SYMPOSIUM

Estrogen Regulation of the Rat Anterior Pituitary Gland Proteome

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Estrogen is known to affect the regulation of all six of the established anterior pituitary gland (AP) hormones, but little is known of the specifics of its regulation of the AP hormones, their isoforms, and nonhormonal AP proteins. We used difference gel electrophoresis in conjunction with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and peptide mass fingerprinting to quantify the effects of estrogen on the AP-soluble protein fraction in rats. Two-month-old rats were ovariectomized and used at 6 months of age. They were injected subcutaneously with sesame oil vehicle or 50 μg estradiol valerate in vehicle and studied 48 hrs later, approximately 3 hrs before the time of the anticipated onset of the estrogen-induced surges of gonadotropins in blood. The APs were pooled, and the soluble protein fraction was examined in replicate analyses. After DeCyder software analysis, we identified 26 protein spots that had a 1.5-fold or greater average increase in the experimental group relative to the controls. Nineteen showed a 1.5-fold or greater decrease. Estrogen increased levels of the more acidic isoforms of growth hormone and prolactin and of proteins involved in protein synthesis, folding, and secretion (e.g., eukaryotic translation elongation factor 2, ERp57, ERp29, Hsc70-ps1, calreticulin, coatomer delta subunit, and secretogranin II) and of some metabolic enzymes (e.g., arginosuccinate synthetase, enolase 1, creatine kinase B, phosphoglycerate mutase, malate dehydrogenase, pyruvate kinase, and aldolase A). The majority of the downregulated proteins were involved in RNA or DNA interactions (e.g., five heterogeneous nuclear ribonucleoproteins, DEAD-box proteins 17 and 48, ssDNA binding protein PUR-alpha, PTB-associated splicing factor, and Pigpen protein), but isovaleryl coenzyme A dehydrogenase, mitochondrial aldehyde dehydrogenase, stathmin 1, vinculin, radixin, and secretogranin III were also reduced. Our results indicate that estrogen acts *in vivo* within 48 hrs to modulate levels of a significant number of AP proteins. Exp Biol Med 230:800–807, 2005

Key words: anterior pituitary gland; DIGE; estradiol; growth hormone; mass spectrometry; prolactin; proteome; rat pituitary

Introduction

Estrogen is an important regulator of the function of the anterior pituitary gland (AP). Changes in ovarian status accompanying puberty, the estrous or menstrual cycle, pregnancy, lactation, aging, ovariectomy, and disease can all modify secretion of the AP hormones (1–5), at least in part, due to altered estrogen secretion. Estrogen can affect hormone secretion by acting directly on the AP, by altering AP responsiveness to hypothalamic factors, or by mediating its actions at the level of the brain where it can alter the secretion of the hypothalamic factors themselves. Pituitary expression of estrogen receptors (ER) and ER mRNA are also modulated by estrogen (6–8), and the ERs have been localized to different AP cell types (9). Estrogen also regulates pituitary mRNA for progesterone receptors (10). It is often difficult to differentiate between these modes of action (3).

Not only does estrogen affect AP hormone synthesis and release, but it can also influence the proportions of hormone isoforms. Postpubertal gonadal regulation of growth hormone (GH) secretion appears to be largely due to estrogens in females and aromatization of androgens to estrogens in males

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(11–13). Estrogens appear to regulate pulsatile secretion of GH by multiple routes, including shifting the proportions of GH isoforms and altering responsiveness to GH-releasing hormone (14–17). Estrogen also enhances proliferation of lactotropes and increases prolactin (PRL) secretion (18–23). In addition, PRL variants differ with gender and glycosylated PRL is increased by estradiol (24, 25). Estrogen is essential for the regulation of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) synthesis and release (3, 26–28). Expression of gonadotropin subunits is suppressed or enhanced by estrogen depending on the conditions (29–31). The relative abundance of specific LH and FSH isoforms and thus their bioactivities may also be regulated by estrogen (32–35). In rats, estradiol administration decreases the adrenocorticotropic hormone (ACTH) response to immobilization stress (36). In gonadectomized rats, estradiol increases ACTH in the plasma (37), and ovariectomy decreases the ACTH response to corticotropin-releasing factor (38). *In vitro* secretion of thyroid-stimulating hormone from pituitaries of ovariectomized rats is increased by low-dose estrogen (39). Estrogen also increased pituitary thyrotropin-releasing hormone receptor mRNA stability and the rate of transcription (40). Together, these data implicate estradiol in the regulation of all six of the established AP hormones. However, little is known of the specifics of its action on the AP hormones, their isoforms, and nonhormonal AP proteins.

Advances in protein separation and mass spectrometry (MS) techniques have markedly enabled our ability to study estrogen-induced effects on proteomes. For example, Molero and colleagues (41) employed 2D gel electrophoresis to demonstrate that 24-hr incubation of bovine vascular smooth muscle cells with 17β-estradiol causes changes in the expression of several proteins involved in smooth muscle cell proliferation (e.g., α-enolase isoform 1, vimentin, and HSP-60), cell contraction (e.g., vimentin and caldesmon), and oxidative stress (e.g., protein disulfide isomerase). Recent advances in protein separation and MS techniques also have made it possible to identify large numbers of pituitary proteins. Zhao and colleagues (42) employed a comprehensive multiple gel-based strategy to identify proteins in human pituitary gland homogenates. Their unique approach allowed them to identify a total of 1449 proteins, including low-abundance proteins, membrane proteins, and proteins at the extremes of isoelectric point (pI) and molecular weight. In our present study, we have employed difference gel electrophoresis (DIGE), matrix-assisted laser desorption/ionization time-of-flight MS (MALDI-ToF MS), and peptide mass fingerprinting (PMF) to analyze the effect of short-term estrogen treatment on the AP proteome of long-term ovariectomized rats. In this initial investigation, we limit our report to selected proteins in the soluble protein fraction of the gland.

Methods

Animals and Collection of Tissue. Two-month-old rats (CD strain) were ovariectomized by Charles River Laboratories, Inc. (Wilmington, MA) and shipped to the University of South Carolina. Rats were housed two per cage in a room with controlled lighting (lights on 0700–1900 hrs daily) and temperature (20°–22°C) and given Teklad Rodent Diet 8604 (Harlan, Madison, WI) and tap water *ad libitum*. They were handled three times per week for cage cleaning and used at 16 weeks after ovariectomy at 6 months of age. Animals were maintained and used in accordance with the standards of the Institutional Animal Care and Use Committee of the University of South Carolina.

Control rats (349–430 g) were injected subcutaneously with a total of 0.1 ml sesame oil vehicle (Sigma Chemical Company, St. Louis, MO) into the right and left inguinal region. Experimental rats (361–430 g) were injected with 50 μg estradiol valerate in vehicle. Rats were injected ip approximately 48 hrs later (1100 hrs) with sodium pentobarbital (75 mg/kg body weight) and then were perfused with PBS as described previously (43). The AP of each rat was collected (43), rinsed in PBS, blotted, weighed, placed in a tube embedded in dry ice, and stored at −80°C. The uterine horns in all vehicle-treated animals were small and threadlike; those in all estrogen-treated rats were markedly swollen, indicating that they had been stimulated by estrogen. Careful gross examination of the area of fatty tissue near the end of the uterine horns revealed no evidence of ovarian tissue.

Preparation of Soluble Protein Fraction. The APs were divided into two pools. Four glands from the vehicle-treated group formed pool A (64.1 mg), and three glands from the estrogen-treated group formed group B (44.0 mg). The APs in a pool were thawed and combined in a 1-ml tissue grinder (Duall 20, catalog no. 885450-0020; Kontes Glass Co., Vineland, NJ). The tissue was homogenized by hand on ice in 10 volumes of buffer (50 m*M* Tris [pH 7.5], 150 m*M* NaCl, 0.1% TX-100 [Surfact-AmpsX-100; Pierce Biotechnology, Inc., Rockford, IL]) in the presence of protease inhibitor cocktail added at twice the recommended concentration (Complete Mini; Roche Diagnostics GmbH, Mannheim, Germany) and phosphatase inhibitor cocktail 1 added at twice the recommended concentration (contains cantharidin, bromotetramisole, and microcystin LR; Sigma). The TX-100 (0.1%) was added to permeabilize any compartments not broken by homogenization and to release soluble components. After homogenization, the sample was treated to digest nucleic acids that interfere with 2D gel resolution (5 m*M* $MgCl_2$, 200 U/ml DNase I [Sigma; catalog no. D 4513], and 10 U/ml RNase A [Sigma; catalog no. R 5500] for 10 mins on ice). The homogenate was then centrifuged (40,000 *g*, 30 mins, 4°C). The supernatant containing the soluble protein fraction was collected for protein precipitation as described in the

following. Membrane and cytoskeleton/chromatin fractions prepared from the pellet were saved for future analysis.

The protein in the soluble fraction was quantitatively precipitated with methanol and chloroform by the method of Wessel and Flugge (44). Briefly, four volumes of aqueous sample were mixed with four volumes of methanol and one volume of chloroform, vortexed vigorously, and centrifuged. The aqueous phase containing salts and nucleotides was discarded. The protein interface and lower chloroform phase were mixed with three volumes of methanol and centrifuged. The top phase containing lipids was discarded, and the protein pellet was dried in a vacuum centrifuge. The dry protein pellet was resolubilized in reaction buffer (7 *M* urea, 2 *M* thiourea, and 4% w/v CHAPS) overnight at room temperature (RT). The dissolved protein was treated with 10 m*M* DL-dithiothreitol (DTT) to reduce cysteines and to increase protein solubilization. The sample was centrifuged (16,000 *g*, 10 mins, RT) to remove any undissolved material, mainly small amounts of connective tissue. An aliquot of the solubilized protein was diluted 50-fold and assayed for protein concentration by the method of Bradford (Protein Assay, Bio-Rad Laboratories, Inc., Hercules, CA), and the sample was then adjusted to 5 mg/ml protein with the reaction buffer.

Protein Labeling and 2D Gel Electrophoresis. Protein was next subjected to minimal CyDye labeling (45–47; CyDye DIGE Fluors [minimal dyes] for Ettan DIGE, GE Healthcare, Piscataway, NJ). For this reaction a 10-µl aliquot (50 µg of total protein) was removed, and the pH was adjusted by addition of pH 8.5 Tris to a final concentration of 30 m*M*. (The pH was checked with pH paper to ensure that it was near optimal for the labeling reaction.) The CyDye (200 pmoles in 1 µl anhydrous dimethylformamide) was added to the protein and the reaction allowed to proceed (30 mins, 4°C, in the dark). Under these conditions approximately 1% of the lysine residues of the protein are covalently conjugated to the CyDyes (45, 46). The reaction was then quenched by the addition of 1 µl of 10 m*M* lysine. For each gel the control sample was labeled with Cy3, and the experimental sample was labeled with Cy5. A pool was prepared from small aliquots of each sample and was labeled with Cy2. This pool was used on all gels as an internal standard to allow for more accurate quantification (47). The samples labeled with Cy3 and Cy5 were mixed with an additional 425 µg of unlabeled protein from each of the two samples to facilitate protein identifications after electrophoresis, and then the Cy2 labeled internal standard (50 µg) was added. The mixture was adjusted to a total volume of 450 µl by adding more reaction buffer (i.e., pI 3–10 Pharmalytes [1% final], bromphenol blue [0.001% final], and hydroxyethyl disulfide [0.1 *M* final]; Destreak; GE Healthcare). Free cysteines reacted with hydroxyethyl disulfide to become disulfide linked to hydroxyethyl groups. During focusing this protects the cysteines from oxidation, which can cause streaking and artifactual spot production in the basic region of 2D gels.

Replicate gels were run (i.e., gel 1 and gel 2). The protein samples (450 µl, 1000 µg of total protein) were passively rehydrated into 24-cm immobilized pH gradient strips (IPG 3–10 NL, GE Healthcare) for 16 hrs and then focused (IPGphor system, GE Healthcare) for 133,000 V·hr (48). Free cysteines were regenerated by reduction. This involves incubating the strips (20 mins, RT) in equilibration solution (6 *M* urea, 50 m*M* Tris pH 8.8, 30% [w/v] glycerol, 2% [w/v] SDS, 0.001% bromophenol blue) containing 0.5% DTT. The cysteines were then alkylated by incubation in equilibration solution containing 4.5% iodoacetamide (20 mins at RT). Second dimension separation was performed at 20 W/gel at 25°C (Ettan DALT12 vertical system, GE Healthcare) using hand-cast 8%–16% gradient gels.

Gel Imaging, Spot Picking, and Protein Identification. The gels were scanned with a Typhoon 9400 Variable Mode Laser Imager (GE Healthcare) set to 100-µm resolution. DeCyder (Differential In-Gel Analysis [DIA]) software version 5.0 (GE Healthcare) was used for spot detection and relative quantification of proteins based on fluorescence images. DeCyder (Biological Variation Analysis [BVA]) software was used to match gels in the study using the internal standard.

For picking spots, gel 2 was poststained with Deep Purple to visualize the unlabeled protein (*ca.* 99.9% of the total), which does not precisely comigrate with the CyDye-labeled protein. The spots were then excised with a 2.0-mm picking head (Ettan Spot Picker, GE Healthcare) and transferred to 96-well plates for washing and trypsin digestion (Ettan Digester, GE Healthcare). The spots were washed twice with 25 m*M* NH_4HCO_3/50% methanol, once with 75% CH_3CN, and once with 100% CH_3CN. The 96-well plate was allowed to dry, and trypsin was added to each well (10 µl, 10 µg/ml in 20 m*M* NH_4HCO_3). The plate was covered and incubated (16 hrs, RT). Digests were mixed with a matrix (α-cyano-4-hydroxycinnamic acid in 80% CH_3CN, 0.1% TFA) and analyzed by MALDI-ToF MS (Voyager DE-PRO; Applied Biosystems, Foster City, CA). Spectral processing included defining the baseline and noise, selection of the monoisotopic peaks, calculation of the signal-to-noise ratios, and calibration on autolytic trypsin peaks. These tasks were performed by ProTS Data (Efeckta Technologies). The output of this program is a list of all peptide masses, excluding trypsin peaks, with a signal-to-noise ratio of >4. The peak list is then submitted to Mascot (Matrix Science Ltd; version 1.9) and PMF performed by matching the entries against the predicted masses of the tryptic fragments generated *in silico* for each entry in the nonredundant protein database (NCBI nr, November 21, 2004). A mass accuracy of 50 ppm was required for each matching peptide, no post-translational modifications were permitted, but a single missed cleavage was allowed. Searches were not constrained by pI or molecular weight. A *P* value less than 0.05 was achieved

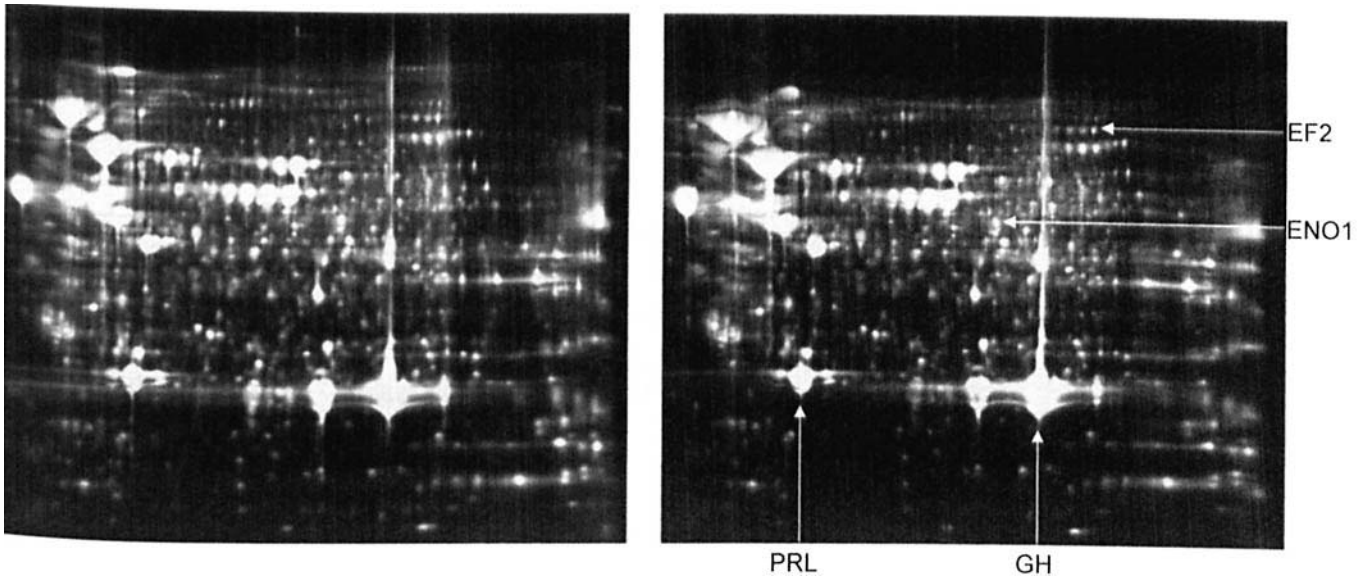

Figure 1. Replicate gels demonstrate method reproducibility. The soluble protein fraction from the control rat AP pool A was labeled with Cy3, and the soluble protein fraction from the AP pool B from estrogen-treated rats was labeled with Cy5. For clarity, the internal standard sample labeled with Cy2 is not shown. The acidic region is to the left and higher molecular weight proteins are to the top. Red = upregulation and green = downregulation in estrogen-treated rats relative to controls. Yellow = proteins not differentially regulated. Somatotropin (growth hormone, GH), prolactin (PRL), enolase 1 (ENO1), and eukaryotic translation elongation factor 2 (EF2) are indicated by arrows.

with a MASCOT score of greater than 76. Minimum requirements for positive protein identification were described previously (48).

Results and Discussion

The DIGE gels are shown in Figure 1. We used standard operating procedures to ensure reproducibility. Resolution was optimized by incorporating (i) nuclease to remove nucleic acids, (ii) a methanol/chloroform extraction method to remove salts and lipids, (iii) hydroxyethyl disulfide to protect cysteines, and (iv) optimal isoelectric focusing and SDS-polyacrylamide gel electrophoresis conditions. To assess analytical variability the labeling was repeated, and a second 2D gel was prepared (Fig. 1, right panel). The gels are remarkably consistent, indicating the reproducibility of all the discrete steps involved in our sample analysis by DIGE.

The DeCyder software package determined spot boundaries and spot ratios (experimental/control) on each gel. The use of the Cy2 channel facilitated spot matching between analytical replicate gels (47) and allowed for more accurate quantification (48). We considered a spot to be regulated in response to estrogen if there was a 1.5-fold or greater average change in its experimental/control ratio. Forty-five regulated gel spots were identified by MALDI-ToF MS and PMF. Of these, 26 were upregulated and 19 downregulated. Protein spots, with the greatest fold change, between the control (pool A) and estrogen-treated (pool B) groups are listed in Table 1.

A major advantage of 2D gel strategy is that multiple isoforms of the same protein can be identified. Several examples are evident (Fig. 1), and of special interest are the AP hormone isoforms. The large yellow spot in the lower-right quadrant is somatotropin (growth hormone, GH), and two isoforms of increasing acidity are evident. Acidic isoforms have the same molecular weight as the parent protein but lower pIs and so appear to the left of the parent spot. In this report they are numbered in order of increasing acidity. The two acidic isoforms of GH exhibit increased expression after estrogen treatment (Table 1). In humans it is known that the acidic GH isoforms are due to phosphorylation of serine and deamidation of glutamine and asparagine (49, 50). Many of the acidic isoforms seen here likely arise *via* the same mechanisms, emphasizing the importance of using phosphatase inhibitors during sample preparation. Acetylation of the N-terminus or lysine residues can also produce a net acidification by neutralizing a positive charge. The large yellow spot in the lower-left corner is PRL, and to its left is an acidic isoform that is more abundant following estrogen. The DeCyder software also displays three-dimensional topographical images where the intensity of each pixel is represented by height (i.e., Z-axis, Fig. 2). This is an important tool for checking the spot boundaries determined by DeCyder DIA and for checking that the pattern of spots is correctly matched between the gels. Here the control sample (Cy3) is shown on the left and the estrogen-treated sample (Cy5) on the right. The 3D representations can be rotated and viewed from any angle so that differences can be clearly seen and evaluated. In this example the increase in the acidic isoform of PRL is clearly evident, while the surrounding spots are unchanged. The large spot of PRL, partially shown, is unchanged.

Other proteins important to AP function also show multiple isoforms. The row of spots indicated to the top right is composed of closely related forms of elongation factor 2 (EF2), a protein involved in protein synthesis. The row of spots near the middle of the gel is composed of

Table 1. Effects on AP protein content of treatment of ovariectomized rats with estrogen. The fold change in spot intensity (estrogen-treated/control) was determined from the Cy5/Cy3/Cy2 images for both gels shown in Figure 1. Only those proteins in the soluble protein fraction that had an average fold change of 1.5 or higher are listed. The nominal molecular weight (mol wt) and nominal isoelectric point (pI) were calculated from the sequence of the protein in the database. The measured mol wt and pI of the protein spots were determined from their position on the 2D gel. Isoforms of the same protein are grouped together and placed in order of increasing acidity. The number of peaks submitted to MASCOT, the number of peptide peaks matched to the protein sequence, and their respective MASCOT scores are indicated. The percentage coverage is the amount of the protein sequence covered by the matched peptides. The proteins that increased with estrogen treatment relative to controls have positive fold changes, and the proteins that decreased with estrogen treatment relative to controls have negative fold changes. The fold changes were remarkably consistent between the two gels, indicating the reproducibility of the DIGE-based proteomic analysis.

Protein name	gi no.	Predicted mol wt	Measured mol wt	Predicted pI	Measured pI	MASCOT score	Fold change gel 1	Fold change gel 2	Fold change average	Percentage coverage	Peaks submitted	Peaks matched
Enolase 1	50926833	47,440	46,000	6.2	6.1	232	4.37	3.75	4.06	64	43	19
Enolase 1, acidic isoform 1	50926833	47,440	46,000	6.2	5.9	212	6.10	5.75	5.93	59	37	17
Enolase 1, acidic isoform 2	50926833	47,440	46,000	6.2	5.8	198	5.07	4.68	4.88	51	28	15
Enolase 1, acidic isoform 3	50926833	47,440	46,000	6.2	5.7	85	2.79	1.86	2.33	17	10	6
ERp57, acidic isoform 2	38382858	57,044	48,000	5.9	5.8	272	1.57	1.68	1.63	51	53	27
ERp57, acidic isoform 4	38382858	57,044	49,000	5.9	5.6	324	2.31	2.14	2.23	51	43	25
ERp57, acidic isoform 5	38382858	57,044	49,000	5.9	5.5	196	2.26	2.08	2.17	37	37	16
Arginosuccinate synthetase	25453414	46,752	46,000	7.6	7.6	159	2.33	2.13	2.23	28	27	13
Arginosuccinate synthetase, acidic isoform	25453414	46,752	47,000	7.6	7.4	107	1.69	1.66	1.68	19	20	9
ERp29	16758848	28,614	30,000	6.2	5.9	161	2.22	2.21	2.22	41	22	11
Somatotropin, acidic isoform 1	1432171	24,892	25,000	5.7	6.3	218	1.91	2.18	2.05	68	57	17
Somatotropin, acidic isoform 2	1432171	24,892	25,000	5.7	5.9	211	1.89	2.46	2.18	68	51	16
Prolactin, acidic isoform	38181555	25,707	21,000	5.4	4.9	215	1.98	2.11	2.05	30	23	7
Hsc70-ps1, acidic isoform 2	56385	71,112	71,000	5.4	5.2	255	2.02	1.98	2.00	43	37	23
DRP-2 (TOAD-64, CRMP-2)	34874349	82,050	58,000	6.0	6.4	204	1.77	2.11	1.94	43	30	17
Creatine kinase-B	203474	42,984	25,000	5.7	5.9	216	1.75	1.89	1.82	68	51	16
Eukaryotic translation elongation factor 2, acidic isoform 2	8393296	96,192	105,000	6.4	7.1	203	1.52	1.43	1.48	25	36	21
Eukaryotic translation elongation factor 2, acidic isoform 3	8393296	96,192	105,000	6.4	7.0	151	1.81	1.74	1.78	18	28	19
Pyruvate kinase, M1/M2	206205	58,314	49,000	7.2	7.2	190	1.62	1.83	1.73	35	29	15
Secretogranin II	12083601	70,987	88,000	4.7	4.6	245	1.69	1.68	1.69	35	39	22
Programmed cell death 6 interacting protein[a]	6755002[a]	96,520[a]	105,000	6.2	6.4	183	1.69	1.65	1.67	15	23	16
Calreticulin	11693172	48,137	45,000	4.3	4.4	149	1.64	1.69	1.67	40	36	13
Malate dehydrogenase	15100179	36,631	37,000	5.9	6.0	176	1.64	1.65	1.65	41	20	12
Phosphoglycerate mutase type B subunit	8248819	28,942	30,000	6.7	6.6	165	1.70	1.48	1.59	51	23	12
Coatomer delta subunit (archain 1)	34863255	57,619	56,000	5.9	6.3	82	1.52	1.60	1.56	18	30	9
Aldolase A	6978487	39,783	45,000	8.3	8.2	88	1.52	1.52	1.52	23	36	9
DEAD-box protein 48[a]	20834678[a]	47,109[a]	46,000	7.0	6.7	78	−1.56	−1.44	−1.50	14	19	7
Radixin	56799432	68,672	86,000	6.0	6.7	97	−1.51	−1.50	−1.51	19	22	10
hnRNP F[b]	16876910[b]	46,013[b]	45,000	5.4	5.2	117	−1.57	−1.47	−1.52	39	29	10
Vinculin	31543942	117,215	110,000	5.8	6.3	195	−1.66	−1.45	−1.56	21	26	18

Table 1. *Continued.*

Isovaleryl coenzyme A dehydrogenase	6981112	46,862	46,000	8.0	6.6	113	−1.56	−1.69	−1.63	29	35	11
Secretogranin III	16758722	53,151	45,000	4.9	4.7	107	−1.56	−1.74	−1.65	22	15	8
Stathmin 1	8393696	17,278	20,000	5.8	5.7	125	−1.81	−1.60	−1.71	47	20	8
Pigpen[a]	7920331[a]	52,870[a]	66,000	9.4	9.0	97	−1.94	−1.52	−1.73	18	25	9
ssDNA binding protein PUR-alpha	34878862	34,976	45,000	6.1	6.5	92	−1.66	−2.03	−1.85	39	42	9
hnRNP K	16923998	51,230	58,000	5.4	5.4	142	−2.31	−2.34	−2.33	24	26	12
Mitochondrial aldehyde dehydrogenase	45737866	56,079	45,000	6.4	6.4	160	−1.94	−3.04	−2.49	32	20	12
hnRNP L	34855641	64,550	58,000	6.7	7.2	83	−2.86	−2.21	−2.54	19	23	8
DEAD-box protein 17[a]	40068493[a]	73,166[a]	73,000	8.9	8.7	77	−2.59	−3.08	−2.84	13	16	7
hnRNP A1	38328245	33,713	34,000	9.3	9.2	144	−2.53	−2.15	−2.34	41	27	11
hnRNP A1, acidic isoform 1	38328245	33,713	34,000	9.3	8.9	109	−2.86	−3.01	−2.94	34	28	9
hnRNP A1, acidic isoform 2	38328245	33,713	37,000	9.2	8.9	167	−2.13	−2.40	−2.27	41	25	12
hnRNP A2/B1	34855868	36,041	37,000	9.0	8.5	259	−3.10	−3.12	−3.11	58	32	18
hnRNP A2/B1, acidic isoform	34855868	36,041	37,000	9.0	8.3	169	−2.29	−3.98	−3.14	41	35	14
PTB-associated splicing factor (PSF)	34871066	65,267	109,000	9.5	9.1	94	−3.96	−2.78	−3.37	20	25	11

[a] Matched to mouse protein.
[b] Matched to human protein.

closely related forms of enolase 1, a protein involved in glycolysis, growth control, and many other functions. Their red color indicates their very strong upregulation by estrogen. Detailed analysis of the isoforms of enolase 1 is shown in Table 1. All four spots have the same molecular weight (slightly less than the predicted molecular weight due to removal of the signal sequence), but only one spot has the predicted pI. The more acidic spots are likely due to phosphorylation or possibly deamidation or acetylation. These changes either add a negatively charged phosphate group to neutral residues such as serine, threonine, and tyrosine; convert neutral residues such as glutamine and asparagine to negatively charged glutamic acid and aspartic acid residues; or neutralize a positively charged residue. As the isoforms become progressively more acidic, the number of peptides matched decreases, and therefore the percentage coverage decreases. The disappearance of some peptides from the MALDI-ToF MS spectrum may be due to the fact that acidic modifications make them more difficult to ionize, but because the acidic isoforms are themselves less abundant, specific peptides may simply be below the signal-to-noise threshold. Acidic isoform 3 was present at a very low level, and this alone accounts for the lower number of matched peptides and sequence coverage. Tandem MS experiments are currently under way to identify the precise nature and site of the modifications responsible for the isoforms of enolase, GH, and PRL.

Our data demonstrate that treatment with estrogen produces numerous changes in the soluble AP proteome, including increases in the more acidic isoforms of GH and PRL. These data indicate that rat studies are relevant to humans because these isoforms have previously been described in humans and are known to have important biological consequences. There were also increases in the levels of several proteins involved in protein synthesis, folding, and secretion, such as EF2, ERp57, ERp29, Hsc70-ps1, calreticulin, coatomer delta subunit (archain 1), and secretogranin II. The EF2, shown in Figure 1, catalyzes the translocation of peptidyl-tRNA from the ribosomal A site to the P site in translational elongation. It has been suggested that the control of the protein translation rate is influenced by phosphorylation/dephosphorylation of EF-2 (51, 52). Several interesting metabolic enzymes were also upregulated, including arginosuccinate synthetase, enolase 1, creatine kinase B, phosphoglycerate mutase, malate dehydrogenase, pyruvate kinase M1/M2, and aldolase A. Arginosuccinate synthetase is involved in the regulation and detoxification of nitric oxide, an important signaling molecule. Enolase 1 has been implicated in growth control and regulation of c-myc. DRP-2 (TOAD-64, CRMP-2) is activated with cell division. Programmed cell death 6 interacting protein was also upregulated. The majority of the downregulated proteins are involved in RNA or DNA interactions. These include five heterogeneous nuclear ribonucleoproteins (hnRNPs), A1, A2/B1, K, L, and F. Also reduced were DEAD-box proteins 17 and 48, ssDNA

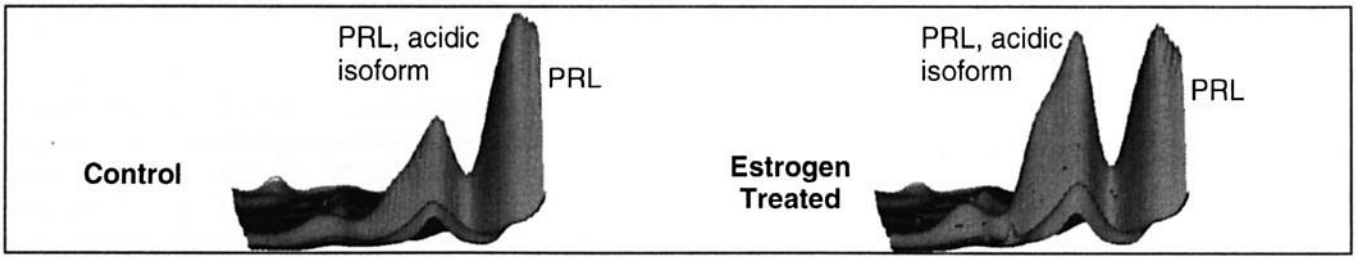

Figure 2. A 3D topographical representation of the PRL region of the 2D gel. The intensity of the CyDye labeled proteins is shown by the height. The control sample (left) and the estrogen-treated sample (right) are similar except for the increase in the level of the PRL acidic isoform.

binding protein PUR-alpha, PTB-associated splicing factor, and Pigpen protein. The changes in this group of proteins may reflect large-scale alterations in gene expression at this early time point following estrogen treatment. Other down-regulated proteins were isovaleryl coenzyme A dehydrogenase, mitochondrial aldehyde dehydrogenase, stathmin 1, vinculin, radixin, and secretogranin III. Downregulation of secretogranin III concurrent with secretogranin II upregulation indicates alterations in the secretory process. Doubtless many other changes will be revealed with analysis of the membrane and cytoskeleton/chromatin fractions.

The results of this study clearly indicate that estrogen acts *in vivo* to regulate a significant number of proteins and some of their acidic isoforms in the AP-soluble fraction. However, the data do not enable us to determine whether estrogen acted directly on the AP or indirectly *via* the hypothalamus or by some other means. We also do not know whether changes in content of an individual protein occurred in one or more of the AP cell types. Compared to a rat with intact ovaries, the experimental design was biased toward analyzing gonadotropes. Ovariectomy causes an increase in AP weight (53), primarily because of an increase in number and size of gonadotropes (54, 55). The APs used in the present study weighed approximately 50% more than sham-operated, aged-matched animals whose AP proteome was not analyzed. We employed a protocol for administering estrogen that exerts a biphasic effect on gonadotropin secretion. Initially estrogen exerts a negative feedback on LH and FSH release (56). This is followed by positive feedback as evidenced by surges of LH and FSH in blood (57). The rats in the present study were studied approximately 3 hrs before the anticipated onset of the surges. Thus, estrogen-induced changes in the AP proteome may be associated with effects on gonadotropes related to negative feedback or associated with prepositive feedback, other AP cell types, or all cell types in the glandular epithelium.

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