

Expression of type 1 and 2A protein phosphatase subunits in the rat corpus luteum across pregnancy

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Abstract

This study was undertaken to test the hypothesis that the reduction in protein phosphatase activity that had been observed at mid-pregnancy in the rat corpus luteum (CL) was due to a decrease in expression of one of the catalytic subunits or an increase in one of the B regulatory subunits of the type 2A protein phosphatase (PP2A). Ovaries were collected from rats on days (d) 1, 3, 7, 14, 20, and 21 of pregnancy, and on day 21 after progesterone treatment on day 20 ($n=6$). Real-time RT-PCR was used to analyze the expression of the α and β isoforms of the catalytic subunit, the structural A subunit,

and three B regulatory subunits of PP2A, as well as the catalytic subunit of PP1. Expression of the α and β catalytic subunits of PP2A was progesterone responsive. Expression of the PP1 catalytic subunit correlated with the previously reported protein phosphatase activity, but PP2A subunits did not. The data suggest that the decreased protein phosphatase activity at mid-pregnancy was due to a decline in expression of the catalytic subunits of PP1 rather than changes in expression of PP2A subunits.

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Introduction

The importance of protein phosphatases in cellular function is increasingly recognized (Cohen 1989, Brautigan 1994, Shenolikar 1994, Gallego & Virshup 2005), although protein kinases continue to receive more attention than protein phosphatases. Our laboratory identified an endogenous inhibitor of protein kinase C (PKC) in rat ovarian cytosols, as well as the reversal of PKC inhibition following partial purification on DEAE chromatography (Eyster 1990, 1993). In subsequent studies, the endogenous inhibitor of PKC was identified as the type 2A protein phosphatase (PP2A; Eyster *et al.* 1993, 1995). Thus, the endogenous inhibitor of PKC in the rat ovary was not a direct inhibitor of the enzyme but rather a separate enzyme, a protein phosphatase, that rapidly dephosphorylated the substrate phosphorylated by PKC.

In our studies of the interactions of PKC and protein phosphatase in the ovaries, we observed a steady rise in the activity of PKC in the ovaries from days 7 to 20 of pregnancy in the rat when the PKC activity was measured in a way that prevented interference by protein phosphatases (Eyster *et al.* 1993). In contrast, protein phosphatase activity declined between days 7 and 10 of pregnancy and remained low for the remainder of pregnancy (Eyster *et al.* 1998). Both the rise in PKC activity and the decline in protein phosphatase activity in the rat ovary occurred near mid-pregnancy at the time that the control of progesterone synthesis shifts from the pituitary to the placenta (Gibori *et al.* 1988). We had reasoned that

since the endogenous inhibitor of PKC is present throughout pregnancy (Eyster 1993), the ovarian protein phosphatase activity must be subject to endogenous regulation in order for changes in PKC activity or content to be recognized by the cell. In support of this concept, we were able to demonstrate hormonal regulation of PP2A in the rat corpus luteum (CL; Eyster *et al.* 1998). In that study, estrogen reduced the activity of ovarian protein phosphatase whereas prolactin had no effect. Others have demonstrated the importance of protein phosphatases in the regulation of progesterone secretion by luteinizing hormone in isolated rat luteal cells (Abayasekara *et al.* 1996).

The PP2A holoenzyme is a heterotrimer composed of a catalytic subunit, a structural A subunit, and a regulatory B subunit (Kamibayashi *et al.* 1994, Wera & Hemmings 1995, Virshup 2000). There are two isoforms of the catalytic subunit, α and β (Cohen 1989, Janssens & Goris 2001). In addition, there are at least four forms of the B regulatory subunit which are not isoforms but distinctly individual proteins. The B regulatory subunits are known as 55kd/PR55/B55, 53kd/PR53/B53, 72kd/PR72/B72, and 110kd/striatin (Hemmings *et al.* 1990, Hendrix *et al.* 1993, Janssens & Goris 2001). The A and catalytic subunits bind to each other and form a scaffold to which the B subunit binds. Only one B regulatory subunit can bind to the A subunit–catalytic subunit dimer at a given time (Mayer-Jaekel & Hemmings 1994). Binding to a B regulatory subunit reduces the catalytic activity of PP2A (Price & Mumby 2000, Janssens

& Goris 2001). The B subunits can also alter substrate specificity and participate in the formation of protein complexes by binding with substrate proteins (Price & Mumby 2000).

The current study was undertaken to test the hypothesis that the reduction in protein phosphatase activity that we had observed from the middle to the end of pregnancy was mediated by a decrease in the expression of one of the catalytic subunits, or by an increase in one of the B regulatory subunits, of PP2A, or by a change in the expression of the catalytic subunit of PP1. Real-time PCR (RT-PCR) was the primary technique used for this study since antibodies were not available for all of the subunits of PP2A.

Methods and Materials

Animal handling and treatment

All protocols for the treatment and handling of animals were approved by the Institutional Animal Care and Use Committee of the University of South Dakota and conform to the NIH Guide for the Care and Use of Laboratory Animals. Timed pregnant Sprague–Dawley rats were obtained from the University of South Dakota Laboratory Animal Services breeding colony. Ovaries were obtained from the pregnant rats on days 1, 3, 7, 14, 20, and 21 of pregnancy ($n=6$ per group; plug date was counted as day 0). An additional set of six rats was treated with progesterone (4 mg/kg) on day 20 of pregnancy and the ovaries were collected on day 21. Animals were anesthetized with isoflurane and decapitated, and trunk blood was collected in heparinized containers.

The ovaries were immersed in the RNA preservation reagent, RNAlater (Ambion, Austin, TX, USA), upon removal from the animals and were stored at -80°C until further processing. The corpora lutea were dissected free from the stroma of the ovaries and weighed. Total RNA was extracted from the corpora lutea using a modification of a previously published method (Rodrigo *et al.* 2002, Eyster *et al.* 2007). The corpora lutea (15–40 mg) were homogenized in TRI reagent (Molecular Research Center, Cincinnati, OH, USA) and the organic and aqueous layers separated as described (Rodrigo *et al.* 2002). The aqueous layer containing total RNA was further purified on a Qiagen RNeasy column (Qiagen) as per company protocol. An on-column DNase treatment (Qiagen) was used to remove any remaining DNA. Total RNA was quantitated by spectrophotometry and quality was examined by ethidium bromide-stained gel electrophoresis.

Progesterone EIA

An immunoassay kit for the measurement of progesterone was obtained from Alpco Diagnostics (Salem, NH, USA). The working range of the assay was 0.3–60 ng/ml; all plasma samples

were diluted 2.5-fold for analysis. The sensitivity of the assay was 0.1 ng/ml. The coefficients of variation of intra- and inter-assay precision were 10.6 and 10.2% respectively. The antibody used in this assay cross-reacts with both progesterone and 11 α -OH-progesterone, but not with 17-OH-progesterone, and exhibits <0.2% cross-reactivity with 20 α -dihydroprogesterone. Progesterone concentration was measured in plasma obtained from animals across pregnancy ($n=5$).

Real-time RT-PCR

Primers and probes for real-time RT-PCR for the α and β catalytic subunits of PP2A, the structural A subunit, the B regulatory subunits p53, p55, and p110/striatin, the catalytic subunit of PP1, and the housekeeping gene, cyclophilin were designed using Primer Express software (Applied Biosystems, Foster City, CA, USA). Primers were obtained from Integrated DNA Technologies (Coralville, IA, USA). Minor groove-binding (MGB) probes were labeled with FAM dye at the 5'-end and with a non-fluorescent quencher at the 3'-end; these MGB probes were synthesized by and obtained from Applied Biosystems. Concentration–response curves for RNA and for the primers and probes were performed for each gene to determine the appropriate concentration of RNA, primers, and probe for each reaction (data not shown). The sequences and concentrations of the primers and probes are shown in Table 1.

The real-time RT-PCRs were conducted with TaqMan Gold One-Step RT-PCR kit (Applied Biosystems) as described (Eyster *et al.* 2007). Total RNA (50 ng) was used for each reaction, conducted in duplicate. The total volume of the reaction mix was 25 μl and included 6 μl RNA, 13.1 μl reaction mix, and 5.9 μl primer/probe mix. The reverse transcription reaction was carried out at 48°C for 30 min, followed by heat inactivation at 95°C for 10 min. The PCR was set for 40 cycles of 95°C for 15 s and 60°C for 1 min.

The fluorescent signal data were analyzed by ABI Prism 7000 signal detection system software (Applied Biosystems). The threshold was set such that it did not overlap any background non-reaction-related fluorescence. The threshold cycle, C_T , for each reaction indicated the fractional cycle number at which the amount of amplified target reached a fixed threshold, and was averaged for duplicate reactions. Cyclophilin was used as the endogenous control. Changes in expression of the protein phosphatase subunit genes were calculated relative to cyclophilin. Data from real-time RT-PCRs were analyzed using qBase software (Hellemans *et al.* 2007). The qBase program uses a $\Delta - C_T$ relative quantitation model with multiple reference gene normalization and PCR efficiency correction.

Immunoblot

Corpora lutea were dissected free from the ovaries of pregnant rats ($n=3$ /day of pregnancy) and homogenized in 20 mM Tris buffer (pH 7.4) containing 1 mM EDTA, 1 mM EGTA,

Table 1 Sequences of the primers and probes for rat protein phosphatase subunits and for the housekeeping gene, cyclophilin, are shown here with sequences 5'–3'

	Forward primer ^a	Reverse primer ^a
PP2A		
Cat α	GAG GCG AGC CAC ATG TCA CT (900 μ M)	CCA TTA GGT CAA CAG ACG GTG TT (900 μ M)
Cat β	GAT GTG CAT GGC CAA TTC C (900 μ M)	CCT CTG TCT ACA TAG TCA CCC ATG A (900 μ M)
A	GTG GTA AGG AAA TAA CCA CTA AGC AAA T (900 μ M)	CTG CAG TGA CTT AGC CAC ATT GA (900 μ M)
p53	GAA AAT CTA GCC AAA GCG AAT CC (300 μ M)	GGG TGA GCC AGG TCC TAT GTC (900 μ M)
p55	CCA TTG TGG CGT CCC TTT C (50 μ M)	CCT AAA TGG CTA GCA GAC ATG GA (900 μ M)
Striatin	CCC TGG CTA AGG TCT TTG TAT GA (900 μ M)	CTC CCT CAG CCA AAC AAA AGG (900 μ M)
PP1	CCC GCA ATT CTG CCA AAG (900 μ M)	CGC TGT GTT CCC CAT GAT G (900 μ M)
Cyclo	CTA CTC CTT GGC AAT GGC AAA (300 μ M)	CAC GTG GTT TTC GGC AAA GT (50 μ M)
	Probe sequence^a	
PP2A		
Cat α	CTG TAA TGA AAG TTT AAC CTT GTA CAG T (100 nM)	
Cat β	CCA GAC ACC AAC TAT (100 nM)	
A	TGC CTA TCG TGT TGA AAA (100 nM)	
p53	TGA GGC TTG CGT GAA T (100 nM)	
p55	CAA CAA CAC ATT TGA TAG CC (100 nM)	
Striatin	CAA GTC AAC CGC ACA CAG (100 nM)	
PP1	AGA AAT AGC CTC CAT GTG C (100 nM)	
Cyclo	CAA TGA TCA CAT CCT TCA (100 nM)	

^aThe concentrations of primers and probes are shown in parentheses.

0.25 M sucrose, 50 mM 2-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride (Eyster *et al.* 1998). Protein concentration was determined by the bicinchoninic acid protein assay (Pierce, Rockford, IL, USA). Proteins (50 μ g per lane) were separated by electrophoresis on 10% polyacrylamide (wt/vol) SDS gels. The samples were transferred from the gel to polyvinylidene difluoride membranes (Bio-Rad Laboratories) by electrophoresis for 1 h on ice at a constant voltage of 100 V. The membranes were blocked in AquaBlock (East Coast Biologics, North Berwick, ME, USA) overnight, then incubated overnight with rabbit anti-PP1 primary antibody diluted in AquaBlock (1:500; Millipore, Temecula, CA, USA). The membranes were washed and incubated with IRDye 800-conjugated secondary anti-rabbit antibody (1:2000; Rockland Immunochemicals, Gilbertsville, PA, USA) for 3 h. The membranes were washed again and imaged on an Odyssey Infrared Imager (Licor, Lincoln, NE, USA). The membranes were then incubated with mouse anti-actin antibody (1:8000; Calbiochem, La Jolla, CA, USA) overnight, washed, and incubated with IRDye 800-conjugated secondary anti-mouse antibody (1:8000, Rockland Immunochemicals) overnight. The membranes were washed and imaged again on the Odyssey Infrared Imager. The PP1 and actin images were analyzed using the Odyssey densitometry program (Licor). Data for PP1 were normalized to the actin loading control.

Statistical analysis

The real-time RT-PCR data from qBase, protein content from immunoblot, and progesterone EIA results were

analyzed by one-way ANOVA followed by Newman-Keuls *post hoc* test when a significant F was found (GraphPad Prism 4, San Diego, CA, USA). Significance was accepted at $P < 0.05$. Data are expressed as mean \pm S.E.M.

Results

The expression of the α catalytic subunit of PP2A was significantly greater in corpora lutea obtained on day 21 of pregnancy than on days 7, 14, and 20 of pregnancy, and on day 21 when the animals had been treated with progesterone on day 20 (21 + P; Fig. 1A). The expression of the β catalytic subunit of PP2A was significantly greater in corpora lutea obtained on day 21 than on all other days of pregnancy tested, including day 21 + P (Fig. 1B). The expression of the A structural subunit of PP2A was significantly greater in the corpora lutea from day 21 of pregnancy than in corpora lutea from day 20 of pregnancy (Fig. 2), but no other significant differences were observed.

The expression of the 53 kDa B regulatory subunit of PP2A was significantly greater in corpora lutea obtained on days 1, 3, 7, and 21 of pregnancy than on day 20 (Fig. 3A). The expression of the 53 kDa B regulatory subunit was also significantly greater on days 3 and 7 than on day 14. However, days 14 and 20 were not different from each other, and day 21 was not different from day 21 + P (Fig. 3A). The expression of the 55 kDa B regulatory subunit of PP2A was significantly greater in corpora lutea obtained on day 7 than on days 14 and 20 of pregnancy (Fig. 3B). The expression of the 55 kDa B regulatory subunit on each of days 1, 3, 21, and 21 + P was not different from any other days of pregnancy (Fig. 3B).

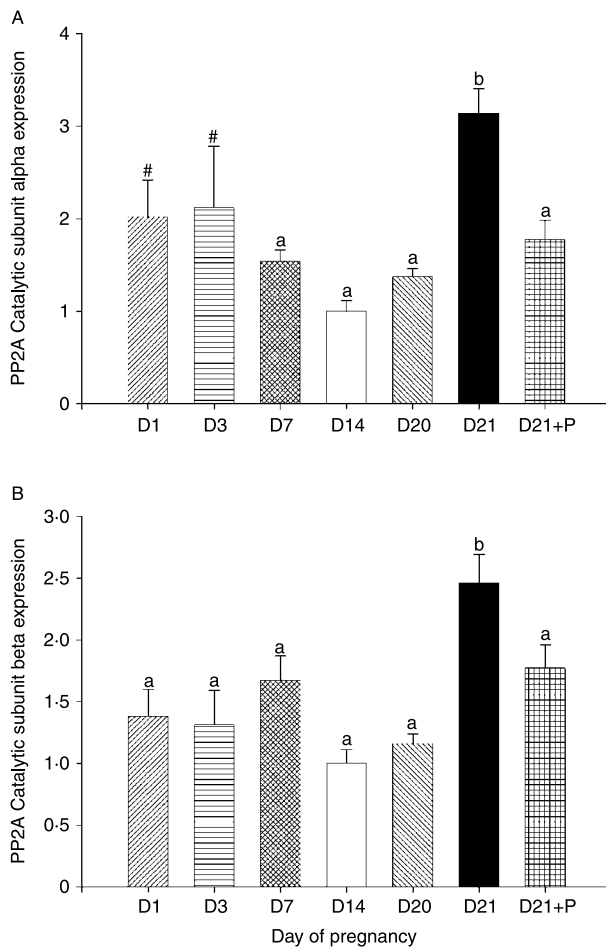


Figure 1 Expression of the two isoforms of PP2A catalytic subunits, α (A) and β (B) in the rat corpus luteum on days (D) 1, 3, 7, 14, 20, and 21 of pregnancy and on day 21 after progesterone was administered to pregnant rats on day 20 (D21+P). Real-time RT-PCR was used to analyze gene expression. Data are expressed as mean \pm S.E.M., $n=6$ for all groups. Statistical analysis utilized ANOVA followed by Newman-Keuls *post hoc* test. Bars with different letter superscripts denote that the data for those groups are significantly different from each other. Groups for which the means are not significantly different from any other group are designated by the symbol, #.

The expression of the 110 kDa B regulatory subunit/striatin of PP2A was significantly greater in corpora lutea obtained on days 7 and 21+P of pregnancy compared with day 14 (Fig. 3C), but the expression of striatin on each of days 1, 3, 20, and 21 was not significantly different from any other day of pregnancy tested (Fig. 3C). The data illustrate that the expression of all three B regulatory subunits of PP2A was significantly lower on day 14 than on day 7.

The gene expression of the catalytic subunit of PP1 was significantly greater in corpora lutea obtained on day 7 of pregnancy than on all other days of pregnancy tested (Fig. 4A). In addition, gene expression was significantly lower on day 20 than on days 1, 3, 7, and 21+P (Fig. 4A).

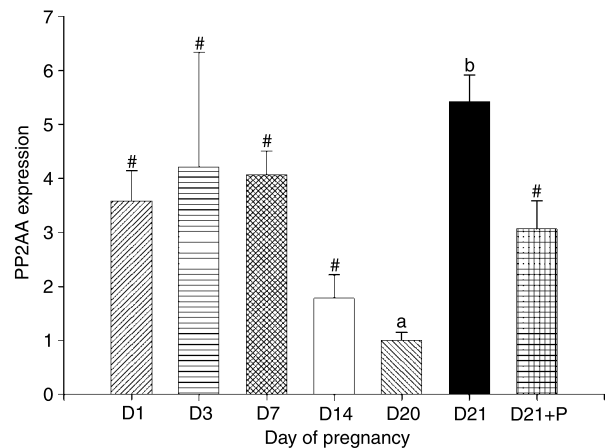


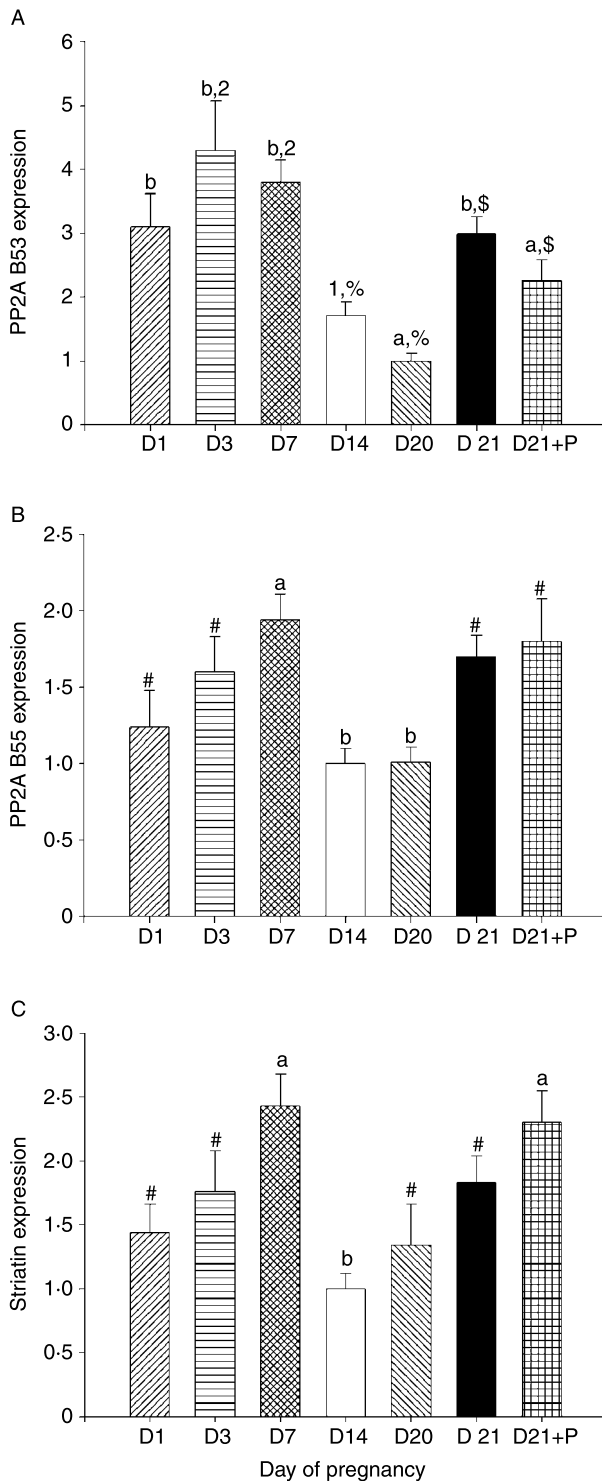
Figure 2 Expression of the A structural subunit of PP2A in the rat corpus luteum on days (D) 1, 3, 7, 14, 20, and 21 of pregnancy and on day 21 after progesterone was administered to pregnant rats on day 20 (D21+P). Real-time RT-PCR was used to analyze gene expression. Data are expressed as mean \pm S.E.M., $n=6$ for all groups. Statistical analysis utilized ANOVA followed by Newman-Keuls *post hoc* test. Bars with different letter superscripts denote that the data for those groups are significantly different from each other. Groups for which the means were not significantly different from any other group are designated by the symbol, #.

The expression of PP1 catalytic subunit protein was also examined (Fig. 4B). PP1 protein showed the same general pattern of expression as the PP1 gene. PP1 protein expression was significantly greater on day 7 than on day 20.

The plasma progesterone concentration rose between days 3 and 7, was not significantly different between days 7 and 20, then dropped significantly between days 20 and 21 (Fig. 5). Injection of progesterone (4 mg/kg) on day 20 returned progesterone concentrations on day 21 to the levels observed between days 7 and 20 (Fig. 5).

Discussion

Previous data from our laboratory demonstrated significant protein phosphatase activity in the rat CL (Eyster *et al.* 1993, 1995, 1998). The phosphatase was identified as the PP2A based on its ability to preferentially dephosphorylate certain specific substrates over others and based on the activity of protein phosphatase inhibitors (Cohen 1991) against the luteal protein phosphatase activity (Eyster *et al.* 1993, 1995). The protein phosphatase activity in the corpora lutea of the pregnant rat decreased between days 10 and 12 of pregnancy; enzyme activity levels then remained low through day 20 of pregnancy (Eyster *et al.* 1998). These data led us to hypothesize that the decline in enzyme activity was due to a decrease in the expression of the catalytic subunits of PP2A, or to an increase in one of the B regulatory subunits of PP2A. This hypothesis was based on the heterotrimeric structure of the PP2A holoenzyme in which the B regulatory subunits inhibit enzyme activity of the catalytic



subunits (Price & Mumby 2000, Janssens & Goris 2001). The data presented herein show that the mRNA expression of the PP2A subunits did not follow the predicted pattern. The expression of the catalytic subunits of PP2A and of PP2A A did not change at mid-pregnancy, and the expression of the B regulatory subunits fell significantly between days 7 and 14. In contrast, the expression patterns of PP1 gene and protein followed enzyme activity more closely than the expression patterns of PP2A and its subunits. These data suggest that PP1 may make a more substantial contribution to the protein phosphatase activity in the CL of the rat ovary of pregnancy than previously recognized.

Neither substrate preferences nor phosphatase inhibitor preferences are absolute markers of enzyme identity (Eyster 2000), so it is possible that our initial identification of the dominant protein phosphatase in the rat CL was inaccurate and PP1 is as important, or more important, than PP2A. An alternative explanation lies in the fact that this study examined mRNA expression rather than protein expression of PP2A subunits. Protein expression is not always a direct reflection of mRNA expression; thus, it is possible that the expression of the protein subunits of PP2A follow protein phosphatase enzyme activity more closely than the mRNA patterns. Antibodies are not available for all of the subunits of PP2A so this possibility was not tested. Other potential explanations for the difference between protein phosphatase enzyme activity and PP2A mRNA expression are potential splice variants of the B regulatory subunits of PP2A (Hemmings *et al.* 1990, Shenolikar & Nairn 1991, Akiyama *et al.* 1995, Tehrani *et al.* 1996, Nagase *et al.* 1997, Janssens & Goris 2001), phosphorylation of PP2A catalytic subunit (Chen *et al.* 1992, Guo *et al.* 1993, Shenolikar 1994), or inhibition of PP2A activity by ceramide 1-phosphate (Chalfant & Spiegel 2005) or the endogenous inhibitors, inhibitor 1 or inhibitor 2 of PP2A (I₁PP2A and I₂PP2A; Li *et al.* 1995). None of these regulatory mechanisms would have registered in this experimental design.

The mRNA expression of the α and β catalytic subunits and the A subunit of PP2A was highest on day 21 of

Figure 3 Expression of three forms of the B regulatory subunits of PP2A in the rat corpus luteum: the 53 kDa B subunit (A), the 55 kDa B subunit (B), and the 110 kDa subunit, also called striatin (C). Corpora lutea were obtained on days (D) 1, 3, 7, 14, 20, and 21 of pregnancy and on day 21 after progesterone was administered to pregnant rats on day 20 (D21 + P). Real-time RT-PCR was used to analyze gene expression. Data are expressed as mean \pm s.e.m., $n=6$ for A and B, $n=5$ for C. Statistical analysis utilized ANOVA followed by Newman-Keuls *post hoc* test. In A, significant differences between day 20 and other groups are designated by letters (a versus b). Significant differences between days 3, 7, and 14 are designated by numbers (1 vs 2). Within those comparisons, groups for which the means are significantly different are shown by different letter or number superscripts. In A–C, groups for which the means are not significantly different from each other are designated by the same symbol (\$, %), letter, or number, and groups for which the means are not significantly different from any other group are designated by the symbol, #.

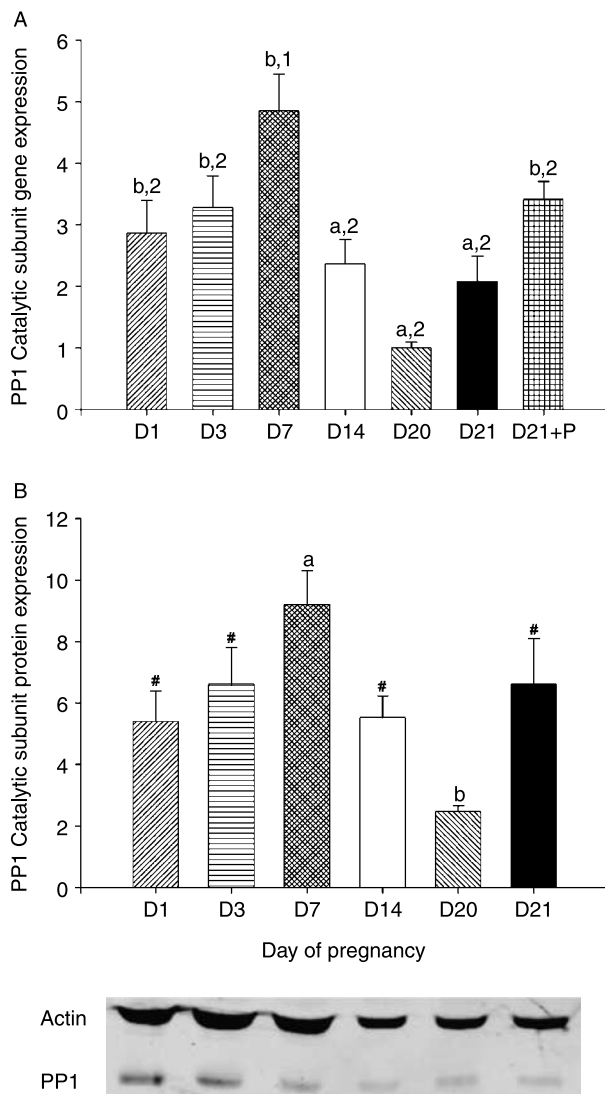


Figure 4 Gene and protein expression of the catalytic subunit of the type 1 protein phosphatase (PP1) in the rat corpus luteum. Corpora lutea were obtained on days (D) 1, 3, 7, 14, 20, and 21 of pregnancy, and on day 21 after progesterone was administered to pregnant rats on day 20 (D21 + P). Statistical analysis utilized ANOVA followed by Newman–Keuls *post hoc* test. (A) Real-time RT-PCR was used to analyze gene expression. Data are expressed as mean \pm S.E.M., $n=6$ for all groups. In A, number superscripts denote significant differences of day 7 compared with other groups, and letter superscripts denote significant differences of day 20 compared with other groups. Within those comparisons, bars with different numbers or letters denote that the data for those groups are significantly different from each other. (B) Analysis of protein expression of PP1 catalytic subunit on days 1, 3, 7, 14, 20, and 21 of pregnancy by immunoblot. Data are expressed in the bar graph as mean \pm S.E.M. for PP1 expression normalized to actin expression. The experimental $n=3$ for all groups. A representative immunoblot for PP1 and the loading control, actin, are also shown in B. For B, bars with different letters denote that the data for those groups are significantly different from each other. Groups for which the means were not significantly different from any other group are designated by the symbol, #.

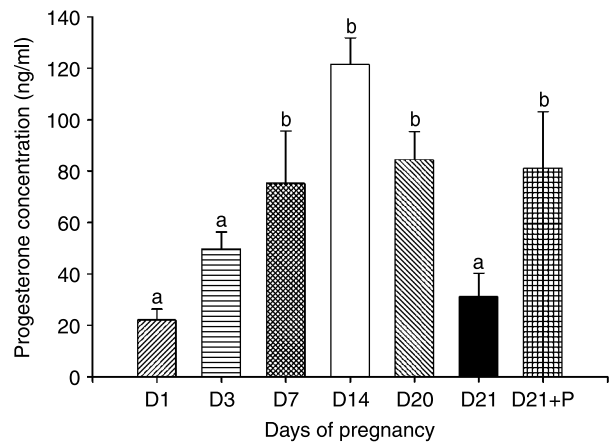


Figure 5 Plasma progesterone concentration on days (D) 1, 3, 7, 14, 20, and 21 of pregnancy, and on day 21 after progesterone was administered to pregnant rats on day 20 (D21 + P) was measured by EIA. Statistical analysis utilized ANOVA followed by Newman–Keuls *post hoc* test. Bars with different letter designations denote that the data for those groups are significantly different from each other. The experimental $n=5$ for all groups except $n=4$ for day 21.

pregnancy when progesterone levels had dropped. To test whether PP2A subunits were regulated by progesterone, a group of pregnant rats was treated with progesterone on day 20 and the corpora lutea were obtained on day 21. This artificial maintenance of progesterone concentration returned the expression of the α and β subunits of PP2A to the day 7–20 levels. These data suggest that progesterone may negatively regulate the expression of the α and β catalytic subunits of PP2A. In contrast to the catalytic subunits of PP2A, treatment with progesterone on day 20 did not significantly affect the expression of the A and B subunits of PP2A or the catalytic subunit of PP1 on day 21.

In summary, the expression patterns for PP2A subunits did not change at mid-pregnancy in accordance with enzyme activity patterns. Thus, the decline in protein phosphatase activity may be due to differential expression of protein versus mRNA, alternative splicing of the B regulatory subunits, inhibition of catalytic activity by phosphorylation or by inhibitory factors such as ceramide 1-phosphate, I_1 PP2A, or I_2 PP2A. Alternatively, PP1 may make a more substantial contribution to protein phosphatase activity in the rat CL of pregnancy than previously recognized, as its levels mimicked the enzyme activity profile more closely than did the isoforms and subunits of PP2A. An additional important observation in these studies was that the α and β catalytic subunits of PP2A were regulated by progesterone.

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