate nonbound conjugates. Afterward, cell medium (medium 199 for HUVECs or RPMI medium for REN and REN/PECAM cells) supplemented with 10 mg/ml of glucose was added to the wells. At the indicated time, aliquots of the supernatants were collected, and radioactivity was determined. After 20 h of incubation of the cells in a CO2 incubator at 37°C, total radioactivity in the wells was determined by collecting the supernatants and cell lysates. 51Cr release was determined as the percent radioactivity in the supernatants.

In separate wells, H2O2 was added to the wells to final concentrations of 1–30 mM (this range of H2O2 concentrations has been used because of a relatively high resistance of REN cells to oxidative injury). To determine the effect of inactivation of the cellular catalase, the cells were incubated with 50 mM ATZ 30 min before treatment with the conjugate or H2O2. To determine the effect of extracellular catalase, 50 µg of bovine catalase (20,000 U/mg) were added to the cell medium before the addition of glucose or H2O2. In the experiments with glucose deprivation, the cells were supplemented with 5 mM fructose to avoid cell starvation.

RESULTS

Antigen-binding capacity and enzymatic activity of anti-PECAM-GOX conjugate. To demonstrate that SA cross-links murine b-MAb (anti-PECAM) and enzymatically active b-GOX, we first incubated anti-PECAM-GOX conjugate in wells coated with anti-IgG. Control wells were coated with BSA. As shown in Fig. 1A, subsequent incubation with a glucose solution led to H2O2 generation only in wells containing anti-PECAM-GOX bound to anti-IgG and not in control wells. No H2O2 was detected in the wells coated with anti-IgG or BSA and incubated with unconjugated GOX.

To characterize the antigen-binding activity of the conjugate, we incubated the anti-PECAM-GOX or its nonimmune counterpart IgG-GOX in wells coated with either purified PECAM-1 (in the form of a PECAM-Ig fusion protein) or BSA. As shown in Fig. 1B, subsequent incubation with a glucose solution led to H2O2 generation only in the PECAM-coated wells containing anti-PECAM-GOX and not in the control wells. IgG-GOX produced no H2O2 in the wells coated with either PECAM or BSA, thus confirming the specificity of anti-PECAM-GOX binding to immobilized PECAM.

These results indicate that the conjugation procedure yields a trimolecular complex (anti-PECAM-GOX conjugate) composed of an active murine b-MAb to PECAM-1, SA cross-linker, and enzymatically active b-GOX.

Immunotargeting of anti-PECAM-GOX to PECAM-expressing cells. To examine the specific targeting of anti-PECAM-GOX to PECAM-expressing cells, we determined the uptake of the conjugate by a human endothelial-like cell line (REN cells derived from a malignant mesothelioma) that does not normally express PECAM-1 (REN/PECAM−) as well as by REN cells genetically modified to express PECAM-1 (REN/PECAM+).

To determine the binding specificity, we incubated cells with anti-PECAM-125I-GOX or IgG-125I-GOX. As shown in Fig. 2A, significant binding occurred when anti-PECAM-125I-GOX was added to REN/PECAM+ cells but not to control REN cells. IgG-125I-GOX did not bind to either cell type. Importantly, elution of the surface-bound 125I-GOX by acidic buffer showed that 71 ± 8% of the anti-PECAM-125I-GOX that bound to the REN/PECAM+ cells was internalized after 90 min of incubation at 37°C.

To determine the enzymatic activity of the bound conjugates, REN/PECAM+ or REN/PECAM− cells were incubated with anti-PECAM-GOX or Ig-GOX conjugates, exposed to glucose for 60 min, and then lysed. Figure 2B shows that H2O2 was detected in the lysates...
of REN/PECAM+ cells treated with anti-PECAM-GOX but not with IgG-GOX. There was no significant \( \text{H}_2\text{O}_2 \) production in control REN/PECAM− cells treated with either conjugate.

To assess the biological consequences of anti-PECAM-GOX targeting, REN/PECAM+ and REN/PECAM− cells were first loaded with \( ^{51}\text{Cr} \) and then reacted with either anti-PECAM-GOX or IgG-GOX. Analysis of \( ^{51}\text{Cr} \) release revealed that after exposure to glucose-containing medium, anti-PECAM-GOX caused a significant increase in cell death in REN/PECAM+ cells but not in control REN cells (Fig. 2C). The results of \( ^{51}\text{Cr} \) release were confirmed by observation of the morphological signs of cellular death, including shape changes, vacuolization of the cytoplasm, disruption of the cellular monolayer, and cell detachment. In contrast, \( ^{51}\text{Cr} \) release from either cell type treated with IgG-GOX was indistinguishable from the control level (14 ± 5%). Neither nonconjugated anti-PECAM nor \( \beta \)-anti-PECAM-SA conjugated with biotinylated ferritin or catalase caused cellular damage or death in HUVECs or in REN cells transfected with PECAM (data not shown). In addition, nonconjugated anti-PECAM did not affect the sensitivity of REN/PECAM cells to a reagent, \( \text{H}_2\text{O}_2 \). These data indicate that the cytotoxic effect of anti-PECAM-GOX is mediated by the enzymatic activity of GOX.

To confirm that the cytotoxic effect of anti-PECAM-GOX was dependent on enzymatic conversion of glucose to \( \text{H}_2\text{O}_2 \), but glucose starvation is not responsible for the conjugate toxicity.

The study of transfected cells versus nontransfected cells offers a valuable system to define specificity of the targeting and the effects of anti-PECAM-GOX. However, although the REN/PECAM+ cells displayed levels of PECAM expression and localization in the plasma membrane similar to those in endothelial cells (1), they may not behave identically to endothelial cells with regard to their sensitivity to \( \text{H}_2\text{O}_2 \) and their ability to bind and process the conjugate. We therefore repeated these experiments with cultured HUVECs. Although the absolute values of the conjugate binding varied (depending on the HUVEC donors, passage, density, and confluence of the culture), binding of anti-PECAM-\( ^{125}\text{I} \)-GOX (for example, 5.3 ± 0.2 vs. 1.3 ± 0.2 ng/well in HUVECs of fourth passage). Similar to the REN/PECAM+ cells, 60 ± 4% of anti-PECAM-\( ^{125}\text{I} \)-GOX bound to HUVECs was internalized after 90 min of incubation at 37°C. Again, like in the REN/PECAM+ cells, cell-bound anti-PECAM-GOX generated \( \text{H}_2\text{O}_2 \) in glucose-containing medium and caused cellular damage: 84 ± 7% of \( ^{51}\text{Cr} \) release was detected in cells treated with anti-PECAM-GOX vs. 26 ± 3% of \( ^{51}\text{Cr} \) release in cells treated with IgG-GOX.

Therefore, these results show that anti-PECAM-GOX specifically binds to PECAM-positive target cells, enters the cells, generates \( \text{H}_2\text{O}_2 \) from glucose, and kills the target cells. Because anti-PECAM-GOX had similar effects in REN/PECAM+ cells and HUVECs, we utilized the more uniform REN/PECAM+ cells in the remainder of our studies.

Intracellular generation of \( \text{H}_2\text{O}_2 \) by anti-PECAM-GOX. Although the experiments described in Immunotargeting of anti-PECAM-GOX to PECAM-expressing cells indicated that the majority of the cell-bound
anti-PECAM-GOX was internalized and that the cell-bound conjugate generated H$_2$O$_2$, these studies did not exclude the theoretical possibility that only the non-internalized conjugate is active and H$_2$O$_2$ was produced extracellularly.

To define the site of H$_2$O$_2$ generation, we used the dye DHR 123 as a probe (25). DHR forms a fluorescent dye, rhodamine, when oxidized by H$_2$O$_2$ in the presence of peroxidases or metals. DHR is also rapidly oxidized by peroxynitrite but not by superoxide anion. Therefore, its oxidation to rhodamine is indicative of the production of ROS with a high oxidative potential. DHR is particularly useful in the monitoring of intracellular oxidant production. Both DHR and its oxidation product rhodamine are membrane permeable. However, unlike DHR, rhodamine is a charged lipophilic compound and therefore is retained in negatively charged intracellular compartments such as the mitochondrion (25).

REN/PECAM+ cells loaded with DHR were incubated with anti-PECAM-GOX for 1 h at 37°C to allow for conjugate uptake by the cells. The cells were then washed and incubated in RPMI medium containing 10 mg/ml of glucose for a further 30 min in either the absence or presence of catalase (1,000 U) in the medium. In parallel wells, nonconjugated GOX (extracellular GOX; 1 µg/well) was added to REN/PECAM+ cells in glucose-containing medium to generate H$_2$O$_2$ extracellularly in the presence and absence of catalase in the medium.

The results of this experiment were assessed qualitatively by observation with epifluorescence microscopy. Intracellular rhodamine fluorescence was observed in the wells treated with anti-PECAM-GOX (Fig. 3A). In contrast, diffuse fluorescence was seen in the medium but not in the cells treated with extracellular GOX, indicating that H$_2$O$_2$ was produced primarily in the medium (Fig. 3C). The addition of catalase to the medium had no detectable effect on the intracellular fluorescence in the PECAM-GOX-treated cells (Fig. 3B), whereas it diminished fluorescence in the cell medium produced by the addition of extracellular GOX (Fig. 3D). This effect of catalase confirms the intracellular location of H$_2$O$_2$ generated by anti-PECAM-GOX.

To gain more quantitative data about the relative production of H$_2$O$_2$ in the intracellular and extracellular compartments, cell lysates and medium were harvested postincubation, and relative fluorescence was directly measured with a spectrofluorimeter. Figure 4A shows the distribution of rhodamine fluorescence in the cell lysates compared with that in the medium obtained from the cells incubated with either anti-PECAM-GOX or nonconjugated GOX in the absence of catalase in the medium (corresponding to conditions in Fig. 3, A and C, respectively). Although the total fluorescence was higher in the wells with GOX-treated than with PECAM-GOX-treated cells (880 ± 39 vs. 500 ± 49 AFU), the majority of fluorescence was intracellular in the anti-PECAM-GOX-treated cells (70% of total fluorescence), whereas only 11% of the total fluorescence was intracellular in the GOX-treated cells. Figure 4B shows the distribution of rhodamine fluorescence in the cell culture when catalase was added to the medium. Under these conditions (corresponding to those in Fig. 3, B and D), total fluorescence recovered from the wells with anti-PECAM-GOX-treated cells was higher than that recovered from the wells with GOX-treated cells (406 ± 2 vs. 307 ± 38 AFU). Importantly, in the cells treated with anti-PECAM-GOX, 96% of the fluorescence was intracellular, whereas in the cells incubated with GOX, only 10% of the fluorescence was intracellular.

These results indicate that the major portion of the H$_2$O$_2$ generated by the cell-bound anti-PECAM-GOX

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**Fig. 3.** Intracellular generation of H$_2$O$_2$ by anti-PECAM-GOX in REN/PECAM cells as visualized by rhodamine fluorescence. Cells were loaded with dihydrorhodamine (DHR) 123 before incubation with conjugate. Images show rhodamine fluorescence in wells. A and B: cells incubated for 1 h with anti-PECAM-GOX (10 µg/well). After non-bound conjugate was washed, cells were incubated further for 30 min in glucose-containing medium. C and D: control cells incubated for 30 min with GOX added to medium (1 µg/well). B and D: catalase was added to glucose-containing medium. Note that background in C is lighter due to H$_2$O$_2$ generated by free GOX in medium. Fluorescence of untreated (control) cells loaded with DHR 123 was similar to that of cells treated with extracellular GOX in presence of extracellular catalase (D).
was formed in the intracellular compartment. Rhodamine fluorescence detected in the catalase-free medium of anti-PECAM-GOX-treated cells (Fig. 4A) implies that either the noninternalized portion of anti-PECAM-GOX (~30% of the cell-bound conjugate according to a tracing of 125I-GOX) generates H2O2 on the external surface of the cells or H2O2 generated intracellularly diffuses to the medium. Because cellular antioxidants (catalase, GSH, and peroxidases) do not degrade extracellular H2O2 unless it diffuses into the intracellular compartment, extracellular H2O2 accumulating in the medium may thus contribute in some way to cellular oxidative stress (see Role of extracellular catalase in protection against oxidative stress caused by extracellular H2O2 or anti-PECAM-GOX). Catalase added to the medium degrades extracellular H2O2 in anti-PECAM-GOX-treated cells and thus may be useful in eliminating the extracellular component of oxidative stress.

Role of intracellular catalase in protection against oxidative stress caused by extracellular H2O2 or anti-PECAM-GOX. To further characterize the cytotoxic effects of extracellular H2O2 versus anti-PECAM-GOX, we determined the release of 51Cr from labeled cells as a measure of irreversible plasma membrane damage. Use of this technique in conjunction with ATZ treatment (a potent irreversible inhibitor of catalase) allowed us to examine the role of cellular catalase in the defense against these forms of oxidative stress.

First, we characterized the cytotoxic effect of extracellular H2O2 added to the cell medium. Figure 5A shows that 30 mM H2O2 caused a rapid and massive 51Cr release, whereas 1 mM H2O2 caused a delayed 51Cr release evident only after an overnight incubation of REN/PECAM+ cells exposed to H2O2. Reports from several groups (4, 19) documented that 1 mM H2O2 causes marked cell death in HUVECs as soon as a few hours postexposure. Thus our data imply that REN/PECAM+ cells are less sensitive to H2O2 than HUVECs. Pretreatment of the cells with ATZ accelerated the time course and increased the amplitude of 51Cr released (Fig. 5B). This result indicates that intracellular catalase plays an important role in the defense against extracellular H2O2 that diffuses through the plasma membrane into the intracellular compartment. This is illustrated more graphically by comparison of 51Cr release at 3 h in ATZ-treated versus control cells (Fig. 5C). Under these conditions, 10 mM H2O2 caused no detectable 51Cr release in control cells and a marked 51Cr release in ATZ-treated cells.

Figure 6A shows the kinetics of 51Cr release from REN/PECAM+ cells treated with anti-PECAM-GOX. Incubation of anti-PECAM-GOX-treated REN/PECAM+ cells in glucose-containing medium led to cell death, whereas IgG-GOX had no effect on cell viability. The time course of 51Cr release caused by anti-PECAM-GOX at a dose of 10 µg/well was similar to that for treatment with 10 mM extracellular H2O2 (compare Fig. 5A with Fig. 6A). The cytotoxic effect of anti-PECAM-GOX was dose dependent and also facilitated by ATZ (Fig. 6B). As with the reagent H2O2, the effect of ATZ was more evident when 51Cr release was assessed at a short time interval after exposure to anti-PECAM-
GOX in the presence of glucose. Figure 6C shows that anti-PECAM-GOX caused >80% cellular death in ATZ-treated REN/PECAM cells within 3 h, whereas cellular death in the control cells was at the background level (~20%). In addition, this result indicates that IgG-GOX did not cause significant cellular death even in ATZ-treated REN/PECAM cells, thus confirming a high specificity of the anti-PECAM-GOX cytotoxicity. The aggravation of injury by the catalase inhibitor ATZ indicates that H2O2 produced from glucose causes oxidative stress and cellular death.

Role of extracellular catalase in protection against oxidative stress caused by extracellular H2O2 or anti-PECAM-GOX. To define the role of extracellular generation of H2O2 in the cellular toxicity of anti-PECAM-GOX conjugate, 51Cr-labeled REN/PECAM + cells were exposed to medium (control), anti-PECAM-GOX, or 10 mM extracellular H2O2 in the absence and presence of extracellular catalase (Fig. 7). When the cells were examined after 9 h of incubation in glucose-containing medium (Fig. 7A), catalase added to the medium abrogated 51Cr release caused by either anti-PECAM-GOX or 10 mM extracellular H2O2. This result suggests that H2O2 generated by the surface-bound portion of the conjugate (see Fig. 4B) accumulated in the medium and accelerated the cytotoxic effects of anti-PECAM-GOX. Importantly, extracellular catalase had no significant effect on the late manifestations of cellular injury seen 20 h after incubation with cell-bound anti-PECAM-GOX in glucose-containing medium (Fig. 7B). This result supports the conclusion that a major portion of H2O2 generated by anti-PECAM-GOX is intracellular and thus is inaccessible for the extracellular catalase. In contrast, catalase completely abrogated the late manifestations of the cytotoxic effect of extracellular H2O2. Therefore, in the absence of catalase in the medium, anti-PECAM-GOX causes oxidative stress with both intracellular and extracellular components. Catalase added to the medium degrades extracellular H2O2, diminishes an extracellular component, and delays manifestation of the oxidative stress and cell injury.

We also characterized the protective effect of extracellular catalase in REN/PECAM + cells treated with the intracellular catalase inhibitor ATZ (Fig. 8). Although catalase completely rescued ATZ-treated cells when they were exposed to extracellular H2O2, extracellular catalase had no protective effect against anti-PECAM-GOX in ATZ-treated cells. These results confirm that...
H₂O₂ produced by anti-PECAM-GOX causes intracellular oxidative stress, which cannot be prevented by extracellular catalase.

**DISCUSSION**

The causes, mechanisms, manifestations, and methods for therapy of oxidative stress have been the focus of enormous interest for several decades. The overwhelming antioxidant defenses or the pathological overproduction of ROS result in modification and inactivation of functionally or structurally important components in cells and in the extracellular milieu, thus leading to tissue injury, cell death, or functional abnormality.

The site of ROS formation determines the targets of oxidative injury and is critical for their action. For example, more reactive ROS (e.g., the hydroxyl radical formed from H₂O₂) have a very limited radius of action because they react promiscuously with target molecules in close proximity to the ROS formation site. Less reactive ROS (such as H₂O₂) are more diffusible, although the radius of their action is limited due to degradation by enzymes (e.g., catalase) or scavengers (e.g., GSH). Intracellularly generated ROS attack intracellular components and the inner leaflet of the plasma membrane, whereas extracellularly generated ROS attack blood elements, glycocalix components, surface proteins, and the outer leaflet of the plasma membrane. Therefore, intracellular and extracellular ROS may cause different effects and forms of oxidative stress in the cells.

Both extracellular and intracellular ROS may cause endothelial oxidative stress in vivo. For example, activated leukocytes adhere to endothelium at the site of inflammation and release ROS. Although the ROS released from the activated leukocytes are initially extracellular, they may act as intracellular ROS after diffusion through the endothelial plasma membrane. Extracellular oxidative stress in endothelium can be studied in cell culture by direct exposure of endothelial cells to ROS (such as H₂O₂) or ROS-generating enzymes (such as GOX or xanthine oxidase) or by addition of activated leukocytes to the cell culture medium (4, 19, 24). It also may be modeled in vivo by systemic activation of circulating leukocytes in animals (9).

Another scenario is the oxidative stress caused by excessive ROS generation within the endothelium. Endothelial cells can generate ROS (e.g., H₂O₂ or O₂⁻) via enzymatic pathways including 1) xanthine oxidase formed from xanthine dehydrogenase on hypoxia (24, 32); 2) lipoxygenase and cyclooxygenase activated by hormones, cytokines, or inflammatory mediators (7, 23); 3) univalent reduction of molecular oxygen by respiratory chain enzymes in the mitochondria (22, 26); 4) uncoupled superoxide anion generation by endothelial nitric oxide synthase (23); and 5) NADPH oxidase and/or NADH oxidase activated on ischemia and/or by cytokines (13, 31). The first four of these enzymatic pathways generate ROS intracellularly, whereas the latter one is postulated to generate ROS in close proximity to the plasma membrane. Therefore, a substantial (perhaps major) fraction of endothelium-derived ROS is generated inside the endothelium (intracellular ROS). The amplitude of ROS generation by endothelial cells is lower than that of leukocytes, but the duration of endothelial ROS generation may be longer.

Intracellular oxidative stress in the endothelium has been difficult to study, although numerous approaches have been described. Chemicals such as quinones (e.g., menadione) enter cells and cause ROS generation (2, 27). Anoxia-reoxygenation, cytokines, phorbol 12-myristate 13-acetate, growth factors, bradykinin, and lipoproteins stimulate ROS generation by the endothelium in culture (7, 11, 23, 29–31). These models, however, have certain limitations. First, commonly used (patho)physiological inducers of ROS generation, such as cytokines, bradykinin, or hypoxia, cause complex alterations in the endothelium. Therefore, it is difficult to dissect out the effects due directly to ROS versus ROS-unrelated and/or secondary effects of ROS inducers. Second, most of these inducers cause concomitant generation of several ROS (i.e., both H₂O₂ and O₂⁻), thus confusing the analysis of the role for a particular ROS. Third, there are no known methods to provide a controlled rate of intracellular flux of ROS. Finally, some of them is specific to the endothelium. For example, injection of cytokines or quinone would cause ROS generation by a variety of cell types, including leukocytes. Therefore, these previously described strategies would not be applicable to study cell-specific endothelial intracellular oxidative stress in vivo.

In the present study, we have developed a novel approach that allows intracellular generation of a defined ROS, H₂O₂, within endothelial cells by using intracellular immunotargeting of GOX. Our results indicate that GOX conjugated with a monoclonal antibody to the endothelial cell surface antigen PECAM-1 binds selectively to endothelial cells, enters the cells, generates H₂O₂ intracellularly, and induces an oxidative stress, which leads to irreversible damage to the endothelial plasma membrane. Intracellular catalase plays an important role in the cellular defense against anti-PECAM-GOX. It also protects cells against extra-
cellular H$_2$O$_2$, presumably via degradation of H$_2$O$_2$ diffusing through the plasma membrane into the intracellular compartment. Oxidative stress induced by anti-PECAM-GOX markedly differs from that induced by extracellular H$_2$O$_2$: extracellular catalase fails to protect cells against anti-PECAM-GOX, whereas it effectively protects either normal or ATZ-treated cells against extracellular H$_2$O$_2$.

Failure of extracellular catalase to provide protection against anti-PECAM-GOX, taken together with fluorescent visualization of the intracellular H$_2$O$_2$ generated by anti-PECAM-GOX, provides strong evidence that the internalized conjugate is enzymatically active. This is the first direct experimental evidence of the intracellular delivery of an active ROS-generating enzyme. This result, therefore, provides important support for the strategy of intracellular delivery of enzymes (and perhaps other compounds and drugs) by immunotargeting (15–17).

Although anti-PECAM-GOX was readily internalized, ∼30% of the cell-bound conjugate remained on the cell surface and produced H$_2$O$_2$ in the extracellular compartment. Analysis of DHR fluorescence showed that ∼30% of the total H$_2$O$_2$ generated by the cell-bound anti-PECAM-GOX was also located in the extracellular compartment. Thus production of H$_2$O$_2$ by the surface-associated conjugate and/or diffusion of H$_2$O$_2$ from the intracellular to the extracellular compartment occurs in our model. In the absence of extracellular catalase, both processes lead to accumulation of extracellular H$_2$O$_2$. Because extracellular H$_2$O$_2$ is not decomposed by the cellular antioxidant defense (unless it diffuses back to the intracellular compartments), its concentration may reach toxic levels. Thus extracellularly accumulated H$_2$O$_2$ contributes to the oxidative stress, augments the toxic effect of intracellular H$_2$O$_2$, and aggravates cellular injury caused by anti-PECAM-GOX.

In the presence of extracellular catalase, the manifestation of the conjugate cytotoxicity is delayed because anti-PECAM-GOX requires longer time to generate enough purely intracellular H$_2$O$_2$ to cause cell death. In addition, lower doses of H$_2$O$_2$ require a longer time to reveal the toxicity (Fig. 5). We conclude that extracellular catalase allowed us to model a pure intracellular oxidative stress caused by anti-PECAM-GOX. Under physiological conditions, the endothelial extracellular luminal compartment is blood, a tissue possessing an extremely high antioxidant potential. Thus cell culture experiments with anti-PECAM-GOX in the presence of catalase in the cell medium adequately models the situation in vivo. Such an adequate design is not possible in cell culture experiments with exposure of cells to extracellular ROS because antioxidants in the cell medium degrade these readily accessible ROS and completely abrogate oxidative stress. Therefore, intracellular immunotargeting of ROS-generating enzymes may permit a better approximation to pathophysiological conditions in cell culture experiments.

In addition to providing a way to study a defined intracellular oxidative enzyme in endothelial cell culture, two key features of immunotargeting (specific recognition of the endothelial cells and intracellular delivery of ROS-generating enzymes to the vascular endothelium) allow application of this strategy in intact animals. We have recently found that an intravenous injection of anti-PECAM-GOX, but not of IgG-GOX, in mice causes oxidative vascular injury in the pulmonary endothelium (M. Christofidou-Solomidou, G. Pietra, E. Argiris, D. Harshaw, G. FitzGerald, S. Albelda, and V. Muzykantov, unpublished data). Therefore, immunotargeting offers a new modality for the investigation of the intracellular oxidative stress in endothelial cells in vivo. Immunotargeting of xanthine oxidase (that generates both O$_2^-$ and H$_2$O$_2$) or other ROS-generating enzymes may help to define the contribution of a specific ROS in the stress and will provide further flexibility to the models.

Like other approaches, an Immunotargeting strategy has some potential limitations. First, intracellular generation of ROS without concomitant cellular alterations caused by ROS inducers (e.g., cytokines) represents a significant departure from pathophysiological settings. We view this as an inevitable "cost" for a well-defined experimental system allowing us to isolate a role for a specific intracellular ROS.

Although GOX represents a relatively simple system for ROS generation, glucose and H$_2$O$_2$ are not the only components of the reaction. GOX consumes oxygen along with glucose and coproduces uric acid with H$_2$O$_2$. Uric acid is a weak antioxidant; hence it unlikely contributes to the cytotoxic effect of the conjugate. More importantly, 1) GOX may cause hypoxia via oxygen consumption and 2) the tissue level of oxygen may modulate enzymatic activity of anti-PECAM-GOX. Both factors may potentially confuse the results of experiments in cell culture or in vivo. At the present time, in collaboration with Dr. Donald Buerk (University of Pennsylvania, Philadelphia), we are directly characterizing the levels of H$_2$O$_2$ and oxygen in glucose-GOX mixtures utilizing selective electrodes. The goal of this ongoing study is to quantify the efflux of H$_2$O$_2$ in the cells as well as the consumption of oxygen and regulation of enzymatic activity of GOX (e.g., inhibition by the products). Preliminary results of our measurements indicate that 1 µg of GOX causes no more than a 10% reduction of oxygen level in the presence of 10 mg/ml of glucose. Apparently, oxygen diffusion compensates for the further decline in oxygen level and diminishes the "hypoxic" effect of anti-PECAM-GOX. The amount of cell-associated anti-PECAM-GOX in our experiments does not exceed 0.5–0.7 µg/well. We conclude, therefore, that oxygen consumption plays only a minor, if any, role in anti-PECAM-GOX action. Also, results of our in vivo study (Christofidou-Solomidou et al., unpublished data) directly indicate that anti-PECAM-GOX does cause an oxidative lung injury in the pulmonary vasculature. Most likely, continued ventilation and oxygen transport/diffusion compensate for a low consumption of oxygen by anti-PECAM-GOX.

The amplitude of ROS generation by the intracellularly delivered enzymes may exceed that occurring at
physiological and even pathological conditions in vivo. This represents a potential problem because the supra-
physiological levels of H2O2 in model studies require careful interpretation. The actual levels of ROS gener-
ation by the endothelium in various pathological set-
tings are still to be determined. Utilization of radiola-
beled GOX or xanthine oxidase may help to quantitate
the amount of ROS-generating enzymes delivered to
the target cells (either in cell culture or in vivo) and
may thus provide a correlation between the effects of
ROS-generating enzymes and the rate of ROS flux.

The issue of dose effects may be particularly interest-
ning when one begins to differentiate the cytotoxic
effects of intracellular H2O2 from lower doses that may
affect signaling pathways. H2O2 is becoming increas-
ingly recognized as a signal transduction molecule with
widespread effects on the cytoskeleton, arachidonic acid
metabolism, antioxidant defenses, apoptosis, and
other physiological parameters of endothelial cells.
Delivery of lower amounts of the conjugates coupled
with careful measurements of H2O2 production may
provide a very useful system for studying signaling
events.

Finally, we can envision endothelial GOX immunotar-
geting as a potential therapeutic agent. For example,
by replacing the anti-PECAM carrier with an antibody
recognizing antigens upregulated in the tumor vascula-
ture (i.e., the vascular endothelial cell growth factor
receptor), one could potentially generate an agent
useful for the eradication of tumors. This approach
could complement antitumor therapy based on the
immunotargeting of toxins or procoagulants to the
tumor vascular endothelium (8).

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