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1 Myeloperoxidase and Eosinophil Peroxidase Inhibit the *in vitro* Endotoxin Activities of
2 Lipopolysaccharide (LPS) and Lipid A and Increase Survival in an *in vivo* Mouse LPS LD90
3 model

4

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10

11 Running Head: Haloperoxidases inhibit endotoxin *in vitro* and *in vivo*

12

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21 **Abstract:** Myeloperoxidase (MPO) and eosinophil peroxidase (EPO) are cationic leukocyte
22 haloperoxidases with potent microbicidal and detoxifying activities. MPO selectively binds and
23 kills specific Gram-positive bacteria (GPB) and all Gram-negative bacteria (GNB) tested.
24 Endotoxin, i.e., lipopolysaccharide (LPS) comprising a toxic Lipid A component, is a
25 characteristic of all GNB. The possibility that haloperoxidases bind to and inhibit the endotoxin
26 of GBN was considered and tested by contacting MPO and EPO with LPS and Lipid A and
27 measuring for inhibition of endotoxin activity using either the *in vitro* gel or chromogenic
28 Limulus amebocyte lysate (LAL) assays. Contacting MPO and EPO with LPS purified from
29 *Escherichia coli* O55:B5 and with diphosphoryl Lipid A purified from *E. coli* F583 inhibited
30 their endotoxin activities in proportion to the natural log of the MPO or EPO concentration.
31 Although MPO is less cationic than EPO, MPO consistently demonstrated inhibition of
32 endotoxin activity that is about threefold superior to EPO. Haloperoxidase enzymatic activity
33 was not required for inhibition, and MPO haloperoxidase action did not increase endotoxin
34 inhibition. MPO and EPO inhibition of LPS endotoxin activity was also measured using a 90%
35 lethal dose (LD_{90}) mouse model studied over a five-day period. Based on Kaplan Meier survival
36 analysis, MPO significantly increased mouse survival in a dose-dependent manner. EPO was less
37 effective. In conclusion, contacting MPO and EPO with LPS and Lipid A inhibits *in vitro*
38 endotoxin activities, but inhibition is independent of haloperoxidase enzymatic function. MPO
39 significantly increases mouse survival against LPS in an *in vivo* LD_{90} endotoxin model.

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43 **Introduction:**

44 Myeloperoxidase (MPO) is a unique dimeric heme A glycoprotein produced by neutrophil and
45 monocyte leukocytes (1, 2). Eosinophil leukocytes produce a monomeric eosinophil peroxidase
46 (EPO) with moderate homology to MPO (72.4% at the nucleotide and 69.8% at the amino acid
47 level) (3, 4). Both MPO and EPO are cationic, but EPO is more cationic than MPO (5). Both
48 enzymes are haloperoxidases; i.e., MPO and EPO catalyze the oxidation of chloride and
49 bromide, respectively. Both enzymes have haloperoxidase activities that are highly microbicidal
50 (6, 7). MPO production in neutrophils is abundant and influenced by the extent of stimulated
51 myelopoietic turnover (8, 9).
52
53 MPO selectively binds and kills some specific gram-positive bacteria (GPB), but binds and kills
54 all gram-negative bacteria (GNB) tested (10). In addition to microbial killing, the haloperoxidase
55 activity of MPO has been reported to inactivate microbial toxins, including diphtheria toxin,
56 tetanus toxin, and *Clostridium difficile* cytotoxin (11, 12). Like microbicidal action, the toxin-
57 destroying activities of MPO require haloperoxidative enzymatic action that is hydrogen
58 peroxide and halide-dependent. There are no reports of MPO binding or inhibition of the
59 endotoxin activity of lipopolysaccharide (LPS) or Lipid A.
60
61 The cell envelope of GNB is composed of a cell wall with an inner cytoplasmic cell membrane
62 and an outer cell wall membrane presenting LPS with its toxic Lipid A component (13). Release
63 of endotoxin secondary to GNB lysis causes severe toxemia, i.e., septic shock. The MPO binding
64 observed for all GNB tested (10, 11) suggests the possibility that endotoxin, a characteristic
65 component of all GNB (14-16), might be responsible for such binding. The present studies were

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66 designed to determine if MPO binding to GNB might involve direct binding to endotoxin, and if
67 such binding might inhibit the endotoxin activity of LPS and Lipid A.
68
69 The haloperoxidase activity of MPO is potent, and might be considered potentially toxic. Our
70 contention is that MPO lethal action is binding-specific and focused, and that the highly reactive
71 products of haloperoxidase action are temporally and physically restricted to the proximate site
72 of enzyme binding (11). Recently, interesting evidence has been presented that blockade or
73 genetic deletion of MPO increases mortality associated with LPS toxicity, and as such, MPO
74 appears to protect against the adverse effects of endotoxin (17). The *in vitro* and *in vivo* research
75 described herein provides direct empirical evidence for MPO and EPO contact inhibition of LPS
76 and Lipid A endotoxin activities.
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89 **Materials and Methods:**

90 **Enzymes**

91 The haloperoxidases, porcine myeloperoxidase (MPO) and porcine eosinophil peroxidase (EPO),
92 were produced by Exoxemis Inc. The porcine MPO used was 98.9% pure by ultraperformance
93 liquid chromatography (RP-UPLC) and 100% pure by molecular size exclusion high
94 performance liquid chromatography (SEC-HPLC). The MPO had a reinheitszahl (RZ;
95 A_{430nm}/A_{280nm}) of 0.79. The porcine EPO used was 99.2% pure by reverse phase high
96 performance liquid chromatography and had a Reinheitszahl (RZ; A_{415nm}/A_{280nm}) of 0.96.
97 Glucose oxidase (GO) was isolated from *Aspergillus niger* and purified by Exoxemis Inc. Its
98 final purity was 99.8% by RP-HPLC and 99.9% by SEC-HPLC.
99

100 **Endotoxins**

101 Lipopolysaccharide (LPS) purified from *E. coli* O55:B5 was purchased from Sigma-Aldrich
102 (L4524). The manufacturer specification for LPS endotoxin activity was 3×10^6 endotoxin units
103 (EU) per mg. Using standard LPS dilutions, our results were consistent with the activity value
104 described by the manufacturer using the clot-based Limulus amebocyte lysate (LAL) assay E-
105 Toxate test (Sigma-Aldrich) or using the microplate chromogenic LAL assay (LAL
106 Endochrome-K, Endosafe; Charles River). Diphosphoryl Lipid A purified from *E. coli* F583 (Rd
107 mutant) was purchased from Sigma Aldrich (L5399). The manufacturer specification for Lipid A
108 endotoxin activity was 1×10^6 EU per mg. Using a set of standard LPS dilutions, our results
109 were lower than the activity described by the manufacturer, but Lipid A activity was consistently
110 replicated using either the E-Toxate clot-based LAL or the Endochrome-K, Endosafe
111 chromogenic LAL assay.

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112

113 **Limulus amebocyte lysate gel assay**

114 Detection and semi-quantitation of the endotoxin activities of LPS and Lipid A were performed
115 using the tube-based E-Toxate test (Sigma-Aldrich). The LAL reagent, prepared from a lysate of
116 circulating amebocytes of the horseshoe crab *Limulus polyphemus*, changes viscosity and opacity
117 on contact with minute quantities of endotoxin (18). Endotoxin in the presence of calcium ions
118 activates a trypsin-like enzyme that proteolytically modifies a "coagulogen" to produce clotted
119 protein (19). The limit of sensitivity of the test is 0.05-0.10 endotoxin units (EU)/mL. The LAL
120 measured activity is proportional to the pathophysiologic activity of LPS (20).

121

122 **Gel LAL inhibition testing**

123 Detoxification of endotoxin by exposure to sodium hydroxide (NaOH) can be quantified using
124 the E-Toxate test to measure *Limulus* amebocyte gel activity (20). In similar manner, the
125 inhibitory effects of MPO and EPO on lipopolysaccharide (LPS) was investigated by contacting
126 varying quantities of MPO or EPO with a given quantity of LPS and measuring for inhibition of
127 endotoxin activity using the LAL gel assay.

128

129 **Chromogenic Limulus amebocyte lysate assay**

130 Endotoxin was also quantified using a microplate chromogenic Limulus amebocyte lysate (LAL)
131 assay (LAL Endochrome-K, Endosafe) purchased from Charles River (21). Endotoxin activation
132 of the LAL clotting enzyme was quantified by measuring endotoxin-activated enzymatic
133 cleavage of a synthetic chromogenic substrate releasing p-nitroaniline (pNA). Activity was
134 measured as change in absorbance at a wavelength of 405 nm in a microplate spectrophotometer

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135 (Tecan). Kinetic measurement of the time to maximum color change was used to gauge the
136 activity of endotoxin present. The limit for detection was set at 1680 seconds (28 min).
137 Calibration standards were prepared and a standard curve was used to generate an equation with
138 a coefficient of determination (R^2) for each experiment performed.
139

140 **Chromogenic LAL inhibition testing**

141 The modified chromogenic LAL method served as an *in vitro* assay of haloperoxidase inhibition
142 of endotoxin. This inhibition assay included a pre-incubation step where either LPS or Lipid A
143 was contacted with the test enzyme (MPO, EPO, or GO) for a period of 30 minutes at 37˚C. Test
144 agents were diluted in low endotoxin reagent water (LRW). Following incubation Limulus lysate
145 solution was added and chromogenic activity was measured as the time (in seconds) to maximum
146 color change. Inhibition was calculated as the difference between the activity expected for the
147 quantity of endotoxin present in the absence of haloperoxidase, and the actual measured
148 endotoxin activity of LPS or Lipid A contacted with MPO or EPO. Inhibition was expressed as a
149 percentage of the activity of LPS or Lipid A alone. MS Excel software was used for data
150 analysis, curve fitting, coefficient of determination (R^2) calculations and graphic construction.
151 The R^2 statistic tests the fit of the measured observations in proportion to total variation of
152 outcomes predicted using the empirically generated equation; i.e., the proportion of the variance
153 in the dependent variable predictable from the independent variable (22).
154

155 **Mice lethal dose 90 (LD_{90}) model**

156 Experimentally naïve, healthy BALB/c female mice with a weight range of 16.2 to 19.7 g were
157 divided into dose groups. Treatment of the animals (including but not limited to all husbandry,

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158 housing, environmental, and feeding conditions) was conducted in accordance with the
159 guidelines recommended in Guide for the Care and Use of Laboratory Animals. All mouse
160 testing was performed according to the protocols and standard operational procedures of
161 Concord Bioscience LLC, Concord, OH 44077.

162 Except for one group containing 15 mice, each test group contained 20 mice. For all groups each
163 mouse received a 0.5 mL total volume with different doses of purified LPS injected
164 intraperitoneally (IP). Low endotoxin reagent water (LRW) was used to adjust the concentrations
165 of LPS. Groups 1, 2, 3 and 4 received 0.20, 0.35, 0.50 and 0.65 mg of LPS per mouse,
166 respectively. After 5 days of observation, the censored (live) and the event (dead) mouse counts
167 were tabulated. Group 1 had 20 live with 5 dead for a 75% survival (25% mortality); Group 2
168 had 5 live with 15 dead for a 25% survival (75%mortality), and Groups 3 and 4 had 20 dead with
169 0 live for 0% survival (100% mortality). The 90% lethal dose (LD_{90}), i.e., the dose estimated to
170 produce 90% mortality, was set at 0.40 mg per mouse, i.e., about 22 mg/kg.

171 The mouse LD90 testing was done in two parts. The first part tested the inhibitory action of 0.5
172 mg MPO combined with the 0.4 mg LPS (LD_{90} dose). The second part expanded the testing to
173 include doses of 2.5 and 5.0 mg MPO, as well as doses of 2.5 and 5.0 mg EPO in combination
174 with the 0.4 mg LPS (LD_{90} dose). The appropriate concentrations of LPS plus MPO or EPO
175 were vortexed vigorously for about a minute then incubated at 37°C for 45 min. The mix was
176 vortexed again prior to IP injection of each animal.

177

178 Survivor analysis was performed with IBM SPSS software using the Laerd Statistics guide for
179 Kaplan Meier survival analysis.

180

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181 **Results**

182 **MPO and EPO inhibit LPS endotoxin activity measured by the clot-endpoint LAL**

183 Endotoxin was quantified using the E-Toxate clot-endpoint Limulus amebocyte lysate (LAL)
184 assay. Following testing of LPS standards and confirmation of endotoxin unit activity, LPS at
185 0.0125 EU per tube was tested in combination with a set of MPO and EPO dilutions to determine
186 the lowest haloperoxidase dilution sufficient to completely inhibit clot-formation.

TABLE 1. Haloperoxidase inhibition of endotoxin activity (EU) activity measured as Limulus amebocyte lysate (LAL) gelation.

EU required for LAL gelation

LPS	EU/tube	None	0.0250	0.0125	0.0060	0.0030	0.0015
		Neg	Pos	Pos	Pos	Neg	Neg

Haloperoxidase inhibition of EU activity measured as LAL gelation

LPS	EU/tube	None	0.0125								
MPO	mg/tube	0.560	0.560	0.420	0.320	0.240	0.180	0.130	0.100	0.075	0.056
		Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos
EPO	mg/tube	0.560	0.560	0.420	0.320	0.240	0.180	0.130	0.100	0.075	0.056
		Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos

187

188 The results presented in **Table 1** demonstrate that both MPO and EPO inhibit the EU activity of
189 LPS measured by the E-Toxate LAL gelation test. Based on the haloperoxidase mass required
190 for inhibition, MPO is about 3.2 times more potent than EPO.

191

192 **Chromogenic LAL assay measurement of MPO and EPO inhibition of LPS**

193 Endotoxin was also quantified using the chromogenic Limulus amebocyte lysate (LAL) assay
194 (21). Endotoxin activated cleavage of the chromogenic substrate releases p-nitroaniline that is
195 measured at a wavelength of 405 nm with microplate spectrophotometer. Temporal kinetic
196 measurement of the time-to-maximum-slope was used to gauge the endotoxin activity.

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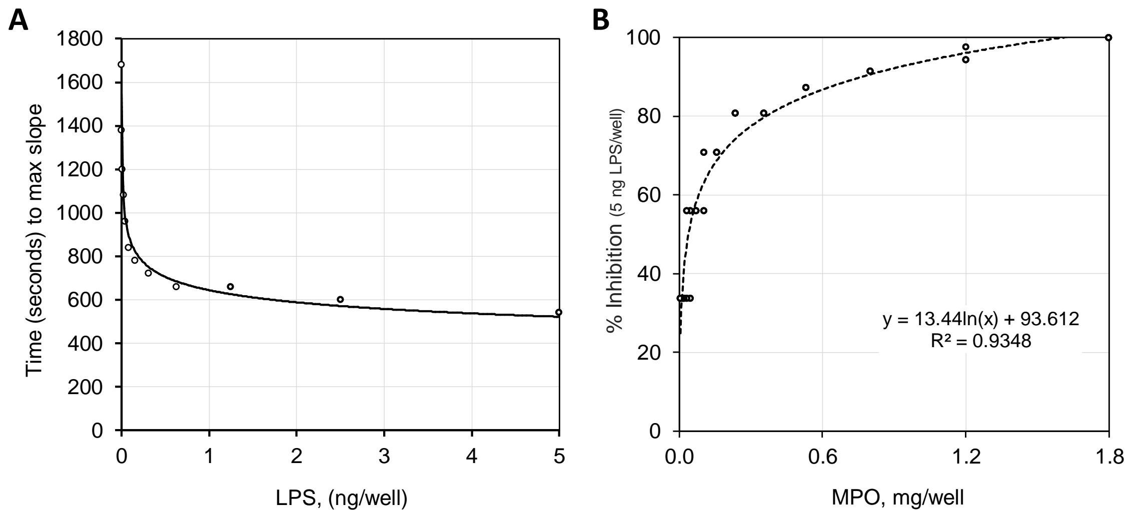
197 Calibration standards were run and a standard curve with equation was generated for each set of
198 measurements as shown in Fig. 1 Graph A.



199

200 **Fig. 1 Graph A.** Regression plot of LPS standards (ng/well) against time (sec) to maximum slope. For the
201 range: 0.0012 - 5 ng LPS/well the derived equation was $y = 643.96x^{-0.131}$ with an $R^2 = 0.982$; for the
202 range: 0.0012 - 0.625 ng LPS/well the equation was $y = 600.99x^{-0.146}$ with an $R^2 = 0.991$. **Graph B.**
203 Percent inhibition of the LAL activity for 5.0 ng LPS/well plotted against varying concentrations of MPO
204 (mg/well). Testing was run in quadruplicate with all data points depicted. The regression equation shows
205 inhibition proportional to the natural log of the MPO mass with R^2 is included.

206

207 Using a set of standards, the endotoxin mass/well is plotted against the time point of maximum
208 color change (max slope). The manufacturer specified activity for this lot of LPS was 3×10^6
209 endotoxin units (EU) per mg, i.e., 3 EU/ng. As expected, the 0.01 ng LPS/well standard had a
210 time to max slope of 1200 sec, a value equivalent to about 0.03 EU. This chromogenic LAL test
211 was modified to serve as an *in vitro* assay of haloperoxidase inhibition of endotoxin. The
212 inhibition assay included a pre-incubation step where LPS or Lipid A was exposed to a test
213 enzyme (MPO, EPO, or GO) for a period of 30 minutes at 37˚C. The reaction conditions

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214 contained no hydrogen peroxide, and as such, inhibition reflected protein binding in the absence
215 of haloperoxidase enzymatic action. Following incubation, reaction was initiated by addition of
216 the Limulus lysate solution, and the time (in seconds) to maximum color change was measured.
217 Based on the standards-derived equation, the time-to-max-slope was converted to equivalent LPS
218 mass. Inhibition was calculated as the difference between the activity of the expected mass, e.g.,
219 endotoxin without MPO, and the actual measured LPS activity, e.g., the percentage of endotoxin
220 activity measured in the presence of MPO. The plot of MPO mass versus % of LPS endotoxin
221 activity is illustrated by the plot of the data shown in **Fig. 1 Graph B**.

222

223 Inhibition of the endotoxin activity of 5.0 ng LPS was proportional to the log of MPO
224 concentration (mass) present. One mg MPO inhibited about 91% of the endotoxin activity of 5
225 ng LPS; stated differently, 1 mg MPO inhibited about 4.5 ng LPS. However, at one-hundredth
226 the MPO mass (0.01 mg), MPO was still capable of inhibiting 28% of the activity of 5 ng LPS,
227 i.e., about 1.4 ng LPS. Note that with LPS mass held constant and MPO mass varied, the percent
228 inhibition of LAL activity is directly proportional to the natural log of MPO concentration. As
229 the ratio of the MPO to LPS mass increases, the percent inhibition of endotoxin activity
230 increases, but the efficiency of MPO inhibition decreases.

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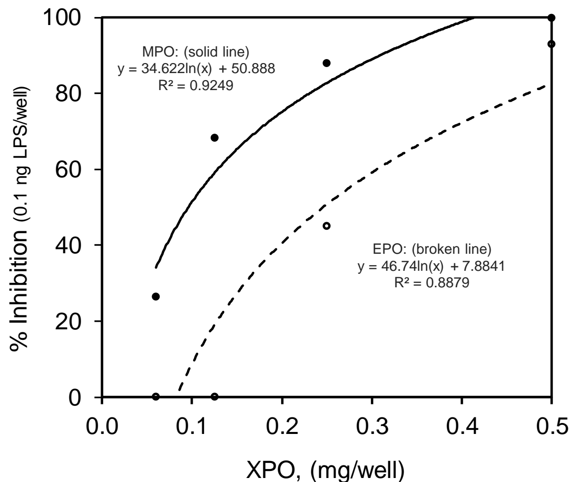

238

239 **Fig. 2.** Percent inhibition of LAL endotoxin activity for 0.1 ng LPS/well plotted against mg/well of MPO,
240 EPO and GO (not shown). The regression equations are shown for MPO and for EPO. LPS inhibition is in
241 proportion to the natural log of the MPO and EPO mass contacted. GO was not inhibitory and not
242 included.

243

244 For comparison, the endotoxin inhibition activities of MPO and EPO were measured and the
245 results are plotted in **Fig. 2**. Both MPO and EPO inhibited LPS, but MPO was superior to EPO in
246 inhibition capacity on a mass or molar basis. For a constant mass of LPS, inhibition of endotoxin
247 activity was proportional to the log of the mass of MPO or EPO. At a mass of 0.25 mg, MPO and
248 EPO inhibited 88% and 45% of the endotoxin activity of 0.1 ng LPS, respectively. Glucose
249 oxidase (GO) did not inhibit LPS endotoxin activity within the range of concentration tested.

250

251 **MPO and EPO inhibition of Lipid A endotoxin activity by the chromogenic LAL**

252 Lipid A, the toxic component of LPS (14), has two glucosamine units with anionic phosphate
253 groups attached and typically six hydrophobic fatty acids that anchor it into the outer membrane
254 of GNB. Purified Lipid A is toxic and its endotoxin activity is measurable with the LAL assay.

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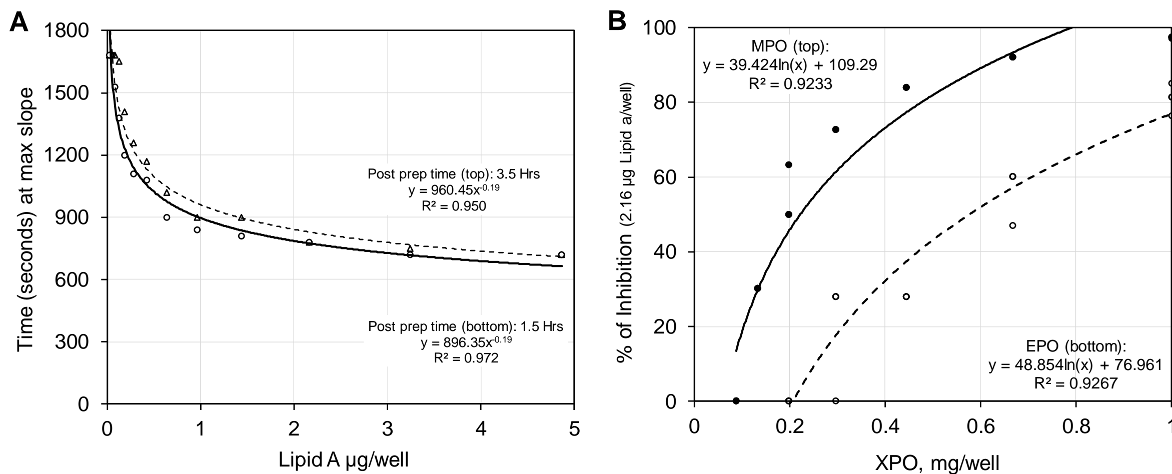
255 The inhibitory actions of MPO and EPO were tested on diphosphoryl Lipid A from *E. coli* F583
256 (Sigma Aldrich, L5399) using the same chromogenic LAL assay described above. The results of
257 Lipid A standards testing over time are presented in **Fig. 3 Graph A**.
258



259

260 **Fig. 3. Graph A.** Regression plot of Lipid A standards (ng/well) against time (sec) to maximum slope with
261 derived equations and R^2's. The top and bottom curves were obtained from the same set of standards
262 prepared 1.5 and 3.5 hours post preparation of the Lipid A standards as indicated. **Graph B.** Percent
263 inhibition of LAL activity for 2.16 µg Lipid A/well plotted against varying concentrations of MPO or EPO in
264 mg/well. The regression equations for MPO and EPO inhibition of Lipid A are shown. Lipid A standards
265 were simultaneously run for each MPO or EPO inhibition experiment.
266

267 The hydrophobic character of Lipid A complicated preparation of aqueous standards (13), and
268 the endotoxin unit activity per Lipid A mass was lower than the manufacture reported value (i.e.,
269 $\geq 1 \times 10^6$ EU/mg) based a coagulation LAL assay. As illustrated by the data of **Fig. 3 Graph A**,
270 Lipid A endotoxin activity quantified using the chromogenic LAL assay was reasonably stable
271 over several hours. Using the equation generated from simultaneously run Lipid A standards,
272 MPO and EPO inhibition of Lipid A endotoxin activity was measured as shown in **Fig. 3 Graph**

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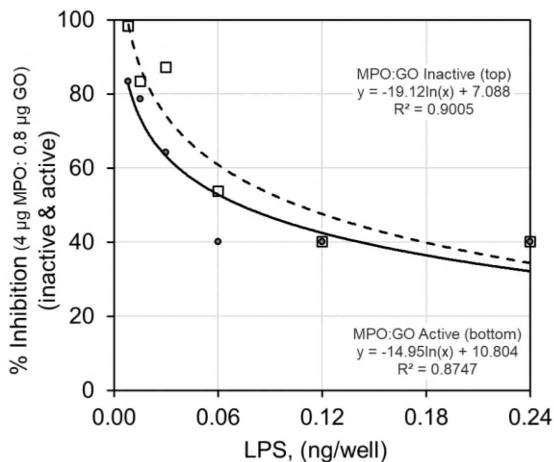
273 **B**. Consistent with the finding for LPS, the endotoxin activity of Lipid A was inhibited by both
274 MPO and EPO, and inhibition was proportional to the natural log of the MPO or EPO
275 concentration. Likewise, MPO was more inhibitory than EPO. GO did not inhibit the endotoxin
276 activity of Lipid A.
277

278 **Haloperoxidase enzymatic action not required for endotoxin inhibition**

279 The microbicidal and antitoxin activities of MPO require enzymatic action, i.e., haloperoxidase
280 activity (6, 12, 23). All of the experiments described above were conducted in the absence of
281



282

283 **Fig. 4.** Percent inhibition of LPS endotoxin activity for a formulation composed of 4 µg MPO and 0.8 µg
284 GO with 23 µg Cl^-/well measured against varying concentrations of LPS using the LAL assay. The
285 formulation was tested without D-glucose, i.e., no haloperoxidase activity, and with a non-limiting
286 concentration (0.2 mg/well) of D-glucose, i.e., haloperoxidase activity. The regression equations describe
287 that the percentage inhibition of LPS endotoxin activity for a constant concentration of MPO plus GO with
288 or without D-glucose is proportional to the negative log of the LPS concentration.

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289 hydrogen peroxide or a peroxide generating system, i.e., in the absence of haloperoxidase
290 enzymatic action. This experiment was devised to measure possible differences in
291 endotoxin inhibition with regard to haloperoxidase activity. A formulation containing MPO plus
292 GO as a hydrogen peroxide generating enzyme, i.e., 4 μg MPO and 0.8 μg GO. The enzyme
293 complex was tested for LPS endotoxin inhibitory activity in the absence of D-glucose, i.e., no
294 haloperoxidase action, and in the presence of D-glucose, i.e., high haloperoxidase activity. The
295 microbicidal action of the fully active MPO-GO-glucose preparation has been previously
296 described (10, 24). The results are presented in **Fig. 4**.
297
298 With the concentration of MPO plus GO held constant, i.e., MPO held constant, and the
299 concentration of LPS varied, the percent inhibition of endotoxin activity is proportional to the
300 negative natural log of the LPS concentration. This is the expected inverse relationship to that
301 observed when LPS or Lipid A is held constant and MPO or EPO is varied. As the mass ratio of
302 LPS to MPO increases, the percent inhibition of endotoxin activity decreases. The functional
303 enzymatic MPO-GO complex, with a non-limiting concentration of D-glucose as substrate and
304 chloride as cofactor, was slightly less effective than the non-enzymatically active MPO-GO
305 complex without D-glucose. Contacting MPO with LPS inhibited its endotoxin activity in the
306 absence or presence of oxidative enzymatic function. Haloperoxidase activity did not improve
307 endotoxin inhibition.
308

309 **Effect of MPO and EPO on mouse survival in an endotoxin LD_{90} model**

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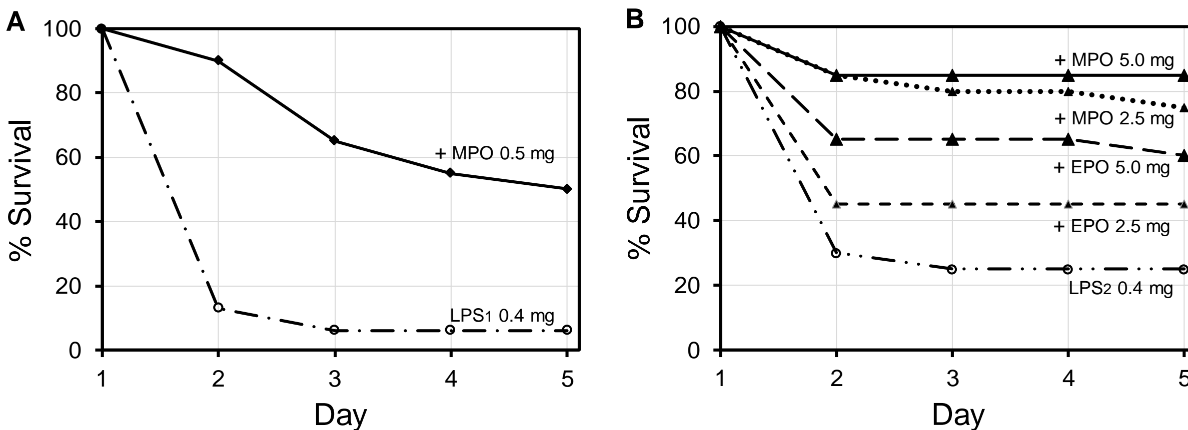
310 Intraperitoneal (IP) injection of a 0.5 mL volume containing 0.4 mg LPS per mouse produced
311 about 90% lethality (LD_{90}) in the naïve, healthy BALB/c female mice tested as described in
312 **Methods**. The LD_{90} dose, equivalent to about 22 mg/kg, was used for all testing. Highly purified
313 porcine MPO and EPO were used for testing as described in **Materials**. The indicated
314 concentrations of LPS and MPO were mixed for about a minute, incubated at 37°C for 45 min,
315 then vortexed again prior to intraperitoneal injection (IP) of 0.5 mL per mouse. No hydrogen
316 peroxide was present in the preparation, and as such, there was MPO contact without
317 haloperoxidase action.

318 LD_{90} testing consisted of two studies. The initial study determined the effect of 0.5 mg of MPO
319 on the survivability of mice treated with 0.4 mg of LPS. As depicted in **Figure 5 A** and described
320 in **Table 2**, this quantity of MPO significantly improved the survival of mice in the LPS LD_{90}
321 model (25-29). Contacting the LD_{90} dose of 0.4 mg LPS with 0.5 mg MPO resulted in a 50%
322 survival rate over the expected 10% survival rate. MPO-treatment did not appear to change the
323 incidence of LPS-associated clinical observations, but LPS-associated mortality was clearly and
324 significantly decreased in the MPO-treated animals, as depicted in the Kaplan Meier plot of **Fig.**
325 **5 Graph A.**



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327 **Fig. 5. Graph A.** Initial study of the effect of 0.5 mg MPO/mouse on survival depicted as Kaplan Meier
328 survivor curves for BALB/c female mice over a 5-day period following IP injection of an LD90 (0.4 mg
329 LPS/mouse; i.e., about 22 mg/kg) dose on Day 1. **Graph B.** Follow-up study showing Kaplan Meier
330 survivor curves using 2.5 and 5.0 mg MPO/mouse, and also, 2.5 and 5.0 mg EPO/mouse.

331

TABLE 2. Mean and median survival times plus overall statistical comparisons for the first and second LD90 mouse studies.

First Study

Intervention, treatment	Total Number (N)	N Events (deaths)	N Censored	Percent	Mean[a] Estimate	Mean[a] Std. Error	Mean[a] 95% Confidence Interval Lower Bound	Mean[a] 95% Confidence Interval Upper Bound	Median[b] Estimate	Median[b] Std. Error	Median[b] 95% Confidence Interval Lower Bound	Median[b] 95% Confidence Interval Upper Bound
LPS 0.4_1st study	15	14	1	6.7%	2.200	0.193	1.821	2.579	2.000			
LPS 0.4 + MPO 0.5	20	10	10	50.0%	4.100	0.257	3.596	4.604	5.000			

[a] Estimation is limited to the largest survival time if it is censored.

[b] Median is estimated as the time at when the cumulative survival is 0.50 or less. Time of first observation less than 50% is used. If cumulative survival does not reach 0.50 or below, no median survival time is calculated.

Overall Comparisons

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	17.143	1	0.000035
Breslow (Generalized Wilcoxon)	21.011	1	0.000005
Tarone-Ware	19.566	1	0.000010

Test of equality of survival distributions for the different levels of Intervention, treatment.

Second Study

Intervention, treatment	Total Number (N)	N Events (deaths)	N Censored	Percent	Mean[a] Estimate	Mean[a] Std. Error	Mean[a] 95% Confidence Interval Lower Bound	Mean[a] 95% Confidence Interval Upper Bound	Median[b] Estimate	Median[b] Std. Error	Median[b] 95% Confidence Interval Lower Bound	Median[b] 95% Confidence Interval Upper Bound
LPS 0.4_2nd study	20	15	5	25.0%	2.800	0.288	2.235	3.365	2.000			
LPS 0.4 + MPO 2.5	20	5	15	75.0%	4.450	0.279	3.903	4.997				
LPS 0.4 + MPO 5.0	20	3	17	85.0%	4.550	0.240	4.081	5.019				
LPS 0.4 + EPO 2.5	20	11	9	45.0%	3.350	0.334	2.696	4.004	2.000			
LPS 0.4 + EPO 5.0	20	8	12	60.0%	3.950	0.342	3.280	4.620				

[a] Estimation is limited to the largest survival time if it is censored.

[b] Median is estimated as the time at when the cumulative survival is 0.50 or less. Time of first observation less than 50% is used. If cumulative survival does not reach 0.50 or below, no median survival time is calculated.

Overall Comparisons

	Chi-Square	df	Sig.
Log Rank (Mantel-Cox)	19.375	4	0.00066
Breslow (Generalized Wilcoxon)	20.058	4	0.00049
Tarone-Ware	19.814	4	0.00054

Test of equality of survival distributions for the different levels of Intervention, treatment.

332

333

TABLE 3. Pairwise comparison of LPS alone and in combination with different concentrations of MPO and EPO.

Intervention, treatment		LPS 0.4 + MPO 2.5 Chi-Square	LPS 0.4 + MPO 2.5 Sig.	LPS 0.4 + MPO 5.0 Chi-Square	LPS 0.4 + MPO 5.0 Sig.	LPS 0.4 + EPO 2.5 Chi-Square	LPS 0.4 + EPO 2.5 Sig.	LPS 0.4 + EPO 5.0 Chi-Square	LPS 0.4 + EPO 5.0 Sig.
Log Rank (Mantel-Cox)	LPS 0.4 (2nd study)	10.782	0.0010	14.421	0.0001	1.668	0.1965	5.131	0.0235

334

335 A second LD90 study was designed to confirm the initial results, expand the concentration range
336 of MPO, and also include the same range of EPO for comparison. The LPS only LD90 group for
337 the second study showed less than the expected mortality, i.e., 75% mortality. Overall

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338 comparison of the LD90 controls for the initial and second studies using Log Rank (Mantel-Cox)
339 analysis yields a chi-square of 2.248 with 1 degree of freedom (df) for a significance (p value) of
340 0.134, i.e., no significant difference in the LD_{90} controls (27). As an additional control, a group
341 of twenty mice were treated with high dose (5 mg/mouse) MPO without LPS. The mice treated
342 with this high dose of MPO alone (without LPS) showed no abnormal clinical observations and
343 no mortality. However, an EPO only control group of twenty mice treated with high dose (5
344 mg/mouse) EPO without LPS showed 10% lethality by day 2. The surviving EPO only group
345 mice showed no abnormal clinical observations.
346

347 As depicted in **Figure 5 B** and described in the overall analysis of **Table 2** and in the pairwise
348 analysis of **Table 3**, the increasing the concentrations of MPO to 2.5 and 5.0 mg/mouse yielded
349 significantly improved mouse survival in this LPS LD_{90} model, and survival was increased in a
350 dose dependent manner. The EPO group tested at concentrations of 2.5 and 5.0 mg/mouse also
351 showed improved mouse survival in this LPS LD_{90} model, and survival was increased in a dose
352 dependent manner as depicted in **Figure 5 B**. However, the small increase in survival for the 2.5
353 mg EPO/mouse group was not significant; the pairwise Kaplan Meier analysis of **Table 3**
354 showed a p value of 0.1965 (27). At the higher EPO concentration of 5.0 mg/mouse, a
355 statistically significant increase in mouse survival was obtained, i.e., p value of 0.0235.

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361 **Discussion**

362 MPO binds to a broad spectrum of microbes. Strong binding has been observed with many
363 Gram-positive bacteria (GPB), including *Staphylococcus aureus*, and with all Gram-negative
364 bacteria (GNB) tested. Members of the lactic acid family of GPB show relatively weak binding
365 (30-32). The strength of MPO binding is proportional to the efficiency of haloperoxidase-
366 mediated microbicidal action. When the concentration of MPO is limiting, strong MPO-binding
367 to bacteria such as *S. aureus*, *Escherichia coli*, or *Pseudomonas aeruginosa* in combination with
368 weak MPO-binding to hydrogen peroxide-producing viridans streptococci results in restriction of
369 microbicidal action to MPO-bound bacteria and sparing of streptococci. This selectivity is
370 observed in the absence and in the presence of strongly catalase-positive human erythrocytes at a
371 high ratio of erythrocytes to MPO-binding bacteria, further supporting the requirement of
372 proximity with regard to MPO microbicidal action. The primary and secondary microbicidal
373 products of MPO action, i.e., hypochlorite and singlet molecular oxygen with its microsecond
374 lifetime, favor the selective killing of MPO-bound microbes (10, 32).

375

376 MPO inactivates microbial toxins including diphtheria toxin, tetanus toxin, and *Clostridium*
377 *difficile* cytotoxin (11, 12, 23). The toxin-destroying activities of MPO required haloperoxidase
378 enzymatic action. Enzymatic or non-enzymatic action of MPO or EPO on endotoxin by MPO or
379 EPO has not been described. The moderate to strong MPO binding reported for all GNB might
380 be related to the fact that endotoxin is a characteristic of GNB (14-16). All GNB tested to date
381 show MPO binding (10, 31), suggesting a possible commonality with regard to the mechanism of
382 binding. The cell envelope of a GNB is composed of a cell wall with an inner cytoplasmic cell
383 membrane and an outer cell wall membrane presenting lipopolysaccharide (LPS) with its toxic

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384 Lipid A component (13). The toxic action of LPS remains even after the death of the GNB.
385 Release of endotoxin secondary to GNB lysis produces severe toxemia, i.e., septic shock.
386
387 The present studies were designed to determine if haloperoxidase binding, in addition to
388 improving the potency and selectivity of microbicidal action, might provide additional protection
389 against the endotoxin activity of residual LPS and Lipid A. The presented results demonstrate
390 that MPO, and to a lesser extent EPO, bind to and inhibit the endotoxin activity of LPS measured
391 by either the gel or chromogenic LAL assay. Electrostatic interaction is reasonably expected to
392 play a role in MPO and EPO binding to the anionic phosphate groups of LPS and its Lipid A
393 component. Electrostatic binding alone is insufficient to explain the threefold greater inhibitory
394 action of MPO relative to more cationic EPO. MPO is significantly less cationic than EPO.
395 Although the mechanism of inhibition likely involves electrostatic interaction, electrostatic
396 binding provides an incomplete explanation.
397
398 The toxic activities of LPS and Lipid A are measured in endotoxin units (EU). Measuring EU
399 activity using the chromogenic LAL assay, the percent inhibition of a constant concentration of
400 LPS or Lipid A is directly proportional to the natural log of the MPO or EPO concentration as
401 shown in **Figures 1-3**. Likewise, if the concentration of MPO is held constant and the LPS
402 concentration is varied, the percent inhibition of endotoxin activity is proportion to the negative
403 natural log of the LPS concentration as shown in **Figure 4**.
404
405 MPO or EPO binding is sufficient for inhibition of LPS and Lipid A endotoxin activity. Unless
406 stated otherwise, no hydrogen peroxide was available to either MPO or EPO. As described in

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407 Figure 4, when endotoxin inhibition was tested in the absence and in the presence of a hydrogen
408 peroxide generation, the enzyme-functional haloperoxidase system produced slight decreased
409 inhibition. MPO and EPO haloperoxidase enzymatic function are not required for endotoxin
410 inhibition.

411

412 Although the clot-based and chromogenic assays for MPO and EPO inhibition of endotoxin
413 activities provide good *in vitro* evidence, we extend testing to include an *in vivo* LD90 mouse
414 pathophysiology model of LPS endotoxicity. Initial study showed strong evidence for increased
415 survivability using MPO at 0.5 mg relative to the LD_{90} dose of 0.4 mg LPS. The follow-up study
416 increased the doses to 2.5 and 5.0 mg MPO and expanded testing to include testing of 2.5 and 5.0
417 mg EPO; the same LD_{90} dose of 0.4 mg LPS was used. As depicted by the Kaplan Meier plots of
418 **Figure 5**, MPO, and to a lesser extent EPO, increased survivability in in a dose dependent
419 manner. As indicated by statistical analysis of the results presented in Tables 2 and 3, increased
420 survival was statistically significant at all MPO concentrations tested. However, statistically
421 significant increase in survival was only achieved using 5.0 mg EPO.

422

423 The *in vitro* and *in vivo* results presented herein unambiguously demonstrate that MPO, and to a
424 lesser extent EPO, inhibit endotoxin activity. Thus, in addition to the potent haloperoxidase
425 microbicidal action of MPO and EPO, these proteins also provide non-enzymatic protection
426 against endotoxin, and this protection is independent of microbicidal enzymatic action. Our
427 position is that the haloperoxidase action of MPO is pH-restricted, and that the selective binding
428 characteristic of MPO physically focuses its potent but temporally transient oxygenation
429 activities. The enzymatic action of MPO is focused to exert maximum microbicidal action and

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430 produces minimal host damage (10). The direct *in vitro* and *in vivo* evidence of MPO binding
431 inhibition of endotoxin activity presented herein is consistent with the recently reported evidence
432 that blockade or genetic deletion of MPO increases the mortality associated with LPS toxicity,
433 and that MPO protects against the adverse effects of endotoxin (17).

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