LH/chorionic gonadotropin signaling pathway involves protein tyrosine phosphatase activity downstream of protein kinase A activation: evidence of an obligatory step in steroid production by Leydig cells

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Abstract
Our recent reports indicate that protein tyrosine phosphorylation is an obligatory component of the mechanism of action of ACTH in its stimulatory action of corticosteroid production in adrenal zona fasciculata (ZF). The role of protein tyrosine phosphatase (PTP) activity in the regulation of steroidogenesis by LH/chorionic gonadotropin (CG) was tested using cell-permeable PTP inhibitors. Thus, PTP inhibition blocks LH- and 8-bromo-cAMP-stimulated testosterone production by Leydig cells without affecting 22(R)OH-cholesterol-supported steroidogenesis, similar results to those obtained in the adrenal ZF/ACTH system, leading us to propose that PTP action is an obligatory and common step in the cascade triggered by both hormones. Then, we continued the study testing whether LH modulates PTP activity in MA-10 cells, a Leydig cell line. In this regard, we observed by an in-gel PTP assay two PTPs of 110 and 50 kDa that are activated by hormone and 8-bromo-cAMP activation of the cells. Moreover, there is a transient increase by the second messenger in total PTP activity that correlates with the higher activity displayed by the 110 and 50 kDa proteins in the in-gel assay. In accordance with these results, analysis of tyrosine phosphorylated proteins showed the LH-induced dephosphorylation of proteins of 120, 68 and 50 kDa. The results of this study indicate that PTPs play an important role in the regulation of Leydig cell functions and that there exists a cross talk between serine/threonine phosphorylation and tyrosine dephosphorylation mediated by hormone-activated cAMP-dependent protein kinase and PTPs. These results are the first evidence of PTP having a role in LH/CG-stimulated steroidogenesis.

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Introduction
Protein tyrosine phosphatases (PTPs) comprise a structurally diverse family of enzymes which includes both transmembrane receptor-like and non-transmembrane forms (Charbonneau & Tonks 1992, Walton & Dixon 1993, Neel & Tonks 1997). Despite growing evidence suggesting important roles for PTPs in the regulation of a large number of diverse cellular events involved in growth, division and differentiation (Mauro et al. 1994, Florio et al. 1996, Zhang et al. 1999), little is known about the function of these enzymes in the mechanism of action of steroidogenic hormones.

We have recently described the involvement of PTP in the mechanism of action of adrenocorticotropin (ACTH) at a stage following cAMP-dependent protein kinase (PKA) activation and before cholesterol delivery to the inner mitochondrial membrane (Paz et al. 1999), the rate-limiting step of the pathway (Crivello & Jefcoate 1980, Privalle et al. 1983). Moreover, both in vivo treatment with ACTH and in vitro stimulation with 8-bromo-cAMP (8Br-cAMP), a permeable analog of the second messenger, increase PTP activity in the adrenal gland. Consequently, we postulate the existence of one or more tyrosine-phosphorylated proteins exerting an inhibitory action on steroidogenesis. Hormone action promotes the activation of PTPs and the dephosphorylation of those regulatory proteins, releasing the steroidogenic cascade. In order to test this hypothesis, studies of PTP activity were extended to another steroidogenic system, as Leydig cells, using fresh interstitial testicular and MA-10 cells, an established and well-characterized tumor Leydig cell line (Ascoli 1981, Stocco & Chaudhary 1990, Stocco & Ascoli 1993, Weiss-Messer et al. 1998). Testosterone production by Leydig cells in the testis is triggered by luteinizing hormone (LH) through cell surface receptors coupled to a
signaling transduction mechanism based on PKA-mediated phosphorylation (Dufau et al. 1977, Clark et al. 1994), a similar pathway to ACTH-activated cortico-steroid production in adrenal zona fasciculata (ZF) (Podestá et al. 1979, Sala et al. 1979).

PTP activity was found to be a requisite for hormonal stimulation of steroidogenesis in both systems (interstitial testicular and MA-10 cells). In addition, we detected PTP activation by a PKA-dependent mechanism, involving at least two PTPs (110 and 50 kDa). Moreover, hormone action produces the tyrosine dephosphorylation of endogenous proteins in MA-10 cells. These results are the first evidence of PTP having a role in LH/chorionic gonadotropin (CG)-stimulated steroidogenesis.

Materials and Methods

Materials

Purified human CG (hCG) (batch CR–127, potency 14 900 IU/mg) was a gift from the National Hormone and Pituitary Program, National Institute of Diabetes, Digestive and Kidney Diseases (National Institutes of Health, Bethesda, MD, USA). 22(R)-hydroxycholesterol, 8Br-cAMP, collagenase, BSA, methyl-isobutyl-xanthine, acrylamide, bis-acrylamide, phenylarsine oxide (PAO), sodium orthovanadate, poly(glutamic acid-tyrosine) random copolymer (4:1 ratio) (poly(Glu:Tyr)), catalase, epidermal growth factor (EGF) fragment 20–31, EGF receptor, insulin receptor (from rat liver) and insulin (from bovine pancreas) were from Sigma Chemical Co. (St Louis, MO, USA). $\gamma$-32P]ATP was from New England Nuclear (Boston, MA, USA). Cell culture supplies and supported nitrocellulose-1 membrane were obtained from Gibco-BRL Technologies (Rockville, MD, USA), and plasticware was from Corning-Costar (Corning, NY, USA). Specific polyclonal anti-phosphotyrosine antibody was purchased from Transduction Laboratories (Lexington, KY, USA). Electrophoresis supplies and second antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG) were from BioRad Laboratories (Hercules, CA, USA). Chemiluminescence detection was performed with reagents provided by Amersham Pharmacia Biotech (Little Chalfont, Bucks, UK). All other reagents were commercial products of the highest grade available.

Preparation of interstitial rat testis cells

Adult male Wistar rats (90 days old) were used throughout. Animals were killed by decapitation and testes rapidly removed and decapsulated. Isolated interstitial cells were prepared by collagenase dispersion of testes as previously described (Catt et al. 1974). Cells were resuspended in Medium 199 containing 1·2 g/l NaHCO3, 0·1 mM methyl-isobutyl-xanthine (a phosphodiesterase inhibitor), and 0·5% BSA, at a density of $10^6$ cells/ml and were maintained under a carbogen (95% O2:5% CO2) atmosphere throughout the entire procedures.

Cell culture of MA-10 Leydig cells

MA-10 Leydig cells were handled as originally described (Ascoli 1981). MA-10 is a clonal strain of cultured mouse Leydig tumor cells that produces progesterone rather than testosterone as the major steroid. The cell line, generously provided by Dr Mario Ascoli (University of Iowa College of Medicine, Iowa City, IA, USA), was kept growing in 75 cm2 flasks and experiments were performed in 6 × 35-mm well plates. The growth medium consisted of Waymouth MB752/1 containing 1·1 g/l NaHCO3, 20 mM Hepes, 50 µg/ml gentamycin and 15% horse serum. Flasks and well plates were maintained at 36 °C in a humidified atmosphere containing 5% CO2.

Conditions for incubation of testicular interstitial and MA-10 cells with PTP inhibitors

Testicular interstitial cells ($10^6$ cells) were incubated with hCG, 8Br-cAMP or 22(R)OH-cholesterol at final concentrations of 2 ng/ml, 10 µM and 5 µM respectively. Stimulation of MA-10 cells was performed with hCG, 8Br-cAMP or 22(R)OH-cholesterol at final concentrations of 20 ng/ml, 1 mM and 50 µM respectively, in serum-free medium supplemented with 1 mg/ml BSA.

In both cases, stimulations were performed in the presence or absence of two cell-permeable PTP inhibitors, PAO and pervanadate (PV) (Garcia Morales et al. 1990, Secrist et al. 1993). PV was generated by oxidizing vanadate with equimolar quantities of the oxidant agent, hydrogen peroxide (H2O2), using catalase to remove H2O2 excess, as described by Evans et al. (1994).

Following the appropriate additions, incubation of interstitial cells proceeded for 3 h at 37 °C with gentle shaking, while MA-10 cells were placed in an incubator in 5% CO2 at 36 °C for 1 h, when steroid production was evaluated. Shorter periods (10 and 20 min) were also assayed when assessing PTP activity in MA-10 cells. Media and cells were collected at the end of each experiment. Media were stored at −20 °C until steroid determination. Testosterone and progesterone contents were determined in the incubation media of interstitial and MA-10 cells respectively, by RIA. MA-10 cells were kept in order to perform Western blots and to determine PTP activity.

Preparation of sonicates and subcellular fractionation

The MA-10 cell cultures were washed with PBS and scraped into 10 mM Tris, pH 7.4, 1 mM sodium orthovanadate (Western blot analysis of phosphotyrosine

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content) or 25 mM imidazole, pH 7.4, 0.1% 2-mercaptoethanol (total PTP activity determination) with the help of a rubber policeman. Sonicates were obtained by three 15 s cycles in a Branson 250 sonicator (Branson Ultrasonics Corp., Danbury, CT, USA) at 70 W and stored at −20 °C.

In order to perform the subcellular fractionation, the cell cultures were washed with PBS, scrapped into 10 mM Tris, pH 7.4, 250 mM sucrose, 0.1 mM EDTA, homogenized in a glass Teflon homogenizer, and centrifuged at 600 g for 15 min. A second centrifugation at 10 000 g for 15 min gave a supernatant in which PTP activity was assessed as total PTP activity or in the in-gel assay.

Western blot analysis

Western blotting was used to test phosphotyrosine content in sonicates of MA–10 cells. Proteins (80 µg) were separated by SDS-gel electrophoresis on 10% acrylamide gels as described by Laemmli (1970) and electrophoretically transferred to nitrocellulose membranes according to the procedure described by Towbin et al. (1979). Membranes were blocked in 20 mM Tris–HCl pH 7.4, 500 mM NaCl containing 0.5% Tween 20 (TTBS), and 0.1% BSA (blocking solution) and then incubated overnight with polyclonal anti-phosphotyrosine antibodies in blocking solution. Membranes were washed five times in TTBS prior to incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG for 2 h. Immunoreactive bands were detected by enhanced chemiluminescence.

Determination of total PTP activity

32P-poly(Glu:Tyr) was used as substrate in the total PTP activity determination and in the in-gel assay. Poly-(Glu:Tyr) (0.3 mg) was labeled with [32P]phosphate using commercially available EGF and insulin receptors as tyrosine kinases (2.5 U EGF receptor and 17.5 U insulin receptor, in a final volume of 0.17 ml) following the procedure described by Burridge & Nelson (1995).

Total PTP activity was measured by the release of [32P]orthophosphate from phosphorylated poly(Glu:Tyr) in a 60 µl reaction mixture containing 25 mM imidazole, pH 7.4, 0.1% 2-mercaptoethanol, 1 mg/ml BSA, 20 000 c.p.m. 32P-poly(Glu:Tyr) (specific activity 2 × 107 c.p.m./mg), and the sample (2 µg proteins). The reaction was allowed to proceed for 10 min at 30 °C then stopped by addition of 180 µl ice-cold 20% trichloroacetic acid (TCA) and 20 µl 25 mg/ml BSA as a carrier. The liberated inorganic [32P]phosphate was assessed in a scintillation counter as TCA-soluble radioactivity. One unit of PTP activity is defined as the amount which releases 1 nmol phosphate/min at 30 °C.

In-gel PTP assay

An in-gel PTP assay, using 32P-poly(Glu:Tyr) as substrate, was used to identify the hormonally regulated PTP(s) in MA–10 cells. The 32P-poly(Glu:Tyr) was incorporated into the regular polyacrylamide gel mixture prior to polymerization at approximately 105 c.p.m./ml. SDS-PAGE was performed as described by Laemmli (1970) on 10% acrylamide gels. PTP activity was detected in-gel, following the procedure described by Burridge & Nelson (1995). Briefly, after separating the samples (10–20 µg of proteins per lane) by electrophoresis, the SDS was removed, the gel was first incubated in denaturing buffer (6 M guanidine chloride, 0.3% 2-mercaptoethanol, 50 mM Tris–HCl, pH 8), and then in renaturating buffer (50 mM Tris–HCl, pH 8–0, 1 mM EDTA, 0.04% Tween 20, 0.3% 2-mercaptoethanol and 4 mM dithiothreitol). After drying, the gel was exposed to X-ray film.

Proteins with PTP activity become evident as clear bands on a dark background due to the removal of inorganic [32P]phosphate from the radiolabeled substrate incorporated into the matrix gel. This method has been demonstrated to be specific for PTP activity based on the PTP-specific substrate incorporated into the gels and the loss of the signal by addition of PTP inhibitors during the development of the enzymatic activity (Burridge & Nelson 1995).

Protein determination

Protein concentration was determined by the method of Lowry et al. (1951), using BSA as standard.

Statistical analysis

Each experiment was performed at least four times under identical conditions, analyzed separately, and a representative experiment is shown in the figures. Values represent the means ± s.d. of determinations from triplicate wells or tubes in the same experiment. Statistical analysis of the data was performed using ANOVA, with P<0.05 considered significant. Western blots and in-gel PTP assays were performed at least three times and a representative result is shown in the figures.

Results

Effect of PAO and PV on steroidogenesis in Leydig cells

The involvement of PTP in the steroidogenic response of Leydig cells was tested by incubation of interstitial rat testis and MA–10 cells with two powerful cell-permeable PTP inhibitors (PAO and PV), followed by evaluation of steroid production upon stimulation by hCG and 8Br-cAMP (permeable cAMP analog).

Freshly isolated interstitial cells (106 cells/tube) were incubated with various concentrations of the inhibitors, PAO and PV, together with hCG or 8Br-cAMP, and testosterone production was measured (Fig. 1). Results show a dose-dependent inhibitory effect of PAO on
hormone and 8Br-cAMP actions, reaching 100% inhibition in both cases (Fig. 1A). PV also inhibited steroid production, reaching 70% inhibition at the maximal concentration used (Fig. 1B). Higher concentrations of this PTP inhibitor extended the inhibition of agonist-induced steroid production to almost basal levels. However, the use of PV in concentrations beyond those shown in Fig. 1B also produced an inhibition of mitochondrial steroidogenic enzymes. Such concentrations are unsuitable to explore
Coomassie Blue staining of the gel. Analyzed by in-gel PTP assay. Molecular masses of hormonally active proteins obtained from control (lane 1), hCG (lane 2), hCG plus 0·5 µM PAO (lane 3) and 8Br-cAMP (lane 4) treated cells were analyzed by in-gel PTP assay. Molecular masses of hormonally activated PTPs are indicated by the arrows on the right. Inset, Coomassie Blue staining of the gel.

![Gel Image](image)

Figure 3: Electrophoretic profile of PTP in MA-10 cells. Autoradiography of a representative 13·P-poly(Glu:Tyr)-containing gel processed to develop PTP activity. Twenty micrograms of proteins obtained from control (lane 1), hCG (lane 2), hCG plus 0·5 µM PAO (lane 3) and 8Br-cAMP (lane 4) treated cells were analyzed by in-gel PTP assay. Molecular masses of hormonally activated PTPs are indicated by the arrows on the right. Inset, Coomassie Blue staining of the gel.

The effects of PV on LH/CG regulation of the rate-limiting step. Finally, neither of the two inhibitors affected basal steroidogenesis (Fig. 1A and B).

The effects of PTP inhibitors on hCG- and 8Br-cAMP-stimulated progesterone production in MA-10 cells (2 × 10⁶ cells/35-mm well) were also studied. Following preincubation (10 min) with the inhibitors, hCG or 8Br-cAMP was added to the medium and stimulation proceeded for 1 h. PAO and PV significantly inhibited both hCG- and 8Br-cAMP-induced progesterone production; a concentration of 2 µM PAO inhibited 95% of hCG- and 8Br-cAMP-stimulated progesterone production seen in cultures in the absence of the inhibitor (Fig. 2A). The second PTP inhibitor, PV, also inhibited both hCG- and 8Br-cAMP-induced progesterone production (Fig. 2B) by MA-10 cells. Addition of PTP inhibitors alone to MA-10 Leydig cells had no significant effect on progesterone production (Fig. 2A and B).

In order to check the inhibitory capacity of PAO and PV on MA-10 cells, total PTP activity was determined as described in Materials and Methods on sonicates of the cells obtained after the incubation in the presence and absence of PAO and PV. An incubation time as short as 10 min with 1 µM PAO or 5 µM PV was enough to inhibit PTP activity by 50 and 35% respectively. These results confirm that these two compounds are in fact acting in intact cells, as already described (García Morales et al. 1990, Secrist et al. 1993, Huyer et al. 1997).

22(R)OH-cholesterol-supported steroid production was used to determine whether the inhibitory effects of PAO and PV on steroid production might be due to inhibition of the activities of the mitochondrial steroidogenic enzymes. There was no significant difference in steroid production among the treatments when 22(R)OH-cholesterol was used as substrate (Figs 1 and 2), indicating that the inhibition described above is localized upstream of the cholesterol side-chain cleavage.

The results of steroid production by Leydig cells obtained using PTP inhibitors suggest the involvement of these enzymes in the signal transduction pathway of LH/CG. Moreover, analysis of the 8Br-cAMP-stimulated and 22(R)OH-cholesterol-supported steroidogenesis allows the restriction of the site of action of PTP in the signal transduction pathway to a point located after PKA activation, and before cholesterol delivery to the inner mitochondrial membrane.

Subsequent studies focused on obtaining evidence of the potential activation of PTP in LH/CG-stimulated androgenesis. Two strategies were followed: evaluation of both PTP activity and phosphotyrosine content.

Effect of hCG and 8Br-cAMP on PTP activity in MA-10 Leydig cells

An in-gel assay was used to determine the PTP profile of MA-10 Leydig cells, and to evaluate the modulation of the activity on individual PTP after hCG and 8Br-cAMP action (10 min). Multiple bands were detected in MA-10 Leydig cells, indicating the presence of several PTPs (Fig. 3, lane 1), two of which (molecular masses of 110 and 50 kDa) were hormonally stimulated (Fig. 3, lane 2 vs lane 1). Incubation of the cells with PAO abolished the observed bands (Fig. 3, lane 3), indicating again the effective inhibitory action of this compound in intact cells. The fact that 8Br-cAMP produces a similar profile of activated PTPs to that obtained with the hormone (Fig. 3, lane 4 vs 2) confirms that PTP activation is mediated by PKA action. Indeed, 8Br-cAMP stimulation of the cells produces a transient increase in total PTP activity determined in the 10 000 g supernatants of MA-10 cells (Fig. 4A). This effect on total PTP activity correlates with the higher signal of the 110 and 50 kDa bands displayed by the same samples in the in-gel assay (Fig. 4B). Thus, the increase in total PTP activity may be attributed at least to these two PTPs.

hCG-dependent dephosphorylation of endogenous tyrosine-phosphoproteins in MA-10 Leydig cells

Further evidence that there is hormone-dependent activation of PTP in Leydig cells was obtained comparing phosphotyrosine–phosphoprotein profiles in samples of MA-10 cells. Western blots developed with anti-phosphotyrosine antibodies revealed that hCG treatment decreased the phosphotyrosine signal in several bands. Endogenous proteins of 120, 68 and 50 kDa showed reduced phosphotyrosine contents after hCG treatment (Fig. 5, lane 2 vs lane 1). Cell treatment with...
PAO blocked the effects of hCG (Fig. 5, lane 3 vs lane 2), indicating again that this compound effectively inhibits PTP in intact cells.

**Discussion**

Protein phosphorylation in serine/threonine residues is a primary mode of regulation of hormone-stimulated steroidogenesis (Dufau et al. 1977, Sala et al. 1979, Clark et al. 1994). Recently, we demonstrated that the ACTH signaling pathway also includes tyrosine dephosphorylation (Paz et al. 1999). The present study provides evidence that tyrosine dephosphorylation would be an essential event in the signaling pathway of steroidogenic hormones; we demonstrate that LH- and cAMP-stimulated steroidogenesis is dependent on PTP activity. In addition, LH increases the activity of two PTPs, and

![Figure 4](image.png)

**Figure 4** Effect of 8Br-cAMP on PTP activity in MA-10 cells. Total PTP activity determination (A) (values represent the means ± S.D. of triplicates) and in-gel PTP assay (B) were performed with the 10,000 g supernatants of MA-10 cells after incubation with 8Br-cAMP (1 mM) for different periods: 0 min (lane 1), 10 min (lane 2), 20 min (lane 3) and 60 min (lane 4). In (B), the upper section shows the 110 kDa band and the lower section shows the 50 kDa band. Coomassie Blue staining of the complete 32P-poly(Glu:Tyr) gel is shown in the bottom part of (B). ***P<0·001, **P<0·05 vs control, with ANOVA followed by a Tukey test.
induces tyrosine dephosphorylation of endogenous proteins in MA-10 Leydig cells.

The fact that PTP inhibition by cell-permeable inhibitors blocks hCG- and 8Br-cAMP-stimulated steroidogenesis without affecting 22(R)OH-cholesterol-supported steroidogenesis indicates that PTP activity plays a crucial role in the hormonal regulation of the rate-limiting step in the steroidogenic pathway. The possibility that the observed effect is due to a toxic action of PTP inhibitors can be ruled out, since they do not affect either cell viability or the mitochondrial enzymes involved in steroid synthesis. The effect can be attributed to PTP inhibition since these compounds indeed reduce PTP activity in MA-10 cells as seen by the PTP activity determination and the in-gel assay. These methods also provided evidence of PTP activation by LH: an analog of the second messenger produced a rapid increase in total PTP activity and two of the PTPs present in the cells displayed higher activity by hormone and cAMP stimulation. In adrenal ZF, we detected three ACTH-activated PTPs, two of which migrate with molecular masses similar to the two hCG-activated PTPs (Paz et al. 1999).

Based on the results obtained with the second messenger analog, 8Br-cAMP, we suggest that PKA-mediated phosphorylation is involved in the PTP activation process. In this regard, the regulation of PTPs in Leydig cells by LH seems to involve a mechanism similar to the regulation of adrenal ZF PTPs, since in this latter tissue we described an increase of total PTP activity under 8Br-cAMP stimulation (Paz et al. 1999). Moreover, we recently demonstrated that the activity of one of the ACTH-activated PTPs (115 kDa) is modulated by in vitro phosphorylation with PKA (Paz et al. 2000). In addition, it was recently demonstrated that the ACTH-dependent activation of PTP1D in bovine adrenal ZF involves serine phosphorylation (Rocchi et al. 2000).

Another finding supporting PTP activation by LH is that this stimulus induces endogenous dephosphorylation of tyrosine residues of Leydig cell proteins. It is particularly noteworthy that one of the hormonally dephosphorylated proteins migrates with a molecular mass similar to that of paxillin (approximately 68 kDa), a cytoskeletal protein, described as being dephosphorylated by ACTH in Y1 cells (an adrenal cortical tumor cell line), bovine and rat adrenal ZF (Han & Rubin 1996, Vilgrain et al. 1998, Paz et al. 1999). Western blot analysis of paxillin, with either sonicates or immunoprecipitates of the samples with antipaxillin antibodies, repeatedly showed changes in the intensity of the signal and in the electrophoretic mobility by treatment of the cells with the agonist (data not shown). These changes are associated with highly phosphorylated proteins when they become dephosphorylated (Flint et al. 1993, Shifrin et al. 1997). Unfortunately, assays to detect a change in phosphotyrosine content in paxillin immunoprecipitates were not successful.

Figure 5 Phosphotyrosine profile in MA-10 cells. Eighty micrograms of proteins obtained from control (lane 1), hCG- (lane 2) and hCG plus 0.5 μM PAO- (lane 3) treated cells were analyzed by Western blotting with anti-phosphotyrosine antibody. Molecular masses of hormonally tyrosine-dephosphorylated proteins are indicated by the arrows on the right. Ponceau red staining of the nitrocellulose membrane is shown on the extreme right.
According to the results presented in this report, we conclude that PTPs are hormonally activated in Leydig cells through a mechanism that involves PKA activation, that they belong to the steroidogenic activating pathway and that they act at a point located before cholesterol delivery to the inner mitochondrial membrane. Consequently, our hypothesis is that there is at least one protein basally phosphorylated in tyrosine that acts negatively on steroid synthesis. This phosphoprotein would have to be dephosphorylated by hormone-activated PTP(s) in order to activate steroidogenesis. Based on these and previous results, we postulate that PTP activation is an obligatory event in hormone-induced steroidogenesis. In order to elucidate the mechanism by which tyrosine dephosphorylation is involved in steroid production, our current efforts are directed to evaluate the relationship between PTP and steroidogenic acute regulatory protein (StAR), widely characterized as a regulatory protein in hormone-activated steroidogenesis.

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