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Ascorbic Acid-Dependent Cytoprotection of Ovarian Cells by Leukocyte and Nonleukocyte Peroxidases

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ABSTRACT. Luteal cells contain high levels of ascorbic acid that is secreted by stimulation with agents like luteinizing hormone (LH) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). One role for interstitial ascorbic acid, we propose, may be the detoxification of H_2O_2 by regeneration of catalytically active peroxidase. By serving as a preferred secondary substrate, ascorbic acid regenerates the catalytically active peroxidase that is inhibited irreversibly by H_2O_2 alone. To test this hypothesis, luteal cells were incubated in the absence and presence of peroxidases and H_2O_2, and the maximal cyclic AMP and steroidogenic response to LH was examined. In luteal cells, H_2O_2 is known to severely inhibit LH-sensitive cyclic AMP accumulation and steroidogenesis, and the addition of lactoperoxidase, myeloperoxidase, eosinophil peroxidase, or ascorbic acid (1 mM) alone had no effect on these responses to H_2O_2. However, co-incubation of ascorbic acid and the peroxidases completely reversed the inhibition of cyclic AMP accumulation and steroidogenesis produced by H_2O_2. These findings and the results that show direct oxidation of ascorbic acid in the presence of peroxidase and H_2O_2, but not with H_2O_2 alone, support the conclusion that ascorbic acid released from cells may detoxify H_2O_2 by regenerating the catalytically active state of peroxidases. BIOCHEM PHARMACOL **55**;9:1497–1503, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. ascorbic acid; hydrogen peroxide; leukocyte; ovary; luteal cells; peroxidase; cytoprotection

The corpus luteum differentiates from the follicle after ovulation and serves as the major source of progesterone in the luteal phase and in early pregnancy. In the absence of a successful pregnancy, the corpus luteum undergoes regression, which is characterized initially by a loss of steroidogenesis followed by eventual involution [1]. Luteal steroidogenesis is under strict endocrine control by gonadotropins, particularly LH,† and an early event in luteal regression is the loss of gonadotropic hormone action [1]. $PGF_{2\alpha}$ induces luteal regression that is associated with the generation of O_2^-, H_2O_2, and lipid peroxidation [2–6]. H_2O_2 and lipid hydroperoxides produce an almost immediate interruption of LH action by inhibition of cyclic AMP accumulation and cyclic AMP-dependent steroidogenesis [7–9].

Leukocytes, predominantly monocytes/macrophages, are resident in the corpus luteum, and leukocyte infiltration is one hallmark of luteal regression [10–15]. Phagocytic leukocytes are robust generators of O_2^- and H_2O_2 [16–18], and are likely candidates for the origin of reactive oxygen species in the corpus luteum. Leukocytes also contain abundant levels of peroxidases that are generally thought to be involved in the elimination of pathogens by the generation of cytotoxic oxidants such as hypochlorous acid [19]. Peroxidases become irreversibly inhibited by H_2O_2 in the absence of a suitable oxidizable, secondary substrate [20, 21], which is one reason that peroxidases are not generally thought to serve a cytoprotective role against the actions of H_2O_2. In fact, MPx released *in vitro* is inactivated by the respiratory burst of leukocytes [22]. In the pecking order of biological reducing agents, ascorbic acid serves a preeminent role [23] and is an avid secondary substrate for peroxidases. Thus, ascorbic acid regenerates the catalytically active state of peroxidases [24, 25] until either H_2O_2 or ascorbic acid becomes exhausted.

Certain tissues are endowed with extremely high levels of ascorbic acid, and one such tissue is the corpus luteum in which the intracellular concentration reaches 3–5 mM in contrast to blood levels of about 50 μM [6, 26, 27]. Luteal levels of ascorbic acid vary enormously, as agents like LH and $PGF_{2\alpha}$ cause a rapid and marked depletion *in vivo* that is sustained for hours; lipid peroxidation is one consequence of ascorbic acid depletion in the corpus luteum in response to $PGF_{2\alpha}$ [6, 27]. Though oxidation of ascorbic acid may be one mechanism of depletion, secretion of ascorbic acid in response to LH and $PGF_{2\alpha}$ was also shown recently to occur in luteal cells [27].

The cellular release of ascorbic acid would seem to be a counterproductive process unless extracellular ascorbic acid

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† *Abbreviations:* ABTS, azinobis-3-ethylbenzothiazoline-6-sulfonic acid; Epx, eosinophil peroxidase; hCG, human chorionic gonadotropin; LH, luteinizing hormone; LPx, lactoperoxidase; MPx, myeloperoxidase; O_2^-, superoxide anion; PEG, polyethylene glycol; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; PMSG, pregnant mare serum gonadotropin; and RIA, radioimmunassay.

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serves some role. One possibility, we propose, is that ascorbic acid may permit extracellular leukocyte peroxidases to detoxify H_2O_2 and prevent the formation of other toxic products such as hypochlorous acid, lipid hydroperoxides, or damaged proteins. As a consequence, cytoprotection against leukocyte peroxidases and H_2O_2 may occur by endocrine or paracrine stimulation of ascorbic acid release from cells. To test this hypothesis, the effects of ascorbic acid and peroxidases on the antigonadotropic and antisteroidogenic action of H_2O_2 were examined in ovarian luteal cells.

MATERIALS AND METHODS

Reagents

Highly purified human EPx was a gift from Dr. Gerald Gleich, Department of Immunology, Mayo Clinic, which by direct assay showed 1.5-fold the activity of LPx (76 U/mg). Ascorbic acid and LPx (76–120 U/mg) were purchased from the Sigma Chemical Co., and MPx was purchased from Calbiochem. Luteinizing hormone (oLH 25) was a gift from the National Institutes of Health.

Animals and Treatments

All animals were housed and cared for in the fully accredited facilities operated by Yale University Animal Resources Center. Treatments and procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals under a protocol approved by the Yale University Animal Care Committee. The synchronization of ovulation and corpus luteum formation of immature female rats was achieved by s.c. injection of 50 IU of PMSG, followed 56 hr later by s.c. injection of 25 IU of hCG to induce ovulation as described [28]. Animals were killed 6–8 days after ovulation. Tissues were collected after anesthetizing the animals with a mixture of ketamine (Ketaset, Fort Dodge Laboratories)/xylazine (Rompun, Miles)/saline (1/0.22/0.78; by vol.) given i.p. (1.8 mL/kg) followed by perfusion with saline to remove contaminating blood cells from the ovaries. Luteinized ovaries were collected and either flash frozen on dry ice, and stored at −80° for purification of peroxidase activity, or used immediately for preparation of primary cultures of luteal cells.

Isolation and Incubation of Luteal Cells

Luteal cells were dispersed, enriched, and cultured as described previously with the minor modification of use of phenol red-free, Earl's balanced salt solution for incubation of the cells [7]. In brief, the highly luteinized ovaries were minced and dispersed in medium containing collagenase and DNase and purified on a Percoll gradient column. The enriched cells were counted, and cell viability was determined by trypan blue exclusion as described [7]. Cells were allowed to recover for a period of 3 hr before utilization in experiments. Prior to the addition of luteal cells ($1.5–3 \times 10^5$), the medium was preincubated in the absence and presence of H_2O_2 (100 μM), peroxidases, or ascorbic acid (1 mM) for 10 min followed by incubation for an additional 60 min in the presence of LH (1 μg/mL).

Assays

Progesterone and cyclic AMP were determined by RIA as described [7]. Ascorbic acid oxidation was determined spectrophotometrically by following the decrease in absorbance of ascorbic acid based on methods described earlier [24]. Lipid peroxidation [6] and protein concentration [29] were determined as described.

Soluble peroxidase activity was determined using ABTS as described [30] with slight modification. The ABTS peroxidase assay buffer was 0.2 M of sodium acetate (pH 5.5), and the reaction (total volume = 200 μL) was initiated by the addition of H_2O_2. Absorbance (410 nm) was measured continuously for 5 min in 96-well microtiter plates maintained at 37° using a microtiter plate reader (Molecular Devices Corp.) Guaiacol was used for the assay of leukocyte peroxidase activity as described [31] with slight modification for kinetic analysis with a microtiter plate reader. The guaiacol assay did not detect soluble peroxidase activity. The guaiacol peroxidase assay buffer was 10 mM of Tris-HCl (pH 7.4), and the assay included 5 mM of guaiacol and 1.25 mM of H_2O_2. The reaction was initiated by the addition of H_2O_2, and absorbance was monitored continuously at 450 nm for 15 min at 37°.

Distribution of Peroxidase Activity

Luteal tissue was homogenized (100 mg/mL) in 50 mM of sodium phosphate buffer (pH 7.4), centrifuged at 39,000 *g* for 30 min (4°), and the supernatant fraction retained for assay (39,000 *g*; SN1). The pellet was rehomogenized in sodium phosphate buffer, recentrifuged (39,000 *g*; 30 min), the supernatant fraction retained (SN2), the pellet extracted with 10 mM of Tris-HCl (pH 7.4) supplemented with 0.5 M of $CaCl_2$, and the extract collected by centrifugation (30,000 *g*; $CaCl_2$ extract). The supernatant fractions and the extract were assayed for ABTS and guaiacol activity as described above.

Purification and Characterization of Soluble Peroxidase Activity

Luteal tissue was homogenized (100 mg/mL) in 10 mM of sodium phosphate (pH 7.4) with phenylmethylsulfonyl fluoride (1 mM) and centrifuged at 100,000 *g* for 60 min (4°). A solution of 50% polyethylene glycol (PEG-8000; Sigma Chemical Co.) was added to the supernatant fraction to achieve a final concentration of 10% and incubated for 30 min at room temperature before centrifugation (15,000 *g*; 15 min; room temperature). Further PEG (50%) was then added to the 15,000 *g* supernatant fraction to achieve a final concentration of 20%, and the mixture was incubated

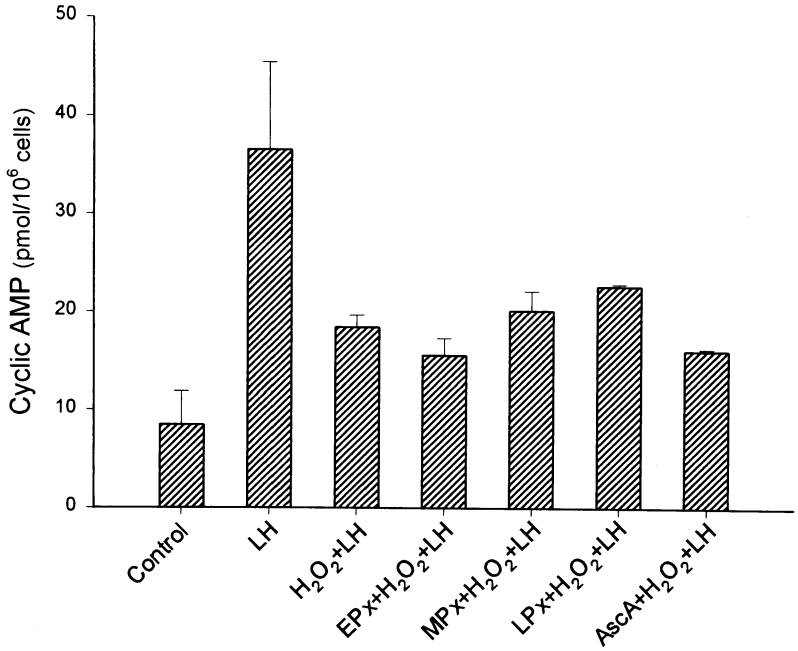

FIG. 1. Effect of peroxidases and ascorbic acid on LH-stimulated cyclic AMP accumulation in luteal cells. Cells (2–3 × 10^5) were incubated in the presence of LH (1 μg/mL) or in a combination of LH and H_2O_2 (100 μM). The individual effects of Epx (3 μg/mL), MPx (0.5 U/mL), LPx (0.5 U/mL), or ascorbic acid (AscA; 1 mM) on the response to H_2O_2 were determined. Cells were incubated as described in Materials and Methods. Values are the means ± SEM of at least three independent replicated experiments.

for 30 min at room temperature before centrifugation (15,000 *g*; 15 min; room temperature). The precipitate was dissolved in homogenization buffer with a final concentration of 20% glycerol at a volume 1/5 of that used for homogenization and loaded onto a Sephadex G-100 (Pharmacia Biotech) column (2.5 cm i.d. × 40 cm) equilibrated in 10 mM of sodium phosphate buffer (pH 7.4) containing 20% glycerol. The column was calibrated with standards obtained from Pharmacia Biotech. Fractions that contained peroxidase activity (single peak; 19.7 to 21.4 kDa) were pooled and diluted with column buffer supplemented with 4 M of NaCl to achieve a final concentration of 2 M of NaCl and loaded onto a Phenyl-Sepharose CL-4B (Pharmacia Biotech) column (1 cm i.d. × 12 cm) equilibrated with 10 mM of sodium phosphate buffer (pH 7.4) containing 2 M of NaCl and 20% glycerol. Peroxidase activity was eluted with a linear gradient from 2 to 0 M of NaCl in loading buffer and the single peak of activity was pooled. The pooled activity was concentrated and desalted using a Centricon D concentrator (Amicon) and fractionated on a 15% polyacrylamide gel, transferred to an Immobilon membrane, and stained with Coomassie blue [32, 33]. Two major

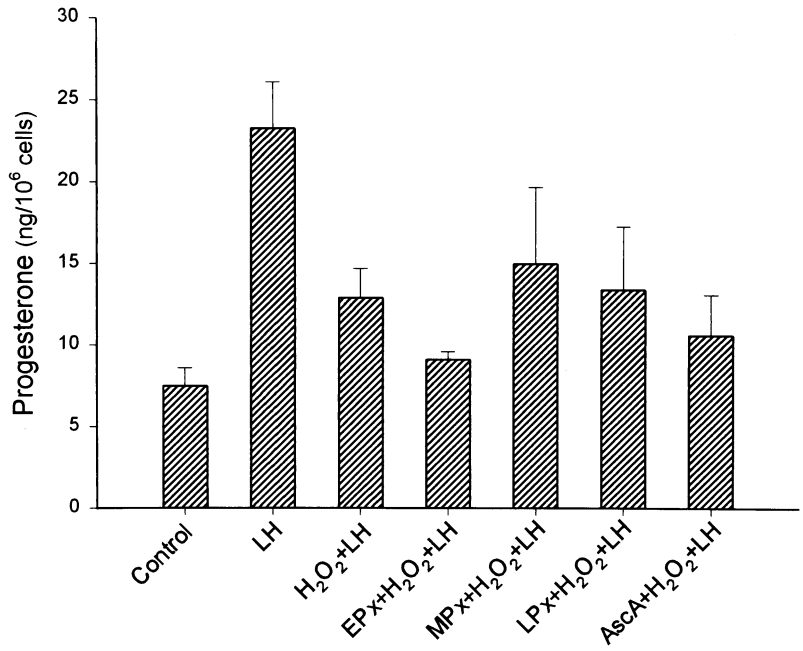

FIG. 2. Effect of peroxidases and ascorbic acid on LH-stimulated progesterone synthesis in luteal cells. Cells (2–3 × 10^5) were incubated in the presence of LH (1 μg/mL) or in a combination of LH and H_2O_2 (100 μM). The individual effects of Epx (3 μg/mL), MPx (0.5 U/mL), LPx (0.5 U/mL), or ascorbic acid (AscA; 1 mM) on the response to H_2O_2 were determined. Cells were incubated as described in Materials and Methods. Values are the means ± SEM of at least three independent replicated experiments.

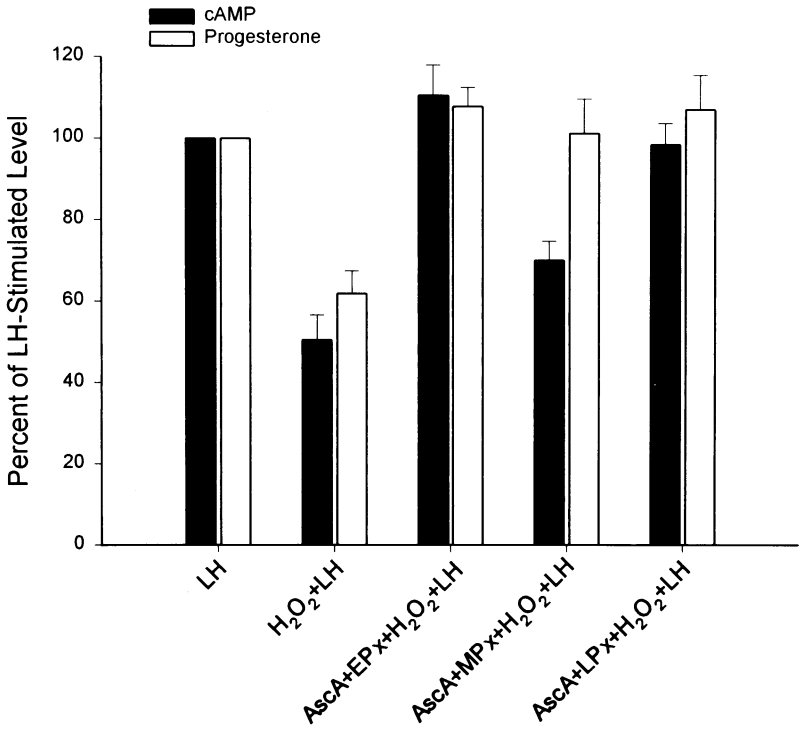

FIG. 3. Permissive role of ascorbic acid on the reversal of H_2O_2 inhibition of cyclic AMP accumulation and progesterone synthesis in luteal cells. Cells (2–3 × 10^5) were incubated in the presence of LH (1 μg/mL) or in a combination of LH and H_2O_2 (100 μM). The combined effects of Epx (3 μg/mL), MPx (0.5 U/mL), or LPx (0.5 U/mL) with ascorbic acid (AscA; 1 mM) on the response to H_2O_2 were determined. Cells were incubated as described in Materials and Methods. Control values for cyclic AMP and progesterone production were 8.5 ± pmol/10^6 cells and 7.5 ng/10^6 cells, respectively. Values are means ± SEM of at least three independent replicated experiments.

bands with apparent molecular weights of 14–15 kDa were excised, and their N-terminal amino acid sequences were determined as described [32].

Statistical Analysis

Luteal cells from several rats were pooled, and equal aliquots were exposed to each treatment in triplicate. Each experiment was repeated at least three times except where noted. Statistical significance between treatments within an experiment was determined by one-way repeated measures analysis of variance. Post hoc comparisons of multiple treatments were made by the Bonferroni *t*-test method. Treatment differences between experiments were determined by one-way analysis of variance, and post hoc comparisons were made with Dunnett's method. A difference of $P < 0.05$ was considered statistically significant.

RESULTS

LH stimulated a 4-fold increase ($P < 0.01$) in cyclic AMP accumulation and progesterone synthesis in luteal cells, and these responses were inhibited to unstimulated levels by H_2O_2 (Figs. 1 and 2). Previous studies showed that these levels of LH and H_2O_2 are maximal and that cell viability is unaffected [7]. The addition of peroxidases or ascorbic acid alone had no significant effect on the H_2O_2 inhibition of either LH-sensitive cyclic AMP accumulation (Fig. 1) or progesterone secretion (Fig. 2). The identical concentration of peroxidases used in these studies had no effect on basal cyclic AMP accumulation or progesterone synthesis (data not shown).

Co-incubation of ascorbic acid with peroxidases completely reversed inhibition of both cyclic AMP and progesterone synthesis produced by H_2O_2 in luteal cells (Fig. 3). Concentration–response studies with lactoperoxidase under conditions identical to those used in the studies summarized in Fig. 3 showed complete reversal of H_2O_2 inhibition of both cyclic AMP and progesterone synthesis at 5 μg/mL,

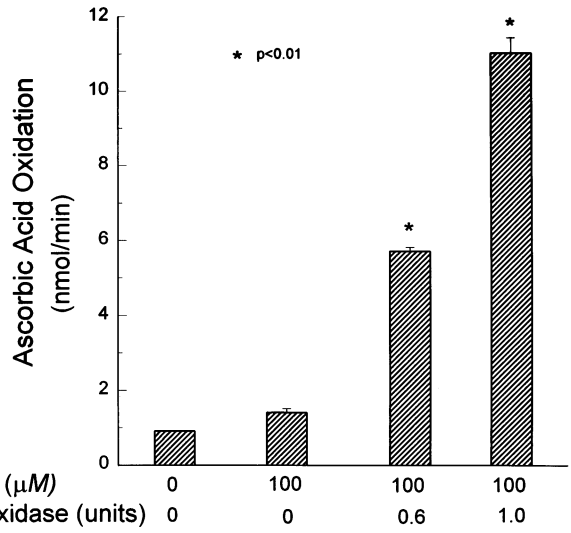

FIG. 4. Ascorbic acid oxidation in the presence but not the absence of H_2O_2. Ascorbic acid oxidation was determined by kinetic analysis, over an interval of 30 sec of the loss of absorbance at 265 nm of a solution (200 mM of sodium acetate; pH 5.5) of ascorbic acid (100 μM) in the absence and presence of H_2O_2 and lactoperoxidase. Values are means ± SEM of three experiments.

TABLE 1. Distribution of ABTS and guaiacol peroxidase activity in the luteinized rat ovary

Fraction	Peroxidase activity (μmol/min/mg)	
	ABTS	Guaiacol
39,000 *g* SN1	4540	0
39,000 *g* SN2	125	0
$CaCl_2$ extract	62	62

Ovarian tissue was homogenized and the extracts were assayed for peroxidase activity, as described in Materials and Methods. Shown are the mean values of at least three independent experiments.

whereas 1 μg/mL showed an approximately 50% reversal; 0.5 μg/mL was ineffective (4 separate replicated experiments). Concentration–response studies with the other peroxidases were not carried out due to their limited availability.

Ascorbic acid was depleted by incubation of peroxidase with H_2O_2 (Fig. 4). A marked increase in ascorbic acid oxidation occurred in the presence of lactoperoxidase and H_2O_2, whereas only minimal oxidation of ascorbic acid occurred with H_2O_2 alone.

Table 1 shows the distribution of peroxidase activity in ovarian fractions. No guaiacol peroxidase activity was detectable in the soluble fraction, but both guaiacol and ABTS peroxidase activity were evident in the membrane-bound fraction that was extractable with $CaCl_2$. The latter procedure is known to solubilize leukocyte peroxidase [34]. The greatest amount of ABTS peroxidase activity was soluble, as it was readily extractable into the homogenization buffer (100,000 *g* supernatant fraction; Table 2), whereas about 1.5% of the total ABTS activity was membrane-bound. The same amount of ABTS and guaiacol peroxidase activity was found in the membrane-bound fraction. Purification (42.4-fold) of the soluble enzyme activity was achieved by sequential steps of precipitation with polyethylene glycol, gel-exclusion chromatography with Sephadex G-100 (single peak of activity of 19.7 to 21.4 kDa), and hydrophobic affinity chromatography with Phenyl-Sepharose (Table 2). The single peak of soluble peroxidase activity showed a double band on PAGE (14–15 kDa). Electrophoresis electrophoretic transfer, and N-terminal amino acid sequence analysis (20 cycles) of the larger and smaller bands showed complete homology with the β-chain and the α-chain, respectively, of rat hemoglobin (data not shown).

DISCUSSION

The present results confirm that ascorbic acid may serve as a secondary substrate for peroxidase and become oxidized in the process [24]. It is by this mechanism, we propose, that ascorbic acid blocked the antigonadotropic and antisteroidogenic actions of H_2O_2 when peroxidases were present, but not in their absence. These findings support the conclusion that peroxidases of leukocyte and nonleukocyte origin may serve as an avenue for the detoxification of H_2O_2 if ascorbic acid is available.

Membrane-bound peroxidases are released into the extracellular space upon activation and degranulation of leukocytes [19, 22], whereas LPx is secreted into milk and saliva [35]. Similarly, O_2^- and H_2O_2 are released into the extracellular space following triggering of the respiratory burst of leukocytes [36]. Ascorbic acid has been shown to be released from neural tissue [37] and from luteal cells in response to agents such as $PGF_{2\alpha}$ and LH [27]. Thus, all of the elements for detoxification of extracellular H_2O_2 by peroxidases and ascorbic acid are present in the corpus luteum and probably a number of other tissues, which indicates the broader implications of this cytoprotective pathway.

While the corpus luteum is endowed with very high levels of ascorbic acid, leukocytes have been reported to have even higher levels of 4–14 mM [38, 39]. Thus, ascorbic acid could also serve an important role in detoxifying H_2O_2 within leukocytes by interaction with an intracellular peroxidase that is abundant in these cells and, thereby, could protect leukocytes against H_2O_2 generated during the respiratory burst. It is doubtful whether such a cytoprotective mechanism would be operative within luteal cells, as the present results indicate that the corpus luteum peroxidase has the characteristics of an enzyme of leukocyte origin.

Abundant peroxidase activity has been reported to be present in the luteinized rat ovary and to be up-regulated by LH [30]. We have confirmed the presence of this peroxidase activity in the present studies, but found that the activity was reduced drastically by saline perfusion to remove much

TABLE 2. Purification of the soluble peroxidase activity in the luteinized rat ovary

Fraction	Total			Yield		
	Protein (mg)	Activity (O.D./min)	Specific activity (O.D./min/mg)	Protein (%)	Activity (%)	Purification (Fold)
100,000 g SN fraction	213	706	3.3			
PEG 10–20%	37.4	877	23.5	17.6	124	7.0
Sephadex G-100	11.6	782	67.4	5.5	111	20.2
Phenyl-Sepharose	1.2	167	139.2	0.56	23.6	42.4

Purification of the soluble peroxidase activity was as described in Materials and Methods. The yield for each fraction is expressed as the percent of the starting protein or activity. Purification is expressed as the fold increase in specific activity. Shown are the mean values of at least three independent experiments.

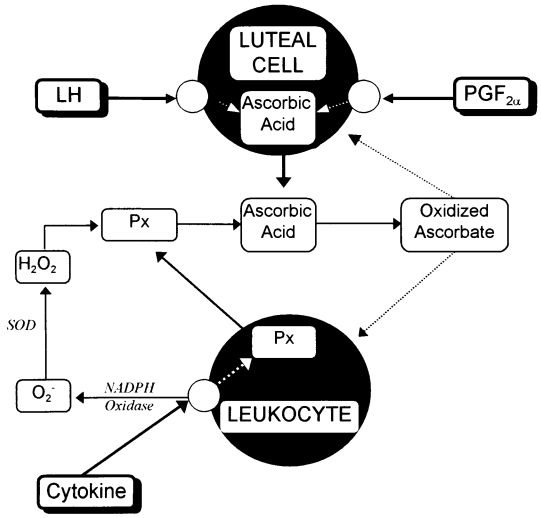

FIG. 5. Leukocyte-luteal cell interactions in detoxification of H_2O_2 by peroxidase–ascorbic acid coupled reactions. Activated leukocytes release superoxide and peroxidases. Superoxide is reduced to H_2O_2 by extracellular superoxide dismutase (SOD). Peroxidase interacts with H_2O_2 to form the intermediate enzyme complex that becomes irreversibly inactivated in the absence of a secondary substrate [20, 21]. Ascorbic acid release is stimulated by LH or $PGF_{2\alpha}$ in luteal cells and serves as an avid electron donor (secondary substrate) for peroxidase, thereby regenerating catalytically active enzyme. The reaction continues until either ascorbic acid or H_2O_2 becomes exhausted. Oxidized ascorbic acid is taken up by leukocytes [40] and luteal cells (Kodaman PH and Behrman HR, unpublished findings) as depicted in the dotted lines.

of the residual blood (Kolodecik TR and Behrman HR, unpublished findings); the remaining soluble ovarian peroxidase activity was characterized as hemoglobin as described in the present studies. Also, De *et al.* [40] reported no detectable soluble peroxidase related to the lactoperoxidase family in the rat ovary. Our findings indicate that the soluble ovarian peroxidase is heme, which is well known to evoke peroxidase activity [24, 25], and we have confirmed identical characteristics of hemoglobin with the soluble peroxidase activity that is present in the luteinized ovary. It would seem reasonable to conclude, however, that hemoglobin probably does not serve a physiological role as a peroxidase. Nevertheless, membrane-bound peroxidase activity was detected in the luteinized ovary, which is probably derived from resident monocytes/macrophages that are known to be present in luteal and other tissues.

To our knowledge this is the first report of a cytoprotective role for ascorbic acid by interaction with peroxidases, although a similar role has been proposed for thiocyanate, which can also act as a secondary substrate for peroxidases [41, 42]. Figure 5 summarizes a proposed mechanism for such a role of ascorbic acid. Ascorbic acid is released from luteal cells, and this process is known to be regulated by LH and $PGF_{2\alpha}$, as we have shown [27]. Peroxidases are known to be released from activated leukocytes at the same time the respiratory burst results in the generation of H_2O_2, and the production of H_2O_2 is known to occur in the corpus luteum at regression [3]. Thus, all of the components are present for ascorbic acid to serve as a secondary substrate for the detoxification of H_2O_2 by peroxidase. Cytoprotection would continue until the levels of ascorbic acid became exhausted, and it is interesting that lipid peroxidation of the corpus luteum does not occur until ascorbic acid is depleted *in vivo* [6]. While this scenario depicts these cytoprotective events as occurring in the extracellular space, a similar process may also occur within resident leukocytes, as these cells are also notable for their high levels of ascorbic acid [38, 39], but probably not in luteal cells, as these cells do not appear to contain peroxidase activity.

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