The International Journal of Biochemistry & Cell Biology 43 (2011) 1402–1411

Contents lists available at ScienceDirect

The International Journal of Biochemistry & Cell Biology

journal homepage: www.elsevier.com/locate/biocel

Progesterone synthesis by human placental mitochondria is sensitive to PKA inhibition by H89

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ARTICLE INFO

Article history:
Received 4 March 2011
Received in revised form 27 May 2011
Accepted 6 June 2011
Available online 12 June 2011

Keywords:
Steroid biosynthesis
Protein kinase A
Human placenta
Syncytiotrophoblast mitochondria
Phosphorylation/dephosphorylation system

ABSTRACT

The transfer of cholesterol to mitochondria, which might involve the phosphorylation of proteins, is the rate-limiting step in human placental steroidogenesis. Protein kinase A (PKA) activity and its role in progesterone synthesis by human placental mitochondria were assessed in this study. The results showed that PKA and phosphotyrosine phosphatase D1 are associated with syncytiotrophoblast mitochondrial membrane by an anchoring kinase cAMP protein-121. The ^{32}P-labeled of four major proteins was analyzed. The specific inhibitor of PKA, H89, decreased progesterone synthesis in mitochondria while in mitochondrial steroidogenic contact sites protein-phosphorylation was diminished, suggesting that PKA plays a role in placental hormone synthesis. In isolated mitochondria, PKA activity was unaffected by the addition of cAMP suggesting a constant activity of this kinase in the syncytiotrophoblast. The presence of PKA and phosphotyrosine phosphatase D1 anchored to mitochondria by an anchoring kinase cAMP protein-121 indicated that syncytiotrophoblast mitochondria contain a full phosphorylation/dephosphorylation system.

1. Introduction

Protein phosphorylation/dephosphorylation by protein kinases and protein phosphatases is a ubiquitous mechanism in eukaryotes and procaryotes to modulate the activity of some intracellular proteins (Krebs, 1994). This phosphorylation/dephosphorylation system has a specific cell distribution and responds to extracellular signals. Among all protein kinases, cAMP-dependent protein kinase (PKA) has been classified into two types: a soluble form which is found in the cytoplasm (Type I) and a form associated to some cellular membranes or organelles like mitochondria (Type II). This organelle association is mediated by a cAMP-dependent A-kinase anchor protein called AKAP (Feliciello et al., 2001; Affaitati et al., 2003; Carlucci et al., 2008).

In this sense, the phosphorylation of several mitochondrial proteins has been described: the α-subunit of pyruvate dehydrogenase and the α-subunit of branched-chain 2-oxoacid dehydrogenase complex, both located in the mitochondrial matrix (Bradford, 1986); four phosphoproteins in the inner membrane: the 18 kDa AQDQ subunit of complex I (Papa et al., 1996); the 17 kDa subunit IV of cytochrome c oxidase (Steenaart and Shore, 1997); and the 22 kDa NP-subunit and the 28 kDa L-subunit of the F_0F_1-ATPase (Struglics et al., 1998). The phosphorylation of a number of unidentified proteins has also been demonstrated in bovine heart mitochondria (Ferrari et al., 1990; Technikova-Dobrova et al., 1993) and in potato tuber mitochondria (Struglics et al., 1998; Sommarin et al., 1990; Pical et al., 1993).

In adrenal glands there is evidence of protein phosphorylation; in particular, PKA catalyzes the phosphorylation of steroidogenic acute regulatory (StAR) protein. This phosphorylation increases StAR activity (Arakane et al., 1997) and promotes its binding to PAP7, a peripheral-type benzodiazepine receptor, and PKA (RIalpha)-associated protein (Li et al., 2001; Rone et al., 2009). In this sense, protein phosphorylation in mitochondria is a key control point for the regulation of steroid hormone biosynthesis.

Our group has been interested in mitochondrial physiology of human placenta, which does not contain StAR and does not have an acute steroidogenic response to hormonal stimulation as adrenal glands and gonads do (Thomson, 1998); however, the rate-limiting step in steroidogenesis is still the transfer of cholesterol into the mitochondrial membranes. Placental mitochondria,

Abbreviations: AKAPs, anchoring kinase cAMP proteins; BSA, bovine serum albumin; DMPK, myotonin protein kinase; KCN, potassium cyanide; PCP, pentachlorophenol; PDVF, poly (vinylidene difluoride); PKA, protein kinase cAMP dependent; PKC, protein kinase C; PKI, protein kinase inhibitor; PMSF, phenylmethylsufonyl fluoride; PTPD1, phosphotyrosine phosphatase D1; P-Ser, phosphoserine; P-Thr, phosphothreonine; P-Tyr, phosphotyrosine; P450/Cyp11A (P450scc), cytochrome P450 side chain cleavage; SDS-PAGE, sodium dodecil sulphate-polyacrylamide gel electrophoresis; StAR, steroidogenic acute regulatory protein.

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doi:10.1016/j.biocel.2011.06.005

just like bovine adrenocortical mitochondria (Cherradi et al., 1994, 1998) contain the cytochrome P450/Cyp11A and the 3β-hydroxy steroid dehydrogenase $\Delta^5 - \Delta^4$-isomerase in the inner membrane; therefore, cholesterol could be transformed into progesterone by this organelle (Martinez et al., 1997). In addition to this, there is evidence suggesting that the phosphorylation of mitochondrial proteins may regulate the development of steroidogenic placental cells (Albrecht and Pepe, 1990).

The production of placental progesterone by syncytiotrophoblast mitochondria is required to suppress maternal uterine contractions in order to maintain pregnancy, implying that a genetic lesion in any factor required for progesterone synthesis will cause spontaneous abortion (Miller, 1988; Duan et al., 2010).

Here we present the study of phosphorylation activity and its role in progesterone synthesis in human placental syncytiotrophoblast mitochondria. The results showed the presence of a PKA and a PTPD1 tightly associated with mitochondrial membranes through an AKAP-121. PKA activity was inhibited by H89, which was detected on mitochondrial steroidogenic contact sites. The role of H89 in steroidogenic biosynthesis is discussed here.

2. Materials and methods

2.1. Isolation of human syncytiotrophoblast mitochondria

Following governmental and ethics committee approval, placentas were collected from a hospital. Full-term human placentas were collected immediately after normal delivery. Mitochondria from placental syncytiotrophoblast were prepared as previously reported (Martinez et al., 1997). Placental cotyledons were isolated and kept in ice-cold 250 mM sucrose, 1 mM EDTA, pH 7.4. The tissue was washed three times with fresh sucrose-solution, and then minced into small pieces. The tissue suspension was homogenized with Polytron at 3000 rpm for 1 min for two cycles at intervals of 1 min in a cold room. The pH of the homogenate was adjusted to 7.4 with Tris–HCl and then centrifuged at 1500 × *g* for 15 min at 4 °C. The supernatant was recovered and centrifuged at 4000 × *g* to obtain a pellet of cytotrophoblast mitochondria and then the supernatant was centrifuged again at 16,000 × *g* for 15 min at 4 °C. The pellet containing syncytiotrophoblast mitochondria was suspended in fresh sucrose-solution and centrifuged at 1500 × *g* for 10 min to remove the remaining erythrocytes. Finally, the mitochondria in the pellet were recovered at 12,000 × *g* for 10 min at 4 °C. To purify syncytiotrophoblast mitochondria, the suspension was loaded in a 35% sucrose solution (25 ml) and centrifuged at 15,000 × *g* for 45 min at 4 °C. The pellets were recovered and diluted with 250 mM sucrose, 1 mM EDTA, pH 7.4, centrifuged at 16,000 × *g* for 15 min at 4 °C, and the mitochondria were suspended at the smallest possible volume and used immediately.

2.2. Mitochondrial oxygen consumption

Oxygen uptake was estimated polarographically using a Clark type electrode in a mixture at pH 7.4, consisting of 250 mM sucrose, 10 mM HEPES pH 7.4, 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) 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 (Navarrete et al., 1999). Mitochondrial respiratory control (RC) was determined as the rate between oxygen uptake in the state 3 and oxygen uptake in the state 4; only mitochondria with a value of RC higher than 4 were used to assure mitochondrial integrity (Fig. 1S).

2.3. Mitochondrial progesterone synthesis

Progesterone synthesis was determined at 37 °C in a mixture made up 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, in a final volume of 50 μl with 1 mg/ml of mitochondrial protein. Where indicated, 10–400 μM H89, 1 mM cAMP, 500 μM KCN, 5 mM sodium azide, 20 μM antimycin A or 25 μM 22(*R*)-hydroxy-cholesterol, were added. After 20 min incubation, the reaction was stopped with 75-μl methanol. Progesterone was detected by radioimmunoassay (Coat-A-Count® Progesterone kit from Siemens Healthcare Diagnostics Inc., USA). The concentration of progesterone at time zero was subtracted from the amount of progesterone in each experiment and the net progesterone synthesis was reported.

2.4. Determination of protein

Samples were treated with 0.017% deoxycholate and precipitated with 6% trichloroacetic acid (Bensadoun and Weinstein, 1976). After centrifugation at 5000 × *g* for 30 min at 4 °C, the protein content was determined as described by Lowry et al. (1951). BSA was used as standard.

2.5. Electrophoretic techniques, Western blot analysis and autoradiography

SDS-PAGE was performed according to Laemmli (1970). Mitochondrial proteins (50 μg per lane) were separated in a 10% polyacrylamide gel under denaturing conditions. After the run, the proteins were stained with Coomassie© Brillant Blue R-125, or with silver using a commercial kit (Bio-Rad) or electrotransferred to polyvinylidene difluoride membrane (Immobilon P; Millipore) in a semi-dry electroblotting system (Bio-Rad) at 25 V for 50 min. Membranes were blocked by incubation in 20 mM Tris-Base, pH 7.5, 500 mM NaCl, and 0.05% Tween-20 (TTBS buffer) containing 5% blotting grade blocker non-fat dry milk (BioRad) and then analyzed with rabbit polyclonal anti-human PKAα, C-terminus (sc-903, Santa Cruz Biotechnology) or with polyclonal anti-human AKAP-121 protein or PTPD1 protein antibody (AKAP and PTPD1 antibodies were kindly supplied by Dr. Antonio Feliciello, Dipartimento di Biologia e Patologia Molecular e Cellulare Instituto di Endogrinologia ed Oncologia Sperimentale, CNR, Napoli, Italy). The presence of phosphoserine, phosphothreonine and phosphotyrosine were determined with the Omni-Phos assay kit (92590, Chemicon International). Membranes were incubated for 1 h with the primary antibody at adequate dilution in TTBS buffer. After several washes with TTBS buffer, bands were visualized using horseradish peroxidase-conjugated goat antimouse IgG (Pierce) at a dilution of 1:35,000 and the enhanced ChemiLuminescence assay (Amersham Life Science) according to the manufacturer's instructions.

Syncytiotrophoblast mitochondria (1 mg/ml) were incubated in progesterone synthesis medium plus 0.32 μM (γ^{32}P)-ATP in the absence or presence of H89 (100 μM), Go7696 (200 μM), staurosporine (2 μM), cAMP (5 mM), oligomycin (28.5 μg/ml) or sodium orthovanadate (2 mM). At the times indicated, an aliquot was taken and the reaction was stopped with 1.5 volume of cold methanol. Phosphorylated proteins were resolved by SDS-PAGE and analyzed by Western blot or gels were dried for autoradiography. Radioactive bands were visualized by exposure to BioMax film from Kodak.

Additionally, for visualizing the proteins phosphorylated on Tyr- His- or Lys-residues, the PVDF membranes were incubated in 1 M KOH at 55 °C for 2 h, dried and autoradiographed. The remaining bands represented phosphotyrosine and phosphohisti-