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The Role of Mitochondria in Syncytiotrophoblast Cells: Bioenergetics and Steroidogenesis

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1. Introduction

Human placenta maintains pregnancy. The mitochondria of this tissue synthesize pregnenolone (P5) from cholesterol through a transport chain formed by adrenodoxin, adrenodoxin reductase and cytochrome P450_{scc} (CYP11A1; EC 1.14.15.6), which breaks up the lateral chain of cholesterol. P5 is transformed into progesterone (P4) within mitochondria by the 3 β -OH-steroid-dehydrogenase- Δ^5 - Δ^6 -isomerase (3HSD). The particular hormone(s) or substance(s) that modulate P4 synthesis during pregnancy is currently unknown (Strauss et al., 1996; Martinez & Strauss, 1997); nevertheless, the presence of cAMP analogues stimulated P4 synthesis in trophoblastic cells, suggesting that a hormonal signal or another kind of signal may modulate the concentration of this second messenger into the cells (Ringler et al., 1989; Strauss et al., 1992). Although P4 synthesis was suggested to be the main function of the placenta, the analysis of P450_{scc} cytochrome concentration shows that placental mitochondria have a lower content of P450_{scc} than respiratory chain cytochromes (Table 1), even when it is compared to adrenal gland mitochondria, suggesting that placental mitochondria participate in other functions different to that from steroidogenesis.

Cytochrome	Human Placenta			Adrenal Glands			
	nmol/mg of mitochondrial protein			nmol/mg of mitochondrial protein			
$a_1 + a_3$	0.140	0.10	-	0.23	0.75		
b	0.089	0.07	-	0.17	0.32		
$c + c_1$	0.125	0.13		0.29	0.67		
P450 _{scc}	0.110	0.10	0.123	1.50	1.30	0.39	1.0
Reference	Négrié et al., 1979	Simpson & Miller, 1978	Meigs & Ryan, 1968	Cammer & Estabrook, 1967	Harding & Nelson, 1966	I. Hanukoglu & Z. Hanukoglu, 1986	Jefcoate et al., 1973

Table 1. Concentration of cytochromes in human placenta and adrenal gland mitochondria

It seems that ATP synthesis in placental mitochondria is not related to its consumption at the cytoplasm, but rather it is related to the mitochondrial metabolism and, although seemingly contradictory, this allows pregnancy to reach full-term delivery.

2. Transport

2.1 Transport of carbohydrates, lipids, and amino acids

The development of both placenta and fetus is metabolically related. The mother supplies the essential nutrients for the fetus and the placenta, and their transport is strictly controlled in the placenta by the expression and the activity of specific transporters in the plasma membrane of syncytiotrophoblast (Angiolini et al., 2006). When the development of the fetus is optimal, some transporters of nutrients and ions are downregulated in the placenta; then a decrease of cellular receptors for specific molecules is observed (Hahn et al., 1999).

The placenta uses mainly glucose for its metabolism and at the same time transports it to the fetus that requires 4-8 mg/kg/min of glucose for the oxidative phosphorylation (OXPHOS) process (Aldoretta & Hay, 1995). Two isoforms of GLUT have been identified: GLUT1 and GLUT3 (Kahn & Flier, 1990). GLUT 1 is responsible of glucose uptake from the maternal circulation and its activity is independent of insulin. Glucose itself down-regulates the placental GLUT1 providing a fetal protection mechanism when maternal glucose is high (Hahn, 1998). GLUT3 is relevant during implantation and establishment of the placenta, and thereafter GLUT3 is not required; suggesting that GLUT3 has no relevance in glucose uptake after the first trimester (Clarson et al., 1997).

Glucose placental transport is accomplished by facilitated diffusion through GLUT1 transporter, but its expression is not affected by the concentration of extracellular glucose (3.7 mM or 70 mg/dl maternal and 3.2 mM or 61 mg/dl fetal) (Ingermann, 1987). It has been reported that the placenta is in constant hypoxia, for which it has a glycolytic metabolism, where 70% of total glucose is used to produce ATP through anaerobic glycolysis (Hanguel et al., 1986).

Glycogen is synthesized by the human placenta only as a primary response to maternal hyperglycemia. Although glycogen during pregnancy has been reported (Ville, 1953), the placenta does not synthesize glycogen efficiently (Barash et al., 1991). There are data showing the presence of gluconeogenic enzymes (Matalon & Michals, 1984), and Prendergast et al. demonstrate that the placenta synthesizes glucose, and evidence of the presence of glucose-6-phosphatase has been published (Matsubara et al., 1999; Prendergast et al., 1999); however, it is unknown whether the glucose synthesis might have any physiologic relevance.

The incubation of trophoblastic cells, perfused explants or placentas, produces lactic acid as a result of anaerobic glycolysis used by the fetus (Battaglia, 1989; Piquard et al., 1990). The radioactivity of glucose labeled with ^{14}C showed the following distribution: lactate 60-69%, glycogen 1.3-4.8%, pentose pathway 5%, fatty acids 0.7-1.4% and CO_2 1.6-2.4% (Desoye & Shafrir, 1994). Similarly, in slices of human placenta from middle gestation, 73% of glucose was degraded through glycolysis, 10% through pentose pathway and the rest through lipids formation and glycogen synthesis. Although pentose pathway has been described in the placenta (Shelley, 1979), reports suggest that it does not make an important contribution to

glucose metabolism (Moe et al., 1991). The placenta can also use fructose or lactate as energy sources.

Condition	Glucose consumption	Lactate production
	mmol/h/g	
95% O ₂ + 5% CO ₂	6.96 ± 2.50	21.23 ± 7.79
95% N ₂ + 5% CO ₂	12.50 ± 3.36	24.06 ± 5.24

Table 2. Glucose consumption and lactate production in human placenta (Modified from Schneider et al., 1988).

The placenta transports 50% of fatty acids to the fetus during the last trimester of pregnancy; that is the reason why β -oxidation is not a good candidate for ATP generation. In addition, placental membranes have a lipoprotein lipase which catalyzes the degradation of lipoproteins, mainly VLDL (Coleman, 1986). Fatty acids and glycerol are transported by simple diffusion and, once inside the trophoblast, they are bound to proteins and are transported to the basal stratus to diffuse to the fetus.

The following proteins have been related to the transport of lipids, the plasma membrane fatty acid binding protein (pFABPpm), fatty acid transporter protein (FATP) and the cytoplasmic fatty acid binding protein family (FABP) (Hornstra et al., 1995). pFABPpm makes up about 4% of cytosolic proteins, it is responsible for the intracellular distribution of fatty acids and to the membrane, and mainly binds essential long chain fatty acids. Also, the transport of essential fatty acids by the placenta is important, since these acids are implicated in cell-cell signaling and contribute to the development of the fetal brain, as well as cardiovascular and lung development (Wollet, 2005; Cunningham et al., 2009).

Cholesterol is the precursor of steroid hormones but it is not synthesized by the human placenta. Trophoblast cells express receptors for lipoproteins like LDL, VLDL, and class A scavenger receptors, as well as for the LDL receptor-related protein (LRP), the apolipoprotein E (apoE) receptor 2, and the scavenger receptor class B type (SR-BI). The cholesterol required for the synthesis of P4 is provided by maternal lipoproteins (Strauss et al., 1996; Palinski, 2009) (Fig.1).

Protein synthesis is essential for fetal development; the human placenta has at least three different amino acid transporters (neutral, cationic and anionic) coupled to an ionic energy dependent process. Two cationic amino acid transporter systems called γ^+ (Hoeltzli & Smith, 1989) and γ^+L (Fei et al., 1995) are specific for cationic amino acids and are widely expressed in both maternal and fetal sides. System " γ " is Na⁺-independent (Moe, 1995) and system γ^+L has higher affinity for lysine and neutral amino acids. Transport of neutral amino acids involves the transporters A, ASC and L. Type A is a Na⁺-dependent transporter with affinity for amino acids with short polar or linear side chains. System ASC is a Na⁺-dependent transport for serine, cysteine, threonine and glutamine. The system L is a Na⁺ independent transporter for large aromatic or non-polar branched side chains of amino acids (Yudilevich & Sweiry, 1985).

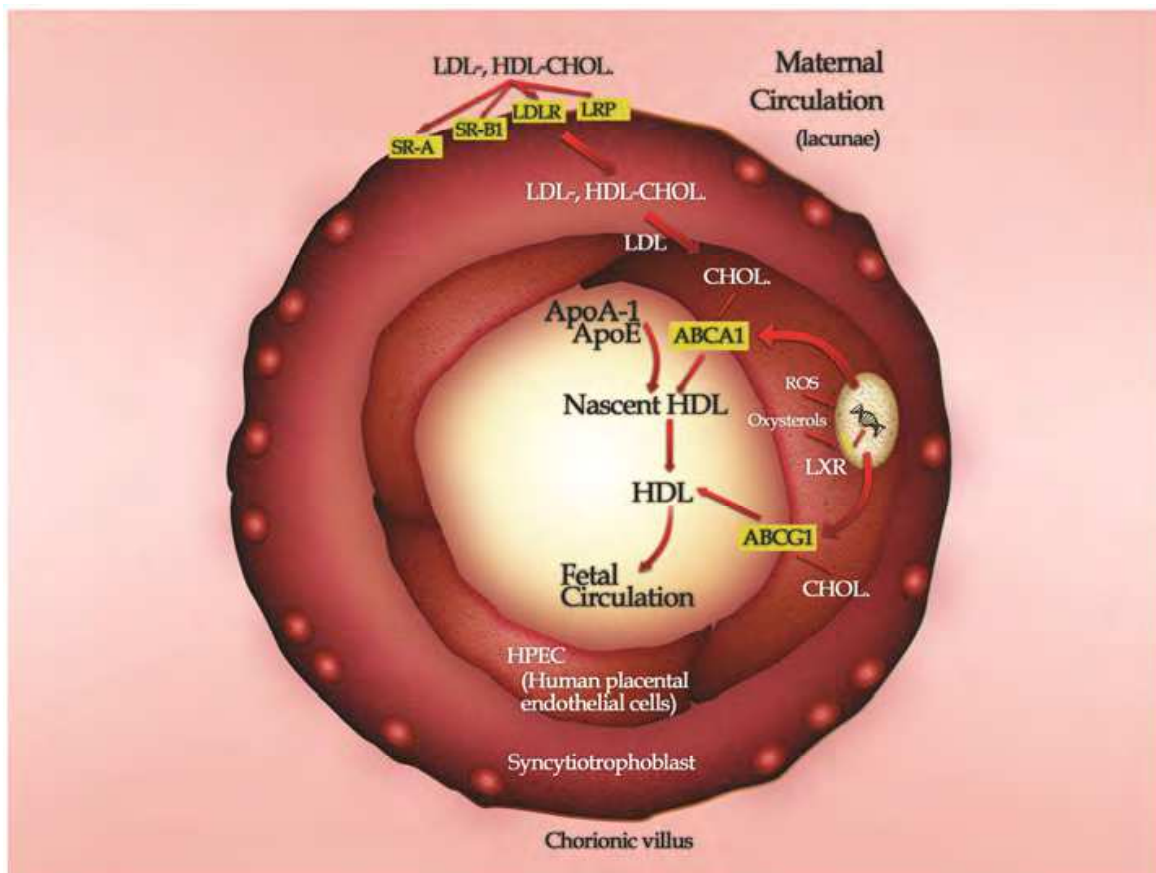


Fig. 1 Maternal-fetal cholesterol transport in the placenta (Modified from Palinski, 2009).

2.2 Transport of relevant ions

Ions are needed for fetus growth and metabolism, due to their contribution to cell homeostasis. The ions are not only related to amino acid transport, because ion transporters are necessary for other cellular functions. Na^+ is transported actively (Stulc et al., 1995). Carter has reported that 30% of the ATP produced by the placenta is used for the synthesis of proteins and steroids; while Na^+/K^+ ATPase consumes another 20-30%, making these processes the most costly and the main consumers of placental energy (Carter, 2000).

Chloride is co-transported with Na^+ or K^+ ; it is bidirectional and almost symmetrical and apparently associated with the maintenance of cell volume. Calcium is transported to the fetus against a concentration gradient reaching a fetal plasma concentration higher than maternal plasma concentration (Stulc, 1997).

Potassium is taken by the Na^+/K^+ -ATPase and its efflux through the K^+ -channel (K_{ATP}); the fetal transport of K^+ is against a concentration gradient and independent of the maternal potassium status (Stulc, 1997).

The movement of ions in the cell has relevance in the mitochondrial metabolism; the increase of K^+ in the cytoplasm in adrenal cells stimulates aldosterone synthesis (Spät & Pitter, 2004). The mechanism involves the release of Ca^{2+} from its cellular reservoirs modifying the mitochondria metabolism. Human placental mitochondria have a vectorial influx of Ca^{2+} , and changes in its concentration modifies P4 synthesis (García-Pérez et al.,

2002), although this transport can be modified by K^+ . It has been observed that the increases of K^+ also modify mitochondrial steroidogenesis (Martinez, 1995). The transport of K^+ in the mitochondria has not been clarified, but it has been proposed that it could be through the $\text{mitoK}_{\text{ATP}}$ channel described by Garlid (Garlid & Paucek, 2003).

3. Energetic metabolism

3.1 Architecture of the human placental mitochondria

The size, aspect and organization of mitochondrial membranes vary between species, tissues and physiological conditions. Using isolated mitochondria and cultured cells, Hackenbrock revealed a close coupling between ultrastructure and energetic state (Hackenbrock et al., 1971). In the energized state, when low ADP concentrations are limited for OXPHOS (the so-called respiratory state IV), mitochondria display the common, orthodox conformation: filamentous electron-dense cristae within a matrix of intermediate electron density. When high ADP concentrations accelerate OXPHOS (the respiratory state III), mitochondria adopt the condensed conformation: the matrix is condensed and electron dense, while the intermembrane space and cristae-lumen appears swollen and electron light. Reversible changes between the orthodox and condensed conformation are modulated by metabolites, by drugs that inhibit respiratory complexes or by ionophores that uncouple respiration and phosphorylation (Hackenbrock, 1968, 1971). Decades ago, several authors confirmed that stimulation of respiration induces the condensed conformation, with a characteristic electron-dense matrix in mitochondria (Malka et al., 2005; Rossignol et al., 2004). It is reasonable to assume that mitochondrial ultrastructure, morphology and dynamics are linked and thus, that mitochondrial morphology and dynamics are also modulated by OXPHOS.

Although many types of mitochondrial cristae structure have been described (Munn, 1974), from recent electron microscopic tomography studies, the differences between typical mitochondria are now evident; e.g., liver (Mannella, 1994, 1997), neuronal (Perkins, 1997, 2001a), brown adipose tissue (Perkins et al., 1998), fungus (Nicastro et al., 2000; Perkins et al., 2001b), rods and cones (Perkins, 2003), and those from steroidogenic tissues, e.g., Leydig cells (Prince, 2002). In general, cristae from typical mitochondria are lamellar while in steroidogenic cells are tubular, vesicular, or tubulovesicular (Reichert et al., 2002). It has been suggested that due to this particular morphology of the cristae, mitochondria of Leydig cells should not be able to produce ATP, since the narrow gap between lamellae would not allow the location of the F_1 subunit of ATP synthase (Prince, 2002); however, a recent publication indicates that mitochondrial membrane potential ($\Delta\psi_m$), mitochondrial ATP synthesis, and mitochondrial respiration are all required to support Leydig cell steroidogenesis (Allen et al., 2006).

In our laboratory, two kinds of mitochondria were isolated from human placenta: non steroidogenic mitochondria with typical cristae from cytotrophoblast, and steroidogenic mitochondria with vesicular cristae from syncytiotrophoblast. *In situ*, large mitochondria were observed in cytotrophoblast cells, with morphology similar to the typical liver mitochondria, and containing lamellar cristae in an orthodox configuration (Fig. 2). In contrast, the syncytiotrophoblast contains smaller mitochondria with a condensed matrix and cristae composed by vesicular regions connected by narrow tubules. The larger cytotrophoblast mitochondria have a round shape, whereas syncytiotrophoblast

mitochondria display an irregular shape with protuberances of the outer and inner membranes (De Los Rios Castillo et al., 2011).



Fig. 2. Ultrastructure of human syncytiotrophoblast and cytotrophoblast cells. (A) Electron micrograph of term placenta villus showing syncytiotrophoblast and underlying cytotrophoblast. N, nucleus; M, mitochondria. 10,000 X. (B) Isolated mitochondria from human cytotrophoblast and syncytiotrophoblast. Scale bar, 200 nm (Modified from De Los Rios Castillo et al., 2011)

Isolated cytotrophoblast and syncytiotrophoblast mitochondria showed values for respiratory control higher than those previously reported for this tissue (Olivera & Meigs, 1975) and the oxygen uptake was coupled to ATP synthesis, reaching 151 ± 16 and 153 ± 13 nmol ATP/mg/min, respectively (De Los Rios Castillo et al., 2011). These observations demonstrate the presence of functional mitochondria in both cell types, retaining the ability to increase the consumption of oxygen and the synthesis of ATP upon the addition of ADP.

3.2 Energetic pathways

The metabolism of mitochondria involves two major pathways: energy production and P4 synthesis, and several evidences show that both are closely related, as suggested by the similar content of cytochromes from the electron transport chain cytochrome P450_{sc}, suggesting the alternating activity of both pathways, which can generate ATP for cell function and for P4 synthesis to maintain pregnancy. A mechanism to regulate the functioning of both pathways could be the presence of alternative enzymes, e.g. an NADP-dependent isocitrate dehydrogenase is associated to the inner mitochondrial membrane; isocitrate, the substrate for this enzyme supports progesterone synthesis, while succinate promotes the synthesis of ATP.

It has been described that ATP-diphosphohydrolase and ADPase enzymes are tightly bound to mitochondrial membranes and their activities are involved in steroidogenesis. Particularly, the activity of ATP-diphosphohydrolase was described as supporting P4 synthesis, probably providing the energy requirement for cholesterol transport between the mitochondrial

membranes, similar to the activity of mitochondrial GDPase reported in adrenal gland (Fig. 3) Additionally, these enzymes could participate in the transformation of ATP to adenosine, which can be released into the blood vessels to promote oxygenation of the placenta.

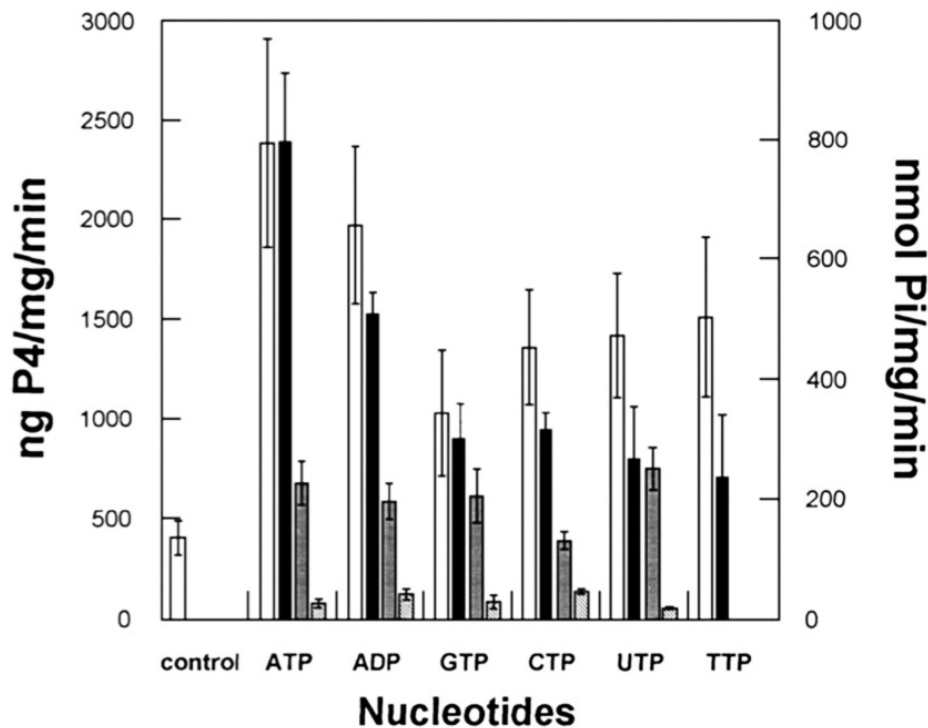


Fig. 3. Effect of several nucleotides on progesterone synthesis and their hydrolysis by human placental apyrase. Mitochondria were incubated in progesterone synthesis medium with or without 1 mM 5'-p-fluorosulfonyl benzoyl adenosine (FSBA). Nucleotide hydrolysis was also performed in progesterone synthesis medium. Progesterone synthesis (\square); nucleotide hydrolysis (\blacksquare); progesterone synthesis + FSBA (hatched); nucleotide hydrolysis + FSBA (dotted) (Modified from Flores-Herrera et al., 2002).

The concentration of adenine nucleotides is another way to analyze the pathways that produce energy in the form of ATP. The total amount of nucleotides at the time of expelling is 0.766 a 0.816 mmol/g wet weight, ATP 0.49, ADP 0.23, and AMP 0.12 nmol/g wet weight (Young & Schneider, 1984). In mitochondria isolated from full-term human placenta the concentrations of nucleotides are 1.24 5.78 y 1.09 nmol of ATP, ADP, and AMP/mg of protein, respectively (Martinez et al., 1987). These data suggest that in spite of the anoxia produced by labor, the placenta synthesizes mainly ATP, whereas glycogen levels do not vary significantly until 60 minutes after labor (Bloxam & Bobinski, 1984). The fact that mitochondria synthesize ATP does not imply that they supply the trophoblast with ATP for its cellular functioning in an important amount.

3.3 The relevance of nucleotide hydrolysis

With regard to their steroidogenic role, syncytiotrophoblast mitochondria synthesize P4 (35.7 ± 0.9 ng P4/mg/min) due to the presence of 3HSD in their inner membrane (Cherradi et al., 1994; Martinez et al., 1997; Brand et al., 1998). Their steroidogenic activity was ten times higher than cytotrophoblast mitochondria (3.6 ± 1.34 ng P4/mg/min). In both cases,

22(R)-hydroxycholesterol, a soluble substrate used to assess maximal steroidogenesis (Tuckey, 1992) increased steroidogenic activity to 92.2 ± 3.4 and 10.1 ± 3.95 ng P4/mg/h in syncytiotrophoblast and cytotrophoblast mitochondria, respectively (De Los Rios Castillo et al., 2011). Additionally we demonstrated that ATP is essential for progesterone synthesis (Flores-Herrera et al., 2002). These results agree with the specialized role of each placental cell (Martinez et al., 1997) and demonstrate that isolated mitochondria from syncytiotrophoblast are intact and retain their physiological function.

Nevertheless, human syncytiotrophoblast mitochondria have bioenergetics and steroidogenic functions, which raise an interesting question: why do they have an irregular shape with tubular, vesicular, or tubulovesicular cristae? At present, there is interest concerning proteins that govern mitochondrial ultrastructure, but few of such proteins have been identified (Pellegrini & Scorrano, 2007). It has been speculated that the dimer of F_0F_1 -ATP synthase (complex V for oxidative phosphorylation of ATP) may play a major role in determining cristae formation (Paumard et al., 2002; Minauro-Sanmiguel et al., 2005; Dudkina et al., 2005; Strauss et al., 2008). Dimerization of F_0F_1 -ATP synthase in the mitochondrial inner membrane has been described in yeast (Arnold et al., 1998) and bovine mitochondria (Schägger & Pfeiffer, 2000). A critical role in the stability of the mammalian dimeric complex V has been proposed for the inhibitory F_1 moiety protein (IF_1) (García et al., 2006). IF_1 is known to dimerize in solution (Gordon-Smith et al., 2001), and this dimer has been recently shown to interact with two molecules of soluble F_1 simultaneously (Cabezón et al., 2000; Dominguez-Ramirez et al., 2001). If the same interaction occurs in the membrane, it might be responsible for the dimerization of the F_0F_1 -ATP synthase complex, and in mammalian cells, changes in IF_1 concentration affect the degree of F_0F_1 -ATP synthase dimerization, which in turn could alter the formation of cristae (García et al., 2006). In this sense, the density of mitochondrial cristae in HeLa cells is increased by IF_1 overexpression and decreased by IF_1 suppression (Campanella et al., 2008); interestingly, IF_1 overexpression increases both the formation of dimeric F_0F_1 -ATP synthase and F_0F_1 -ATP synthase activity (Campanella et al., 2008).

The analysis of the electron transport chain and oxidative phosphorylation complexes from human syncytiotrophoblast and cytotrophoblast mitochondria allow us to demonstrate that the dimeric form of the F_0F_1 -ATP synthase (complex V) is involved in the cristae architecture in trophoblast cells (De Los Rios Castillo et al., 2011). In this sense, we found that the dimer of mitochondrial F_0F_1 -ATP synthase is scarce in syncytiotrophoblast associated to a low IF_1 concentration. Due to the fact that human placenta cells are ontogenetically related, i.e. cytotrophoblast cells differentiate into syncytiotrophoblast cells, the amounts of IF_1 found in the steroidogenic cells could be the result of cell differentiation and have a significant effect on their mitochondrial architecture (tubulovesicular cristae) and physiology (P4 synthesis). The amount of IF_1 and F_0F_1 -ATP synthase dimer present in these mitochondria is probably related to its physiological functions.

But, why do syncytiotrophoblast cells have mitochondria with atypical cristae morphology? Since the human placenta does not express StAR (Tuckey, 2005) and TSPO (Maldonado-Mercado et al., 2008), two proteins involved in mitochondrial cholesterol flow, it has been suggested that the reduction in the size of syncytiotrophoblast mitochondria and the change in the structure of cristae may improve the steroidogenic activity of syncytiotrophoblast cells (Martinez et al., 1997). In this sense, the translocation of cholesterol to P450scc has been

well known to be the rate-limiting step in steroidogenesis; thus, a greater surface could enhance the movement of cholesterol to the inner membrane where P450_{scc} is located. It is tempting to speculate that the non-orthodox cristae structure in mitochondria from steroidogenic tissue allows cholesterol to flow from the outer to the inner mitochondrial membranes and improves hormone production.

3.4 Mitochondrial accessory proteins involved in progesterone synthesis

For P4 synthesis by human syncytiotrophoblast mitochondria, the cristae architecture could not be considered as the only and most important issue; we have identified a set of different proteins involved in hormone production as a heat shock protein-60 kDa (HSP60), associated to the increases of progesterone synthesis through its association with the MLN64-like protein (Olvera-Sanchez et al., 2011); an ADPase, and an ATP-diphosphohydrolase associated to mitochondrial membranes (Uribe et al., 1999; Flores-Herrera et al., 1999).

ATP-diphosphohydrolase is anchored to mitochondrial membranes with its nucleotide-hydrolyzing activity oriented to the intermembrane space. The kinetic characterization of its activity in a detergent solubilized fraction revealed that it can use ATP, ADP, GTP, GDP, UTP, UDP, CTP, CDP, TTP and TDP in a cation (Mg^{+2} , Ca^{+2} , and Zn^{+2}) dependent fashion as substrates (Flores-Herrera et al., 1999) (Fig. 4). ATP hydrolysis by this ATP-diphosphohydrolase can stimulate oxygen uptake in intact mitochondria from syncytiotrophoblast coupling with ATP synthesis (Martinez et al., 1993). Since one main function of mitochondria is the synthesis of ATP through the F_0F_1 -ATPase activity, the presence of external mitochondria ATP-diphosphohydrolase and an ADPase in the human

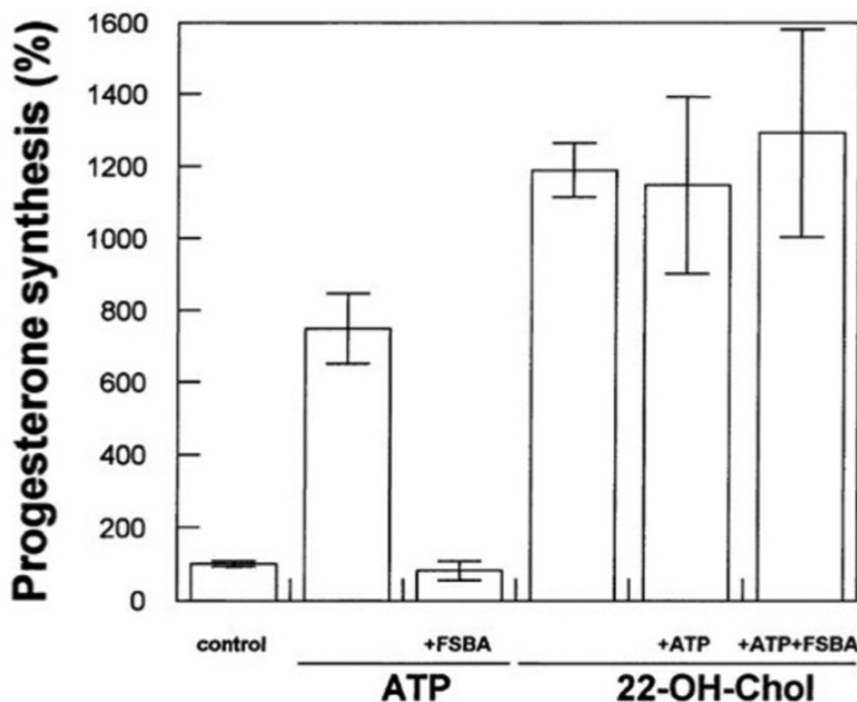


Fig. 4. Effect of 22(R)-hydroxycholesterol (22-OH-Chol) on progesterone synthesis. Mitochondria were incubated in progesterone synthesis medium with or without 1 mM FSBA. Where indicated, 1 mM ATP or 15 μ M 22-OH-Chol was added. 100% progesterone synthesis = 142 ng progesterone/mg/min (Modified from Flores-Herrera et al., 2002).

placental mitochondria could produce a futile cycle due to the combination of the F_0F_1 -ATPase and ATP-diphosphohydrolase activities, being lethal for trophoblast cells. However, the futile cycle is not observed, because the addition of ATP (or ADP) to isolated mitochondria induces oxygen consumption without uncoupling the respiration, suggesting the presence of mechanisms that regulate the activity of ATP-diphosphohydrolase in a way that trophoblast cells remain alive (Martinez et al., 1993). Indeed, ATP-diphosphohydrolase activity and its substrate specificity seem to be regulated by the proton electrochemical potential ($\Delta\mu_{H^+}$), i.e. if mitochondrial inner membrane is energized, ATP-diphosphohydrolase selectively hydrolyzes ATP, while dissipation of $\Delta\mu_{H^+}$ by CCCP produces a loss of substrate specificity, and is able to hydrolyze ATP and GTP equally (O. Flores-Herrera, et al., manuscript in preparation).

With respect to ATP-diphosphohydrolase role in syncytiotrophoblast mitochondria steroidogenesis, its activity in presence of ATP is involved in cholesterol transport between mitochondrial membranes (Flores-Herrera et al., 1999; Flores-Herrera et al., 2002). In addition, other nucleotide hydrolase activities have been determined in mitochondria from other steroidogenic tissues; in particular, in adrenal mitochondria GTP enhances steroidogenesis, a process modulated by a GTPase (X. Xu & T. Xu, 1989); however, no conclusion can be drawn yet in this steroidogenic tissue. However, syncytiotrophoblast mitochondria hydrolyze several nucleosides tri- and di-phosphatides increasing P₄ synthesis, which is sensitive to ATP-diphosphohydrolase inhibition by FSBA, a non-hydrolysable ATP analog (Flores-Herrera et al., 2002) (Fig. 4). Finally, although the mechanisms of regulation of this enzyme have to be elucidated, we conclude that ATP-diphosphohydrolase is anchored to syncytiotrophoblast mitochondrial membranes, which nucleotide hydrolysis activity is involved in cholesterol transport between mitochondrial membranes and in oxygen uptake by mitochondrial electron transport chain (Flores-Herrera et al., 2002).

4. Signaling pathway in placental steroidogenesis

4.1 The role of mitochondria in the phosphorylation cell signaling

Progesterone synthesis by human placenta is essential for the maintenance of pregnancy. In the human being, P₄ is produced in the corpus luteum cells during the secretory phase of the menstrual cycle; whereas in the early stages of pregnancy its production continues due to the stimulus of the Chorionic Gonadotropin hormone (hCG); however, between the 6th and 8th week of gestation, the corpus luteum decreases its production of P₄, which is now synthesized by the trophoblastic cells of the placenta (Tuckey, 2005).

Unlike the other steroidogenic tissues, placental steroidogenesis is chronically regulated. Both regulation systems are under the control of diverse factors or hormones that activate signal transduction pathways in different forms, allowing either short-term or long-term regulation. In general, the chronic response is started within hours after the initiating stimulus and involves the activation of certain signaling pathways, among them the cAMP-dependent protein kinase (PKA) cascade is the most important.

The effect mediated by PKA/cAMP in long-term and short-term regulation of steroidogenesis is the main pathway stimulated by trophic hormones which acts through G protein-coupled receptors, which in turn activates the enzyme adenylate cyclase, which increases the content of cAMP. cAMP has pleiotropic effects such as the activation of PKA,

which phosphorylates proteins and transcription factors such as the steroidogenic factor (SF-1) and the protein cAMP response element binding (CREB), the latter being the main mediator of positive changes in gene expression (Sands, 2008); for example, the activation of StAR gene (Stocco et al., 2005) or even its phosphorylation (Fig. 5).



Fig. 5. The model illustrates the activation of the transduction pathway mediated by PKA/cAMP in the postranscriptional and postranslational regulation of StAR protein (Modified from Manna, 2009).

Although other PKA-dependent and -independent signaling pathways have been reported, the responses generated are generally less potent than those mediated by PKA/cAMP (Manna et al., 2006).

During pregnancy, the production of P4 gradually increases with no substantial variations observed (Tuckey, 2005). It has been reported that a plasmatic decrease of P4 is associated with a higher probability of abortion (Duan et al., 2010).

The addition of 8Br-cAMP or dibutyryl-cAMP to cultured trophoblast cells stimulated the P4 synthesis by increasing the transcription and translation of P450_{scc} and adrenodoxin. Nevertheless, independent of the factors involved in modulating the intracellular concentration of cAMP, the likely participation of different hormones and the trophic factors and cytokines that could have endocrine, paracrine, autocrine or intracrine effect in the regulation of steroidogenesis through the signaling pathway mediated by PKA is still unknown; since, as it has been said, there are other pathways that can participate in the regulation of P4 synthesis (Manna et al., 2006; Stocco et al., 2005) (Fig. 6).

In trophoblast choriocarcinoma-derived cells of human placenta (BeWo) the addition of hCG increased P4 synthesis through PKA. Other factors also increased in a variable way the production of P4; nevertheless, it has been suggested that its main effect would be predominantly as a regulator and just in a few cases as a stimulant of steroidogenesis, although through the activation of cAMP-dependent pathways and PKA activation (Manna et al., 2006).

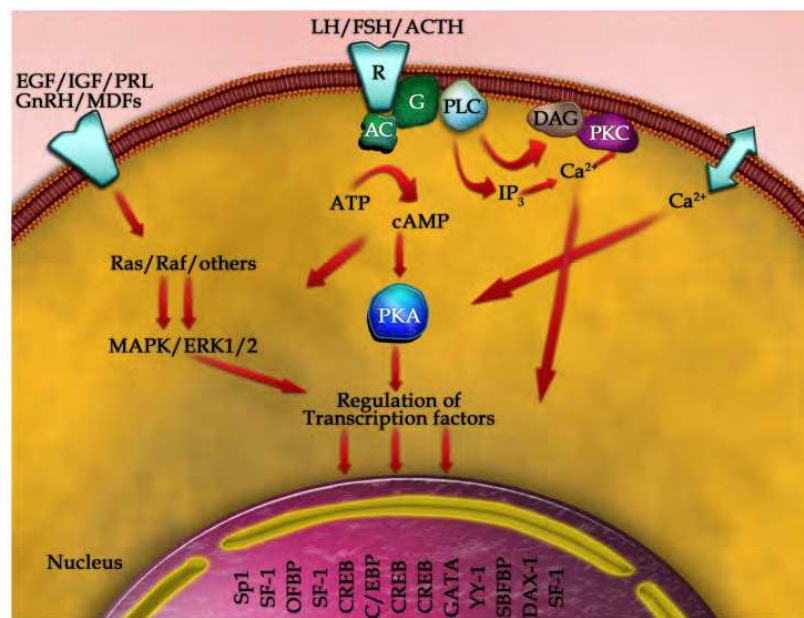


Fig. 6. Model proposed to illustrate the cAMP-dependent and -independent pathways in the regulation of StAR expression and steroidogenesis in adrenal gland and gonads (Modified from Manna, 2009).

Although the signaling pathways associated with P4 synthesis in human placenta are unknown, one of the best studied mechanism is the one mediated by PKA activation. Unlike gonads, adrenal cortex, and the corpus luteum, P4 synthesis by placental cells in the presence of cAMP soluble derivatives produce no acute response, not even in cells obtained from first-trimester placentas (Zosmer et al., 1997).

Previous data confirm that trophoblast cells have the machinery to produce cAMP, which may increase its concentration in cytoplasm through the stimulation of β_2 adrenergic receptors in full-term placentas (Kasugai et al., 1987). Interestingly, in first-trimester placentas, norepinephrine increased the content of cAMP mediated by α_1 -adrenergic receptors (Shi & Zhuang, 1993). There is also evidence of the potential effect of other hormones that may stimulate placental P4 synthesis through cAMP-dependent pathways, just like it happens in the case of estrogens (Pepe & Albretch, 1999), insulin (Lavy et al., 1987), insulin-like growth factor 1 (Nestler, 1987) and epidermal growth factor (Ritvos, 1988). The results suggest the possibility of different stimuli for specific effectors according to the gestational age.

The function of PKA in cells is vital, since its activity is aimed at specific functions, which include the differential expression of regulatory and catalytic subunits in different tissues. Thus, anchor proteins (AKAP's, **A**-kinase **a**nchor **p**roteins) in certain cellular locations place PKA close to its substrates, making its activity more efficient (Fig. 7). For instance, in Leydig tumoral cells from mice the association of AKAP 121 with the regulatory subunit α of PKA II in mitochondria in response to cAMP was observed, which was interpreted as a powering effect of steroidogenesis on directing the synthesis and activation of StAR to the mitochondrion in response to cAMP (Dyson et al., 2008).

In a similar way, mitochondria isolated from human placenta show the interaction of PKA, AKAP-121, and PTPD1 (Gómez-Concha et al., 2011) (Fig. 8). In addition, it was seen that in the presence of radioactive ATP several proteins were phosphorylated in less than 5 min on

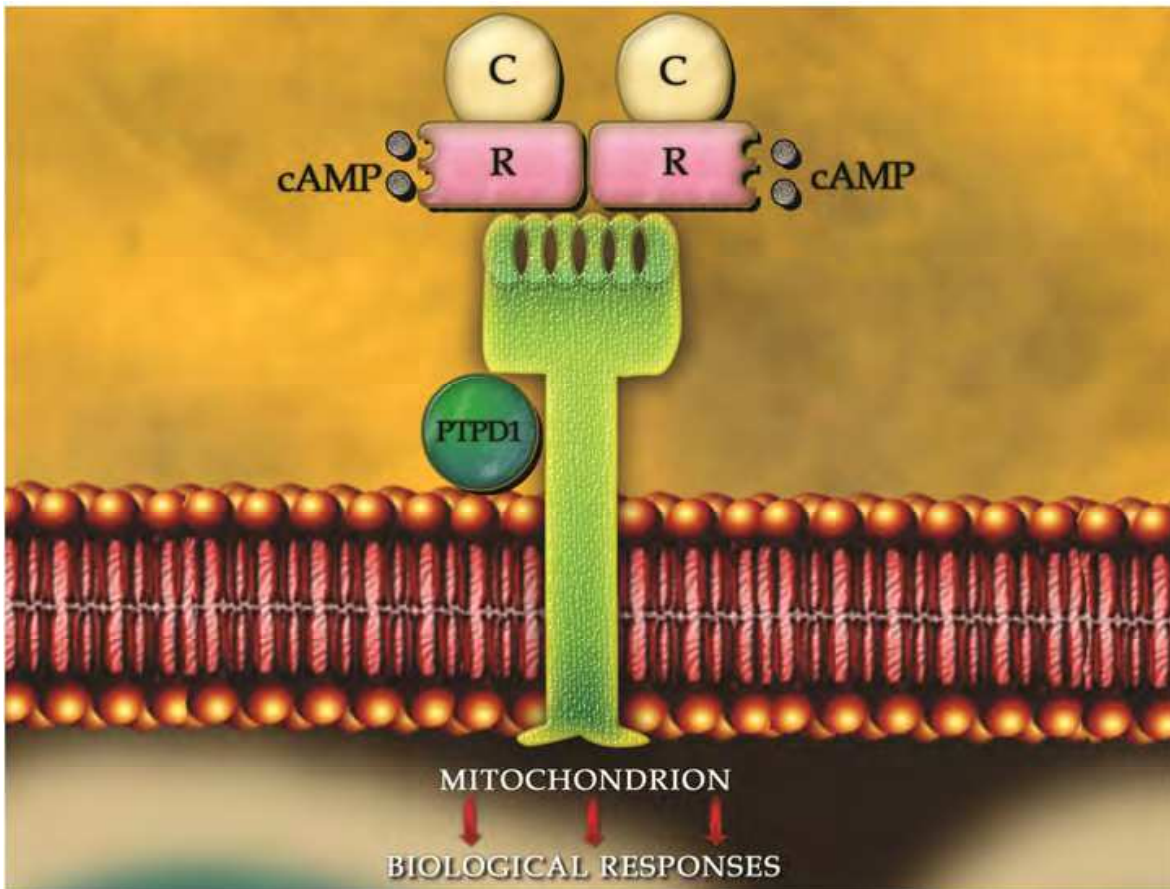


Fig. 7. A signaling complex made up by AKAP 121, which anchors PKA close to the mitochondrion, thus facilitating the phosphorylation of mitochondrial proteins (Modified from Feliciello, 2005).

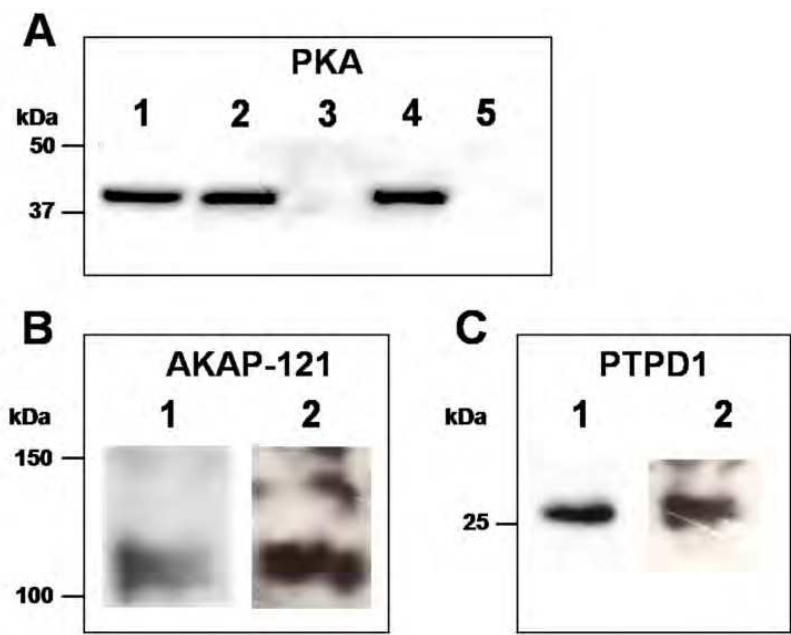


Fig. 8. Immunodetection of PKA (A), AKAP-121 (B) and PTPD1 (C) (see Material and Methods from Gómez Concha, 2011.)

serine and threonine, and to a lesser degree on tyrosine, suggesting the potential activity of kinases. Likewise, the importance of cAMP-PKA pathway in BeWo cells and in mitochondria isolated from human placenta was shown using H89, an inhibitor of PKA activity (Maldonado-Mercado et al., 2008). In mitochondria isolated from syncytiotrophoblast, steroidogenesis was inhibited 50% with 100 μM of H89, whereas in BeWo cells with concentrations of 10 and 20 μM , an inhibition of steroidogenesis of 70% and 90% was found, respectively. The addition of 22(*R*)-hydroxycholesterol to the isolated mitochondria previously inhibited with H89, reestablishes P4 synthesis, thus confirming that the activities of P450_{scc} and 3HSD are not affected by H89.

The results show that to the maximal concentration of H89 inhibited the synthesis of P4 in 99%, but only 50% of protein phosphorylation, which suggests that there are other kinase activities in mitochondria isolated from the human placenta, suggesting the pathway mediated by PKA could be associated to another pathways that ensures the production of P4; then it is relevant to determine the kinases that participate and their associations with the steroidogenesis by the human placenta as it was proposed (Maldonado-Mercado et al., 2008; Gómez-Concha et al., 2011) (Fig. 9).

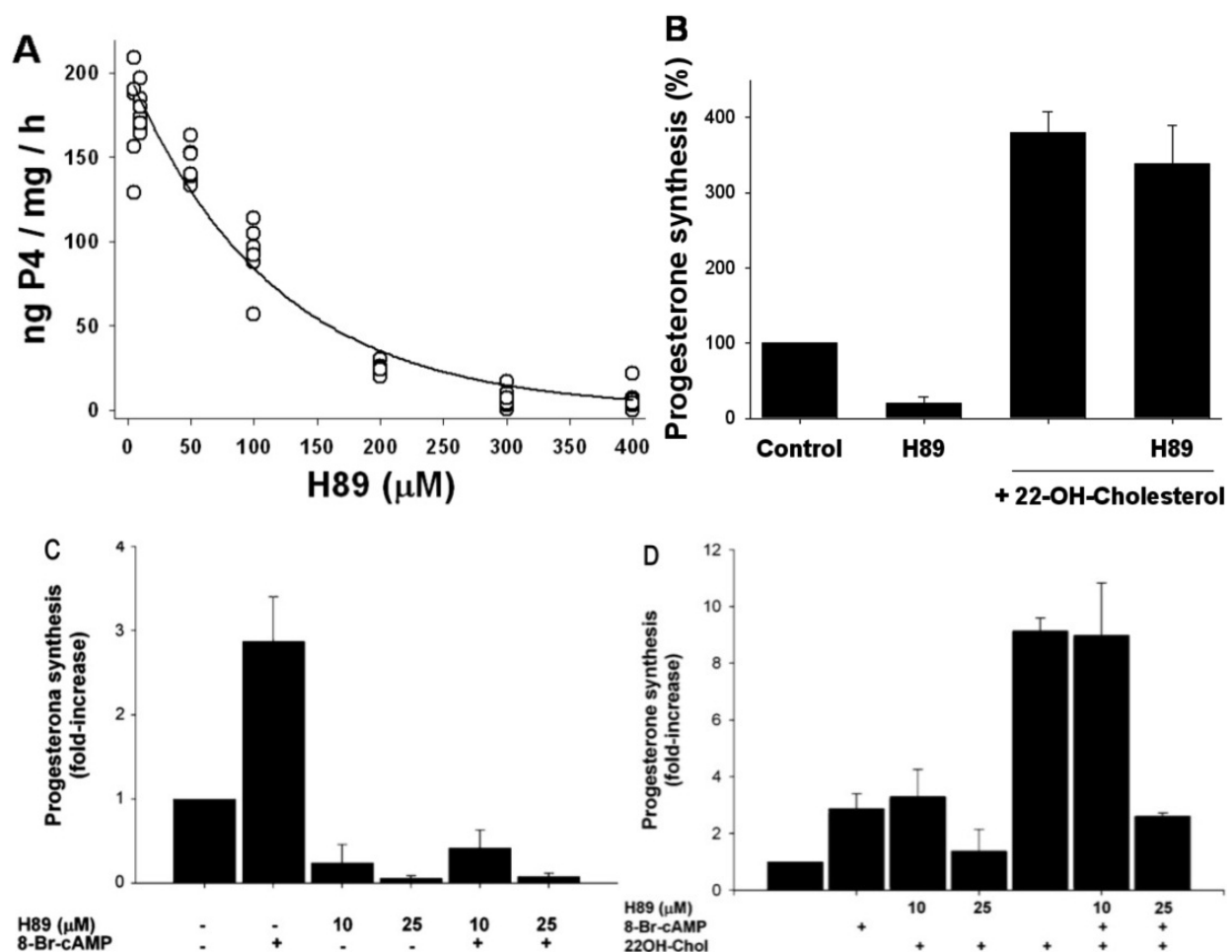


Fig. 9. Progesterone syntheses by syncytiotrophoblast mitochondria (A and B) and progesterone synthesis in BeWo cells (C and D) (see Material and Methods from Maldonado-Mercado et al., 2008, and Gómez-Concha et al., 2011). 22(*R*)-hydroxycholesterol (22-OH-Cholesterol or 22OH-Chol).

Interestingly, in cultured human placenta cells, the stimulus of an acute signaling pathway associated with steroidogenesis as the one observed in isolated mitochondria has not been described; nevertheless it has been suggested that the pathway mediated by PKA/cAMP plays an important role in the regulation of placental steroidogenesis, perhaps with the potential participation of other signal transduction pathways, as it has been observed in other tissues (Dodge-Kafka & Kapiloff, 2006). The endocrine, paracrine, autocrine, and even intracrine mechanisms that modulate this process are still to be described. No matter which one is the signaling pathway activated, the importance of the events of dynamic phosphorylation of proteins with pleiotropic effects in cellular functions as hormonal synthesis is the most relevant (Corso & Thomson, 2001; Gorostizaga et al., 2007; Thomson, 2002).

4.2 Protein phosphorylation in the control of steroidogenesis

Protein phosphorylation is one of the most studied postranslational modifications. The modification of target proteins on specific residues allows structural changes, changes in the protein-protein interaction, and favors their activation or inactivation (Chang & Karin, 2001), thus achieving the regulation of their functions (Matthews, 1995; Klumpp & Krieglstein, 2005; Puttick et al, 2008).

It has been shown that the activity of certain proteins modulated by phosphorylation/dephosphorylation has a temporary effect in specific cellular regions such as the plasma membrane, endoplasmic reticulum, and nucleus, regulating the cellular metabolism (Trost, 2010; Bauman & Scott, 2002), a system that seems to be associated with steroidogenesis (Gómez-Concha et al., 2011), similar to StAR protein (Steroidogenic acute regulatory protein) in adrenal glands and gonads. StAR protein phosphorylation mediated by PKA stimulates the transport of cholesterol from the cytoplasm into mitochondrial membranes (Stocco, 2000; Thomson, 1998; Manna & Stocco, 2005), apparently through a multiprotein complex associated to this organelle (Thomson, 2002) (Fig.10).

It has been suggested that a tyrosine phosphatases is the key for the regulation of StAR and the transport of cholesterol, where phosphorylation/dephosphorylation of intermediary proteins is fundamental in the regulation of steroids biosynthesis (Cooke et al, 2011). Although the placenta does not synthesize StAR protein, data suggest that trophoblast cells have a phosphorylation/dephosphorylation system associated with steroidogenesis.

It has been described that the PKA activity associated with mitochondria is different from that of cytoplasm, suggesting a differential regulation according to their subcellular location, maybe related to the complex formed by PKA, AKAP 121, and PTPD1 (Felicciello et al, 2001; Gómez-Concha et al., 2011). This way, the identification of proteins phosphorylated on their serine, tyrosine, and threonine residues suggests that the metabolism of placental mitochondria has important kinase activity (Gómez-Concha et al., 2011).

In BeWo cells and in isolated mitochondria from placenta, the phosphorylated proteins of 46, 42 and 36 kDa appear to be a potential target of kinases and phosphatases system, where the H89 change the balance of phosphorylation/ dephosphorylation between proteins of 42 and 36 kDa (Maldonado-Mercado et al., 2008; Gómez-Concha et al., 2011) (Fig. 11 and 12).

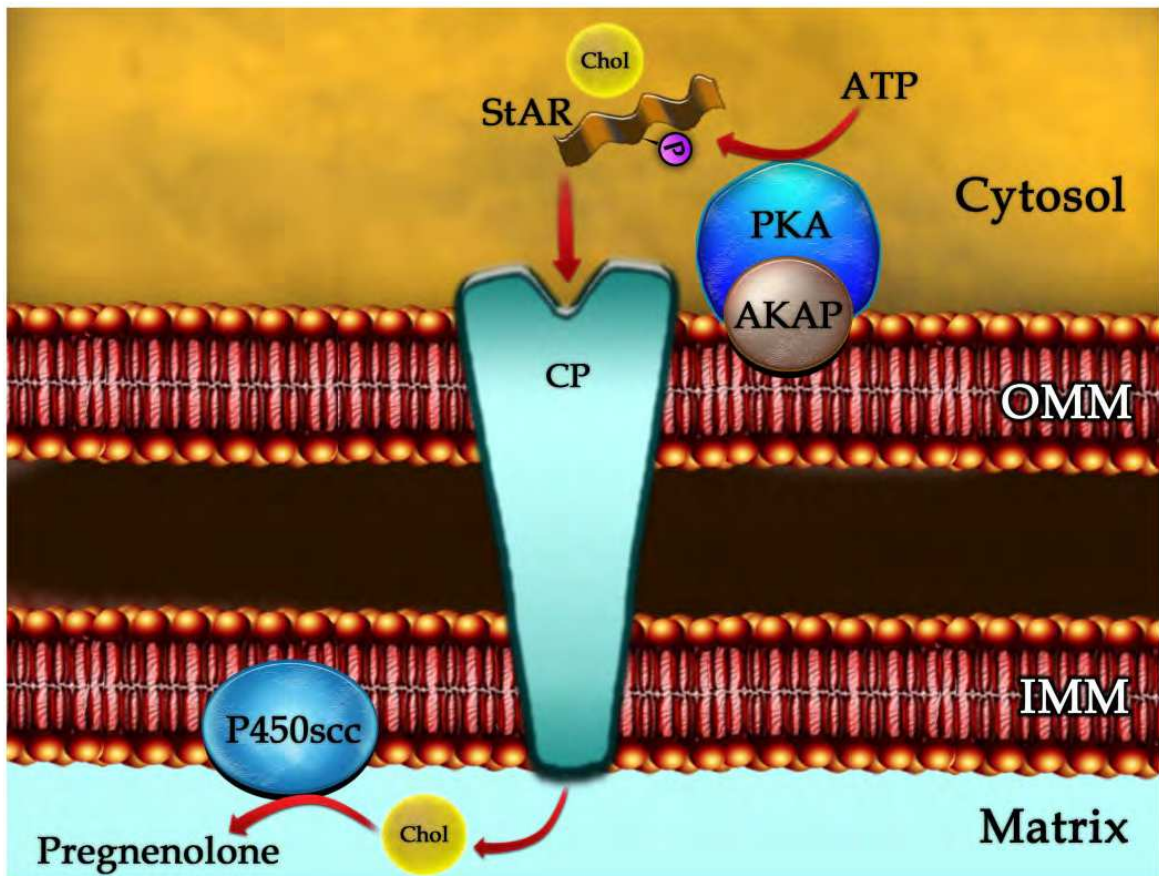


Fig. 10. Proposed model for steroidogenic modulation by mitochondrial PKA (Modified from Thomson, 2002).

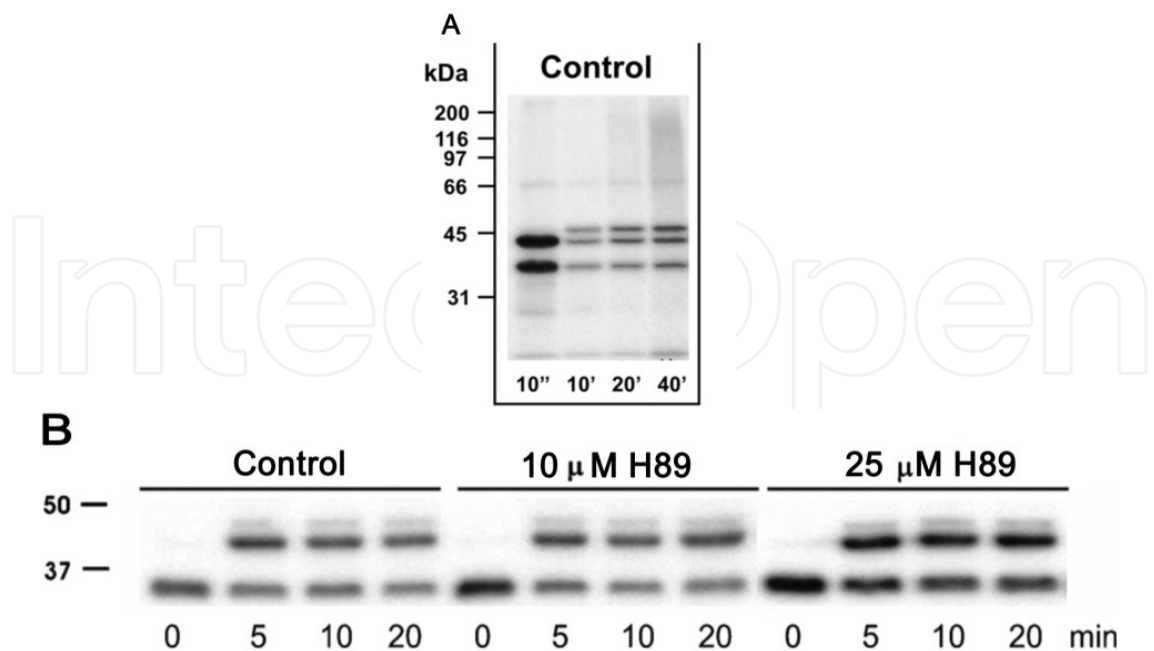


Fig. 11. Incorporation of ($\gamma^{32}\text{P}$) to syncytiotrophoblast mitochondria proteins (A) and isolated mitochondria from BeWo cells in the presence of H89 (B). (see Material and Methods from Maldonado-Mercado et al., 2008; Gómez -Concha et al., 2011)

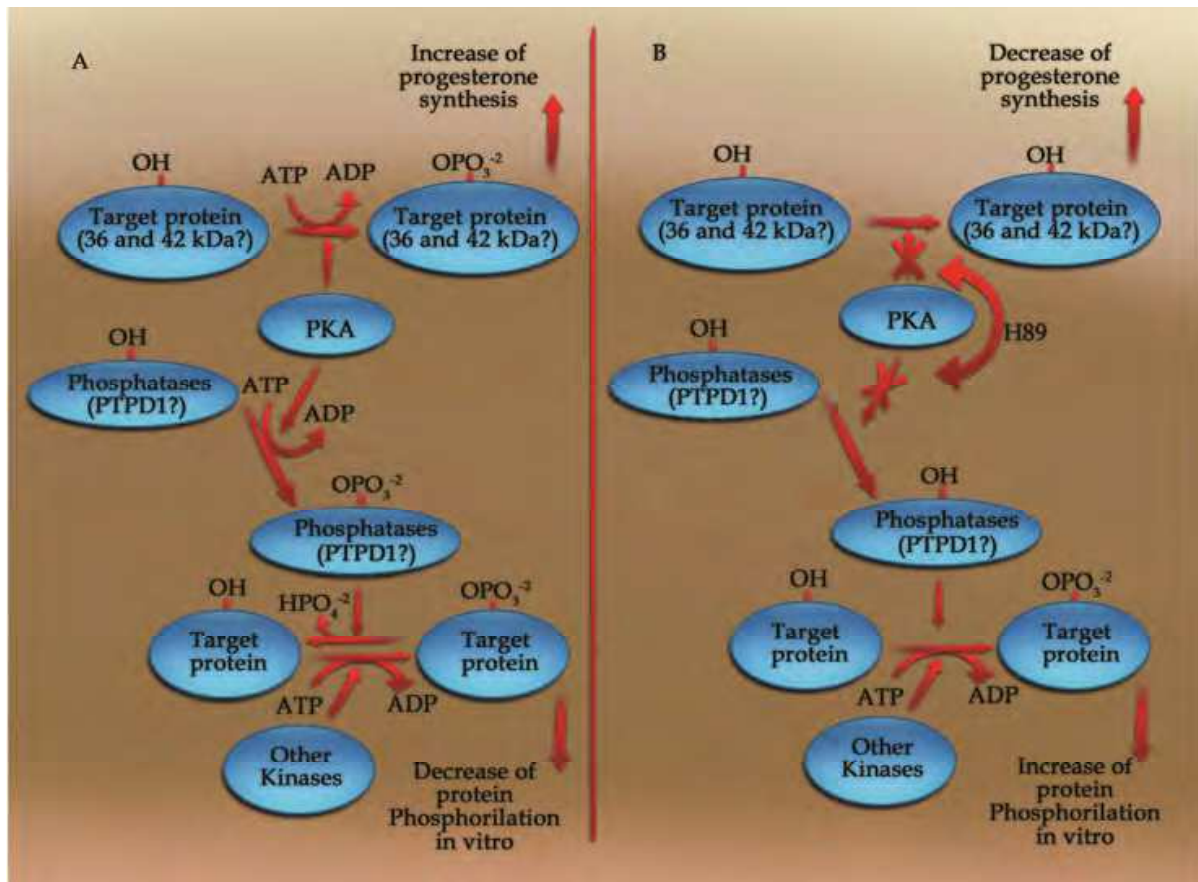


Fig. 12. Model proposed for the participation of PKA in steroidogenesis by syncytiotrophoblast mitochondria (Modified from Gómez -Concha et al., 2011).

5. Steroidogenesis

The transport of cholesterol to mitochondria by proteins is required for steroidogenesis. Deep differences between the placenta and adrenal glands have been observed, which are tightly associated to the permanent production of progesterone.

Meanwhile the cellular movement of cholesterol from cytoplasm to different organelles is accomplished by StAR, i.e. adrenal glands, gonads, liver, brain and others; human placenta does not express the StAR protein. A metastatic lymph node 64 protein (MLN64), which has similar characteristics to StAR protein to recognize cholesterol and transport it to the mitochondria, has been implicated in the movement of cholesterol in the human placenta; also, other proteins have been associated with cholesterol transport, such as the heat shock protein 60 (HSP60) (Olvera-Sanchez et al, 2011) and porine (Espinosa-Garcia et al., 2000).

In the previous sections of this chapter, several aspects of the trophoblast cells have been described. As it was observed, the human placenta possesses special characteristics basically oriented to maintain the relationship between mother and fetus in order to reach a successful delivery. The human placenta works as a mechanical barrier, being highly specific about the molecules that can cross it. In a sense, it controls by itself the mechanisms that regulate the metabolism and hormone production to assure nutrient supply by using different metabolic and signaling pathways, also modulating the mitochondrial activity

through ATP-diphosphohydrolase and other enzymatic activities due to the relevant role that mitochondria play in both ATP synthesis and steroidogenesis.

As mentioned before, cholesterol is the source of steroid hormones, but the human placenta is unable to synthesize it, so the cholesterol must be obtained from mother's lipoproteins. During pregnancy, the amount of progesterone required is high, and during the first trimester its production is responsibility of the corpus luteum, while the egg implantation in the maternal epithelium of the uterus occurs.

5.1 Electron transport chain coupled to cytochrome P450scc

Placental steroidogenesis is an essential process for reproduction. Syncytiotrophoblast cells are the P4-producing cells in the human placenta (Martinez et al., 1997). By full-term pregnancy, placenta produces about 300 mg of P4 per day (Strauss et al., 1996). The first enzymatic stage in its production is the conversion of cholesterol into P5 by P450scc type I (CYP11A1; EC 1.14.15.6), composed by approximately 530 amino acids, including the signal peptide necessary for its association to the mitochondrial inner membrane and only one heme group. P450scc receives six electrons from 3 moles of NADPH through a 54 kDa flavoprotein, ferredoxin reductase (adrenodoxin reductase), and ferredoxin (adrenodoxin) a 2Fe-2S protein with a molecular weight of 13.5 kDa. Both are found in the mitochondrial matrix. Studies of the molecular mechanism about the formation of this complex and electron transport have proposed a stoichiometry for proteins 1:1:1 or 1:2:1, and it has been suggested that adrenodoxin behaves as a mobile electron transporter from adrenodoxin reductase to P450scc (Miller, 2005), and the interaction between CYP11A and adrenodoxin reductase has been shown by molecular biology technics (Payne & Hales, 2004; Strushkevicha, 2011) (Fig. 13).

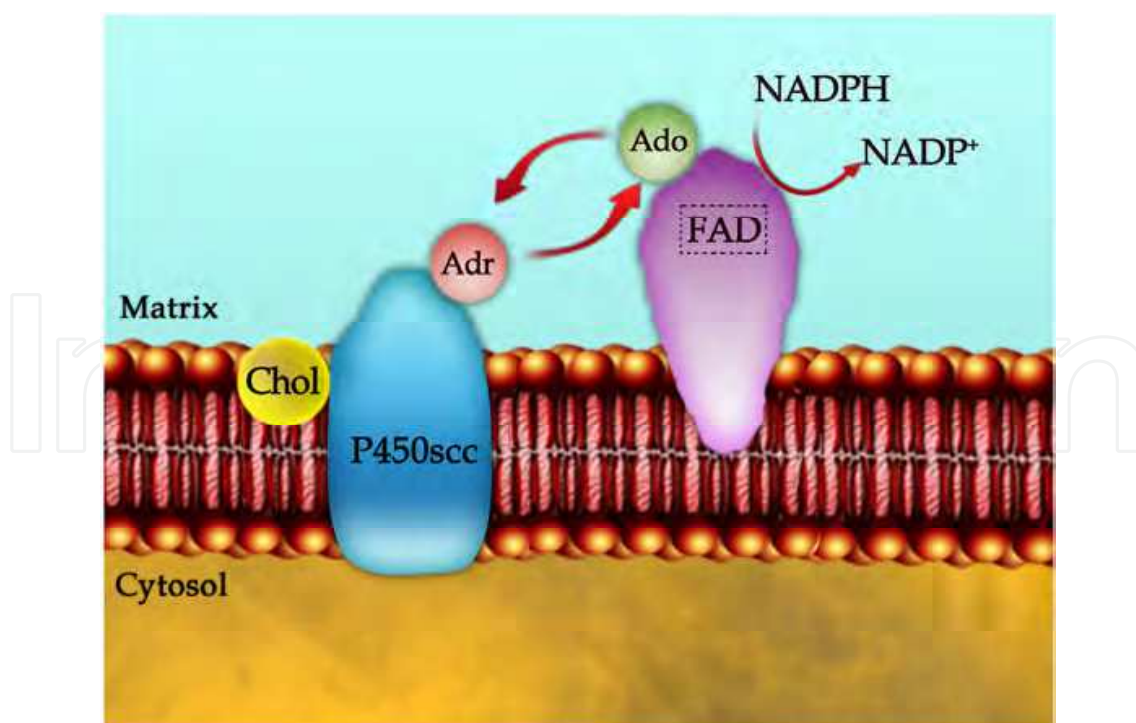


Fig. 13. Electron transport from adrenodoxin reductase to P450scc. Ado = adrenodoxin; Adr = Adrenodoxin reduced; FAD = adrenodoxin reductase (Modified from Payne and Hales, 2004).

The transformation of cholesterol into P5 requires three mono-oxygenation reactions, using molecular oxygen, involving two stereo-specific hydroxylations with the formation of 22(*R*)-hydroxycholesterol and 20(*R*),22(*R*)-dihydroxycholesterol followed by the breaking of the bond C-C between carbons 20 and 22 to release the lateral chain, yielding isocaproaldehyde and P5, which is changed into P4 through two consecutive reactions that require NAD⁺ and are catalyzed by the same enzyme 3HSD type 1 (EC 5.3.3.1) with no release of intermediaries. Two isoenzymes of 3HSD are known in humans, product of different genes (Payne & Hales, 2004). In the placenta, 3HSD is found in the mitochondria, unlike other steroidogenic tissues in which it is found in the endoplasmic reticulum. The activity of this enzyme is higher than P450_{scc} activity; therefore, it is not a limiting step in P4 synthesis (Tuckey, 2005). On the other hand, no disease involving the loss of 3HSD activity in the placenta is known, suggesting that its absence is incompatible with pregnancy.

5.2 Systems for cholesterol transport and mitochondrial contact sites

The cholesterol that participates in P4 synthesis comes from maternal circulation as lipoprotein complexes (LDL or HDL) which bind to their receptors in the plasma membrane. LDLs are released from their endosomal receptors to make late endosomes/lysosomes and obtain free cholesterol as substrate for P450_{scc} (Hu et al., 2010).

The transport of cholesterol from the cytoplasm into the outer mitochondrial membrane in most steroidogenic tissues is associated to many proteins; the StAR, is the first protein identified as part of a family that contains the START domain (StAR-related lipid transfer domain) of about 210 amino acids. It is synthesized as a 37 kDa protein with a signal peptide aimed to the mitochondrion to yield cholesterol to the outer mitochondrial membrane and, then is transformed into a 30 kDa intramitochondrial protein (Manna & Stocco, 2005). This protein is phosphorylated and activated in response to hormonal stimulation in steroidogenic cells (Arakane et al., 1996). The constructs lacking the 62 amino acid residues from the amino-terminal of StAR yields a truncated protein still able to participate in steroidogenesis. These results suggest that the translocation of the StAR protein to the interior of the mitochondria is not a requisite for cholesterol transport, and suggests that cholesterol may be transferred to another soluble acceptor protein or transporter in the outer mitochondrial membrane which finally allows it to reach P450_{scc} for P4 synthesis (Bose et al., 2000; Alpy & Tomasetto, 2006). Nevertheless, the human placenta does not express StAR, and it has been proposed that the protein MLN64 (Moog-Lutz, 1997) could perform the activity of cholesterol transporter. MLN64 is a 54 kDa protein (445 amino acids), isolated from a metastatic nodule of breast cancer. MLN64 is found in late endosomes and has two functional domains, one in the amino end with four transmembrane domains and another at the carboxyl end, corresponding to the START domain, oriented towards the cytoplasm, composed by 227 amino acids with an identity of 37% of StAR sequence. The tridimensional organization of its crystals shows the formation of a hydrophobic tunnel which allows the collocation of one molecule of cholesterol. This location supports the theory of MLN64 substituting StAR in human placenta to promote the flow of cholesterol.

Full-term human placenta isolated mitochondria synthesize P4 without the addition of exogenous cholesterol (Martinez et al., 1997). It has been reported that cholesterol transport between human placenta mitochondrial membranes requires proteins, since when treated with trypsin they are unable to transport cholesterol and, therefore, synthesize P4.

Nevertheless, mitochondria treated with trypsin were able to efficiently transform 22(R)-hydroxycholesterol into P₄, a substrate that does not need a protein membrane transport system, showing that the P450_{scc} chain is not modified by such treatment; thus making human placenta isolated mitochondria an adequate model for the study of cholesterol transport and steroidogenesis (Espinosa-García et al., 2000).

The transport of cholesterol towards the inner mitochondrial membrane requires many proteins associated with the contact sites; these are dynamic structures formed by proteins coming from both the outer and inner membrane and work as complexes that are assembled and degraded according to specific mitochondria conditions. Hence, contact sites might represent the most efficient route for cholesterol to reach P450_{scc} (Thomson, 2003).

It has been reported that during the isolation of mitochondrial contact sites from full-term human placenta, 3 fractions were obtained from the outer membrane and 4 from the inner membrane. The protein composition was specific for each one of them and only one fraction of the inner membrane was able to transform cholesterol into P₄. In such fraction, reported as steroidogenic site, porine, creatine kinase, the translocator of adenine nucleotides, ATP-diphosphohydrolase, MLN64, and HSP90, HSP72, HSP40 and HSP27, enzymes of the P450_{scc} chain, and NADP⁺-dependent isocitrate dehydrogenase were identified. These results support the theory that binding sites are an efficient system for cholesterol transit in the human placenta mitochondria (Uribe et al., 2003) (Fig. 14).

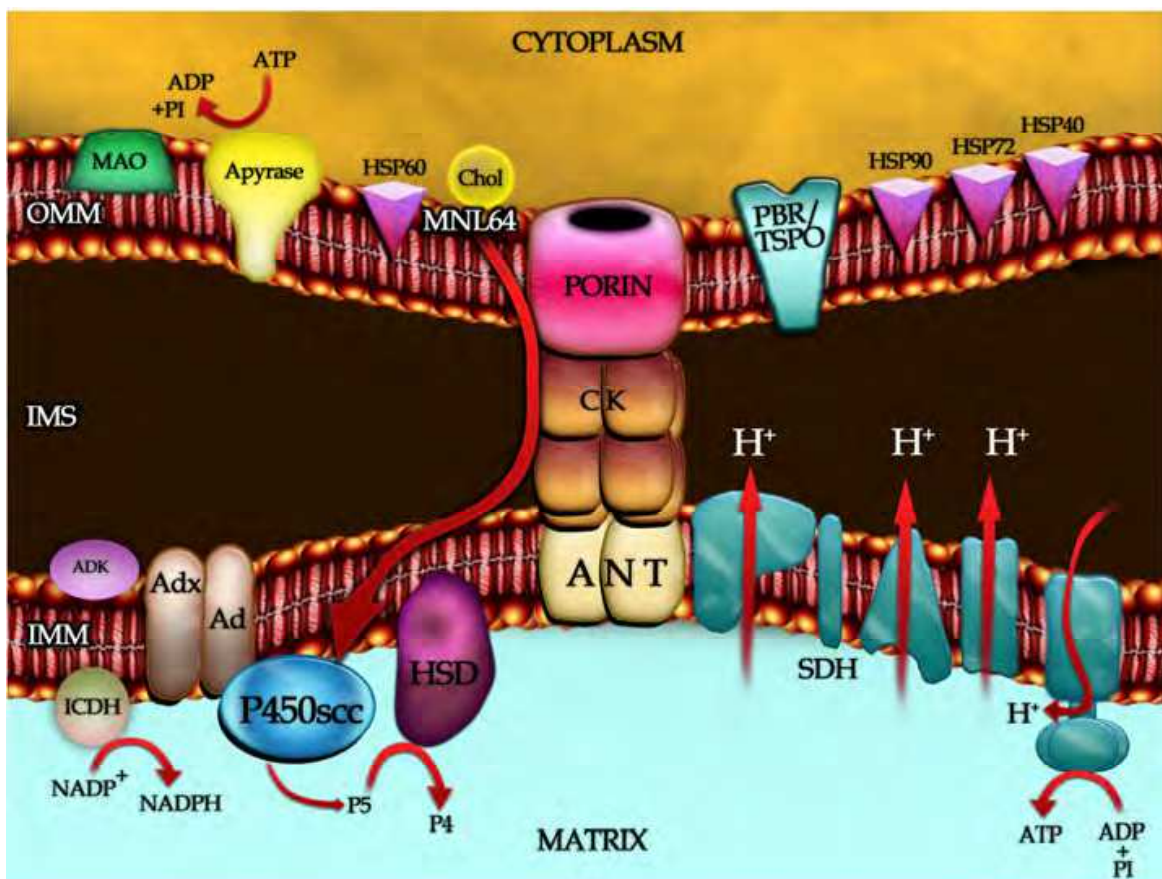


Fig. 14. Model of isolated contact sites from human placental syncytiotrophoblast mitochondria. (Modified from Uribe et al., 2003).

The use of MLN64 antibodies allowed the recognition of a 60 kDa protein identified as an HSP and another 30 kDa protein corresponding to the START domain of MLN64, in human placenta isolated mitochondria. Results suggest that both proteins participate in placental steroidogenesis, favoring both cholesterol movement towards mitochondrial membranes and the release of P4 from mitochondria (Olvera-Sanchez et al., 2011).

5.3 Steroidogenesis regulation

P4 biosynthesis regulation in placenta seems to be at two levels. One is related to hormones and/or factors currently unknown, which initiates a signal transduction cascade involving PKA activation cAMP mediated, as explained before, and another level involving the mitochondria.

As for the mechanism at mitochondrial level, it has been proposed that the activity of P450_{scc} might be regulated by the concentration of adrenodoxin reductase which causes a decrease of P450_{scc} affinity for cholesterol, and makes it work just at 16% of its capacity (Tuckey & Headlam, 2002). Nevertheless, the results obtained from mitochondria isolated from the placenta in the presence of 22(*R*)-hydroxycholesterol show no limitation in their capacity to produce P4. Therefore, it is unlikely that in physiologic conditions the activity of adrenodoxin reductase is a controlling factor.

Just like other steroidogenic tissues, the limiting step in P4 production is the access of cholesterol to mitochondria. So far, no evidence of any protein limiting transport is available, surely because it would be incompatible with pregnancy. Data available suggest that placental cells have the necessary mechanisms to allow cholesterol to reach mitochondria constantly, making P4 synthesis a constitutive metabolic pathway that assures, independently of nutritional conditions, physical or related to mother's health, that the fetus reaches the full term of pregnancy. In this context, the knowledge of the endocrine, paracrine, etc., signaling pathways would allow the development of therapeutic strategies that favor the integral development of the fetus.

Nevertheless, it is important to mention that acute regulation at mitochondrial level is necessarily accompanied by a chronic modulation mediated by the control of the transcription/translation of the genes that encode for the different steroidogenic enzymes, in a tissue-specific fashion. As for the placenta, it has been observed that there are mechanisms controlling the expression of the genes of the steroidogenic enzymes in which cAMP has no prominent role.

In gonads or adrenal glands, mutations of the genes encoding for proteins STARD1, CYP11A1 or 3HSD affects steroid production, being SF-1 the main factor regulating P450_{scc} expression (Schimmer & White, 2010). Nevertheless, SF-1 factor is not found in human placenta. It has been suggested that P450_{scc} expression is regulated by AP-2 factors that bind to cis elements overlapped to the sequences required for the recognition of SF-1 in other steroidogenic tissues (Ben-Zimra, 2002). It has also been proposed that LBP (Long Terminal Repeat Binding Proteins) identified in the syncytiotrophoblast might assume the regulator role of SF-1, binding to the region -155 to -131 of the promoter of the genes that allow the expression of P450_{scc}. LBP-1b would act as an activator of the expression of P450_{scc}, whereas LBP-9 and LBP-32 would act as repressors (Henderson et al., 2008; LaVoie & King., 2009).

6. Conclusion

On the whole, the information shows that mitochondria isolated from human placenta are an appropriate model to study the mechanisms of cholesterol transport. The physiological difference between human placenta and other steroidogenic organs make the human syncytiotrophoblast a particular tissue, which maintains a functional independence while allowing the interaction between the mother and the fetus to successfully reach the full term of pregnancy in order to perpetuate the species.

7. Acknowledgements

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