REGULATION OF INFLAMMATION: THE ROLE
OF THE ENDOTHELIAL CELLS

by

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ABSTRACT

The endothelium plays an important role during the inflammatory response. Through the interaction with leukocytes, endothelial cells (EC) can be exposed to numerous pro-inflammatory mediators including peroxidases resulting in enhancement of the inflammatory process. In this study, EC were exposed to various peroxidases to determine their effects on cytokine secretion and respiratory burst (RB). Chemiluminescence was employed to determine the effects of various peroxidases on EC RB. Recombinant enzymatically inactive human myeloperoxidase (iMPO) and enzymatically inactive horseradish peroxidase (dHRP) increased RB. Enzymatically active porcine eosinophil peroxidase (pEPO) demonstrated a rapid increase in RB followed by no effect over the course of time. Other peroxidases tested had no effect on RB by EC. Recombinant enzymatically active human myeloperoxidase (MPO) and iMPO are derived from neutrophil degranulation and have been reported to augment cytokine secretion by macrophages. Endothelial cells exposed to iMPO in vitro exhibited dose- and time-dependent increases in interleukin-6 (IL-6), interleukin-8 (IL-8), and granulocyte-macrophage colony-stimulating factor (GM-CSF); however, MPO did not have a significant effect. A ribonuclease protection assay (RPA) indicated this enhancement by iMPO was due to an increase in mRNA levels. Also, EC were exposed to pEPO and an enzymatically inactive fragmented form of human eosinophil peroxidase; both pEPO and fEPO observed a slight decrease in IL-8 secretion by EC. Taken as a whole, these data provide further insight into the effects of peroxidases on the development of inflammation.
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>dHRP</td>
<td>horseradish peroxidase (enzymatically inactive)</td>
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<td>endothelial cells</td>
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<td><em>Limulus amoebocyte</em> lysate test</td>
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<td>RB</td>
<td>respiratory burst</td>
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<td>RPA</td>
<td>ribonuclease protection assay</td>
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<tr>
<td>S.E.M.</td>
<td>standard error of the mean</td>
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<tr>
<td>TMB</td>
<td>tetramethyl benzidine</td>
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<tr>
<td>TNF-α</td>
<td>tumor necrosis factor-alpha</td>
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CHAPTER I
INTRODUCTION

Inflammation is the body's reaction to invasion by an infectious agent, antigen challenge, or physical damage. It represents the consequence of capillary dilation with accumulation of fluid (edema) and the immigration of leukocytes (1). Once the initiating event has occurred, the persistence of the inflammatory process has been attributed to a variety of events including superantigen expression (2), antigen-antibody complexes (3), activated T cells (4, 5, 6) and abnormal cytokine expression (5, 6, 7, 8). This persistent immune activation can result in various pathological consequences such as rheumatoid arthritis (RA), Crohn's disease, chronic asthma, and various allergic diseases (e.g., allergic rhinitis) (9, 10, 11).

At the site of inflammation, both neutrophils (PMNs) and macrophages (Mφ) predominate. After an initiating event, PMNs are the first cells to arrive at the site of inflammation (12). Activation of PMNs results in the release of myeloperoxidase (nMPO) into the extracellular environment (13). Macrophages usually infiltrate sites of inflammation several hours after lymphocytes. They are responsible for killing aberrant cells and removing debris (12), in addition to their ability to release numerous "pro-inflammatory" cytokines. It has been reported that Mφ directly influence a PMN-dependant inflammatory response and that PMNs, in turn, recruit and activate Mφ (14).

Endothelial cells (EC) provide an interface between the blood and extravascular space. Under normal conditions, they provide a barrier to the release of various macromolecules into the tissues. However, they have also been shown to be involved in inducing inflammation by secreting "pro-inflammatory" mediators and interacting with leukocytes (15). Specifically in response to tumor necrosis factor-alpha (TNF-α), EC secrete "pro-inflammatory" cytokines such as interleukin-1 (IL-1), interleukin-8 (IL-8), and granulocyte-macrophage colony-stimulating factor (GM-CSF), which further enhance the inflammatory response (16).

Peroxidases are hydrolytic heme enzymes found in both plants and animals. They belong to a group of enzymes that utilize hydrogen peroxide (H₂O₂) as a substrate. The
peroxidases have a porphyrin ring structure composed of nitrogen atoms surrounding an iron atom, which is the active center (17). For the present study, the following peroxidases were employed: recombinant enzymatically active human myeloperoxidase (MPO) and enzymatically inactive human myeloperoxidase (iMPO) and enzymatically active porcine eosinophil peroxidase (pEPO) and enzymatically inactive fragmented human eosinophil peroxidase (fEPO).

Naturally occurring myeloperoxidase (nMPO), which is present in the azurophilic granules of neutrophils, makes up 5% of their dry weight (18). Bradley et al. reported that neutrophils found in the synovial fluid of RA patients degranulated and released approximately 50% of their nMPO into the microenvironment. Also, at the site of inflammation approximately 40% of the nMPO is rapidly inactivated via oxidation (19). In addition, Edwards et al. have reported the presence of 16-29 µg/ml of iMPO in an arthritic joint (20). Therefore, at the site of inflammation, there is a mixture of MPO and iMPO. Also, it has been reported that binding of leukocytes including monocytes and neutrophils to the endothelium is enhanced by nMPO (21). Therefore, the EC would be exposed to both MPO and iMPO at a site of inflammation.

Eosinophil peroxidase (EPO) is the most abundant substance present in the crystalloid-containing granules of eosinophils (22), and at the site of inflammation there may be approximately 100-200 ng/ml of EPO (23). It has been reported that upon exposure to certain stimuli, eosinophils degranulate and release their contents into the microenvironment (22) and as such EC would be exposed to the products of eosinophil degranulation including EPO and fEPO.

Other investigators have reported that radicals induce cytokine secretion (24, 25). Studies completed in this laboratory have shown that exposure of murine Mφ to various peroxidases resulted in an increase in respiratory burst (RB) with a concomitant increase in H₂O₂ and other oxygen species (26, 27). If an increase in RB by EC occurred after exposure to peroxidases, this would explain another means by which EC contribute to the inflammatory process.

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The interaction of peroxidases and EC has not been previously investigated. To fill this void in our understanding of inflammation, this study was undertaken with the following objectives:

1. Could MPO, iMPO, pEPO, or fEPO enhance the RB of EC,
2. Would the above peroxidases enhance EC secretion of cytokines, which would enhance the inflammatory process.
CHAPTER II
MATERIALS AND METHODS

Reagents

Both forms of myeloperoxidase, MPO and iMPO, were generously supplied by Dr. Nicole Moguilevsky and Dr. Alex Bollen from the Universite Libre de Bruxelles, Belgium. Protein determination of both MPO and iMPO were performed by the Lowry method and are as follows: MPO at 1180 μg/ml (177 U/ml) and iMPO at 1400 μg/ml (<10 U/ml). Dr. R.C. Allen, ExOximis Corp., Little Rock, Arkansas, supplied pEPO, and fEPO was supplied by Gerald Gleich, Mayo Clinic, Rochester, Minnesota. The Lowry method of protein determination was employed on both of these and was as follows: pEPO at 1184 μg/ml and fEPO at 2000 μg/ml. Fetal bovine serum (FBS) was purchased from Intergen (Purchase, NY). Guinea pig complement (serum) and Auto-POW MEM were purchased from Gibco (Long Island, NY). The following reagents were obtained from Sigma (St. Louis, MO): gentamycin sulfate, heparin, HEPES, enzymatically inactive horseradish peroxidase (dHRP), bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), and endothelial cell growth supplement from bovine neural tissue. A 20mM stock solution of luminol (Eastman Kodak, Rochester, NY) dissolved in DMSO was made and stored at 4°C until needed. All reagents were tested for endotoxin contamination using the Limulus amoebocyte lysate test (LAL) (Associates of Cape Cod, Woods Hole, MA). Preparations of MPO, iMPO, pEPO, and fEPO contained ≤ 8.0, ≤ 64.0, ≤ 0.02, and ≤ 0.01 ng/ml of endotoxin, respectively.

Cell Culture

Human umbilical vein endothelial cells (HUVEC) were obtained from American Type Culture Collection (Rockville, MD). Cells were maintained in Kaighn’s nutrient mixture F-12K (Gibco BRL Products, Grand Island, NY) supplemented with 10% FBS, 50 mg/ml gentamycin sulfate, 100 μg/ml heparin, and 30 μg/ml endothelial cell growth supplement from bovine neural tissue.
Chemiluminescence Assay

Methods used were modified from chemiluminescence assay described by Lefkowitz et al. (27). Briefly, HUVEC were suspended in Kaighn's nutrient mixture F-12K supplemented with 2% FBS at 4 x 10^5 cells/ml. Each well of a 96-well microtiter plate was seeded with 100 μl of the HUVEC suspension. Following a two hour incubation at 37°C, under 5% CO₂, the monolayers were washed three times with media without phenol red (Auto-POW) but supplemented with 0.6 g/dl HEPES, 0.2 g/dl sodium bicarbonate, and 1.0 g/dl BSA. This mixture was termed CL media. One hundred μl were added to each of the wells, and culture fluids were allowed to equilibrate for an additional 20 minutes at 37°C, under 5% CO₂. Subsequently, media were removed, and 100 μl of one of the following was added to each well: media alone or media containing MPO, iMPO, pEPO, fEPO, or dHRP. In addition, 50μl of zymosan opsonized with guinea pig complement, and 50 μl of luminol (80 mM working concentration) were added to all of the wells. The plate was placed in a Dynatech ML 3000 plate luminometer, and luminescence was recorded at 2 sec. over a 2-min. interval. The results were plotted as time vs. relative light units. The mean of triplicate treatments ± S.E.M. was determined. Each experiment was repeated at least twice.

Macrophage Collection

Murine resident peritoneal MΦ were collected as described previously (28). Mice were sacrificed by cervical dislocation and MΦ collected by peritoneal lavage using PBS at 4°C. Cells were washed and resuspended in DMEM supplemented with gentamycin and 25 mM HEPES. Macrophage cell number was adjusted to 1 x 10^6 MΦ/ml and 100 μl of the cell suspension were added to each well of a 96-well Costar microtiter plate (Fisher Scientific, Pittsburgh, PA). Following incubation for 2 hr. at 37°C under 5% CO₂, cell monolayers were washed to remove non-adherent cells. The MΦ population was ≥ 99% as determined by microscopy.
Enzyme-Linked Immunosorbent Assay (ELISA)

After HUVEC were adjusted to $2.0 \times 10^5$ cells/ml, 100 μl of the suspension were added to a 96 well Falcon Primaria plate (Becton Dickinson, Lincoln Park, NJ). Following incubation overnight, the cells were washed with F-12K/ 0% FBS/ no supplement. Different concentrations of peroxidases were added to the cells and cultures incubated for various time intervals. Supernatants were collected and stored at -70°C until assayed. Sandwich ELISA mini-kits for the detection of various cytokines were purchased from the following sources and utilized per manufacturer’s instructions: hIL-8 OptEIA kit (PharMingen, San Diego, CA); hIL-6 OptEIA kit (PharMingen, San Diego, CA); hGM-CSF (Endogen, Woburn, MA); and mouse TNF-α OptEIA kit (PharMingen, San Diego, CA). Briefly, Maxi-sorb™ 96 well microtiter plates (Nunc, Inc., Naperville, IL) were coated with monoclonal antibody specific for the cytokine of interest and incubated overnight. Wells were then blocked and samples were added and incubated for 1 hour. Wells were then washed, and a secondary polyclonal antibody for the cytokine of interest along with an avidin-horseradish peroxidase (HRP) conjugate were added. Following the incubation with the conjugate, the amount of HRP-labeled antibody was detected with tetramethyl benzidine (TMB). Absorbances were read at a wavelength of 450 nm on a microtiter plate reader (Dynatech Laboratories, Chantilly, VA).

Ribonuclease Protection Assay (RPA)

HUVEC were cultured in 6-well plates with MPO or iMPO for 3 hours. Cells were lysed using TriZol (Gibco, Long Island, NY) and total cell RNA was collected by phenol:chloroform extraction and ethanol precipitation. A RNase protection assay was then performed on equal amounts of total cell RNA from each treatment group using the RiboQuant multi-probe RNase protection assay kit (PharMingen, San Diego, CA) per manufacturers instructions. Briefly, $^{32}$P-labeled anti-sense RNA probes, specific for Mφ inflammatory protein-1 beta (MIP-1β), GM-CSF, IL-1β, monocyte chemotactant protein-1 (MCP-1), IL-8, and the two housekeeping genes L32 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were hybridized to sample RNA. A ribonuclease specific for single-stranded RNA was added to digest non-hybridized RNA, and the final products
resolved on an 8M urea polyacrylamide gel. The bands on the gel were then visualized by autoradiography. To determine differences between treatments, the amount of mRNA loaded in the lanes was normalized based on GAPDH or L32 band intensities using densitometry.

**Statistical Analysis of Data**

All of the above experiments were repeated at least 2 times in order to ensure reproducibility. A Student's t-test was used to determine the significance between two treatments. A one-way analysis of variance and Tukey post-test were used to determine significance among multiple groups. All values are reported as mean ± S.E.M. Differences were considered significant at P ≤ 0.05. In general, significance is reported between control and experimental values unless otherwise noted.
CHAPTER III
RESULTS

Effect of Peroxidases on Respiratory Burst in HUVEC

The RB is an important component of the inflammatory response. To assess the effects of various peroxidases on the RB of HUVEC, chemiluminescence experiments were conducted. Cells were incubated with dHRP, HRP, MPO, iMPO, pEPO, or fEPO and stimulated to undergo RB by the addition of opsonized zymosan. The luminol-enhanced light emissions were measured on a luminometer and expressed in relative light units. Figure 1a shows that cells exposed to fEPO did not display a change in the amount of light emitted, indicating no change in RB. HUVEC exposed to 0.057 µM pEPO produced a rapid increase in reactive oxygen intermediates (ROI) that then returned to values close to control. However, iMPO induced a slow generation and release of ROI, with MPO producing only a modest transient increase (Figure 1b). It can also be seen in this figure that dHRP stimulated a rapid and potent increase in RB.

Determination of Cytokine Secretion by HUVEC in Response to LPS

Previous experiments indicated that various lots of MPO and iMPO had LPS levels sufficient to stimulate cytokine secretion in Mφ. Experiments were therefore conducted to determine the concentration of LPS capable of inducing IL-8 secretion in HUVEC. HUVEC were cultured with control media or 0.1, 0.3, 1.0, or 3.0 µg/ml LPS for 6–48 hr before supernatants were collected and analyzed for IL-8 via ELISA. Figure 2 shows that a statistically significant (P ≤ 0.005) increase in IL-8 secretion was detected only when cells were incubated with 3.0 µg/ml LPS. This level is well in excess of the highest concentration of LPS found in the working concentrations of peroxidases employed in this study (≤ 4.5 ng/ml).
Effect of Various Lots of iMPO and MPO on IL-8 Secretion by HUVEC

Previous research in the current lab has indicated that MPO and iMPO affect cytokine secretion in Mφ. However, no research has been conducted as to the effects these peroxidases have on HUVEC functions. Recombinant human MPO and iMPO were assessed for their ability to stimulate IL-8 secretion in HUVEC. Cells were incubated with one of the MPO or iMPO mutants for 6 hr before supernatants were collected and analyzed for IL-8 by ELISA. The MPO preparations (OPT-3, -4, and -5) did not reproducibly affect IL-8 secretion, but all iMPO mutants tested enhanced IL-8 secretion with the Cys mutant inducing the greatest increase (Figure 3). While figure 3 does indicate a reduction in IL-8 by MPO, this was not a reproducible result.

Effect of MPO on Murine Peritoneal Mφ TNF-α Secretion

Experiments were conducted to determine if the OPT-5 variant of MPO could stimulate pro-inflammatory cytokine secretion in Mφ. Because Mφ have been used previously in this lab to assess the efficacy of new variants of MPO, experiments utilized this cell type to ensure that OPT-5 had the capacity to stimulate TNF-α secretion, a cytokine routinely analyzed in this lab for its pro-inflammatory nature. Macrophages incubated with 33 μg/ml OPT-5 displayed a significant increase in TNF-α secretion at 6 hr, indicating that this form of MPO was capable of stimulating cytokine secretion (Figure 4).

Determination of Appropriate Media for Cytokine Stimulation in HUVEC

Preliminary experiments were conducted to determine the media that would provide the best conditions for the stimulation of cytokines. HUVEC require complex growth conditions, some of which may interfere with cytokine stimulation. Therefore, HUVEC were cultured in standard growth media and then stimulated with 20 μg/ml MPO for 6 hr. Certain groups of cells were stimulated in the presence of growth supplements and FBS, while others received media conditioned with FBS alone. These
results indicated that the greatest increase in IL-8 secretion was observed in cells stimulated without supplements and in the presence of 10% FBS (Figure 5).

Effects of Different Peroxidases on IL-8 Secretion in HUVEC

Since none of the MPO preparations induced IL-8 secretion, the majority of the future studies only employed iMPO. In order to determine if iMPO induced a dose-dependent increase in IL-8, HUVEC were incubated with different concentrations of iMPO for 6–24 hr. Subsequently, culture media were collected and analyzed for IL-8 secretion by ELISA. Cysteine iMPO exhibited a dose- and time-dependent increase in IL-8 secretion with 100 μg/ml inducing the highest titers (Figure 6). At 6 hr, 100 μg/ml iMPO induced a 3.5-fold increase over control; however, at 24 hr, an 18-fold increase was observed. Eleven μg of iMPO, the lowest concentration employed, induced a 2.6-fold increase after as early as 12 hr of incubation time.

Eosinophil peroxidase has also been demonstrated to affect cytokine secretion. However, only fEPO proved effective at reducing cytokine secretion in LPS-stimulated Mφ. When HUVEC were incubated with 1.36–13.6 μg pEPO or fEPO for 6 hr, a slight decrease in IL-8 was observed by both fEPO and pEPO (Figure 7). The Cys mutant of iMPO was used as a positive control.

Secretion of IL-6 by HUVEC in Response to MPO and iMPO Stimulation

Interleukin-6 is a cytokine that has the capacity to enhance the inflammatory cascade. To determine if either MPO or iMPO were capable of stimulating IL-6, HUVEC were incubated with 11 or 33 μg/ml of OPT-5 MPO or Cys iMPO for 6–24 hr. Supernatants were then collected and analyzed for IL-6 via ELISA. As was seen in assays for IL-8, neither concentration of MPO was capable of inducing IL-6 secretion (Figure 8). However, 11 and 33 μg/ml iMPO induced a 2-fold and 4-fold increase in IL-6 secretion at 6 hr, respectively. A time-dependent effect was also observed with both concentrations of iMPO, but not with MPO.
Effects of iMPO on GM-CSF Secretion by HUVEC

Granulocyte-macrophage CSF is a cytokine that has been shown to have numerous functions including the induction of neutrophil degranulation. Because HUVEC have been reported to secrete GM-CSF, experiments were conducted to determine the effects of iMPO on GM-CSF secretion. HUVEC were incubated with 0, 11, 33, or 100 µg/ml of the Cys iMPO for 6-24 hr. At each time period, a dose-dependent increase was observed (Figure 9). The highest 2 concentrations both produced statistically significant (P < 0.05) time-dependent increases in GM-CSF.

Analysis of MPO- and iMPO-induced mRNA in HUVEC

To determine if the increase in cytokine secretion observed with iMPO stimulation was the result of an increase in mRNA, an RPA was performed on HUVEC incubated with 33 µg/ml of either MPO (OPT-5) or iMPO (Cys) for 3 hr. The total cell RNA was then purified and hybridized to anti-sense probes specific for MIP-1β, GM-CSF, IL-1β, MCP-1, IL-8, and the two housekeeping genes L32 and GAPDH. The RNase-digested samples were then resolved on a denaturing acrylamide gel and exposed to radiography film. Figure 10a shows that there was an increase in the transcripts for IL-8, monocyte chemotactant protein-1 (MCP-1), and GM-CSF from cells incubated with iMPO, but only a slight effect in the cells exposed to MPO. Densitometry indicated that there was a 16-fold increase in IL-8 mRNA and 10-fold increase in GM-CSF mRNA from iMPO-treated HUVEC over control cells (Figure 10b). No changes were observed in the level of the constitutively expressed transcripts for GAPDH and L32.
Figure 1: Effects of Peroxidases on Respiratory Burst in HUVEC.

HUVEC were added to 96-well luminometry plates and cultured for 2 hr. Media were removed and the cells were treated with various forms of EPO (A) or MPO (B) in Autopow MEM. Opsonized zymosan and luminol were then added and the relative light units were analyzed on a luminometer. Values represent the mean ± S.E.M. of duplicate wells. All experiments were repeated at least twice.
<table>
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<th>Time (hr)</th>
<th>IL-8 (pg/ml)</th>
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<tr>
<td>6</td>
<td>0.1 μg/ml LPS</td>
</tr>
<tr>
<td>12</td>
<td>***</td>
</tr>
<tr>
<td>24</td>
<td>***</td>
</tr>
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<td>48</td>
<td>***</td>
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Figure 2: IL-8 Secretion by HUVEC in Response to LPS Stimulation.

HUVEC were added to the wells of a microtiter plate. After overnight attachment, monolayers were washed and treated with either media alone or media containing various concentrations of LPS. Supernatants were removed at various time periods and assayed for IL-8 via ELISA. Values represent the mean ± S.E.M. of duplicate wells. All experiments were repeated at least twice. Values without indicators demonstrate no significant differences relative to controls. ** p ≤ 0.01, *** p ≤ 0.005.
Figure 3: Screening of Active and Inactive MPO for Optimal HUVEC IL-8 Secretion
HUVEC were added to the wells of a microtiter plate and allowed to attach overnight. Monolayers were then washed and cells were treated with either media alone or media containing 13 U/ml of MPO (light gray) or iMPO (dark gray) at the following concentrations: 140 μg/ml (Cys) and 240 μg/ml (His and Met). Following a 6-hr. incubation, supernatants were removed and assayed for IL-8 via ELISA. Values represent the mean ± S.E.M. of duplicate wells. All experiments were repeated at least twice. Values without indicators demonstrate no significant differences relative to controls. * p ≤ 0.05, *** p ≤ 0.005.
Figure 4: TNF-α Secretion by Murine Mφ in Response to MPO Stimulation.

Resident peritoneal Mφ were added to the wells of a microtiter plate. After a 2-hr. attachment, monolayers were washed and treated with either media alone or media containing MPO (OPT-5). Following a 6-hr. incubation, supernatants were removed and assayed for TNF-α via ELISA. Values represent the mean ± S.E.M. of duplicate wells. All experiments were repeated at least twice. ***p ≤ 0.005.
Figure 5: Effect of Media Supplements on HUVEC IL-8 Secretion in Response to MPO Stimulation.

HUVEC were added to a microtiter plate and incubated overnight. Monolayers were then washed and treated as follows: (a) supplemented media containing 10% FBS alone, or with 20μg/ml MPO; (b) supplemented media containing 2% FBS alone, or with 20μg/ml MPO; (c) media without supplement containing 10% FBS alone, or with 20μg/ml MPO; (d) media without supplement containing 2% FBS alone, or with 20μg/ml MPO. Following a 6-hr. incubation, supernatants were removed and assayed for IL-8 via ELISA. Values represent the mean ± S.E.M. of duplicate wells. All experiments were repeated at least twice.
Figure 6: IL-8 Secretion by HUVEC in Response to iMPO Stimulation.

HUVEC were added to wells of a microtiter plate. After overnight attachment, monolayers were washed and treated with either media alone or media containing various concentrations of iMPO (Cys.). Supernatants were removed at various time periods and assayed for IL-8 via ELISA. Values represent the mean ± S.E.M. of duplicate wells. All experiments were repeated at least twice. Values without indicators demonstrate no significant difference relative to controls. * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.005.
Figure 7: IL-8 Secretion by HUVEC in Response to pEPO and fEPO Stimulation.

HUVEC were added to wells of a microtiter plate. After overnight attachment, monolayers were washed and treated with either media alone or media containing various concentrations of pEPO, fEPO, or iMPO. Cells were cultured with treatments for 6 hr and then supernatants were collected and analyzed for IL-8 via ELISA. Values represent the mean ± S.E.M. of duplicate wells. All experiments were repeated at least twice. Values without indicators demonstrate no significant difference relative to controls. *** p ≤ 0.005
Figure 8: IL-6 Secretion by HUVEC in Response to Stimulation by MPO and iMPO.

HUVEC were added to the wells of a microtiter plate. After overnight attachment, monolayers were washed and treated as follows: (a) media alone, (b) media containing various concentrations of MPO (OPT-5) or (c) media containing various concentrations of iMPO (Cys.). Supernatants were removed at various time periods and assayed for IL-6 via ELISA. Values represent the mean ± S.E.M. of duplicate wells. All experiments were repeated at least twice. Values without indicators demonstrate no significant differences relative to controls. *** p ≤ 0.005.
Figure 9: GM-CSF Secretion by HUVEC in Response to iMPO Stimulation.

HUVEC were added to wells of a microtiter plate. After overnight attachment, monolayers were washed and treated with either media alone or media containing various concentrations of iMPO (Cys.). Supernatants were removed at various time periods and assayed for GM-CSF via ELISA. Values represent the mean ± S.E.M. of duplicate wells. All experiments were repeated at least twice. Values without indicators demonstrate no significant difference relative to controls. * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.005.
Figure 10: Upregulation of mRNA Levels by HUVEC in Response to Stimulation with iMPO.

HUVEC were added to 6-well cluster plates and allowed to adhere overnight. Media were then removed and cells incubated with control media (Lane 1), 33 μg/ml iMPO (Lane 2), or 33 μg/ml MPO for 3 hr. Total cell RNA was then collected and an RPA was performed as described in the materials and methods section. This figure (A) of the autoradiogram is a representative of duplicate experiments. Densitometry (B) was performed to determine relative differences in mRNA levels between treatments.
CHAPTER IV
DISCUSSION AND CONCLUSIONS

Endothelial cells are found throughout the body and participate in many inflammatory reactions. This study demonstrated that peroxidases, proteins with which EC may interact, had different effects on the function of the EC depending on their source and enzymatic activity. One of the first mediators of inflammation is termed the RB. The RB is characterized by the production of ROI, substances that can be used to kill invading pathogens, as well as cause significant tissue damage. Previous studies have indicated that both MPO and EPO induce RB in murine peritoneal Mφ (26, 27). The present study was undertaken to determine if these peroxidases could produce similar results with EC. Peroxidases, such as dHRP and iMPO, demonstrated an upregulation in RB by EC; however, the active forms of peroxidases used in this study showed little effect. The rapid increase in RB upon pEPO stimulation quickly diminished, possibly indicating that the ROI killed the cells, although this has not been confirmed. Approximately 40% of nMPO is rapidly inactivated via oxidation at the site of inflammation (20) and another report stated that there was 16-29 μg/ml of iMPO at an inflammatory site (29); therefore, the presence of iMPO at the endothelium is not only plausible, but these data indicate that it would significantly increase inflammation and tissue damage.

It has been previously reported that LPS is capable of stimulating cytokine secretion by EC (30) and because the peroxidases used in this study contained as much as 4.5 ng/ml of LPS, experiments were conducted to determine the sensitivity of HUVEC to LPS. The results showed that IL-8 secretion was not enhanced by LPS concentrations that were less than 3 μg/ml. This is consistent with a report by Jirik et al. stating that at least 1 μg/ml of LPS was needed to cause secretion of cytokines by EC (31). Therefore, it can be concluded that the effects of the peroxidases used in this study were not due to LPS contamination.

Interleukin-8 secretion was measured in most preliminary studies because of its importance in the development of inflammation and because HUVEC are known to
secrete IL-8 at relatively high levels (32). Interleukin-8 is a potent chemoattractant for neutrophils and eosinophils, the cells responsible for the release of MPO and EPO (33). A correlation between the level of peroxidase exposure to EC and release of IL-8 would suggest a feedback effect exacerbating inflammation and perpetuating its chronicity. It was demonstrated that two different mutants of iMPO induced a significant (P ≤ 0.005) increase in IL-8 production over the control. The histidine mutant of iMPO had only a slight stimulatory effect, indicating the possibility that this site within nMPO plays an important role in the stimulation of cytokine secretion by EC. Because the different mutants of iMPO were not equally stimulatory, the microenvironment could contain a continuum with respect to their binding and stimulatory activities. Despite two of the three different preparations of MPO inducing a slight decrease in IL-8, as depicted in Figure 3, subsequent experiments showed them to have no effect. This was true for all MPO preparations. A possible explanation for the lack of stimulation by MPO in EC lies in the oxidative potential of this enzyme. The receptors for MPO found on the surface of EC may be rendered inactive by this oxidation, thus accounting for the lack of response.

Throughout the studies presented here, preparations of MPO demonstrated no stimulation of cytokines or RB in HUVEC, a finding that was not in accordance with previous experiments showing potent stimulatory effects for them on Mϕ (34). Therefore, an analysis of one of the preparations was performed on Mϕ to determine if it showed similar results to those obtained with previous preparations. The OPT-5 form of MPO was capable of augmenting TNF-α secretion in Mϕ and indicates that this preparation was not ineffective in all cells. The lack of HUVEC response to OPT-5 may be the result of an idiosyncrasy restricted to those cells alone.

A concern of the current investigators in preliminary experiments was the role that the numerous growth factors and supplements found in the conditioned culture media would have on either stimulation of the HUVEC or the analysis of cytokines released into the media. Therefore, an optimal environment for both conditions was investigated. These experiments demonstrated that the presence of growth supplements during stimulation with MPO reduced the levels of IL-8 detected in the media. This may be the result of interference or competition for binding sites between MPO and the various
growth factors or other products found in the growth supplements. However, a more extensive evaluation of this phenomenon is necessary before solid conclusions can be drawn.

After preliminary experiments were conducted to determine the optimal conditions and preparations of MPO and iMPO to be used in this study, an in-depth analysis of the effects of MPO and iMPO on cytokine secretion in HUVEC was initiated. These experiments demonstrated that iMPO enhanced cytokine secretion (IL-8, IL-6, and GM-CSF), MPO did not have an effect. During the course of an inflammatory condition, the increase in IL-8 secreted by EC would serve to recruit PMN to the site of inflammation, while GM-CSF along with IL-8 would stimulate PMN degranulation. The release of IL-6 would stimulate a T helper-2 response that, in turn, would result in an increase in antibody production. The presence of antibody at the site of inflammation is known to stimulate the complement cascade, further enhancing inflammation by generating pro-inflammatory mediators such as C3b.

Because cytokine secretion was upregulated in response to exposure to iMPO, an RPA was performed to determine if this effect was due to an increase in mRNA for IL-8 and GM-CSF. Transcripts for these cytokines as well as for MCP-1 were increased as a result of iMPO stimulation. This cytokine serves to recruit monocytes to the site of inflammation and its upregulation would therefore elevate the inflammatory condition. However, MPO showed only a slight effect, closely paralleling the ELISA results for protein levels. These results suggest the possibility that the regulation in cytokine production by iMPO is at the transcriptional level; still, further experiments are necessary to rule out the possibility of an increase in mRNA stability. With an upregulation in the transcription and protein secretion of IL-8, GM-CSF, and MCP-1, leukocyte recruitment and activation would continue the inflammatory response.

Eosinophil peroxidase is also present at the site of inflammation via degranulation of eosinophils, however, eosinophils arrive much later in the immune response than PMN. Because cytokine secretion was upregulated by iMPO, a similar analysis of EPO was employed. Both pEPO and fEPO were exposed to HUVEC, and supernatants evaluated for IL-8 secretion. It was found that stimulation of HUVEC by pEPO had no
effect on cytokine secretion, thus further supporting the previous data from RB experiments showing that the active forms of the peroxidases (e.g. MPO and pEPO) were incapable of stimulating EC. The results also indicated that there was a slight downregulation of cytokine secretion from HUVEC exposed to fEPO. Previous studies on the effects of fEPO on Mø functions demonstrated a similar decrease in cytokine production (data submitted for publication). These results could indicate that fEPO may regulate cytokine secretion by EC when the inflammatory process has begun to subside.

The above study describes a dichotomy of function for certain peroxidases. Based on previous studies, MPO is known to be involved in the RB by serving as a member of the "cytotoxic triad." This study suggests that iMPO serves as an immunoregulator. In light of the current and previous research conducted in this laboratory, the following paradigm describes the roles of MPO and iMPO in the chronicity of inflammation associated with various inflammatory diseases. The initiating event for inflammation results in the recruitment of PMN to the site of injury or infection. As PMN arrive at the site, they begin to phagocytize foreign substances, releasing MPO into the milieu in the process. Binding of these foreign substances to the PMN results in the release of ROI generated by RB. It has been shown that H₂O₂, a ROI, inactivates MPO (35) and that 16-29 μg/ml of iMPO are found at the site of inflammation (29). Thus, the iMPO generated in this reaction could interact with EC to upregulate their secretion of "pro-inflammatory" and chemotactic cytokines, as well as stimulating the release of more ROI from EC. This in turn would perpetuate the active immune response leading to a state of chronic inflammation.

In conclusion, this study describes an interaction between EC and peroxidases that provides new perspective into the complex nature of inflammation. The following are a summary of the responses of EC to stimulation with various peroxidases and a diagram (Figure 11) explaining the role of EC in the complex inflammatory condition:

1. Enhanced RB when exposed to iMPO.
2. Increase in IL-8, IL-6, and GM-CSF when exposed to iMPO.
3. Downregulation of IL-8 secretion when exposed to fEPO.
4. Augmented IL-8, GM-CSF, and MCP-1 transcript levels upon stimulation with iMPO.

5. The active forms of the peroxidases, such as MPO and pEPO, demonstrated little or no effect on HUVEC functions.

Figure 11: Overview of Endothelial Cell’s Role in Inflammation
REFERENCES


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