Bromide-dependent Toxicity of Eosinophil Peroxidase for Endothelium and Isolated Working Rat Hearts: A Model for Eosinophilic Endocarditis

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Summary

Eosinophilic endocarditis is a potentially lethal complication of chronic peripheral blood hypereosinophilia. We hypothesized that eosinophil peroxidase (EPO), an abundant eosinophil (EO) cationic granule protein, promotes eosinophilic endocarditis by binding to negatively charged endocardium, and there generating cytotoxic oxidants. Using an immunocytochemical technique, we demonstrated endocardial deposition of EPO in the heart of a patient with hypereosinophilic heart disease. Because EPO preferentially oxidizes Br^- to hypobromous acid (HOBr) rather than Cl^- to hypochlorous acid (HOCl) at physiologic halide concentrations, we characterized the Br^--dependent toxicity of both activated EOs and purified human EPO towards several types of endothelial cells and isolated working rat hearts. In RPMI supplemented with 100 μM Br^-, phorbol myristate acetate–activated EOs, but not polymorphonuclear leukocytes, caused 1.8–3.6 times as much ^{51}Cr release from four types of endothelial cell monolayers as in RPMI alone. H_2O_2 and purified human EPO, especially when bound to cell surfaces, mediated extraordinarily potent, completely Br^--dependent cytolysis of endothelial cells that was reversed by peroxidase inhibitors, HOBr scavengers, and competitive substrates. We further modeled eosinophilic endocarditis by instilling EPO into the left ventricles of isolated rat hearts, flushing unbound EPO, then perfusing them with a buffer containing 100 μM Br^- and 1 μM H_2O_2. Acute congestive heart failure (evidenced by a precipitous decrement in rate pressure product, stroke volume work, aortic output, and MVO_2 to 0–33% of control values) ensued over 20 min, which deletion of EPO, Br^-, or H_2O_2 completely abrogated. These findings raise the possibility that EPO bound to endocardial cells might utilize H_2O_2 generated either by overlying phagocytes or endogenous cardiac metabolism along with the virtually inexhaustible supply of Br^- from flowing blood to fuel HOBr-mediated cell damage. By this mechanism, EPO may play an important role in the pathogenesis of eosinophilic endocarditis.

Chronic peripheral blood eosinophilia can be complicated by a morbid and potentially lethal form of endocarditis characterized by eosinophils (EOs)[1] adhering to and infiltrating the endocardium, mural thrombosis, and embolism (1, 2). Progressive endocardial, then myocardial, damage in eosinophilic endocarditis typically culminates in congestive heart failure or endomyocardial fibrosis (1, 3). Such hypereosinophilic heart disease, though rare in Western societies, is estimated to cause 10–20% of all cardiac deaths in tropical Africa and Southeast Asia, where chronic hypereosinophilia (presumably caused by filariasis or other parasitic infestations) is endemic (4). Individuals with hypereosinophilic heart disease have blood EOs with morphologic and metabolic evidence of activation (5) as well as high serum levels of potentially cytotoxic EO granule cationic proteins such as major basic protein (MBP) (6). This suggests that damage to the endothelial lining of the heart, the endocardium, results from misapplication of the potent EO antiparasitic armamentarium, a hypothesis further buttressed by the finding of dense endocardial deposition of two EO cationic granule proteins, MBP and eosinophil cationic protein (ECP), in such patients (7). Why endocardium in particular should be so much more afflicted than other endothelial cell beds in hypereosinophilic states is, however, unknown.

We hypothesized that another EO cationic granule pro-

[1] *Abbreviations used in this paper:* APAAP, alkaline phosphatase-mouse anti-phosphatase; BAEC, bovine aortic endothelial cells; BENDOC, bovine endocardial cells; EBSS, Earle's buffered salt solution; ECP, eosinophil cationic protein; EO, eosinophil; EPO, eosinophil peroxidase; HOBr, hypobromous acid; HOCl, hypochlorous acid; HUVEC, human umbilical vein endothelial cells; K-H, Krebs-Henseleit buffer; MBP, major basic protein; PAEC, porcine aortic endothelial cells; RPP, rate pressure product; SV, stroke volume.

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tein, eosinophil peroxidase (EPO), which plays a pivotal role in killing helminthic parasites (8), might also contribute to the pathogenesis of eosinophilic endocarditis by binding to endocardial cell surfaces and there generating cytotoxic oxidants. EPO (unlike MBP and ECP, which appear to rely mainly on their membrane-perturbant properties to cause cellular disruption [9, 10]) exerts a cytotoxic effect primarily through its peroxidative catalytic action: utilizing H_2O_2 to oxidize a halide to its corresponding hypohalous acids (11). Of note, EPO is a peroxidase unique to EOs that, in contrast to polymorphonuclear myeloperoxidase, preferentially oxidizes Br^- to hypobromous acid (HOBr) rather than Cl^- to hypochlorous acid (HOCl) at physiologically relevant (i.e., serum) concentrations, where Cl^- is in >1,000-fold excess (100 mM Cl^-, 20–100 μM Br^-) (12, 13).

The present investigation defines a potential role for EPO and its unusual halide preference in promoting eosinophilic endocarditis by demonstrating potent Br^--dependent toxicity of activated EOs and purified human EPO towards endothelium, endocardium, and isolated working rat hearts. In the presence of Br^-, even a small amount of EPO bound to the surface of endothelial cells renders them susceptible to complete destruction by micromolar H_2O_2 by localizing and thereby "focusing" HOBr damage to vulnerable cell components. Because endocardial cells coated with EPO could potentially utilize H_2O_2 generated by the active aerobic metabolism of underlying myocardial cells to fuel their own destruction, our findings may also explain the proclivity of hypereosinophilic states to damage endocardium more than other endothelial beds.

Materials and Methods

Materials. 24-well plates (2 cm^2/well) were obtained from Costar (Cambridge, MA), PMA was from Consolidated Midland Corporation (Brewster, NY), and Earle's buffered salt solution (EBSS), HBSS, Dulbecco's modified MEM, and FCS were obtained from Gibco Laboratories (Grand Island, NY). Hetastarch™ (6% hydroxyethyl starch in normal saline) was obtained from DuPont Co. (Wilmington, DE), and ^{51}Cr was from New England Nuclear (Boston, MA). Sodium bromide, methionine, histidine, guaiacol, sodium azide, aminotriazole, catalase, BSA, cetrimide (hexadecyltrimethylammonium bromide), and hydrogen peroxide (30% solution) were obtained from Sigma Chemical Co. (St. Louis, MO). Percoll was obtained from Pharmacia Fine Chemicals (Piscataway, NJ). Murine IgG1 monoclonal AHE-1, which is specific for EPO (14), was kindly supplied by Dr. Keith Skubitz, University of Minnesota (Minneapolis, MN).

Purified human MBP and EPO were kindly provided by Dr. Gerald J. Gleich (Mayo Clinic and Research Foundation, MN) and were isolated from eosinophil-rich cell suspensions obtained by leukapheresis from patients with hypereosinophilic syndrome, as previously described (15). Briefly, EO cationic granule proteins were prepared from a leukapheresis specimen containing 94% eosinophils by disrupting the cells with shear stress, solubilizing granules by free-thawing, and extraction with 0.01 M HCl. Granule extracts were then chromatographed on a Sephadex G-50 column equilibrated with 0.25 M acetate buffer (pH 4.3) with 0.15 M NaCl. The fractions eluting with the void volume were then collected and further purified to an $OD_{415/280\ nm}$ ratio of >0.9 by chromatography on carboxymethyl Sepharose, as described by Carlson et al. (16). The homogeneity of the EPO prep was further corroborated by SDS-PAGE, which revealed only two discrete bands (molecular mass of ~78 and ~14 kD) that correspond to the large and small fragments of the EPO molecule. No significant contaminants were present. EPO activity was assayed by guaiacol oxidation and converted to international units (the amount of enzyme that oxidizes 1 μmol of electron donor/min at 25°C) (17). The specific activity of the preparations utilized in the course of these experiments was 133–250 U/mg protein.

Preparation of Endothelial Cell Monolayers. Human umbilical vein endothelial cells (HUVEC) were prepared by collagenase treatment of umbilical cords as previously described (18). Cells were seeded in 24-well plates (2 cm^2/well) (Costar) and used as primary cultures upon achieving confluence, usually 5 d after seeding. Porcine and bovine aortic endothelial cells (PAEC and BAEC) were obtained by collagenase treatment of fresh porcine aortas as previously described (19). Bovine endocardial cells (BENDOC), kindly supplied by Una Ryan (Miami, FL), were established from cow heart scrapings. The bovine and porcine cell lines were passaged two times weekly and dispensed onto gelatin-coated plastic tissue culture plates before seeding 24-well plates for cytotoxicity experiments, described below, upon achieving confluence.

Immunohistochemical Staining of Heart Sections. Sections from formalin-fixed and paraffin-embedded tissue obtained from the heart of a patient who underwent cardiac transplantation for hypereosinophilic heart disease were stained for the presence of EPO using AHE-1 mAB specific for EPO and the alkaline phosphatase-mouse anti-alkaline phosphatase (APAAP) technique as previously described (14). A 1:100 dilution of AHE-1 or normal mouse ascites (negative control) was used in the primary antibody incubation. Positive controls were provided by the intense staining of intact eosinophils, but not other inflammatory cells present in the heart sections incubated with AHE-1 only (not shown). Normal heart sections stained with AHE-1 and APAAP showed no deposition. AHE-1 does not bind to MBP, permeabilized neutrophils (and therefore, myeloperoxidase), cathepsin G, or elastase (14).

Eosinophil Purification. EOs were isolated from peripheral blood (50–200 m) of individuals with mild to moderate peripheral blood eosinophilia (4–39%), due primarily to allergy. Isolation of EOs was accomplished using our modification (20) of the FMLP-EDTA technique originally described by Roberts and Gallin (21). The cell yield using this technique was 60–90%; EO purity was 85–99.5% (mean 94%); contaminating cells were largely PMNs.

^{51}Cr Release Cytotoxicity Assay. Endothelial cell toxicity was assayed by measuring specific ^{51}Cr release from labeled monolayers as previously described (20). For HUVEC, BAEC, and BENDOC, incubations were for 18 h at 37°C in a 5% CO_2 and 100% humidified atmosphere; for PAEC, incubations were 2.5 h under identical conditions. Percent specific ^{51}Cr release was calculated as follows: $100\times [(a-c)/(b-c)]$, where a = cpm in the supernatant of any given well, b = total counts (pellet + supernatant) in that well, and c = the mean cpm "spontaneous" release of ^{51}Cr into supernatant buffer of four wells containing labeled monolayers and EBSS only. Spontaneous release was typically 15–20% of the total counts per well. All experiments were conducted using triplicate or quadruplicate determinations of each treatment group. Experiments using intact EOs utilized 2.5 × 10^6 cells/well, yielding an E/T ratio of 20–25:1. EO contact with the endothelial cell monolayers was then ensured by gentle centrifugation (50 g for 4 min) of the 24-well plates. Inhibitors, such as azide and aminotriazole, were added before centrifugation and agonists, such as PMA, after centrifugation. For experiments utilizing purified human EPO, 200

μl of a solution containing 5×10^{-8} M EPO (3.5 μg/ml, 0.5 mU/ml) suspended in HBSS was added to monolayers, which were subsequently incubated 15 min at 37°C. Supernatant buffer containing unbound EPO was then aspirated and discarded, and the monolayers were subsequently washed two times in 1 ml of warm HBSS. Monolayers not treated with EPO were sham washed and incubated and washed in a similar fashion.

Hydrogen Peroxide Consumption Assay. Halide-dependent consumption of hydrogen peroxide by EPO was assayed by following the disappearance of H_2O_2 spectrophotometrically at 230 nm (22).

Native Chemiluminescence Assay of Purified EPO System. Native chemiluminescence assays were performed in clear glass scintillation vials in a scintillation counter (LS 230; Beckman Instruments, Inc., Palo Alto, CA) in the out of coincidence mode. All reagents, except EPO, were added to the vials that were subsequently allowed to equilibrate in the dark for 5 min before addition of EPO and sequential 6-s quantitations of light emission every 20 s. Reaction mixtures contained final concentrations of 10 mM potassium phosphate, pH 7.0, 5×10^{-8} M EPO, 10 mM H_2O_2, and halides at the indicated concentrations in a final volume of 250 μl. Peroxidase inhibition studies were performed using 1.5 mM sodium azide or 2 mM aminotriazole.

Isolated and Perfused Rat Heart Studies. The isolated rat hearts were perfused as previously described (23), with the following modifications. Sprague Dawley (male) rats in the range of 325–375 gm were anesthetized with 40 mg pentobarbital and anticoagulated with 200 U of heparin, both administered intraperitoneally. The animals were intubated and supported on a rodent respirator (Harvard Apparatus Co., Inc., S. Natick, MA). The heart was excised and immediately immersed in ice-cold Krebs-Henseleit buffer (K-H; 120 mM NaCl, 6 mM KCl, 1.5 mM KH_2CO_3, 1.2 mM $MgCl_2$, 1.8 mM $CaCl_2$, 97 μM EDTA, and 11 mM glucose). The heart was then cannulated via the aorta, and retrograde perfusion was established with oxygenated K-H buffer (95% O_2, 5% CO_2) at a column height of 100 cm of H_2O within 45 s of cardiectomy. The hearts were thoroughly flushed to remove residual blood, then re-immersed in ice cold K-H. 0.5 ml of either cold K-H alone or buffer containing 10^{-7} M EPO (350 ng) was instilled retrogradely through the aorta, and hypothermic arrest was maintained for 3 min to allow EPO to adhere to the endocardium. Under these conditions, ~50% (175 ng) of the EPO instilled remained heart associated, as quantitated by the guaiacol assay. Aortic perfusion with 37°C K-H was then resumed, flushing out nonadherent EPO and re-establishing regular cardiac contractions.

The left atrial appendage was cannulated and antegrade perfusion started immediately. Baseline determinations of mechanical function were obtained every 5 min for 15 min before infusion of H_2O_2 commenced. Preload was fixed at 10 cm H_2O and afterload at 85 cm H_2O. A pressure transducer (P23Db; Statham, Pasadena, CA) and a recorder (7402A; Hewlett-Packard Co., Palo Alto, CA) were used to record pressure and heart rate. Myocardial oxygen consumption (MVO_2) was calculated by the difference between the pO_2 in the perfusate and the pO_2 in the coronary effluent, continuously monitored using a Clarke-type O_2 electrode (Yellow Springs Instrument Co., Yellow Springs, OH). Rate pressure product (RPP) was calculated as heart rate times peak systolic pressure, and stroke volume (SV) was calculated as: [(aortic + coronary flow)/heart rate].

Five groups, each containing three hearts, were perfused under the following conditions: (*a*) K-H cardioplegia, perfusion with K-H + 100 μM NaBr (control); (*b*) EPO cardioplegia, perfusion with K-H + NaBr + 1 μM H_2O_2 (complete system); (*c*) K-H cardioplegia, perfusion with K-H + NaBr + H_2O_2 (delete EPO); (*d*) EPO cardioplegia, perfusion with K-H + H_2O_2 (delete NaBr); (*e*) EPO cardioplegia, perfusion with K-H + NaBr (delete H_2O_2). The parameters of cardiac performance described above were monitored for 20 min after the initiation of the H_2O_2 infusion. At the end of the experiment, the hearts were assessed for edema by measuring wet-to-dry ratios.

Results

To ascertain whether EPO is detectable in the endocardium of patients with hypereosinophilic heart disease, we stained sections of heart from such a patient with AHE-1, a mAb specific for human EPO (14), using the APAAP technique (14). As expected, EOs, but not other inflammatory cells present in the sections, stained intensely (not shown). As shown in Fig. 1, chromogenic substrate deposition (*dark*) was absent from the endocardial surface in the section incubated with normal mouse ascites (*left*) but prominent in the section incubated with AHE-1 (*right*). Notably, endocardial deposition of EPO was detectable despite the complete absence of overlying intact EOs. No such deposition was seen in sections of normal heart (not shown). The substantial amounts of EPO found in the endocardium of this patient raised the question of whether EPO simply adherent to vasculature might be toxic even in the absence of intact EOs.

First, however, to assess the cytotoxic potential of bromide-dependent and EPO-mediated reactions in activated EOs, we compared the toxicity of PMA-activated EOs in the presence of physiologic amounts of Cl^- alone with that in the presence of physiologic concentrations of both Cl^- and Br^-. We find that adding 100 μM Br^- to RPMI (a "physiologic" tissue culture medium that otherwise contains 100 mM Cl^- but only trace contaminant amounts of Br^-) substantially increases the toxicity of PMA-stimulated EOs, but not neutrophils (PMNs), towards several types of cultured endothelial cells (Table 1). As shown on the left, damage to endothelial monolayers caused by PMA-stimulated EOs, as assayed by ^{51}Cr release, was amplified 1.8–4-fold in RPMI supplemented with a physiologic concentration of NaBr. The considerable toxicity caused by PMA-activated EOs in RPMI without added Br^- reflects potential contributions from H_2O_2, HOCl generated from Cl^-, or HOBr generated from Br^- contaminating RPMI (commercial buffer preparations have by neutron activation analysis been found to contain ~5 μM Br^- [12, 13]. Identical results were obtained with Br^- supplementation of EBSS, a simple glucose buffer that contains 100 mM Cl^- (not shown). Importantly, 100 μM NaBr was itself completely innocuous to endothelium (not shown). In contrast to EOs, no bromide enhancement of killing was seen when purified PMNs were the effector cells (Table 1). Of note, in RPMI alone as well as in RPMI supplemented with 100 μM Br^-, PMA-activated EOs were consistently more toxic than an equivalent number of PMNS. Because both EOs (12, 13) and purified human EPO (13) oxidize Br^- to HOBr rather than Cl^- to HOCl at physiologically relevant serum concentrations (100 mM Cl^- and 100 μM Br^-) and EPO is the only EO enzyme known to use Br^- as a substrate, the enhanced endothelial toxicity of PMA-activated EOs in the presence of Br^- is presumably attributable to their

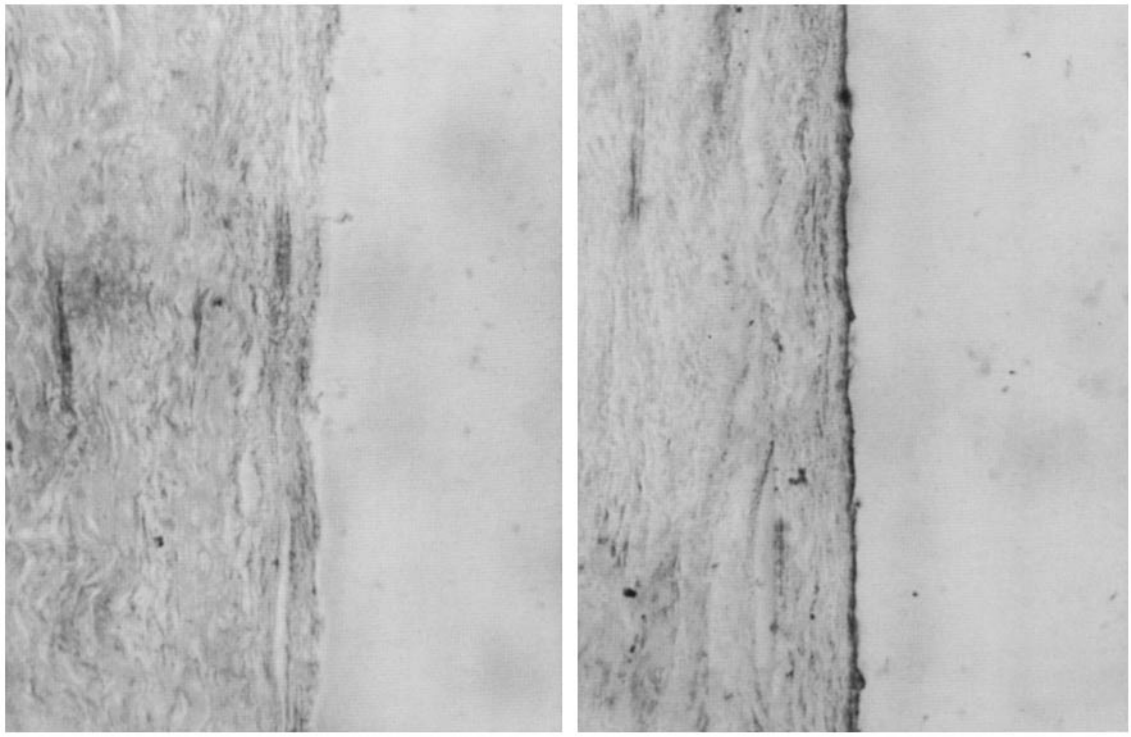

Figure 1. Immunocytochemical localization of EPO in heart of patient with eosinophilic endocarditis. Formaldehyde-fixed and paraffin-embedded tissue sections from a patient with eosinophilic heart disease were incubated with a 1:100 dilution of normal mouse ascites (*left*) or AHE-1 murine mAb specific for human EPO (*right*), then analyzed for the presence of bound antibody by the APAAP technique. The reaction product is dark. In both micrographs, the endocardium is on the left and the ventricular lumen on the right. ×1,200.

generation of cytotoxic (see below) quantities of HOBr: 20–40 nmol/10^6 EOs/h (13, 20).

If the increased endothelial cell damage caused by activated EOs in the presence of Br^- is attributable solely to EPO, then purified EPO in isolation should replicate the findings in intact EOs. However, because EPO, like several other membrane-perturbant EO granule proteins, is extremely cationic (pI > 11 [24]), we first compared the "nonenzymatic" or inherent toxicity of EPO towards endothelium with that of MBP, a major EO granule component with well-docu-

Table 1. *Effect of Bromide on Endothelial Toxicity of PMA-activated EOs and PMNs*

	EOs			PMNs		
	Percent ^{51}Cr release			Percent ^{51}Cr release		
Cell type	+ PMA	+ PMA + Br^-	+ Br^- / − Br^-	+ PMA	+ PMA + Br^-	+ Br^- / − Br^-
BENDOC	28.9 ± 2.8	52.0 ± 6.1	1.79 ± 0.12*	14.4 ± 2.1	17.9 ± 3.2	1.24 ± 0.27
BAEC	46.0 ± 3.5	87.2 ± 3.7	1.90 ± 0.12*	17.0 ± 3.1	16.8 ± 1.9	0.99 ± 0.12
HUVEC	14.9 ± 6.2	26.7 ± 5.2	1.79 ± 0.30*	8.1 ± 3.6	8.3 ± 4.1	1.02 ± 0.40
PAEC	4.0 ± 1.2	14.5 ± 2.1	3.66 ± 0.90*	ND	ND	ND

2-cm^2 confluent monolayers of BENDOC, BAEC, HUVEC, or PAEC in 24-well Costar tissue culture plates were labeled with 1 μCi ^{51}Cr/well, overlaid with 2.5 × 10^6 EOs or PMNs suspended in 1 ml RPMI (100 mM Cl^-, no added Br^-), or RPMI further supplemented with 100 μM NaBr (+Br). Plates were centrifuged 50 *g* for 4 min to ensure contact between phagocytes and endothelium, then 20 ng/ml PMA was added to each well. After 18 h (BENDOC, BAEC, HUVEC) or 2.5 h (PAEC) of incubation in a humidified 5% CO_2 37°C atmosphere, plates were centrifuged 1,000 *g* for 10 min, supernatant medium and 0.1 M NaOH-solubilized monolayers were counted in a gamma counter, and percent specific ^{51}Cr release was calculated as described in Materials and Methods. The ratio of ^{51}Cr release seen in the presence of Br^- (+PMA + Br^-) to that in the absence of Br^- (+PMA) was than calculated (+Br^-/−Br^-). Data shown are ± SEM.

* Ratio significantly different from 1.0 at $p < 0.05$.

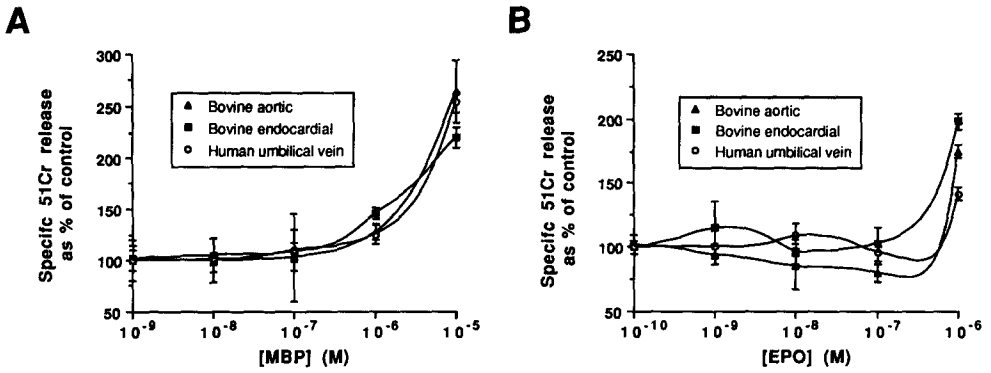

Figure 2. Comparative toxicity of MBP and EPO for endothelium. Endothelial monolayers of the designated types were ^{51}Cr labeled, incubated with either MBP (*A*) or EPO (*B*) at the indicated concentrations for 18 h, then assayed for percent specific ^{51}Cr release as described in Materials and Methods.

mented toxicity for parasitic (25) and mammalian (26) cells. These experiments were performed in EBSS buffer in the absence of Br^- and H_2O_2 to deprive EPO of both substrates necessary to fuel catalytic damage. As seen in Fig. 2, on a molar basis, EPO is at least as toxic as MBP, with ^{51}Cr release above that in buffer controls detectable at $\geqslant 10^{-6}$ M. Therefore, in further experiments assaying the peroxidase-dependent toxicity of EPO, we used concentrations of $\leqslant 10^{-7}$ M, well below its threshold of nonenzymatic toxicity.

We further characterized the previously reported preferential oxidation of Br^- over Cl^- by EPO using purified human EPO, reagent H_2O_2, and halides and by assaying chemiluminescence and H_2O_2 consumption. As shown in Fig. 3 *A*, in the presence of 100 mM Cl^- and 10 mM H_2O_2, addition of 5×10^{-8} M EPO causes a scarcely detectable increase in native (i.e., nonenhanced; EPO can directly oxidize luminol [27]) chemiluminescence, whereas in the presence of 100 μM Br^-, there is a burst of light output that exponentially decays. Addition of 100 μM Br^- to the 100 mM Cl^- vial after EPO has been added elicits a burst of photon output identical to that seen with Br^- alone (not shown); addition of two potent peroxidase inhibitors, 1.5 mM Na azide (Fig. 3 *A*) or 2 mM aminotriazole (not shown), largely obliterates light output. Similar results were obtained by analysis of halide-dependent consumption of H_2O_2 by EPO, a necessary concomitant of its catalytic action (Fig. 3 *B*). Addition of 5×10^{-8} M EPO to a solution of 4 mM H_2O_2 (*open boxes, arrow*) did not cause H_2O_2 consumption, showing that EPO lacks catalatic activity. Subsequent addition of 100 mM Cl^- did not cause sustained H_2O_2 consumption: 65% of the abrupt drop in OD_{230nm} just after addition of Cl^- is ascribable to dilution artifact. In marked contrast, addition of 100 μM Br^- to H_2O_2 and 5×10^{-8} M EPO (*filled boxes*) engenders brisk, exponentially decaying H_2O_2 consumption, indicating that EPO catalyzes rapid oxidation of Br^-. These data describe a nearly absolute preference of EPO for Br^- over Cl^- in buffers of physiologic composition.

Even small amounts of EPO can mediate extraordinary toxicity towards cultured endothelial cells, a toxicity strictly dependent upon the presence of Br^- (Fig. 4). Thus, PAEC monolayers exposed to increasing concentrations of H_2O_2 in EBSS supplemented with 100 μM NaBr showed no signs of toxicity until ~3.3 mM H_2O_2, but in the presence of 0.1 U EPO in solution, they were nearly completely lysed by 33 μM H_2O_2, a 100-fold enhancement (Fig. 4 *B*, *open boxes*). Even greater, 1,000-fold amplification of H_2O_2 tox-

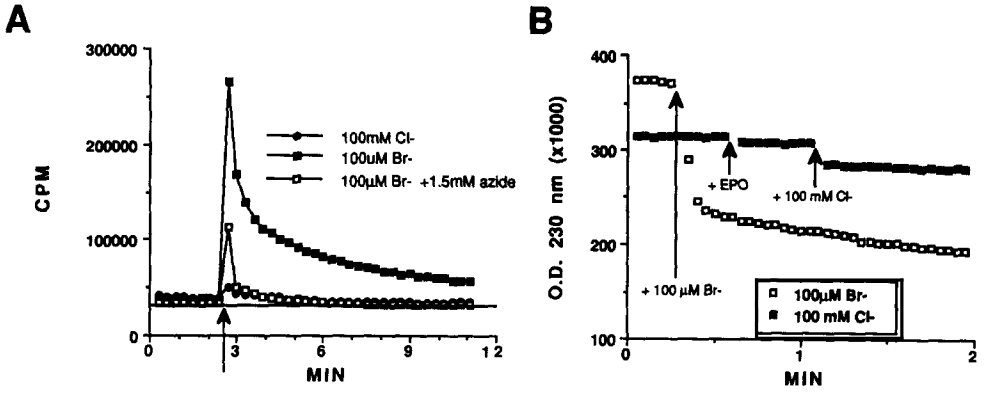

Figure 3. Br-specific chemiluminescence and hydrogen peroxide consumption by EPO. (*A*) Native (i.e., nonenhanced) chemiluminescence was assayed with an out-of-coincidence scintillation counter in clear glass vials containing 10 mM potassium phosphate buffer, pH 7.0, 10 mM H_2O_2, and the indicated halide, with or without 1.5 mm Na azide in a final volume of 200 μl. 6-s counts of photon emission were obtained every 20 s. At the time indicated by the vertical arrow, 10 μl of 10^{-6} M EPO, for a final concentration of 5×10^{-8} M, was added. The horizontal line above the *x*-axis indicates the background ("noise") output of the scintillation counter photomultiplier. (*B*) H_2O_2 consumption was followed spectrophotometrically at 230 nm in a cuvette containing 10 mM potassium phosphate, pH 7.0, and 4–5 mM H_2O_2 in a final volume of 1 ml. (*Open boxes*) At the time designated by the middle vertical arrow (+ EPO), 5×10^{-8} M EPO was added, followed by 100 mM KCl (*right arrow*, + 100 mM Cl^-). (*Filled boxes*) At the time indicated by the arrow on the left (+ 100 μM Br^-), 100 μM KBr was added to a solution containing 5 mM H_2O_2 and 5×10^{-8} M EPO in 10 mM potassium phosphate, pH 7.0.

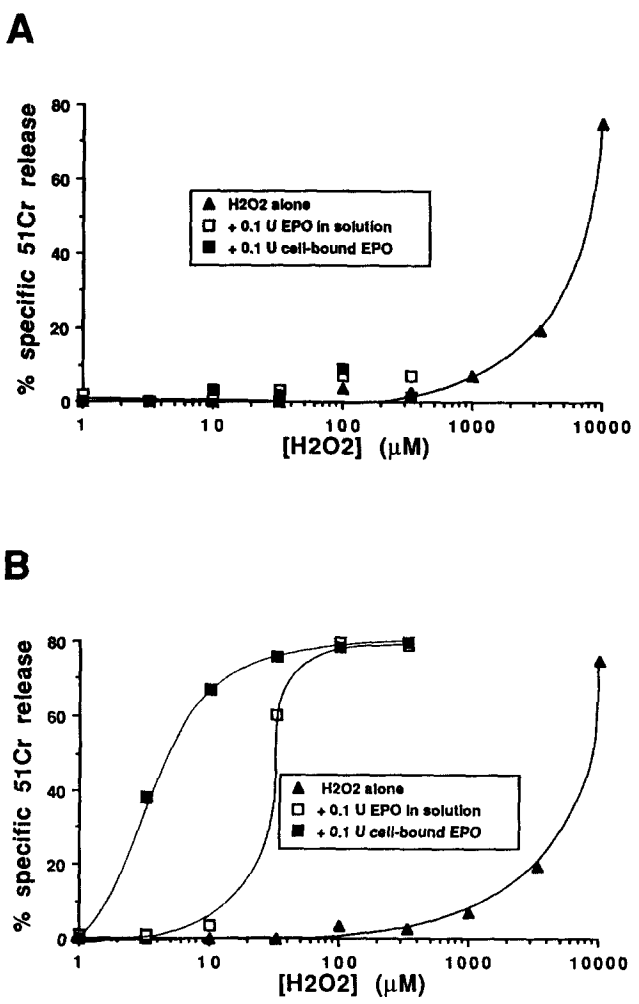

Figure 4. H_2O_2 toxicity for PAEC in the presence of EPO and Br^-. ^{51}Cr-labeled PAEC monolayers were overlaid with 200 μl EBSS buffer (H_2O_2 alone) or EBSS with 5×10^{-8} M (0.1 U) EPO. After 15 min, the wells containing EPO were either supplemented with 800 μl EBSS (+ 0.1 U EPO in solution) or aspirated then washed twice in 1 ml EBSS to remove unbound EPO (+ 0.1 U cell-bound EPO). All wells were made to 1 ml in either EBSS (100 mM Cl^-) (*A*) or EBSS supplemented with 100 μM NaBr (*B*) before adding the indicated amounts of H_2O_2, incubating 2.5 h, then determining percent specific ^{51}Cr release as previously described. Wells containing cell-bound EPO were further supplemented with 25 μg/ml BSA to control for the supernatant protein present in wells containing EPO in solution.

Table 2. *Endothelial Cytotoxicity of the EPO/Br^-/H_2O_2 System: Inhibitor Studies*

	Percent specific ^{51}Cr release	
Preparation	PAEC	HUVEC
EBSS + EPO + Br^- + H_2O_2	75.1 ± 2.7	79.9 ± 5.1
− H_2O_2	−2.1 ± 0.8	−2.0 ± 4.5
− EPO	1.3 ± 2.7	2.0 ± 5.1
− Br^-	4.6 ± 3.1	13.3 ± 6.7
+ 100 μg/ml catalase	−1.6 ± 1.1	ND
+ 1 mM azide	1.4 ± 2.6	ND
+ 10 mM aminotriazole	−1.1 ± 1.0	3.5 ± 3.0
+ 10 mM guaiacol	20.3 ± 4.8	ND
+ 10 mM histidine	16.8 ± 3.2	ND
+ 10 mM methionine	3.9 ± 2.6	ND
+ 1 mg/ml BSA	59.7 ± 6.6	ND

2-cm^2 monolayers of PAEC or HUVEC endothelial cells were ^{51}Cr labeled, then overlaid with 200 μl of EBSS containing 10^{-7} M EPO (1.5 μg or 0.2 guaiacol U) and incubated 15 min, after which supernatant buffer was aspirated, and the monolayers were washed twice to remove unbound EPO. In the complete system (EBSS + EPO + Br^- + H_2O_2), 1 ml EBSS supplemented with 100 μM NaBr, then 10^{-5} M H_2O_2 (10 nmol) was added to each monolayer well. The plates were then incubated 2.5 h in a humidified 5% CO_2 atmosphere at 37°C before determination of percent specific ^{51}Cr release as described in Materials and Methods. The indicated reagents were either deleted (−) or added (+) to the complete system before the addition of H_2O_2.

icity was conferred by the 10% of EPO that remains cell bound (unpublished observation) when monolayers exposed 15 min to 0.1 U EPO were thoroughly washed to remove unbound EPO before being exposed to H_2O_2 (Fig. 4 *B*, *filled boxes*). This greater sensitivity of monolayers with cell-bound EPO over those with 10 times as much EPO (750 ng) in solution was not simply due to the presence of supernatant protein in the latter, because the buffer for the former was supplemented with 25 μ/ml BSA to control for this factor. Alternatively, in the case of EPO in solution, there is cell-bound EPO in addition to EPO in supernatant buffer, so at low H_2O_2 concentrations supernatant EPO could in effect "scavenge" added H_2O_2, limiting its availability for cell-bound EPO. Compatible with this interpretation (and further emphasizing the amplified toxicity of cell-localized HOBr generation) is our observation that reagent HOBr added to supernatant EBSS above PAEC monolayers causes endothelial cell damage with a dose-response curve identical to that of EPO in solution, not cell-bound EPO (not shown). Addition of EPO, whether cell bound or in solution, to PAEC monolayers in the presence of Cl^- alone caused no enhancement whatsoever in their vulnerability to added H_2O_2 (Fig. 4 *A*). These results show that, in comparison with EPO in solution, cell surface–bound EPO more efficiently catalyzes lethal, strictly Br^--dependent damage to endothelium, apparently by localizing and thereby "focusing" damage fueled by freely diffusible H_2O_2 onto critical cellular structures.

The studies summarized in Table 2 show that endothelial cell damage occurring in the presence of EPO, Br^-, and H_2O_2 reflects peroxidative enzymatic activity of EPO rather than some other, nonenzymatic toxic effect. As seen on the top line, the complete system (0.2 U EPO, 100 μM Br^-, and 10 μM H_2O_2) caused nearly complete lysis of both PAEC and HUVEC monolayers. Deletion of any one of the three components almost completely blocked toxicity. Addi-

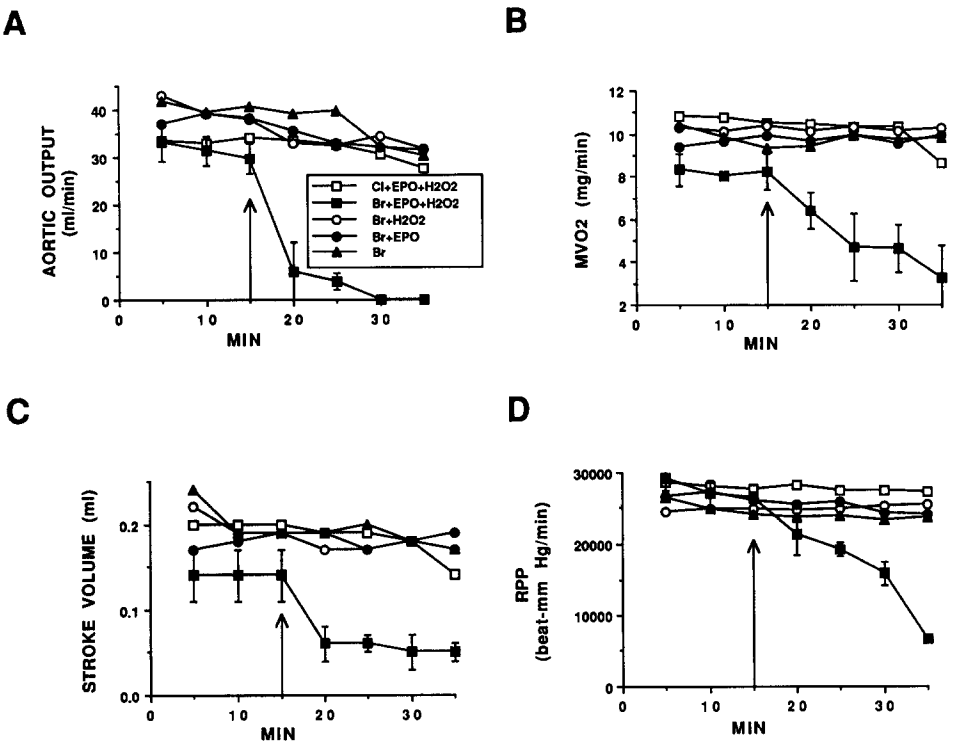

Figure 5. Cardiotoxicity of the EPO/Br^-/H_2O_2 system in isolated working rat hearts. Isolated working Neely rat hearts pre-treated with instillation of 350 ng EPO or K-H buffer into the left ventricle were perfused with K-H (120 mM Cl^-) or K-H supplemented with 100 μM NaBr. Aortic output (*A*), oxygen consumption (*B*), SV (*C*), and RPP (*D*) were then monitored every 5 min for 35 min. At 15 min, as indicated by the vertical arrows, the perfusate was supplemented with 1 μM H_2O_2 in the appropriate groups. Br represents hearts not treated with EPO and simply perfused with Br^--supplemented K-H. Whether the concomitant presence of all three components in the complete system (Br+EPO+H_2O_2) was required to cause cardiotoxicity was assayed by deleting Br^- (Cl+EPO+H_2O_2), EPO (Br+H_2O_2), or H_2O_2 (Br+EPO).

tion of catalase to consume H_2O_2, azide and aminotriazole to inhibit peroxidase enzymatic function, guaiacol to serve as a competitive substrate for Br^-, and the electron-rich amino acids, histidine, and methionine to "scavenge" hypobromous acid (12) completely or partially abrogated the toxicity of the intact system. Interestingly, addition of 1 mg/ml of BSA, a concentration that nearly completely obliterates cytotoxicity in a system in which EPO is free in solution (not shown), produced a statistically significant but minor decrement in toxicity with cell-bound EPO. These results strongly suggest that the enzymatic activity of EPO in generating HOBr accounts for the pronounced toxicity of the complete system.

To test the relevance of our in vitro cell line observations to an intact functioning heart, we established an isolated working rat heart model that mimics the endocardial deposition of EPO demonstrated in tissue sections from human heart biopsies. In this system, as in cultured endothelial monolayers, cell-adherent EPO mediates catastrophic damage, in this case acute heart failure. Fig. 5 shows the time-dependent variation in four parameters of cardiac function in five experimental groups of isolated perfused hearts: aortic output (*A*), oxygen consumption (MVO_2, *B*), SV, (*C*), and RPP (*D*). Hearts whose left ventricles were pre-exposed for 3 min to 350 ng EPO, flushed thoroughly, monitored for 15 min to assure stability, then perfused with 1 μM H_2O_2 in K-H supplemented with 100 μM Br^- (complete system; Fig. 5, *filled boxes*) suffered an abrupt decrement in aortic output and SV as well as a progressive deterioration of MVO_2 and work as measured by RPP. By 20 min after the initiation of the H_2O_2 infusion, at the termination of the experiment, these parameters had all significantly ($p < 0.05$) decreased to between 0% (aortic output) and 33% (MVO_2) that of control hearts, which were sham pretreated with K-H solution without EPO during the cardioplegia, then subsequently perfused with K-H supplemented with 100 μM Br^-. Br^- alone had no discernible effect on cardiac function (not shown). No such decrements were seen when any one of the three components of the complete system (EPO, Br^-, or H_2O_2) was deleted (Fig. 5). Hearts treated with the complete system, but none of the other groups, were edematous, having a wet/dry ratio of 7.63 ± 0.30 vs. 6.22 ± 0.29 ($p < 0.05$) for controls. The failure of heart function occurring with the complete peroxidase system was not attributable to arrhythmias, as heart rates remained regular and constant within a range of 307–336 heart beats/min in all treatment groups (not shown). Thus, cell-bound EPO, in the presence of 100 μM Br^- and a very low (1 μM) concentration of H_2O_2, produces a syndrome of acute congestive heart failure in isolated working rat hearts.

Discussion

Endothelial cells, interposed as they are between the intravascular space and extravascular tissue, bear the brunt of the attack of overly activated phagocytes. EOs participate, as do PMNs, in a variety of inflammatory states involving endothelial cell damage, including vasculitis and pneumonitis (28). For unclear reasons, however, for PMNs the principal "shock" organ is the lung (adult respiratory distress syndrome),

whereas for EOs the target is the endothelial lining of the heart, or endocardium (eosinophilic or Loeffler's endocarditis) (1, 2). The findings of activated EO oxidative metabolism (5), high serum levels of MBP and ECP (6), and dense endocardial deposition of these same EO cationic granule proteins (7) in individuals with eosinophilic endocarditis implicate inappropriate application of EO cytotoxic effector mechanisms (intended to kill parasites) in the pathogenesis of this disorder, but do not explain why the heart suffers disproportionately in hypereosinophilic states.

Our studies suggest a potentially important role for EPO and HOBr in this process by showing that EPO is bound to the endocardium of such individuals and that cell-bound EPO catalyzes from H_2O_2 and physiologically relevant concentrations of Br^- the formation of an oxidant with potent biologic toxicity for endothelium and functioning hearts. EPO is present in large amounts (15 $\mu g/10^6$ cells) in EOs, accounting for as much as 25% of the protein in EO-specific granules (27). Although EPO by itself is at least as potent a toxin for endothelial cells as MBP (Fig. 2), in the catalytic system with 100 μM Br^-, as little as 75 ng (1 pmol) cell-bound EPO catalyzes from 5 to 10 nmol H_2O_2 enough HOBr to lyse an entire 2-cm^2, 200,000-cell monolayer of endothelial cells (Fig. 4). Thus, the amount of EPO present in only 5,000 EOs could, if completely released and evenly spread, theoretically destroy 2 cm^2 of endothelial cell surface. Though complete release and even spread are improbable, we note that absolute EO counts in patients with eosinophilic endocarditis can reach 5×10^7/ml or more, so that even partial EO degranulation could over time easily allow potentially lethal amounts of EPO to accumulate in endocardium. By contrast, 130 μg (10 nmol) of MBP is required to destroy an equivalent endothelial monolayer (Fig. 2). Therefore, on a molar basis, the EPO system is 10,000 times as toxic for endothelial cells as is MBP. Recent similar findings by Klebanoff et al. (29) with respect to killing of schistosomulae further emphasize the remarkable cytotoxic potency of the EPO/Br^- oxidant system.

We recognize that the in vitro models we have developed to study eosinophilic endocarditis are not physiologic, as emphasized by the unusual rapidity of endothelial cell death and heart failure. Several factors probably function in vivo to attenuate the toxicity of this oxidant system. First, whole blood contains large amounts of substances that could potentially scavenge either H_2O_2 (e.g., RBC catalase) or the more reactive HOBr (e.g., proteins, amino acids, and "natural antioxidants" such as ascorbate, uric acid, and bilirubin). On the other hand, when EPO binds to cell surfaces instead of remaining in solution, it not only retains (30–34) but actually increases (15, 35, 36; Fig. 3) its cytotoxic potential in the presence of suitable halides, including Br^-. This increased cytotoxicity may be due to increased enzymatic activity (35), or to focusing of oxidant damage to critical cellular structures with concomitant decreased efficiency of scavenging by supernatant protein (34, 36). Indeed, by virtue of its extreme cationicity and the negative charge of most biologic surfaces, EPO seems ideally endowed to exploit such a strategy. Interestingly, in experiments not shown, we have found that treating PAEC monolayers with myeloperoxidase, even in higher activity amounts than those used in our experiments with cell-bound EPO (Fig. 4), fails to promote killing by 10 μM H_2O_2, a concentration that causes complete destruction in the presence of Br^- and EPO. This difference may be due in part to the greater cationicity and endothelial cell binding of EPO in comparison with myeloperoxidase (37), though peroxidases such as myeloperoxidase are also more prone to autoinactivation by HOCl than by HOBr (38). We also note that in our serum-free systems we are attempting to replicate in hours damage that in vivo likely takes months or even years. It is therefore possible that, despite efficient scavenging by serum factors, enough HOBr escapes over long periods of time to cause significant endocardial damage to accumulate.

Second, though EPO clearly oxidizes Br^- rather than Cl^-, serum contains other potential substrates for EPO that might successfully compete with Br^-, such as iodide (I^-) and the pseudohalide thiocyanate (SCN^-). However, I^- is present in submicromolar concentrations that do not affect HOBr generation by activated EOs (12). We are currently investigating the utilization of SCN^- by EPO. Oxidants produced by EPO in vivo are in fact probably a complex mixture of HOBr, HOSCN, HOI, and the products of other, as yet unidentified, nonhalide substrates, the composition of which is determined by the relative abundance of the potential substrates. Nevertheless, because of its extreme biologic potency, even small amounts of HOBr may mediate significant cytotoxicity without being the sole or even the main reaction product of EPO-catalyzed reactions. Moreover, the principle of potent cytotoxic potential conferred by endothelial cell–bound EPO that is evident in the model we have developed remains relevant regardless of the substrate involved.

What is the oxidant species responsible for the pronounced cytopathic efficacy of the EPO/Br^-/H_2O_2 system? This system clearly generates an oxidized form of Br^- (presumably HOBr in equilibrium with its equivalent oxidation state species Br_2 and OBr^-) capable of covalently modifying proteins (12), trimethoxybenzene (13), and fluoresceinate (39). Another possibility, however, is highly reactive singlet oxygen, which can be generated in the secondary reaction of HOBr with H_2O_2, the so-called "Allen mechanism": $H_2O_2 + HOBr = H^+ + Br^- + O_2(^1\Delta_g)$ (40–42). Unfortunately, though reliable means exist to identify singlet oxygen, to our knowledge, there exists no scavenger specific for HOBr or singlet oxygen, both avid electrophiles, to allow dissection of their relative contributions to cell damage. In any case, singlet oxygen generation, which is kinetically favored in higher H_2O_2 concentrations (41), is less likely in our EPO/Br^-/H_2O_2 systems (1–10 μM H_2O_2) than in the chemiluminescence and H_2O_2 consumption assays (4–10 mM H_2O_2) or from PMA-activated EOs (42).

Our findings may have relevance for the tendency of hypereosinophilic endothelial damage to manifest so prominently in the heart. High (up to 15 μg/ml) concentrations of MBP and ECP circulate in the serum of individuals with hypereosinophilia (6), but there is no evidence to suggest these cationic proteins are preferentially deposited in endocardium

as opposed to other endothelial beds. Nonetheless, generalized endothelial deposition of EPO might have particularly adverse effects in the endocardium because EPO is an enzyme that utilizes Br^-, for which there is an inexhaustible supply in circulating blood, along with H_2O_2 to produce a locally toxic oxidant. Consequently, once bound to an endothelial surface, EPO is constrained only by the lack of H_2O_2 from producing ongoing cellular damage. Endocardium, in contrast to other types of endothelium, is surrounded by incessantly active myocardium, a tissue with unusually rapid aerobic metabolism. It has been estimated that as much as 5% of all O_2 consumption in heart mitochondria is attributable to "leakage" of electrons from the electron transport chain to reduce O_2 to O_2^-, leading to H_2O_2 production (43), and others have documented high rates of myocardial H_2O_2 production (44, 45). Intracellular levels of H_2O_2 in tissue with rapid aerobic metabolism have been estimated to be as high as 10^{-7} M (46), perilously close to the 10^{-6} M we infused to cause acute heart failure in our rat heart model (Fig. 5). Moreover, myocardium is relatively deficient in catalase (46), and it has been suggested that this tissue relies in part upon diffusion of H_2O_2 through endothelial cells to the catalase in circulating RBC as a way of H_2O_2 disposal (46). EPO coating an endocardial cell surface would be ideally situated to exploit such an adventitious H_2O_2 flux to fuel chronic oxidant damage, even in the absence of attached and activated EOs, and so promote progressive endocardial damage and endocarditis. The isolated working heart model we have developed should prove suitable for testing this hypothesis.

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