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PKA tightly bound to human placental mitochondria participates in steroidogenesis and is not modified by cAMP

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ABSTRACT

Introduction: Protein phosphorylation plays an important role in the modulation of steroidogenesis and it depends on the activation of different signaling cascades. Previous data showed that PKA activity is related to steroidogenesis in mitochondria from syncytiotrophoblast of human placenta (HPM). PKA localization and contribution in progesterone synthesis and protein phosphorylation of HPM was assessed in this work.

Methods: Placental mitochondria and submitochondrial fractions were used. Catalytic and regulatory PKA subunits were identified by Western blot. PKA activity was determined by the incorporation of ^{32}P into proteins in the presence or absence of specific inhibitors. The effect of PKA activators and inhibitors on steroidogenesis and protein phosphorylation in HPM was tested by radioimmunoassay and autoradiography.

Results: The PKAα catalytic subunit was distributed in all the submitochondrial fractions whereas βII regulatory subunit was the main isoform observed in both the outer and inner membranes of HPM. PKA located in the inner membrane showed the highest activity. Progesterone synthesis and mitochondrial protein phosphorylation are modified by inhibitors of PKA catalytic subunit but are neither sensitive to inhibitors of the regulatory subunit nor to activators of the holoenzyme.

Discussion: The lack of response in the presence of PKA activators and inhibitors of the regulatory subunit suggests that the activation of intramitochondrial PKA cannot be prevented or further activated.

Conclusions: The phosphorylating activity of PKA inside HPM could be an important component of the steroidogenesis transduction cascade, probably exerting its effects by direct phosphorylation of its substrates or by modulating other kinases and phosphatases.

1. Introduction

Steroidogenesis can be acutely or chronically regulated depending on the tissue and it may occur in the order of minutes and hours, respectively [1]. In acutely regulated tissues such as gonads, the response under the control of trophic hormones, initiates with the mobilization and delivery of cholesterol from the outer to the inner mitochondrial membrane, where it is converted to pregnenolone by the cytochrome P450 side chain cleavage enzyme (P450scc) [2]. In tissues like brain and placenta, the chronic response includes genomic events that initiate within hours after stimuli and result, in general, in a higher expression of enzymes related to steroidogenesis [3]. In the case of adrenals, adrenocorticotropic hormone (ACTH) promotes steroidogenesis over the course of days by increasing the transcription of genes for steroidogenic enzymes. In addition, on a 15–60 min time scale, the same hormone, via cAMP, stimulates the phosphorylation of proteins like the Steroidogenic Acute Regulatory protein (StAR) [4], which is needed for the transfer of cholesterol from the outer to the inner mitochondrial membrane [5].

Both acute and chronic regulations are under the control of factors or hormones that can activate diverse signaling cascades mediated or not by cyclic adenosine monophosphate (cAMP) dependent protein kinase (PKA). However, it has been reported that the induction of steroidogenesis independently of cAMP is less than 1% compared to the cAMP-PKA dependent pathway [1].

The most studied pathway in steroidogenic tissues is mediated by cAMP/PKA, where the stimulation by trophic hormones induces the activation of the enzyme adenylate cyclase, which produces the second messenger, cAMP, which in turn binds to PKA.

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When inactive, PKA is assembled as a tetrameric complex consisting of two regulatory (R) and two catalytic (C) subunits. The regulatory subunits have been described as scaffold proteins; however, they also serve as inhibitors of the C subunits [6]; since the holoenzyme dissociates in response to cAMP and in consequence the active C subunits are released [7]. Two major regulatory isoforms of PKA are known, RI and RII, and the classification is based on the type of the R subunit which binds to A-kinase anchoring proteins (AKAPs) with distinct levels of affinity [8]. Four different genes encoding for the regulatory subunits RIα, RIβ, RIIα and RIIβ, and three genes encoding for the catalytic subunits Cα, Cβ and Cγ [9] have been described. RIα and RIIα are ubiquitously distributed whereas RIβ and RIIβ are predominantly expressed in various endocrine tissues such as brain and fat [7,10].

It is known that PKA phosphorylates proteins like StAR as well as transcription factors associated with the activation of genes related to steroidogenesis [11]. In this sense, it is important to point out that disregarding the signaling cascade activated; the events of phosphorylation/dephosphorylation of proteins play an important role in steroidogenesis [12–14].

The human placenta has been recognized as a chronic response tissue, since there are no substantial short term fluctuations in maternal progesterone during pregnancy [15]. Besides, human placenta does not express the StAR protein, instead, it expresses MLN64, a protein that shares homology with StAR protein and is in charge of maintaining a non-limiting supply of cholesterol for placental progesterone synthesis [16–20]. Mitochondria isolated from human placental syncytiotrophoblast are a useful model to quantify the synthesis of progesterone and concomitantly observe the manifestation of post translational events within minutes (i.e protein phosphorylation).

Protein phosphorylation is an important event for placental steroidogenesis. Previous data obtained by us and other groups working on human placenta and different tissues, have clearly shown that PKA activity is highly regulated and related to steroidogenesis; since the kinase inhibitor H89 decreases progesterone synthesis and increases protein phosphorylation in isolated mitochondria from syncytiotrophoblast of human placenta (HPM) as well as in BeWo cells [21,22].

Even though it has been reported that human placental steroidogenesis can be regulated by luteinizing hormone (LH) and human chorionic gonadotropin hormone (hCG) [3] it still remains poorly understood the role of other key hormones that control cAMP levels [20] as wells as the interplay between the cytosolic signaling cascade mediated by cAMP/PKA and a putative complete intramitochondrial transduction pathway also possibly mediated by the phosphorylating activity of PKA upon specific targets related to progesterone synthesis. The knowledge of the signaling cascades that activate this biosynthetic process will help understand the fine tuning by which the human placenta synthesizes and releases increasing plasmatic concentrations of progesterone throughout pregnancy [20].

In order to assess the overall contribution of PKA in progesterone synthesis and protein phosphorylation in syncytiotrophoblast mitochondria, we searched for the distribution and activity of PKA in different submitochondrial fractions. The PKA Cα subunit has already been identified in mitochondria isolated from human placenta [23] and specifically in both the outer and inner membranes [23].

In this work, we found that the Cα subunit located in the inner membrane has the highest activity. RIIβ subunit was the main isoform detected in both the outer and the inner membranes. Progesterone synthesis was sensitive to inhibitors of the C subunit of PKA but it was insensitive to inhibitors of the R subunit or activators of the holoenzyme. The results suggest that an active intramitochondrial PKA (tightly bound to the membrane fraction) participates in the modulation of steroidogenesis, and this kinase does not appear to be susceptible of being further activated by cAMP in the context of steroidogenesis and even more, its activation cannot be prevented by using inhibitors of the holoenzyme.

2. Materials and methods

2.1. Isolation of human syncytiotrophoblast mitochondria

Following governmental and ethics committee approval, placentas were obtained from the IMSS No 4 Gynecology and Obstetrics hospital. Full-term human placentas from healthy women were collected immediately after normal delivery and processed in the next 30 min. Mitochondria from placental syncytiotrophoblast were prepared as previously reported [24] with modifications previously implemented [25] in order to assure the isolation of syncytiotrophoblast mitochondria. To estimate the contamination degree of mitochondria with other cellular fractions, the activity of various enzyme markers was determined as previously reported by Martinez et al. [26]. Lactate dehydrogenase activity in HPM was less than 10%, and the contamination with plasma membrane was less than 5% [27].

2.2. Mitochondrial oxygen consumption

To assure mitochondrial integrity, oxygen uptake was estimated polarographycally using a Clark type electrode in a reaction mixture containing 250 mM sucrose, 10 mM HEPES, 1 mM EGTA, 1 mM EDTA, 10 mM succinate, 10 mM K_2HPO_4, 5 mM $MgCl_2$ and 0.2% bovine serum albumin (BSA) at pH 7.4 in a final volume of 1.2 ml at 37 °C and 1 mg/ml of syncytiotrophoblast mitochondrial protein. Oxygen consumption was stimulated by the addition of 300–500 nmol ADP [28]. Mitochondria with respiratory control higher than four were used.

2.3. Preparation of submitochondrial fractions

A swelling and shrinking procedure was performed to isolate the outer and inner mitochondrial membrane fractions with modifications to the method previously reported [29]. Briefly, 20–25 mg of mitochondrial protein was incubated for 20 min in an ice bath with 10 mM H_3PO_4, adjusted to pH 7.3 with Tris base in the presence of 10 μg/ml aprotinin, 1 mM phenylmethylsulfonylfluoride and 10 μg/ml leupeptin. After incubation, sucrose was added to attain a concentration of 0.382 M and the mixture was incubated for 20 min in an ice bath. The mixture was centrifuged at 12,500 × *g* for 10 min at 4 °C. The pellet contained the mitoplasts and the supernatant contained the outer mitochondrial membranes. The supernatant was centrifuged at 137,000 × *g* for 1 h to obtain the outer membranes in the pellet, and in the supernatant remained the soluble proteins of the intermembrane space. The mitoplasts were incubated in 1 mM H_3PO_4, adjusted to pH 7.3 with Tris base for 20 min in an ice bath. Then, sucrose was added to reach a concentration of 0.31 M and the mixture was incubated for 20 min in an ice bath and centrifuged at 102,000 × *g* for 1 h. The pellet contained the inner membranes and the supernatant contained the soluble proteins from the matrix. Matrix and intermembrane fractions were concentrated together by using an Amicon® Ultra-15 Centrifugal Filter Device. Where indicated, submitochondrial fractions were washed with a solution containing 2 M KCl. Submitochondrial fractions were used immediately or kept at −70 °C until they were used. With this procedure, inner and outer membrane enriched fractions, as well as outer membranes joined at the contact sites to inner membrane fractions are obtained [29]. The enzyme activities of succinate dehydrogenase (SDH), monoamino oxidase (MAO), adenylate kinase (ADK) and creatine kinase as markers of the inner mitochondrial membrane, outer mitochondrial membrane, intermembrane space and contact sites, respectively, have already been assessed in the inner and outer membrane fractions [30]. However, in order to assure that we are obtaining inner and outer membrane enriched fractions, we assessed the enzyme activities of the chain respiratory complexes I and II as described below.

2.4. Activity determination of complex I and II from HPM and membranes

Activities of complex I (NADH:DCPIP oxidoreductase) and complex II (succinate:DCPIP oxidoreductase) were determined spectrophotometrically at 600 nm by following the reduction of the artificial electron acceptor 2,6-dichlorophenolindophenol (DCPIP; 50 μM; $\epsilon_{DCPIP} = 21\ mM^{-1}\ cm^{-1}$). HPM, inner or outer membranes were permeabilized with 0.3% Zwittergent 3–14 and incubated in 30 mM KH_2PO_4, 5 mM $MgCl_2$, 1 mM EGTA, 120 mM KCl, pH 7.4, and either 100 μM NADH (complex I) or 10 mM succinate (complex II). Complex II was activated by preincubation in the presence of 0.2 mM phenazinemethosulfonate (PMS) during 2 min at 25 °C. The reaction was started by the addition of NADH or succinate. The final relationship was 1 mg of mitochondrial protein/ml.

2.5. Mitochondrial progesterone synthesis

Progesterone synthesis was determined in a medium consisting of 120 mM KCl, 10 mM MOPS pH 7.4, 0.5 mM EGTA, 10 mM isocitrate, 4 μg of aprotinin/ml, 1 μM leupeptin, 5 mM K_2HPO_4 pH 7.4 and 1 mg/ml of mitochondrial protein, in a final