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Effect of Estrogens on the Myeloperoxidase-Mediated Antimicrobial System

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Estradiol 17^{β} prevented the fall in the microbicidal activity of the myeloperoxidase-H_2O_2-halide system induced by high H_2O_2 concentrations. In contrast, when the H_2O_2 (and halide) concentrations were low, the myeloperoxidase-H_2O_2-halide antimicrobial system was inhibited by estradiol. These properties of estradiol 17^{β} were shared by estradiol 17^{α}, estrone, estriol, ethinyl estradiol, and phenol, but not by estradiol-3-benzoate, testosterone, progesterone, hydroxyprogesterone, cortisone, hydrocortisone, or deoxycorticosterone.

A number of peroxidase-catalyzed reactions are stimulated by estrogens (9-12, 20). An intact phenolic hydroxyl group is required, and it was proposed that the estrogen may act as an oxidation-reduction catalyst, being alternately oxidized by peroxidase and H_2O_2, possibly to the phenoxy radical, and then reduced to its original form by an electron donor which is in turn oxidized. In several, but not all, instances, the electron donor is oxidized more rapidly in the presence of the estrogen than in its absence. In the absence of an appropriate electron donor, oxidation of the estrogen can lead to its further degradation with a loss of biological activity (13, 17). In the presence of a protein, firm, presumably covalent binding of the estrogen occurs (6-8, 15), and firm binding of estrogens to peroxidase-positive sites in tissues has been reported (4, 15, 19).

Myeloperoxidase (MPO), H_2O_2, and a halide form a potent antimicrobial system effective against a variety of microorganisms, and this system appears to contribute significantly to the antimicrobial activity of the polymorphonuclear leukocyte (PMN) (for reviews see 14, 16). Estradiol is converted to an alcohol-precipitable form by PMNs during phagocytosis (15). This reaction is inhibited by hypoxia and does not occur in PMNs from patients with chronic granulomatous disease, suggesting a requirement for the phagocytosis-induced respiratory burst. Binding is reduced by catalase and is increased by superoxide dismutase, an enzyme which catalyzes the conversion of the superoxide anion to oxygen and H_2O_2, implicating H_2O_2 as the active product of the respiratory burst. The peroxidase inhibitors azide and cyanide decrease binding under certain experimental conditions, and binding is low when leukocytes which lack MPO are employed, suggesting that MPO also is required.

These findings suggest that estrogens are oxidized by intact PMNs during phagocytosis, in part, as a result of the action of MPO and H_2O_2. This prompted a study of the effect of estrogens on the MPO-H_2O_2-halide antimicrobial system. A dual effect was found: a stimulation of microbicidal activity at high H_2O_2 concentrations and an inhibition when the H_2O_2 and halide concentrations were low.

MATERIALS AND METHODS

Special reagents. *Escherichia coli* (no. 11775, American Type Culture Collection, Rockville, Md.) was maintained on blood agar plates and transferred daily to Trypticase soy broth (Baltimore Biological Laboratory, Cockeysville, Md.). Overnight cultures were washed twice in 0.1 M sodium sulfate and suspended in this solution to the required absorbance at 540 nm just before use. Canine MPO was prepared from pyometrial pus by the method of Agner (1), and human MPO was kindly supplied by I. Olsson, Lund, Sweden. Canine MPO was used unless otherwise indicated. Peroxidase activity was determined by the *ortho*-dianisidine assay (21) before use. One unit of enzyme is the amount which decomposes 1 μmol of H_2O_2 per min at 25°C. Estradiol 17^{β}, estradiol 17^{α}, estrone, estriol, ethinyl estradiol, estradiol-3-benzoate, testosterone, progesterone, hydroxyprogesterone, cortisone, hydrocortisone, and deoxycorticosterone were obtained from Sigma Chemical Co., St. Louis, Mo.

Determination of microbicidal activity. The components of the reaction mixture (see table legends) in a total volume of 0.5 ml were incubated in polystyrene test tubes (12 by 75 mm, Falcon 2052, Oxnard, Calif.) at 37°C for 1 h in a water-bath shaker oscillating 80 times per min. Serial 10-fold dilutions were made in 0.1 M sodium sulfate, and the viable cell count was determined by the pour plate method using Trypticase soy agar. The viable cell count was expressed as organisms per milliliter of original reaction mixture.

Statistical analyses. The geometric mean of the microbicidal data was calculated as previously described (18). Statistical differences were determined

using Student's two-tailed t test for independent means (not significant, $P > 0.05$).

RESULTS

Stimulation by estrogens. Table 1 demonstrates the effect of H_2O_2 alone, the MPO-H_2O_2-chloride system, and the latter system supplemented with estradiol on the *E. coli* viable cell count over a range of H_2O_2 concentrations. Under the conditions employed, H_2O_2 alone did not have a significant inhibitory effect on the viable cell count until a concentration of 4×10^{-3} M was reached ($P < 0.001$). When H_2O_2 was supplemented with MPO and chloride, a microbicidal effect was observed at low H_2O_2 concentrations which disappeared when the H_2O_2 concentration was increased to 5×10^{-4} M. This loss of microbicidal activity was evident until a concentration of H_2O_2 was reached at which H_2O_2 alone was toxic. Peroxidases, including MPO (2), are inhibited by H_2O_2 at high concentrations, and the loss of microbicidal activity of the MPO system presumably reflects this inhibition. When estradiol was added to the MPO-H_2O_2-chloride system, a significant increase in microbicidal activity was observed at H_2O_2 concentrations ranging from 2×10^{-4} to 1×10^{-3} M. Bactericidal activity was stimulated under these conditions by estradiol 17^{β}, estradiol 17^{α}, estrone, estriol, ethinyl estradiol, and phenol, but not by estradiol-3-benzoate, testosterone, progesterone, hydroxyprogesterone, cortisone, hydrocortisone, and deoxycorticosterone (Table 2, system A). Table 3 demonstrates the requirement for each component of the MPO-H_2O_2-chloride-estradiol system.

TABLE 1. *Stimulation of the MPO-H_2O_2-chloride antimicrobial system by estradiol*[a]

H_2O_2 (M)	Viable cell count (organisms/ml $\times 10^{-6}$)[b] in:		
	H_2O_2	MPO + H_2O_2 + Cl^-	MPO + H_2O_2 + Cl^- + estradiol
1×10^{-5}		0.00003	0.0001
1×10^{-4}	7.67	0.002	0.00002
2×10^{-4}	6.92	0.34	0.00003[c]
5×10^{-4}	6.75	6.19	0.00009[c]
1×10^{-3}	6.08	5.95	0.005[c]
2×10^{-3}	2.56	4.26	3.35
4×10^{-3}	0.0009	0.0007	0.0003

[a] The reaction mixture contained 5×10^{-2} M sodium phosphate buffer (pH 7.5), 5×10^{-5} M ethylenediaminetetraacetic acid, 4.4×10^{-2} M sodium sulfate, 6×10^6 *E. coli*, and the supplements indicated below as follows: H_2O_2 at the concentrations indicated; MPO, 8 mU; sodium chloride, 5×10^{-2} M; and estradiol, 10^{-4} M. An isotonically equivalent amount of sodium sulfate (3.3×10^{-2} M) was omitted from tubes containing sodium chloride.

[b] Mean of three to six experiments.

[c] Significance of the difference from the MPO-H_2O_2-Cl^- system, $P < 0.001$; all others not significant.

TABLE 2. *Effect of various agents*[a]

Agent	Viable cell count (organisms/ml $\times 10^{-6}$)[b] in:	
	System A	System B
None	4.32	0.001
Estradiol 17^{β}	0.0[c]	4.03[c]
Estradiol 17^{α}	0.00002[c]	6.09[c]
Estrone	0.00002[c]	0.2[c]
Estriol	0.0004[c]	5.96[c]
Ethinyl estradiol	0.0002[c]	6.13[c]
Phenol	0.0001[c]	4.51[c]
Estradiol-3-benzoate	4.16	0.001
Testosterone	5.67	0.0002
Progesterone	4.47	0.0002
Hydroxyprogesterone	5.35	0.0003
Cortisone	6.93	0.0005
Hydrocortisone	4.62	0.0009
Deoxycorticosterone	4.97	0.001

[a] The reaction mixture was as described in Table 1, except that the supplements were as follows. System A: H_2O_2, 5×10^{-4} M; MPO, 8 mU; sodium chloride, 5×10^{-2} M; steroids (or phenol), 10^{-4} M. System B: H_2O_2, 10^{-5} M; MPO, 8 mU; sodium iodide, 5×10^{-5} M; steroids (or phenol), 10^{-4} M.

[b] Mean of three to four experiments.

[c] $P < 0.001$ steroid (or phenol) versus none; all others not significant.

TABLE 3. *Effect of deletions and halide substitutions*[a]

Additions	Viable cell count	
	Organisms/ml ($\times 10^{-6}$)[b]	P[c]
Control	6.06	
MPO + H_2O_2 + Cl^- + estradiol	0.0002	<0.001
Estradiol omitted	6.21	NS[d]
NaCl omitted	6.49	NS
H_2O_2 omitted	6.44	NS
MPO omitted	6.30	NS
MPO heated	6.30	NS
MPO + H_2O_2 + I^-	4.17	NS
Estradiol added	0.01	<0.025
MPO + H_2O_2 + Br^-	5.34	NS
Estradiol added	0.0008	<0.05

[a] The MPO-H_2O_2-chloride-estradiol system was as described in Table 1, with the H_2O_2 concentration at 5×10^{-4} M. Sodium iodide (10^{-4} M) and sodium bromide (10^{-3} M) were substituted for chloride where indicated. Deletions and additions were made, and the MPO was heated at 100°C for 15 min where indicated.

[b] Mean of three to four experiments.

[c] P value for the significance of the difference from the control which contained *E. coli*, buffer, ethylenediaminetetraacetic acid, and sulfate as described in Table 1.

[d] NS, Not significant.

diol antimicrobial system and the loss of activity on heat-inactivation of MPO. When phenol was the stimulant employed, deletion of chloride decreased but did not abolish antimicrobial activity (data not shown).

Estradiol also increased antimicrobial activity at high H_2O_2 concentration when iodide or bromide was substituted for chloride (Table 3). As for the chloride-dependent system, a bactericidal effect of the MPO-H_2O_2-iodide (or bromide) system was readily observed in the absence of estradiol when the H_2O_2 concentration was lowered (Table 4). Comparable results were observed when MPO was replaced by a purified preparation of guinea pig eosinophil peroxidase, i.e., microbicidal activity was inhibited by high concentrations of H_2O_2, and this inhibition was prevented by estradiol (data not shown). The canine MPO generally employed also could be replaced by human MPO.

Inhibition by estrogens. When the H_2O_2 concentration was 10^{-5} M, the MPO-H_2O_2-halide system was bactericidal at iodide concentrations down to 10^{-5} M under the conditions used in Table 4. In contrast to the findings at high H_2O_2 concentrations, estradiol inhibited antimicrobial activity under these conditions, with a significant inhibitory effect observed at iodide concentrations ranging from 2×10^{-4} M to 2×10^{-5} M. An inhibition by estradiol also was observed when bromide was substituted for iodide; however, when chloride (10^{-1} or 10^{-2} M) was used under the conditions used in Table 4, inhibition by estradiol was not seen. When both chloride (5×10^{-2} M) and iodide (5×10^{-5} M) were employed, the results were comparable to those observed with iodide alone; i.e., estradiol significantly inhibited antimicrobial activity. Those steroids which stimulated antimicrobial activity at high H_2O_2 concentration (Table 2, system A) also inhibited antimicrobial activity at low H_2O_2 concentration (Table 2, system B), although complete inhibition was not observed with estrone under the conditions employed. Phenol also was inhibitory. The canine MPO generally used could be replaced by human MPO.

TABLE 4. *Inhibition of antimicrobial activity by estradiol*[a]

Halide		Viable cell count (organisms/ml × 10^{-6})[b]		
Type	M	−Estradiol	+Estradiol	*P*[c]
NaI	5×10^{-4}	0.00001	0.001	NS
	2×10^{-4}	0.00009	0.10	<0.002
	1×10^{-4}	0.0003	1.20	<0.001
	5×10^{-5}	0.0007	4.78	<0.001
	2×10^{-5}	0.0005	4.51	<0.01
	1×10^{-5}	0.75	4.63	NS
	5×10^{-6}	4.96	4.80	NS
NaBr	1×10^{-4}	0.0002	3.50	<0.005
NaCl	5×10^{-2}	0.00001	0.00001	NS
	1×10^{-2}	0.00009	0.0006	NS
	1×10^{-3}	5.39	5.66	NS

[a] The reaction mixture was as described in Table 1 except that the supplements were as follows: H_2O_2, 10^{-5} M; MPO, 8 mU; sodium iodide and sodium bromide at the concentrations indicated; and estradiol, 10^{-4} M where indicated.

[b] Mean of two to five experiments.

[c] *P* values for the significance of the difference between the presence and absence of estradiol. NS, Not significant.

DISCUSSION

Phenolic estrogens were shown here to have a dual effect on the MPO-H_2O_2-halide antimicrobial system. When the H_2O_2 concentration was increased to a level at which loss of microbicidal activity was observed, presumably due to the inactivation of MPO (2), estrogens increased microbicidal activity. In contrast, when the H_2O_2 and halide concentrations were low, the microbicidal activity evident under these conditions was inhibited by estrogens. The same estrogens which were stimulatory at high H_2O_2 concentrations were inhibitory when the H_2O_2 concentration was low, and non-estrogenic steroids (testosterone, progesterone, hydroxyprogesterone, cortisone, hydrocortisone, deoxycorticosterone) were without effect under both sets of conditions. It is of interest that ethinyl estradiol, which has a substitution in the 17 position, was active, whereas estradiol-3-benzoate, with a substitution on the phenolic hydroxyl group, was ineffective. The importance of the phenolic hydroxyl group was further emphasized by the comparable effects observed when the phenolic estrogen was replaced by phenol. Both the 17^{α} and the 17^{β} stereoisomers of estradiol were active, as were estriol and estrone. In an earlier study, Jacobs et al. (5) observed that estriol and estrone increased the bactericidal activity of a system consisting of the granule pellet from guinea pig PMNs, H_2O_2, and chloride at pH 5.5. The H_2O_2 concentration used was 5×10^{-5} M. In contrast to the findings reported here, stimulation was not observed when estradiol 17^{β} was used or when the granule pellet was replaced by purified human MPO.

MPO has two heme prosthetic groups per molecule. At relatively low concentrations, H_2O_2 forms a dissociable complex with one heme and is utilized for peroxidatic reactions. Both the halide and estrogen are oxidized by MPO under these conditions, with the oxidation of the halide required for microbicidal activity. When the

H_2O_2 and halide concentrations are low, inhibition of microbicidal activity by estradiol may be due simply to the competition between the halide and estrogen for the small amount of H_2O_2 available. It has been proposed (2) that, when the H_2O_2 concentration is increased, complexes are formed with both hemes which interact with the degradation of H_2O_2 to oxygen and water. With a further increase in the H_2O_2 concentration, the iron porphyrin structure is destroyed and the enzyme is denatured. Under the conditions used in Table 1, estradiol was protective of the microbicidal system at concentrations 1/2 to 1/20 that of H_2O_2, indicating that its effect was not due to a stoichiometric interaction with H_2O_2. It is not known whether estrogens function as oxidation-reduction catalysts under these conditions or react directly with MPO to prevent inactivation.

Estrogens are firmly bound by PMNs during phagocytosis, and covalent linkage to cellular components, due in part to oxidation by MPO and H_2O_2, has been proposed (15). It is possible, therefore, that estrogens can influence the microbicidal activity of the MPO system in the intact cell either by competing for the available H_2O_2 or by preventing a fall in activity induced by high levels of H_2O_2. Although effects of estrogens on neutrophil function have been reported (see 3), it is not known whether estrogens are present in adequate amounts or in the proper location to influence microbicidal activity in situ.

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