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PLACENTAL ESTROGEN SYNTHETASE (AROMATASE): EVIDENCE FOR PHOSPHATASE-DEPENDENT INACTIVATION

Francis L. Bellino and Linda Holben

Dept. of Biological Sciences, SUNY/Buffalo, Buffalo, NY 14260

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Summary: The acute regulation of estrogen synthetase (aromatase), the cytochrome P450 enzyme system responsible for estrogen production, is not well explored. We report here that aromatase, but not NADPH-cytochrome c (P450) reductase, activity from human term placental microsomes decreased when incubated in phosphate-free buffer at 37°C. Aromatase activity was stabilized by phosphate buffer or by the phosphatase inhibitors tartaric acid or EDTA, but not NaF, in phosphate-free buffer. Alkaline phosphatase also inhibited aromatase in phosphate-free buffer relative to phosphate buffer, but the inactivation appears to be due primarily to proteolytic solubilization of NADPH-cytochrome c reductase from the microsomes by proteases within the alkaline phosphatase preparation. Based on these data, we suggest that the cytochrome P450 component of aromatase may be regulated acutely by phosphorylation-dependent processes.

Estrogen synthetase (aromatase) is the cytochrome P450 mono-oxygenase enzyme system responsible for the conversion of androgen to estrogen, a key hormone in various physiologic and pathologic processes. Many studies have demonstrated regulation of aromatase activity at the level of gene expression by cAMP, gonadotropins and other peptide factors (1-4), but virtually nothing is known regarding acute regulation of this physiologically crucial enzyme.

Recent publications (5,6) of the cDNA-derived amino acid sequence from the cytochrome P450 component of aromatase revealed a site (RRRIST; positions 263 to 268) in a hydrophilic region (7) that is homologous with the serine phosphorylation consensus sequence for both the cAMP-dependent protein kinase and calcium/calmodulin dependent protein kinase I (8). This observation prompted an examination of the ability of this enzyme to be regulated by phosphorylation. We report here evidence that phosphatases endogenous to placental microsomes inactivate aromatase activity, suggesting that an active phosphorylated form of aromatase is present in placental microsomes and that its activity is lost by phosphatase-dependent processes.

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid; cAMP, cyclic AMP or adenosine 3':5'-monophosphate.

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MATERIALS AND METHODS

Materials: Tris, Hepes, NADPH, 4-androstene-3,17-dione, alkaline phosphatase (Type I-S, from calf intestine), L(+)-tartaric acid, dithiothreitol, cytochrome c, p-nitrophenyl phosphate and azocasein were obtained from Sigma. Trypsin was purchased from Gibco. The aromatase assay substrate, [1β -^{3}H ,4-^{14}C] androstenedione (392 dpm/pmol) was prepared from [1β -^{3}H]- and [4-^{14}C]androstenedione (22.3 and 0.052 Ci/mmol, respectively) from Dupont-New England Nuclear. Lyophilized microsomes were prepared from human term placenta (9).

Methods:

Effect of buffer/phosphatase inhibitor combinations: Lyophilized microsomes and dithiothreitol were homogenized together in distilled water at 22 and 1 mg/ml, respectively. An aliquot (2.2 mg microsomes) was preincubated with the indicated 50 mM buffer containing 20% glycerol (pH values at 21°C were: Tris, 7.8; phosphate, 7.55; Hepes, 7.53) and NaCl (50 mM; control) or phosphatase inhibitor (NaF, tartaric acid at 50 mM, or EDTA at 5 mM), as indicated, at 37°C. Aliquots were removed after 1 h and assayed for NADPH-cytochrome c reductase activity. The microsomes were then collected by ultracentrifugation (180,000 x g, 30 min), suspended in 67 mM phosphate buffer (pH 7.4) and assayed for protein amount and aromatase and NADPH-cytochrome c reductase activities.

Effect of alkaline phosphatase in various buffers: Lyophilized microsomes and dithiothreitol (at 60 and 1 mg/ml, respectively) were homogenized in distilled water. An aliquot (6 mg microsomes) was suspended in 40 mM buffer (as indicated), pH as given above, containing 16% glycerol and 40 mM NaCl in the absence (control) or presence of alkaline phosphatase (6.4 units). After a 10 min incubation at 37°C, aliquots were assayed for NADPH-cytochrome c reductase activity. The microsomes were collected and washed by ultracentrifugation (180,000 x g; 30 min), and suspended in 67 mM phosphate buffer (pH 7.4) before measuring protein amount and aromatase and NADPH-cytochrome c reductase activities.

Enzyme assays: Aromatase activity was determined at 37°C in an incubation containing microsomes, NADPH (0.36 mM), [1β-^{3}H,4-^{14}C] androstenedione (136 nM) brought to 1 ml with 67 mM phosphate buffer (pH 7.4). The reaction was terminated with 0.5 ml of 4% trichloroacetic acid, 1.2% charcoal and 0.06% dextran. The $^{3}H_2O$ produced concomitant with estrogen production (10) was measured by counting an aliquot of the supernatant after centrifugation. NADPH-cytochrome c reductase activity was measured as described (11). Phosphatase activity was determined by incubating 0.24 mg placental microsomes with 5 mM p-nitrophenyl phosphate in 41 mM Tris buffer containing 16% glycerol (pH 7.8) with the indicated buffer/phosphatase inhibitor or NaCl combination in 1 ml for 10 min at 37°C. The A_{410nm} was measured after terminating the assay with 10 ml of 0.02 M NaOH, and the amount of p-nitrophenol phosphate was determined from a standard curve. Protease activity was assayed in placental microsomes (3 mg) and in the alkaline phosphatase preparation (0.5 mg) by incubating these aliquots in 1 ml of each of the buffer/phosphatase inhibitor or NaCl combinations with 1.5 mg azocasein for 1 h at 37°C. The A_{366nm} was measured in the supernatant after terminating the incubation with 3 ml of 3% tricholoracetic acid. The protease activity was determined from a standard curve obtained in the same manner using various amounts of trypsin. All incubations and enzyme assays were conducted in duplicate and all experiments were conducted two or three times with comparable results.

RESULTS AND DISCUSSION

We observed that placental microsomal aromatase was less stable when incubated at 37°C in Tris or Hepes buffers than in phosphate buffer. To determine if this loss of activity was attributable to the presence of endogenous phosphatases in the microsomal preparation, we examined the ability of

several phosphatase inhibitors, tartaric acid (12), EDTA (13,14), or NaF (12-14) to protect aromatase activity. We found (Figure 1) that either tartaric acid or EDTA, but not NaF, protected aromatase activity from the inactivation.

In contrast to the results for aromatase, NADPH-cytochrome c (P450) reductase activities in the original incubation or in the microsomes recovered after the incubation were not affected by the various buffer/phosphatase inhibitor or NaCl combinations (unpublished observations). Furthermore, the reductase activity recovered in the pelleted microsomes relative to the activity prior to centrifugation for the various buffers used averaged ($\pm$SD) for the three phosphatase inhibitors and NaCl incubations (n=4): Tris, 76.5$\pm$4.5%; phosphate, 87.9$\pm$8.6%; Hepes, 58.6$\pm$5.9%. The reductase activities were similar in a particular buffer, as shown by the standard deviation $\leq$ 10% of the mean. Thus, the loss of aromatase activity in the NaCl or NaF incubations relative to the EDTA or tartaric acid incubations does not appear to be attributable to the NADPH-cytochrome c (P450) reductase component of the enzyme, and most of the reductase activity in the starting preparation was recovered in the pelleted microsomes.

To support our interpretation that endogenous microsomal phosphatase activity is responsible for the aromatase instability, we measured phosphatase activity, and the effect of the various buffer/phosphatase inhibitor or NaCl combinations, in placental microsomes under the conditions used to obtain the data in Figure 1. The results in Figure 2 show that considerable quantities of tartrate- or EDTA-inhibitable, but NaF insensitive, phosphatase activity

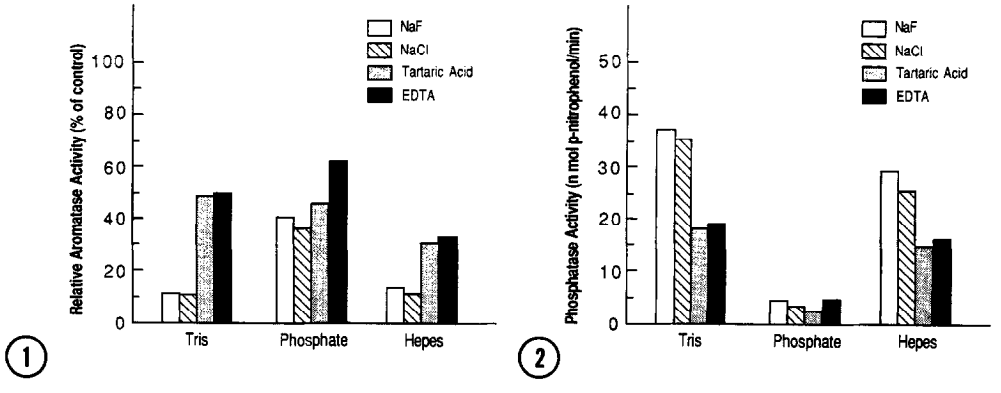

Figure 1. Effect of phosphatase inhibitors on placental microsomal aromatase. Placental microsomes were incubated with the various buffer/phosphatase inhibitor or NaCl combinations, as indicated. Aliquots of pelleted microsomes were assayed for aromatase and NADPH-cytochrome c reductase activities and protein. The 100% aromatase specific activity level in untreated microsomes was 28.1 pmol/min/mg protein.

Figure 2. Phosphatase activity in human placental microsomes. Microsomes were incubated in the various buffer/phosphatase inhibitor or NaCl combinations with p-nitrophenyl phosphate. At the end of the incubation, the amount of p-nitrophenol generated was measured spectrophotometrically.

are present, particularly in Tris or Hepes buffer. These results complement the results shown in Figure 1 for the effect of buffer/phosphatase inhibitor combinations on aromatase activity.

To determine if aromatase is susceptible to inactivation by other phosphatases, we incubated placental microsomes with exogenous alkaline phosphatase in Tris, phosphate, or Hepes buffer. The results (open bars in Figure 3) show that aromatase activity in the pelleted, washed microsomes was greatly reduced by alkaline phosphatase in Hepes or Tris buffer compared with phosphate buffer. NADPH-cytochrome c reductase activity in the original incubation prior to centrifugation was consistently stimulated by 30-50% regardless of the buffer used. The result for aromatase is consistent with observations of the effect of alkaline phosphatase on cholesterol 7α-hydroxylase activity in hepatic microsomes (15,16) or a purified, reconstituted system (17). In the referenced studies, the hydroxylase activity was considered sensitive to the alkaline phosphatase activity.

However, we went one step further and determined the effect of the exogenous alkaline phosphatase preparation on NADPH-cytochrome c reductase activity in the washed microsomes. Aromatase inactivation that appeared to be caused by phosphatase activity might in fact be at least partially attributable to contaminating protease activity via solubilization of the microsomal reductase. The ability to solubilize microsomal NADPH-cytochrome c (P450) reductase by proteolytic cleavage of the small membrane-binding domain (18) is well recognized, and the inability of the proteolytically-derived reductase to reconstitute cytochrome P450 systems in general (19) and aromatase activity in particular (11,20) has been demonstrated. The results (filled bars in Figure 3), showing a loss of NADPH-cytochrome c reductase activity in the washed microsomes after alkaline phosphatase incubation comparable to the loss of aromatase activity, suggest that protease activity rather than phosphatase activity was responsible for the apparent aromatase inactivation. This dependence of microsomal aromatase activity on microsomal NADPH-cytochrome c reductase activity is consistent with the suggestion that the reductase is the rate-determining component of aromatase in placental microsomes (11,21). We independently confirmed the presence of protease activity in the alkaline phosphatase preparation. The data in Figure 4 shows that the protease activity is highest in Tris buffer and lowest in phosphate buffer, and that, except for NaF in Tris buffer, there is no apparent effect of the phosphatase inhibitors. No protease activity was detected by this assay in placental microsomes.

These data strongly suggest (a) that aromatase in human term placental microsomes can be inactivated by microsomal phosphatases, and (b) that the aromatase cytochrome P450 is the affected component of this enzyme system.

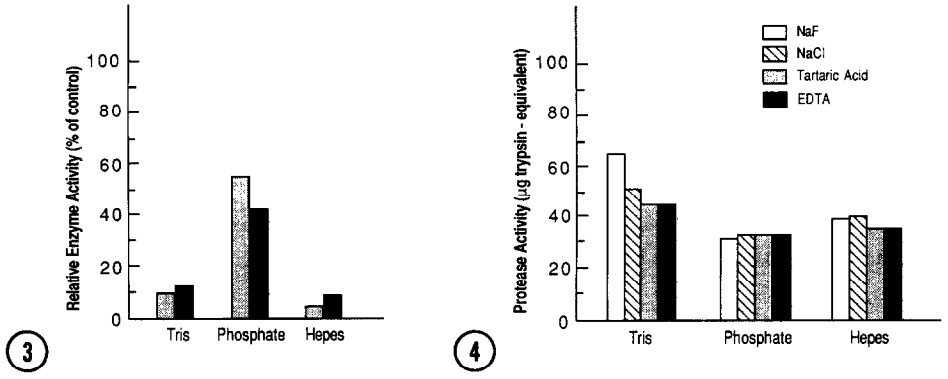

Figure 3. Effect of alkaline phosphatase on placental microsomal aromatase and NADPH-cytochrome c reductase activity. Placental microsomes were first incubated for 10 min at 37°C without (control) or with alkaline phosphatase in the three buffers as shown. The microsomes were recovered and washed in 67 mM phosphate buffer (pH 7.4) by ultracentrifugation. The final pellet was suspended in phosphate buffer and assayed for aromatase and NADPH-cytochrome c reductase activities and protein. The fraction of specific activity recovered in the alkaline phosphatase incubation relative to the control incubation is shown. The 100% specific activity values (±SD) averaged for the three buffers were: aromatase (open bars), 130.5 ± 11.4 pmol/min/mg protein; NADPH-cytochrome c reductase (filled bars), 33.8 ± 1.8 nmol/min/mg protein.

Figure 4. Protease activity in the alkaline phosphatase preparation. Alkaline phosphatase was incubated with azocasein in the various buffer/phosphatase inhibitor or NaCl combinations, as described. The amount of dye released into the acid-soluble fraction was expressed as trypsin-like activity based on a trypsin standard curve determined using various amounts of trypsin in the same assay.

Effects of phosphorylation/dephosphorylation processes, while not described previously for aromatase, are well-documented for several other cytochrome P450 hydroxylating systems. For example, the cytochrome P450 LM2-hydroxylating activity is decreased following phosphorylation by the catalytic subunit of the cAMP-dependent protein kinase (22), concomitant with the conversion of cytochrome P450 to cytochrome P420 (23) and phosphorylation at Ser^{128} (24). In that system, exogenous alkaline phosphatase inhibits the NADPH-cytochrome c reductase activity by affecting its flavin component, but has no apparent effect on cytochrome P450 (25). Other hepatic cytochrome P450's are either phosphorylated by the catalytic subunit of the cAMP-dependent protein kinase, or by protein kinase C or are unaffected by either of these protein kinases (26). Several steroidogenic cytochrome P450's are phosphorylated by a mitochondrial protein kinase (25-hydroxyvitamin D 1α-hydroxylase (27)), the cAMP-dependent protein kinase (11β-hydroxylase (28)), or by protein kinase C (cholesterol side chain cleavage (29)). The most interesting comparison for aromatase, however, is cholesterol 7α-hydroxylase, the rate-limiting enzyme in bile acid synthesis. This activity is inhibited by alkaline phosphatase treatment (15-17), protected from endogenous phosphatase inactivation by fluoride (12,14), but not by EDTA (14) or tartaric acid (12), and stimulated by the cAMP-dependent protein kinase (15-17).

The identity of the protein kinase(s) presumed to act on aromatase, as well as the physiologic implications of acute regulation of aromatase by phosphorylation mechanisms, have yet to be defined. The lag (>6 h) in regulation of aromatase activity by cAMP in human choriocarcinoma (30) or adipose (31) cell culture systems suggests that acute activation of the aromatase cytochrome P450 component by phosphorylation either does not occur in those systems or is not observable by aromatase assay. However, we recently reported a four-fold increase in aromatase activity during the first 3 h of primary culture of cytotrophoblast-like cells from term placenta (32). This rapid modification of activity may involve acute regulatory mechanisms.

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