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Angiotensin II stimulation promotes mitochondrial fusion as a novel mechanism involved in protein kinase compartmentalization and cholesterol transport in human adrenocortical cells.

Katia E. Helfenberger^{1,2}, Ana F. Castillo^{1,2}, Pablo G. Mele^{1,2}, Ana Fiore^{1,2}, Lucía Herrera^{1,2}, Paola Finocchietto^{3,4}, Ernesto J. Podestá^{1,2}; Cecilia Poderoso^{1,2*}

¹Universidad de Buenos Aires, Facultad de Medicina, Departamento de Bioquímica Humana. Paraguay 2155 5th floor. (C1121ABG). Ciudad de Buenos Aires, Argentina

²CONICET-Universidad de Buenos Aires. Instituto de Investigaciones Biomédicas (INBIOMED). Ciudad de Buenos Aires, Argentina

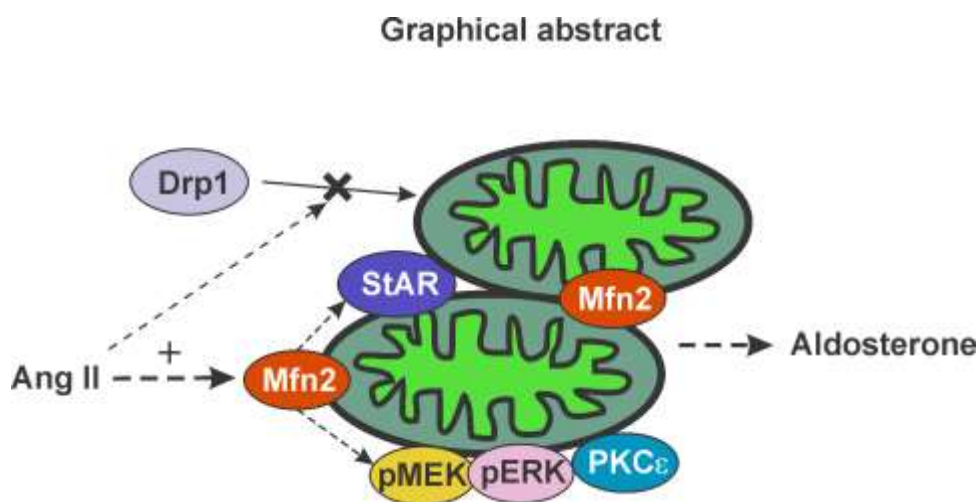
³Universidad de Buenos Aires, Facultad de Medicina, Hospital de Clínicas “José de San Martín”. Laboratorio del Metabolismo del Oxígeno. Av. Córdoba 2351 (C1121ABJ). Ciudad de Buenos Aires, Argentina

⁴CONICET-Universidad de Buenos Aires. Instituto de Inmunología, Genética y Metabolismo (INIGEM). Ciudad de Buenos Aires, Argentina

*Corresponding author: Cecilia Poderoso

E-mail: cepoderos@yahoo.com

Graphical abstract

**Highlights:**

- Angiotensin II increases key steroidogenic enzymes level in adrenal mitochondria.
- Angiotensin II promotes a mitochondrial fused network.
- Mitochondrial mitofusin 2 is upregulated by angiotensin II and potassium.
- Mitofusin 2 is mandatory for mitochondrial localization of key steroidogenic proteins.

Abstract

In steroid-producing cells, cholesterol transport from the outer to the inner mitochondrial membrane is the first and rate-limiting step for the synthesis of all steroid hormones. Cholesterol can be transported into mitochondria by specific mitochondrial protein carriers like the steroidogenic acute regulatory protein (StAR). StAR is phosphorylated by mitochondrial ERK in a cAMP-dependent transduction pathway to achieve maximal steroid production. Mitochondria are highly dynamic organelles that undergo replication, mitophagy and morphology changes, all processes allowed by mitochondrial fusion and fission, known as mitochondrial dynamics. Mitofusin (Mfn) 1 and 2 are GTPases involved in the regulation of fusion, while dynamin-related protein 1 (Drp1) is the major regulator of mitochondrial fission. Despite the role of mitochondrial dynamics in neurological and endocrine disorders, little is known about fusion/fission in steroidogenic tissues. In this context, the present work aimed to study the role of angiotensin II (Ang II) in protein subcellular compartmentalization, mitochondrial dynamics and the involvement of this process in the regulation of aldosterone synthesis.

We demonstrate here that Ang II stimulation promoted the recruitment and activation of PKC ϵ , ERK and its upstream kinase MEK to the mitochondria, all of them essential for steroid synthesis. Moreover, Ang II prompted a shift from punctate to tubular/elongated (fusion) mitochondrial shape, in line with the observation of hormone-dependent upregulation of Mfn2 levels. Concomitantly, mitochondrial Drp1 was diminished, driving mitochondria toward fusion. Moreover, Mfn2 expression is required for StAR, ERK and MEK mitochondrial localization and ultimately for aldosterone synthesis.

Collectively, this study provides fresh insights into the importance of hormonal regulation in mitochondrial dynamics as a novel mechanism involved in aldosterone production.

Keywords: mitochondrial fusion, adrenocortical human cells, mitofusin 2, angiotensin II, protein kinases, StAR.

1. Introduction

Adrenal gland, ovary, testis, placenta and brain are steroidogenic tissues strictly required for normal reproductive function and body homeostasis. Specialized cells from these tissues produce large amounts of steroid hormones from a unique substrate which is cholesterol. Translocation of cholesterol from the outer (OMM) to the inner mitochondrial membrane (IMM) is the rate-limiting step in the production of all steroids and requires a mitochondrial transporter known as steroidogenic acute regulatory protein (StAR) [1–3].

In the adrenal zona glomerulosa, aldosterone secretion is stimulated by angiotensin II (Ang II) and K^+ , in addition to ACTH. Ang II binds to Ang II type 1 (AT1) receptors triggering the activation of the inositol 1,4,5-trisphosphate (IP_3)– Ca^{2+} /calmodulin system [4], while K^+ activates voltage-operated Ca^{2+} channels. Both stimuli promote phosphorylation events, independently of cAMP/PKA pathway activation. It is completely recognized up to date that steroid biosynthesis is modulated by hormones, ions or growth factors through the post-translational phosphorylation of proteins, despite the differences in signal transduction pathways for each steroidogenic hormone.

Steroidogenesis involves the activation of cAMP-dependent protein kinase (PKA), protein kinase C (PKC), Ca^{2+} /calmodulin-dependent protein kinase [5] and mitogen-activated protein kinases (MAPKs) [6–9]. In Leydig MA-10 cells, an interacting module between PKA/MEK/ERK exists in mitochondria to form a multiprotein complex essential for the phosphorylation of intermediary proteins in hormonal action [10]. It is well known that the activation of the extracellular signal-regulated kinases 1/2 (ERK1/2) and its upstream activator–mitogen-activated protein kinase kinase–(MEK1/2) is required for hormonally induced steroid biosynthesis. ERK activity can induce *StAR* gene in adrenocortical cell line Y1 [7] and, specifically, mitochondrial ERK is able to phosphorylate StAR protein exclusively in the presence of cholesterol [6].

The different isoforms of PKC are involved in the signal transduction mechanism triggered by numerous hormones such as Ang II [11,12] to regulate the production of adrenocortical steroids, among others [13]. Ang II activates multiple pathways to MAPK in bovine adrenal glomerulosa cells mainly through a major PKC-dependent pathway [14]. PKC ϵ activation is increased by Ang II to further act as an upstream regulator of ERK1/2 in H295R human adrenocortical cells [11]. It has also been described that PKC ϵ is located in mitochondria of cardiomyocytes where it regulates the formation of a module between ERK and other mitochondrial MAP kinases, favoring cardioprotection [15], while isoform δ of the PKC translocates to mitochondria in the brain of hypertensive rats [16]. Therefore, it could be suggested that the same mitochondrial module is formed with PKC in other steroid-producing tissues. However, neither PKC nor ERK present

domains in their molecules that function as directional signal to mitochondria, nor are there intracellular transport proteins of these kinases. Compartmentalization is also evidenced by localized steroid secretion at the site of hormonal stimulation [17]; however, the mechanisms or proteins involved in this reorganization remain unknown.

Even if mitochondria play an important role in all cell types, they are essential in highly specialized ones such as steroidogenic cells. These essential mitochondrial functions have been attributed to their dynamic nature, i.e. their ability to undergo fusion and fission cycles which result in modifications in morphological changes and movement throughout the cytoskeleton. The key regulators of these mitochondrial dynamics are high molecular weight GTPases, mitofusin proteins (Mfn1 and Mfn2) and optic atrophy 1 (OPA1) as essential modulators of mitochondrial fusion in mammals. Both Mfn1 and 2 are located on the OMM, and Mfn2 in particular is involved in a tight association between mitochondria and the endoplasmic reticulum (ER), a subdomain called mitochondria-associated ER membrane (MAM). In turn, OPA1 plays a role in IMM fusion [18,19]. In contrast, dynamin-like related protein 1 (Drp1) participates in mitochondrial fission. Drp1 is mainly located throughout the cytosol, and a minor fraction of Drp1 localizes to the mitochondrial foci representing future fission sites [20]. The impairment of mitochondrial function has been proven a key factor for the development of several common diseases. For instance, Mfn2 expression levels are diminished in type II diabetes and in some neurodegenerative diseases, like Charcot-Marie-Tooth [21–23] and it is affected in several types of cancer [24]. This decrease depends, at least in part, on the degree of ubiquitination of this protein [25]. In addition, Drp1 regulation is essential to the maintenance of mitochondrial mass by balancing mitogenesis and mitophagy and mainly occurs by phospho/dephosphorylation [26–28].

Despite the importance of these proteins in mitochondrial function in health and disease, little is known about their mechanisms of regulation in endocrine systems. We have reported in our laboratory that mitochondrial fusion is essential for the increase in steroid production, regulated by cAMP/PKA transduction pathways in Y1 murine adrenocortical and MA-10 Leydig cells [29]. Moreover, mitochondrial fusion is required for the localization of key steroidogenic proteins and kinases after activation of G protein coupled-receptors in MA-10 Leydig cells [29]. In this context, the present work evaluates the effect of Ang II stimulation on the compartmentalization of relevant steroidogenic proteins, on the regulation of mitochondrial dynamics and the involvement of this process in steroid biosynthesis. We demonstrate that Ang II promotes the translocation of PKC ϵ and the activation of MEK and ERK specifically in mitochondria, and that mitochondrial fusion increases after Ang II challenge probably due to the upregulation of Mfn2 at transcriptional level. Mfn2 expression induced by Ang II is required for proper mitochondrial protein

compartmentalization and for aldosterone synthesis in H295R human adrenocortical cells. These results reveal the importance of hormone regulation in key protein subcellular localization and highlight mitochondrial dynamics as a novel mechanism involved in aldosterone production.

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2. Materials and methods

Cell line

The NCI-H295R cell line is a clonal strain of human adrenal carcinoma [30]. The cell line was from American Type Culture Collection (ATCC, Manassas, VA) and was handled as originally described [30]. The growth medium consisted of DMEM/Ham's F-12 1:1 containing 1.1 g/liter NaHCO_3 , 20 mM HEPES, 200 IU/ml penicillin, 200 $\mu\text{g/ml}$ streptomycin sulfate, and 5% COSMIC serum. Flasks and multiwell plates were maintained at 37°C in a humidified atmosphere containing 5% CO_2 . Ang II (100 nM) and potassium (14 mM) were used to treat the cells for the indicated times. Prior to treatments, cells were kept in a medium serum-free for 24h. Aldosterone and progesterone production was measured in the culture medium by radioimmunoassay (RIA) developed in our laboratory. Aldosterone and progesterone antibodies were generously provided by Alain Bélanger (Laval University, Quebec, Canada), aldosterone and progesterone standards were purchased to Sigma Aldrich, Inc. (St. Louis, MO, USA) and respective tracers were obtained from NEN Life Science Products Inc. (Massachusetts, USA) [31]

Isolation of mitochondria and post-mitochondrial fractions

Mitochondria were isolated as previously described [32]. Briefly, cell cultures were washed with PBS, scrapped in 10 mM Tris-HCl (pH 7.4), 250 mM sucrose, 0.1 mM EDTA, 10 μM leupeptin, 1 μM pepstatin A, and 1mM EGTA (buffer A), homogenized with a pellet pestle motor homogenizer (Kimble Kontes, Vineland, NJ), and centrifuged at $1000 \times g$ for 10 min. The supernatant was centrifuged at $18000 \times g$ for 20 min and rendered a mitochondrial pellet that was resuspended in buffer A. The supernatant was defined as the soluble, post-mitochondrial supernatant fraction. The purity of each fraction was at least 80%, a value similar to that of previous publications [33]. Control markers of the fractions to determine their purity degree are depicted in Figure S1.

Western blot

Total or mitochondrial proteins (20 μg) were separated on 12% or 10% SDS/PAGE and electrotransferred to polyvinylidene difluoride membranes as previously described [34]. Membranes were then incubated with 5% fat-free powdered milk or 1% bovine serum albumin (BSA) in 500 mM NaCl, 20 mM Tris-HCl (pH 7.5), and 0.5% Tween 20 for 60 min at room temperature with gentle shaking. Membranes were then rinsed twice in 500 mM NaCl, 20 mM Tris-HCl (pH 7.5),

and 0.5% Tween 20 and incubated overnight with appropriate dilutions of primary antibody at 4°C. 1:1000 rabbit polyclonal anti-PKCε, anti-β-tubulin, anti-total MEK1/2, anti-Mfn2, anti-Drp1, anti- full length StAR (Santa Cruz Biotechnology Inc., Dallas, Texas, USA), anti-phospho ERK1/2, anti-phospho MEK1/2, anti-total ERK1/2 (Cell Signaling, Beverly, MA, USA) and 1:10,000 mouse monoclonal anti-OxPhos Complex III core 2 subunit (Invitrogen, Carlsbad, CA, USA) were used. Bound antibodies were developed by incubation with secondary antibody that were obtained from Bio-Rad Laboratories Inc. (1:5000 goat anti-rabbit or 1:5000 goat anti-mouse horseradish peroxidase conjugated) (Hercules, CA, USA) and detected by chemiluminescence (BioLumina, Kalium Tech, BA, Argentina). Immunoblot bands were quantified using Gel-Pro Analyzer software.

RNA extraction

Total RNA from the different treatment groups was extracted using TriZol reagent following the manufacturer's instructions (Life Technologies, Inc.-BRL, Grand Island, NY). Any residual genomic DNA was removed by treating RNA with RQ1 Rnase-free DNase (Promega, Madison, WI, USA) at 37°C for 30 min, which was subsequently inactivated by incubation with 2 mM EGTA for 10 min at 65°C. The RT and PCR analyses were made with 1 µg of Dnase-treated RNA. The cDNA generated were further amplified by qPCR under optimized conditions using the primer pairs named below.

Real time PCR

The specific primers used for real-time PCR were: human Mitofusin 2 cDNA forward, 5'-ATGCATCCCCACTTAAGC -3' and reverse, 5'- GTTCTTCTGTGGTAACGG -3'; and human 18S RNA forward, 5'-ATTCCGATAACGAACGAGACT-3' and reverse, 5'-AGCTTATGACCCGCACTTACT-3' (obtained from RealTimePrimers.com, Elkins Park, PA, USA). Real-time PCR was performed using Applied Biosystems 7300 Real-Time PCR System. For each reaction we used 20 µl of a solution containing 5 µl of cDNA, 10 µM forward and reverse primers, and 10 µl of SYBR Select Master Mix (Applied Biosystems, Carlsbad, CA, USA). All reactions were performed in triplicate. Amplification was initiated by a 2-min preincubation at 50°C, 2-min incubation at 95°C, followed by 40 cycles at 95°C for 15 sec, 55°C for 15 sec and 72°C for 1 min, terminating at 95°C for the last 15 sec. Mfn2 mRNA expression levels were normalized to human 18S RNA expression, performed in parallel as endogenous control. Real-time PCR data were analyzed by calculating the $2^{-\Delta\Delta C_t}$ value (comparative Ct method) for each experimental sample.

Mfn2-shRNA plasmids

We used a murine Mfn2-shRNA which has been already proved in our previous works and reported [10]. BLAST data showed that the 19-mer RNAi sequence is specific against the mRNA sequence of human Mfn2 with 100% identity with the Mfn2 transcript of *Homo sapiens*, without targeting Mfn1, the other isoform that participates in mitochondrial fusion. We also designed a human Mfn2-specific shRNA. We used pSUPER.retro plasmid (OligoEngine, Seattle, WA, USA) containing a 19-bp DNA fragment of the human Mfn2 (GGAAGACATTGAGTTCCAT) named hMfn2-shRNA in the adequate frame shift to generate a shRNA.

Cell transfection

H295R cells were transiently transfected as previously described [29]. In all cases, empty vector was used as control (mock transfection). Briefly, one day before transfection, cells were grown up to 80% confluence onto cover glasses (12 mm) into 24-well plates, for microscopy or into 6-well plates, for Mfn2 knockdown. Transfection was performed according manufacturer's instructions using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). Cells were placed into normal culture medium 6h after transfection and grown for further 24h. Cells were then used as described in the respective figures.

Confocal microscopy

H295R cells cultured on poly-D-lysine-coated cover glasses (12 mm) were transfected with the yellow fluorescence protein targeted to mitochondria (mt-YFP, Clontech). Cells with the indicated mitochondrial morphology characterized as tubular fusion-shape mitochondria were quantified. More than a hundred cells were counted manually in at least four distinct optical fields. Mitochondrial morphology was scored by reference image-based model. For the former, images were assigned two different shapes by comparison to a set of reference images of mitochondrial clustering/punctuated and elongation/fusion [35]. Cell morphology was visualized by actin red staining with the fluorescence dye Phalloidin-TRITC (1:2000), incubated for 1 h at room temperature. Coverslips were mounted onto the slides using Fluorsave antifade reagent (Calbiochem, CA). The images were visualized using a Fluoview 1000 Olympus® confocal microscope (IMEX-Academia Nacional de Medicina, Buenos Aires, Argentina).

Protein quantification and statistical analysis

Protein was determined by the Bradford method [36] using BSA as a standard. Statistical analysis was performed by Student's t test or ANOVA followed by the Tukey's test.

3. Results

3.1 Mitochondrial localization of key protein kinases in cholesterol transport: regulation by angiotensin II

To accomplish the objectives of our work, we used the adrenocortical human cell line H295R, which maintains steroidogenic function and is widely used to study adrenocortical signal transduction mechanisms [30]. The participation of the α and ϵ isoforms of PKC has been demonstrated in H295R adrenocortical cells after stimulation with Ang II [11]. Although there are numerous studies about the role of the different isoforms of PKC in adrenal function, a differential localization of these proteins stimulated by Ang II has not been described so far.

The subcellular fractionation of H295R cells was performed after hormone stimulation and the presence of PKC in the mitochondria was analyzed. The analysis of PKC ϵ was chosen since it plays a key role in this cell line [11].

The stimulation with Ang II caused an increase in mitochondrial PKC ϵ level after 2h of stimulation, which was maintained until the end of the experiment at 6h (Figure 1A). When we analyzed PKC ϵ total levels in cellular lysates, we detected an increase in the expression of this enzyme triggered by Ang II incubation for 1h, earlier than mitochondrial translocation (Figure 1B). These results unveil that the pool of PKC ϵ which translocates to mitochondria after Ang II stimulation is enriched by the up regulation of its expression by the hormone, in a time-dependent manner. These data represent novel results in the regulation and subcellular distribution of this kinase in this steroidogenic system.

Figure 1

Although the activation of ERK by Ang II has been previously described in H295R adrenocortical cells [37], it is not known yet whether the signal triggered by this hormone can lead to a subcellular compartmentalization of the MAPK cascade in adrenal cells. Therefore, we studied the activation kinetics of MEK and ERK, analyzing the cytosolic (post-mitochondrial) fraction for MEK and the mitochondrial fraction for MEK and ERK.

The H295R cells, previously grown without serum for 24h, were stimulated with Ang II for the indicated times and the subcellular fractions were then obtained. Anti-phospho-MEK1/2 (pMEK) and phospho-ERK1/2 (pERK) antibodies were used as a measure of kinase activation. It was observed that pMEK increased gradually in the cytosolic fraction, with a significant increase after 2h of stimulation which was sustained until 6h (Figure 2A). This was clearly observed for

pMEK1, which is the prominent isoform present in the cytoplasm of H295R cells, compared to pMEK2 that just showed an increase in the phosphorylated form at 6h after Ang II challenge. When total MEK levels (tMEK) were analyzed, it can be observed that MEK1 and 2 seem to display an increase in cytosol prior to its phosphorylation, in agreement with AMPc-stimulation in MA-10 Leydig cells [6]. Although both isoforms are present and modulated in cytosolic fraction, just MEK2 is strongly phosphorylated by Ang II, at acute times.

On the other hand, we observed an increase in the mitochondrial pool of pMEK with a peak between 1-2h of stimulation with Ang II and a decrease after 6h (Figure 2B). Conversely, mitochondrial predominant isoform was MEK2. In agreement, a pool of total MEK2 is detected in basal conditions, which is increased after Ang II treatment. These results suggest the existence of upstream signaling for the activation of MAPKs, differentially compartmentalized and triggered by Ang II in adrenocortical cells.

Figure 2

Regarding the activation of ERK, we observed a strong signal from pERK2 and a weaker signal from pERK1 by immunoblot, as detected in previous works [37]. In the case of ERK1, a gradual increase was detected in phosphorylation, starting at 30 minutes and maintained after 6h stimulation with Ang II. About ERK2, we observed a marked increase in phosphorylation at 30 min of stimulation, a peak between 1 and 2h and a decrease at 6h of stimulation, in agreement with the activation kinetics of pMEK in mitochondria. These results suggest differential activation kinetics of ERK1 and 2 isoforms triggered by Ang II, even in the same organelle.

We also evaluated mitochondrial total ERK and we observed a basal mitochondrial localization for ERK in control cells, which is significantly increased during Ang II stimulation (Figure 3), as we described previously in MA-10 Leydig cells [38] and concomitantly phosphorylated, indicating that the pool of ERK that is translocated into mitochondria is effectively activated by Ang II.

Figure 3

3.2. Modulation of mitochondrial morphological changes after hormone stimulation

Given that the synthesis of steroids involves the activation of different signaling cascades through different hormones and cellular receptors, we aimed to investigate changes in the morphology of mitochondria after hormone stimulation with Ang II.

To pursue this goal, we transfected H295R cells with a mitochondria-targeted (mt-YFP) plasmid [29]. Twenty four hours after transfection, the cell medium was changed to serum-free medium for

another 24h. Then, (48h post-transfection) cells were incubated with Ang II for the indicated times. The morphological changes and mitochondrial rearrangements were evaluated according to a characterization of multiple mitochondrial shapes previously published by our group [29].

Under these experimental conditions, two main categories of mitochondrial morphology were clearly distinguished, dotted and elongated, which mainly correspond to mitochondrial fission and fusion, respectively [35].

Confocal microscopy revealed that control H295R cells mostly exhibit a mitochondrial punctate pattern, which switched to the fused pattern after Ang II treatment for 3h (Figure 4A). Mitochondria with the different morphologies were quantified, clearly showing that Ang II stimulation for 3h induced a significant increase in elongated mitochondria in H295R adrenocortical cells (Figure 4B).

Figure 4

3.3 Effect of Ang II and potassium on mitochondrial dynamics key regulator proteins

3.3.1 Hormone regulation of fusion key proteins: Mfn2 and OPA1

Having shown that Ang II promotes mitochondrial fusion, we further analyzed proteins involved in this process. Our group has previously reported the regulation of Mfn2 mRNA expression by hCG/cAMP stimulation in MA-10 Leydig cells [29]. Therefore, we focused on Mfn2 and decided to investigate a possible regulation of this protein by cAMP-independent pathways, such as Ang II and potassium, in steroids biosynthesis.

As observed by quantitative PCR (qPCR) results, 1h of Ang II elicited a robust increase in Mfn2 mRNA, with a prompt and marked decrease to control values thereafter (Figure 5A). In turn, Mfn2 protein analyses showed increase promoted by Ang II stimulation up to 4h and a subsequent decrease, probably due to ubiquitination and proteasome-dependent degradation [25]. Chronic stimulation with Ang II (24h) failed to promote an increase in mitochondrial Mfn2 (Figure 5B). The other classical aldosterone secretagogue of adrenal zona glomerulosa is potassium and its capacity of regulate Mfn2 expression was evaluated. We detected that incubation with KCl positively modulates Mfn2 mitochondrial localization in a time-dependent manner, with a significant increase between 1-4h after KCl incubation and a decrease thereafter (Figure 5C). Then, Mfn2 levels were driven to basal levels, as a possible result of the degradation of this protein, as mentioned before. Taken together, these results demonstrate that Mfn2 expression is

hormonally regulated in an acute manner in adrenocortical cells by several transduction signaling pathways.

Figure 5

In H295R adrenocortical cells, OPA1 has a short and a long isoform and it negatively regulates mitochondrial Ca^{2+} uptake. Although it has been established that OPA1 silencing significantly increased both basal and angiotensin II-induced aldosterone production and extramitochondrial OPA1 does not affect cAMP-mediated aldosterone synthesis; the regulation of OPA1 isoforms by Ang II has not been elucidated yet [39,40,41].

In this regard, Western blot analysis revealed that the short OPA1 (S-OPA1) isoform is hormone-modulated, with a decrease up to 4h of Ang II stimulation followed by an increase after 6h. The long isoform, L-OPA1, is differentially modulated by Ang II with a marked increase after 1h of stimulation. Both isoforms return almost to control levels at 24h of Ang II stimulation (Figure 6). This result shows for the first time a regulation of OPA1 isoforms triggered by Ang II. Even if there is no evidence for the role of OPA1 in cAMP-mediated steroid hormone production [41], Ang II does promote a regulation of the two main OPA1 isoforms detected in H295R cell line.

Figure 6

These results support that mitochondrial fusion is a triggered event in Ang II and potassium signal transduction pathway, a novel mechanism in adrenocortical cells steroidogenesis until now.

3.3.2 Effect of Ang II on Drp1 subcellular localization

Drp1 is mainly a cytosolic protein which is dynamically recruited to mitochondria and peroxisomal membranes, where it oligomerizes and drives membrane constriction in a GTP-dependent manner. Indeed, genetic loss of Drp1 leads to a drastic elongation of both mitochondria and peroxisomes in multiple cell lines and a variety of animal models [20]. As we presented here, Ang II promotes mitochondrial fusion, thus we assessed a possible regulation of Drp1 in adrenocortical cells. Western blot analyses revealed the presence of Drp1 in mitochondria under basal conditions. In contrast, Ang II stimulation promoted a significant time-dependent decrease in Drp1 levels in mitochondria (Figure 7), in agreement with a clear transition to fusion mechanisms.

Figure 7

3.4 Role of mitochondrial fusion in cholesterol transport: localization of StAR, MEK and ERK in mitochondria and steroids synthesis

To address the question whether mitochondrial fusion has a functional role in cholesterol transport, we used the RNA interference technique to transiently knockdown Mfn2, using two different sequences. We used a shRNA directed to murine Mfn2 (pSUPER-Mfn2-shRNA) extensively proved in our previous works [29,38], which showed 100% identity with the Mfn2 transcript of *Homo sapiens*, without targeting Mfn1. We also designed a human Mfn2-specific shRNA (pSUPER-hMfn2-shRNA) to reinforce our down-regulation experiments. The efficiency of Mfn2 knockdown in H295R cells was evaluated by Western blot in mitochondrial proteins (Figure 8A and B). A significant reduction in mitochondrial Mfn2 levels was observed, with both shRNAs, a pronounced effect although the transfection efficiency of this cell line is not a 100% [40].

On the basis of this evidence, further experiments were carried out to evaluate whether mitochondrial fusion and Mfn2 Ang II-dependent regulation have a role in the compartmentalization of cholesterol transport key proteins, also induced by Ang II. We also stimulated with 8Br-cAMP (cAMP permeant analogue) in this experiment (Figure 8A) to compare with previous results obtained in MA-10 Leydig cells [38]. Western blot confirmed that, in H295R cells transfected with both shRNAs, the localization of StAR in the mitochondria was diminished, both at basal level and after stimulation with Ang II (Figure 8A and B) or 8Br-cAMP (Figure 8A), in agreement with previous work [38]. The down regulation of Mfn2 did not affect complex III levels in mitochondria, allowing this OxPhos protein to be used as loading control for these experiments. Although StAR is a classic mitochondrial protein with a targeting leader peptide, mitochondrial fusion appears to be necessary for StAR localization in mitochondria, even in the presence of such directing sequence.

Figure 8

Although it is well known that MAPKs present diverse scaffold proteins to allow the correct subcellular distribution and activation of the cascade, it is not known yet whether these proteins present a selective anchoring to the organelles or the specific mechanism of association to them.

Through Mfn2-knocking down experiments using the two shRNAs described above, the results shows that proper Mfn2 expression is required for Ang II-mediated pMEK and pERK mitochondrial localization (Figure 9A and B), with the consequent physiological role of these kinases in the organelle, probably to form the mitochondrial multiprotein complex essential in steroidogenesis [10]. Interestingly, Mfn2 ablation did not affect total MEK and ERK levels, so

their association to mitochondria is not dependent on mitochondrial fusion but rather phosphorylation and functional activation of these kinases.

Figure 9

Next, we tested the effect of Mfn2 on aldosterone and progesterone synthesis. Stimulation by Ang II has been reported to produce a fast increase in progesterone (after 3h) and a later increase in aldosterone synthesis after 15 hours in H295R cells [42]. The current results further show that the knockdown of Mfn2 caused a significant reduction in progesterone and aldosterone levels produced by H295R cells and released to the medium after 16 hours of stimulation with Ang II (Figure 10). Mfn2 depletion did not modified basal nor aldosterone neither progesterone production levels. Therefore, it is shown that the increase in hormone-stimulated mitochondrial fusion through an increase in Mfn2 mitochondrial levels plays a functional role in the stimulated synthesis of different steroids produced by H295R cells. These results are in agreement with those obtained in MA-10 Leydig cells [29].

Figure 10

4 Discussion

Cellular compartmentalization in membranes and organelles is critical to intracellular signal specificity, as it strategically locates the kinases and their specific substrates. These mechanisms might underlie the localized secretion of steroids, not fully elucidated yet, and arise as a novel approach to the study of cell compartmentalization.

As widely established, steroidogenic hormones act through different message transduction systems to regulate protein phospho-dephosphorylation mechanisms by activating kinases and phosphatases. Transduction of the ACTH and LH/CG signal includes cAMP, PKA and events dependent on phosphorylation by this kinase [43–45], while Ang II and K^+ promote non-cAMP-dependent phosphorylation events, mostly by Ca^{2+} and PKC [4,46].

PKC isoforms are known to take part in the regulation of steroidogenesis at least in two separate enzymatic steps, the transformation of cholesterol to pregnenolone [13] and the conversion of deoxycorticosterone to aldosterone [47]. PKC also participates in the mobilization of cholesterol to the mitochondria mediated by Ang II in H295R cells, which enhances steroid production in its acute stage by increasing substrate availability [48]. PKC ϵ -mediated phosphorylation of the protein kinase D (or PKC μ) is involved in the production of aldosterone stimulated by Ang II through over-expression of 11- β hydroxylase and aldosterone synthase mRNA, in H295R cells [30,49–51]. Here, we have found that PKC ϵ levels are regulated by Ang II specifically in a mitochondrial pool, a novel finding to the best of our knowledge. Interestingly, PKC ϵ appears to be upregulated by Ang II as we observed in cell lysates before translocation to mitochondria. It suggests that newly synthesized enzyme translocates to mitochondria where it is activated by a well established mechanism [52]. Moreover, while total PKC ϵ is degraded by the proteasome according to the literature [53], the association of this enzyme to mitochondria seems to exert a protective mechanism. PKC appears to translocate to mitochondria under numerous conditions [54–56], in agreement with our results in adrenocortical cells. In particular, PKC can form a module in mitochondria with MAPKs with a fundamental function in cardioprotection [15] and the δ isoform of PKC translocates to mitochondria in the brain of hypertensive rats [16]. PKC ϵ translocation was regarded as ischaemic preconditioning-mediated protective effect on mitochondria and cell survival. Although some scaffold proteins have been described for PKC ϵ [57,58], the mitochondrial scaffold proteins for PKC which take part in steroid-producing tissues remain elusive. Future studies will aim to determine putative mitochondrial substrates of PKC and

whether phosphorylation of these substrates is critical for cholesterol transport in adrenocortical steroid biosynthesis.

There is abundant evidence about the location of MAPKs in the mitochondria in multiple cellular models and tissues [15,59,60] and particularly in adrenal gland [46,61]. Ang II causes a rapid and transient increase in mitochondrial phospho and total MEK1/2, particularly for MEK2, which suggests that a phosphatase in the context of the mitochondria dephosphorylates this kinase after 4 hours of stimulation with Ang II, probably as a way to regulate and attenuate the signal. However, the activation of MEK in the soluble fraction shows a different time profile of activation, with a delayed activation of MEK, specially marked for MEK1. These results are in line with that observed in Leydig MA-10 cells after stimulation with 8Br-cAMP, where differential activation of MEK is also observed in different subcellular compartments [6]. Cytosolic and mitochondrial total MEK are increased after Ang II incubation in a time-dependent manner, in fact prior to MEK phosphorylation; it can be suggested that powerful stabilization mechanisms are triggered by Ang II to establish a favored scenario for MEK phosphorylation and MAPK cascade activation. We have observed that after hormone stimulation with Ang II the translocation of total ERK to the mitochondria is acute, at 30 min of stimulation up to 4h with a decline at 6h after Ang II. As soon ERK translocates, is phosphorylated by mitochondrial MEK, which is beginning its activation in mitochondria. It could be argued that shorter times in the presence of Ang II favor the mitochondrial signaling pathway of MEK, possibly for the acute and non-genomic effects of the pathway (i.e. activating ERK to phosphorylate mitochondrial substrates); while after 2h MEK is activated in the cytosol to then phosphorylate cytoplasmic ERK, which translocates to the nucleus and thus regulates the transcription of certain target genes. However, a difference is visualized for the activation of ERK1 and ERK2, with a major phosphorylation for isoform 2. These results are in agreement with the literature, where a major activation of ERK2 is detected against ERK1 in the H295R cell line [37].

With respect to the regulation of ERK phosphorylation, MKP-1, a MAP kinase phosphatase of dual specificity, is increased after stimulation with Ang II in human and bovine adrenal models [62,63]. A study of MKP-1 overexpression has shown inactivation of ERK1/2 leading to a decrease in aldosterone synthesis [64]. Moreover, the fact that MKP-1 is induced approximately 2 hours after stimulation and is located in mitochondria in steroidogenic cells [65] could constitute a mechanism of regulation of the mitochondrial ERK signal in H295R adrenocortical cells. The fact that ERK continues to translocate into the organelle during Ang II treatment suggests that stimulation by Ang II rapidly promotes the phosphorylation of the translocated ERK pool in mitochondria through the activation of MEK and a later translocation of more ERK molecules is

achieved to sustain mitochondrial ERK further activation. ERK is known to phosphorylate StAR in mitochondria, as it does in MA-10 Leydig cells [6], although its action in adrenocortical cells remains to be tested.

As already presented, mitochondrial dynamics are required to preserve the correct functioning of the cell. It is currently accepted that mitochondria are fundamental platforms in the construction of various cellular processes. Despite the importance of mitochondrial dynamics, little has been studied about this process in tissues or cells where mitochondria are involved in specialized functions, such as the synthesis of steroids in endocrine systems. For instance, our group has published results obtained in a line of mitochondrial dynamics research on cAMP/PKA-mediated signal transduction in Leydig cells [29]. Most importantly, in the current work we show for the first time that Mfn2 mRNA and protein levels are modulated by Ang II in a time-dependent manner in adrenocortical cells, which has a strong correlation with the promotion of mitochondrial fusion by Ang II stimulation. The positive regulation on Mfn2 mitochondrial levels is also observed with potassium, suggesting that mitochondrial fusion promotion is a necessary event for steroidogenesis under different steroid secretagogues. It is not known how Mfn2 could be regulated at the transcriptional or post-transcriptional level by Ang II or potassium. Mfn2 promoter is activated by PCG-1 α , ERR α y Sp1 in skeletal and smooth muscle [21,66]. Particularly, Sp1 is a key factor in steroids synthesis [67], therefore it will be of great interest to further study transcriptional factors role, mRNA stabilization and post-traductional mechanisms that could be involved in Mfn2 regulation, in aldosterone synthesis stimulation.

Regarding mitochondrial fusion, OPA1 mediates IMM tethering. OPA1 harbors at least two sites for proteolytic cleavage which generate shorter and soluble fragments. These fragments are detectable by immunoblot and identified mainly as L-OPA1 and S-OPA1. The abundance of the different OPA1 isoforms is cellular context-specific and affects mitochondrial dynamics regulation [20]. The L-OPA1 isoform alone is sufficient to drive fusion and its overexpression is responsible for mitochondrial hyperfusion. Indeed, L-OPA1 accumulation drives fusion during stress-induced mitochondrial hyperfusion [68,69]. Recent advances remark how each OPA1 isoform is able to fulfill "essential" mitochondrial functions, whereas only some variants carry out "specialized" features. Long forms determine fusion, long or short forms alone build cristae, whereas long and short forms together tune mitochondrial morphology [70]. Therefore, the modulation of the balance between both isoforms by Ang II could be a mechanism intended to strongly promote mitochondrial fusion.

In addition to hormone regulation of mitochondrial fusion, we analyzed mitochondrial fission under Ang II stimulation by measuring the levels of Drp1 key fission protein. Drp1 is recruited from the cytosol to the mitochondria, which determine the site for the onset of fission. After stimulation with Ang II for different times, Drp1 levels showed to be decreased from the mitochondria, which is associated with a decrease in fission rates and concomitant balance toward fusion. The regulation of Drp1 includes a great variety of post-translational modifications such as phosphorylation, ubiquitination and sumoylation [28,71–74], which may promote interesting future studies of these modifications in Ang II transduction signaling, in adrenal tissue.

Several studies have now suggested a role for ERK1/2 in regulating Drp1 function. It has been demonstrated that Ser616 of Drp1 is phosphorylated by ERK1/2 in cancer cells and that this event promotes mitochondrial fission [26,75]. So far, our results suggest that the decrease in mitochondrial Drp1 could impair its interaction with ERK1/2 in mitochondria, preventing mitochondrial fission after Ang II stimulation. In agreement, PKC ϵ translocates to the mitochondria and exerts a myocardial protection in mice, promoting the dephosphorylation of Drp1 at Ser616 and avoiding activation of mitochondrial fission process [76]. Interestingly, our results suggest that Ang II-dependent translocation of PKC ϵ could lead to inhibition of Drp1 phosphorylation, loss of Drp1 association to mitochondria and mitochondrial fusion promotion, in H295R adrenocortical cells.

We have clearly demonstrated in this work that mitochondrial fusion through an increase in Mfn2 is strictly required for the localization of StAR in the mitochondria after stimulation with Ang II. It is well established that StAR is directed to the mitochondria by its signal peptide, which is cleaved and then acquires a molten globule shape necessary for its subsequent association with the OMM and the transport of cholesterol to the IMM [77]. Thus, lipid distribution is also a key influence on StAR location. In this sense, mitochondrial fusion could exert changes in the membranes of mitochondria in such a way that interaction with StAR is favored.

In addition, different variants of StAR mRNA are known to be regulated by cAMP-mediated stabilization in Leydig MA-10 cells [78]. StAR mRNA must be associated to the mitochondria through AKAP1, which stabilizes the translational complex in the mitochondria [79]. Thus, mitochondrial fusion could be thought to mediate the approach between StAR mRNA and AKAP1, increasing the level of StAR mRNA and protein in the mitochondria through stabilization. An effect of Mfn2 on the promoter of the *Star* gene, not known until now, should also be considered. The effect of Ang II on MEK and ERK mitochondrial phosphorylation, but not on total kinases levels, is also abolished by Mfn2 down-regulation. This result suggests that

kinases translocation/association with mitochondria have more to do with anchoring or scaffold proteins in organelles while phosphorylation may depend on spatial conformation probably helped by active mitochondrial fusion.

4.1 Conclusions

Mitochondrial fusion mediated by Mfn2 also proved necessary for MEK/ERK1/2 activation in the mitochondria, which indicates that hormone-induced mitochondrial fusion is required for effective MEK interaction with its upstream kinase or association with mitochondria. It can be surmised that decrease of mitochondrial pMEK results in lower phosphorylation and activation of ERK, which leads to a significant decrease in steroid production, together with the decrease in StAR mitochondrial localization.

Reorganization of organelles and contact between membranes can be a primary process in steroid production and secretion through the plasma membrane, with enzyme localization as a key feature to ensure appropriate steroidogenic rates. These regulatory mechanisms that involve mitochondrial fusion are universal to steroid producing tissues, acting by different signal transduction pathways like the Ang II/PKC/Ca²⁺ and potassium in adrenocortical cells, and could be extended to other tissues which metabolize cholesterol, such as the liver in the production of bile salts. Taken together, our findings reveal a novel role of mitochondrial fusion in the re-localization of proteins (as StAR) and kinases that are essential for adrenal steroidogenesis. These results emphasize that the subcellular activation of MAPKs is hormone-dependent on active mitochondrial fusion and Mfn2 expression. The fusion of mitochondria might represent an obligatory mechanism in the onset of processes that require transport of intermediate metabolites to achieve steroids production.

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ACCEPTED MANUSCRIPT

Figure Legends

Figure 1

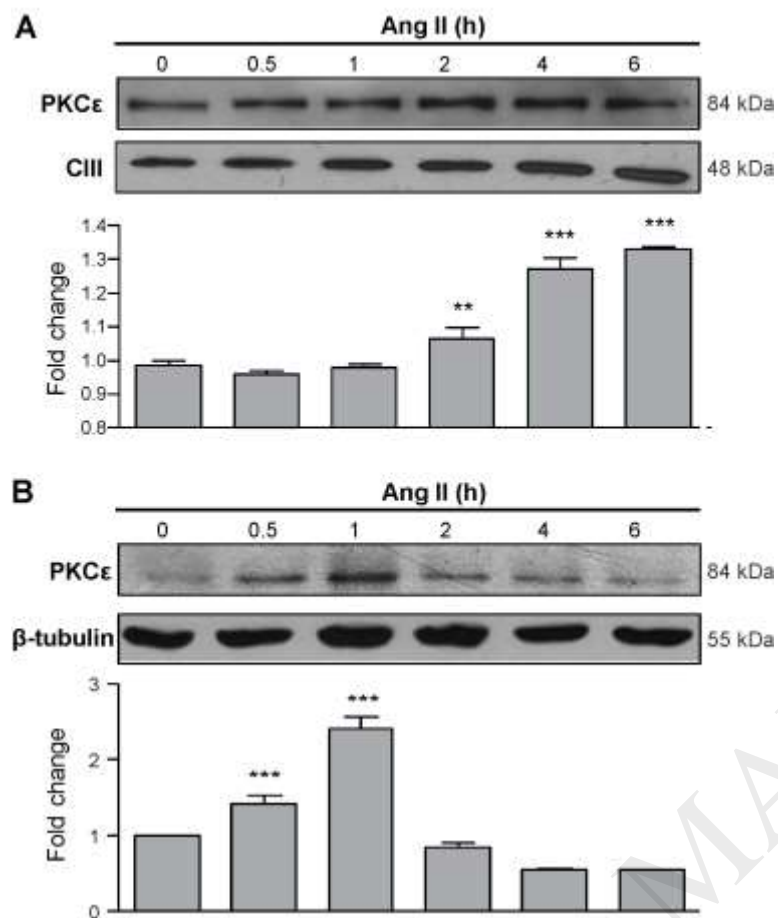


Figure 1: PKCε translocates from the cytosol to mitochondria upon stimulation with Ang II.

H295R cells were incubated with or without Ang II (100 nM) during the times indicated. Mitochondrial (A) and lysate (B) proteins were isolated and analyzed by Western blot. Membranes were sequentially incubated with anti-PKCε and anti-complex III (CIII) or anti-β-tubulin antibodies. Representative images of three experiments are shown and results are expressed as the means \pm SD: *** p <0.001 and ** p <0.01vs control. Control sample intensity was arbitrarily defined as 1 and variations in PKCε/loading control protein are indicated as fold change.

Figure 2

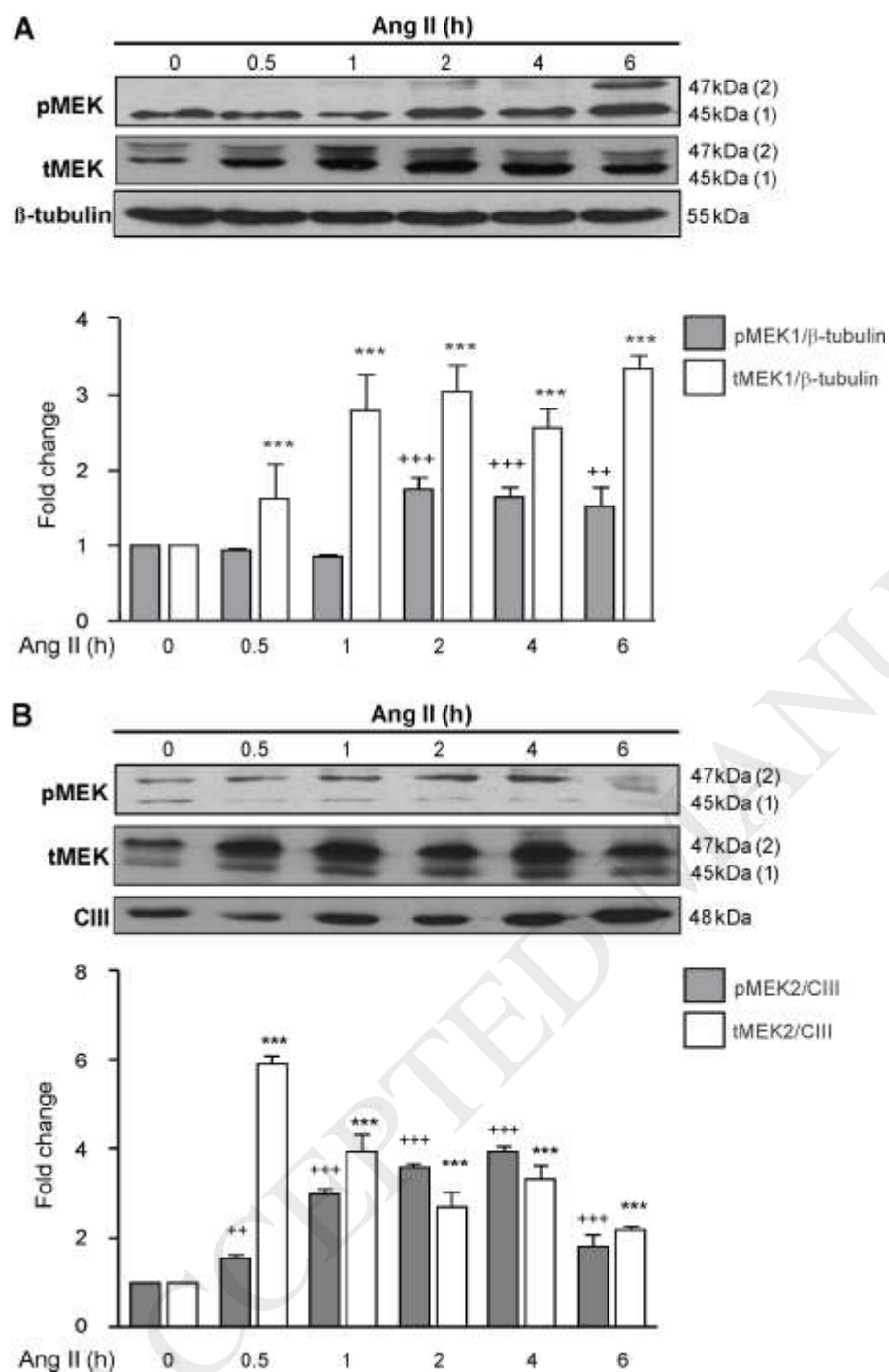


Figure 2: MEK1 and MEK2 are differentially activated in the cytosolic and mitochondrial fractions upon stimulation with Ang II.

H295R cells were incubated with or without Ang II (100 nM) during the indicated times. (A) Cytosolic and (B) mitochondrial proteins were isolated and analyzed by Western blot. Membranes were sequentially incubated with anti-pMEK1/2 (pMEK), total MEK1/2 (tMEK) and anti- β -tubulin or anti-CIII antibodies. The histograms show the normalized densitometric

results of three independent experiments and are expressed as the means \pm SD: *** p <0.001, ++ p <0.01 and +++ p <0.001 vs respective control without Ang II. MEK1/2 phosphorylation and total MEK1/2 levels in control group were taken as 1. For cytosol, pMEK1/MEK1 and for mitochondria, pMEK2/MEK2 were respectively normalized.

Figure 3

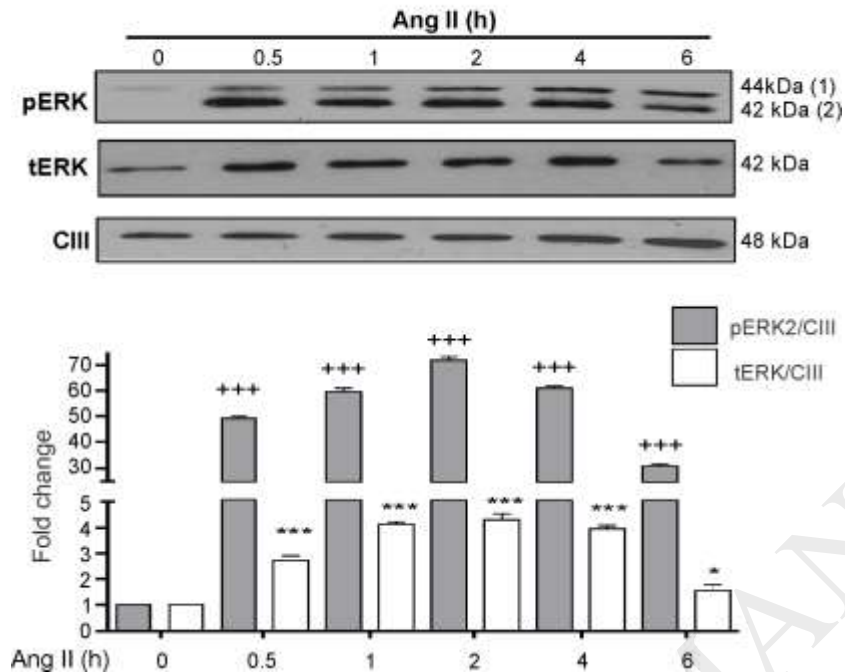


Figure 3: Ang II stimulates differentially ERK1 and ERK2 translocation and further activation in mitochondria.

H295R cells were incubated with or without Ang II (100 nM) during the indicated times. Mitochondrial proteins were isolated and analyzed by Western blot. Membranes were sequentially incubated with anti-phospho-ERK1/2 (pERK), total-ERK1/2 (tERK) and anti-CIII antibodies. The histograms show the densitometric result of the phosphorylation of three independent experiments and results are expressed as the means \pm SD: +++ p <0.001, *** p <0.001, and * p <0.05 vs control without Ang II. ERK phosphorylation and total ERK levels in control group was taken as 1.

Figure 4

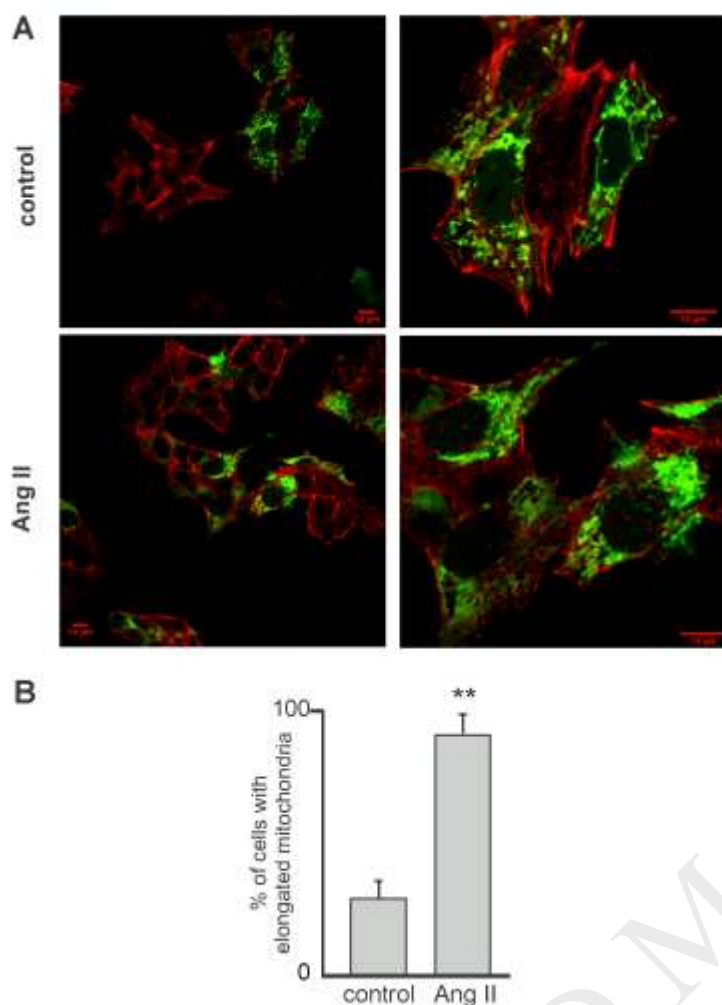


Figure 4: Ang II drives mitochondrial dynamics toward fusion.

H295R cells were transfected with the mt-YFP plasmid and 48h later incubated with or without Ang II (100 nM) for 3h. (A) Representative confocal microscopy images, mt-YFP (green), phalloidin-TRITC (red). Right column shows a magnification of micrographs in the left column. Scale bars are indicated in the figure. (B) Cells were processed as described in materials and methods and observed with a fluorescence microscope. Cells with elongated mitochondrial morphology were quantified. Approximately a hundred cells were counted manually in at least four distinct optical fields. Results are expressed as the means \pm SD of three independent experiments: ** $p < 0.01$ Ang II vs control.

Figure 5

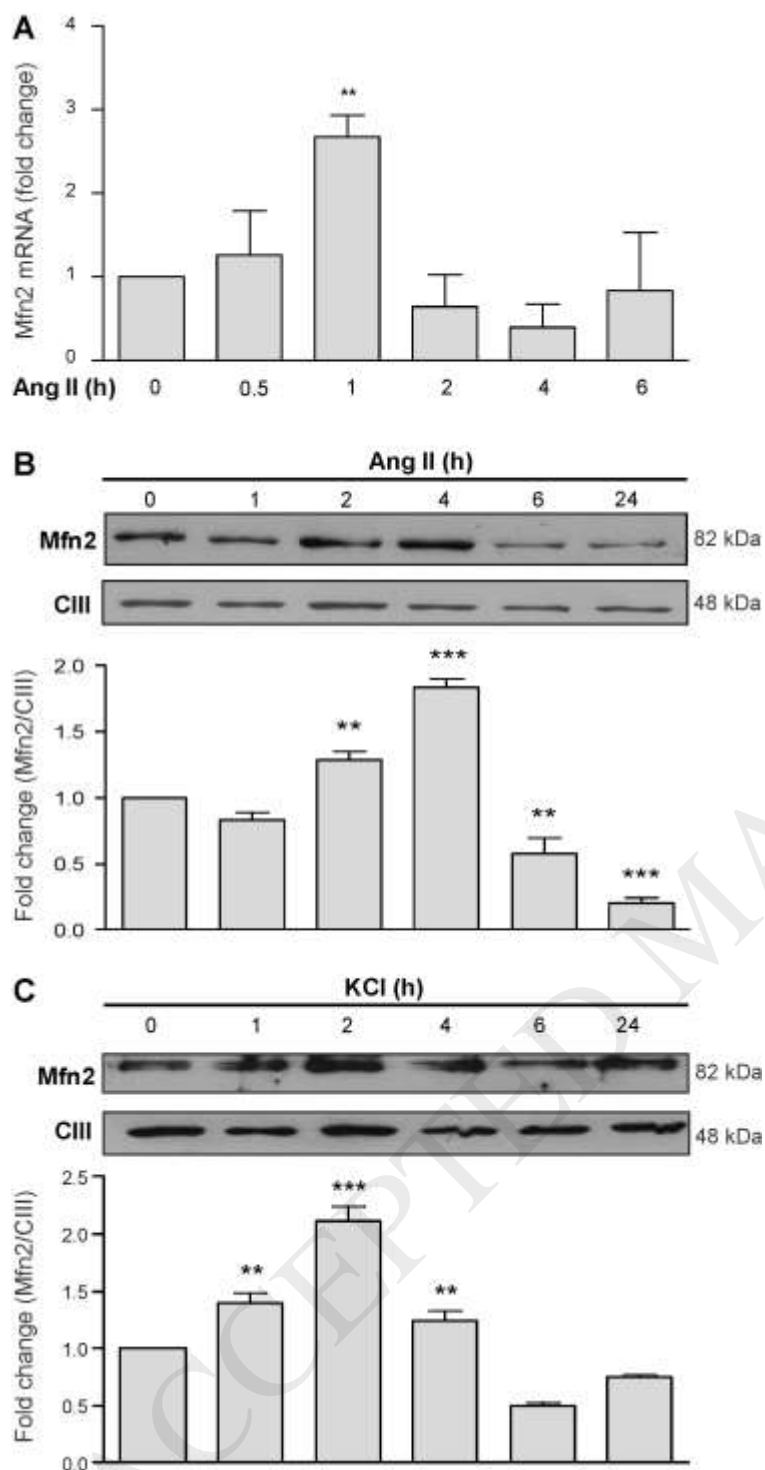


Figure 5: Ang II and potassium induces Mfn2 expression and mitochondrial localization.

H295R cells were incubated with or without Ang II (100 nM) (A and B) or KCl (14 mM) (C) for the indicated times. (A) Total RNA was isolated, treated with DNase, reverse transcribed (RT) and cDNA subjected to qPCR using specific primers. Mfn2 mRNA expression levels were normalized to human 18S RNA expression, performed in parallel as endogenous control. qPCR data were analyzed by calculating the $2^{-\Delta\Delta C_t}$ value (comparative Ct method) for each experimental sample.

Relative expression levels of Mfn2 are shown: $**p < 0.005$. (B and C) Mitochondrial proteins were isolated and analyzed by Western blot. Membranes were sequentially incubated with anti-Mfn2 and anti-CIII antibodies. Control sample intensity was arbitrarily defined as 1 and variations in Mfn2/CIII are indicated as fold change. Results are expressed as mean \pm SD of three independent experiments: $***p < 0.001$, $**p < 0.01$ vs. control.

Figure 6

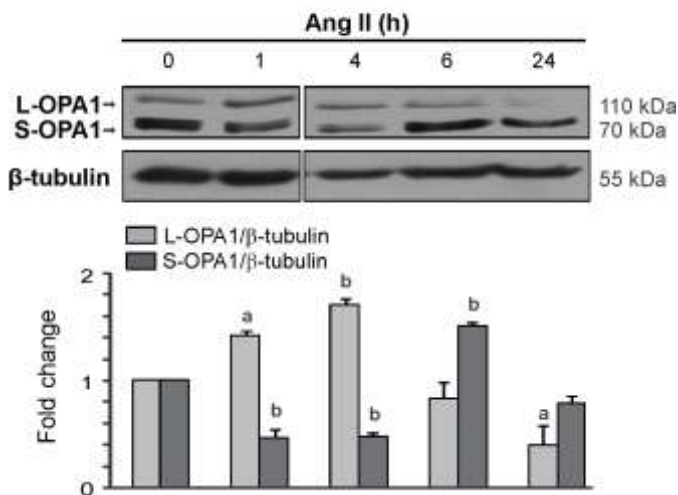


Figure 6: Ang II regulates OPA1 isoforms pattern.

H295R cells were incubated with or without Ang II (100 nM) for the times indicated. Total lysate proteins were isolated and analyzed by western blot. Membranes were sequentially incubated with anti-OPA1 and anti- β -tubulin antibodies. Control sample intensity for each isoform was arbitrarily defined as 1 and variations in OPA1/ β -tubulin are indicated as fold change. a: $**p < 0.01$, b: $***p < 0.001$ vs control of corresponding isoform.

Figure 7

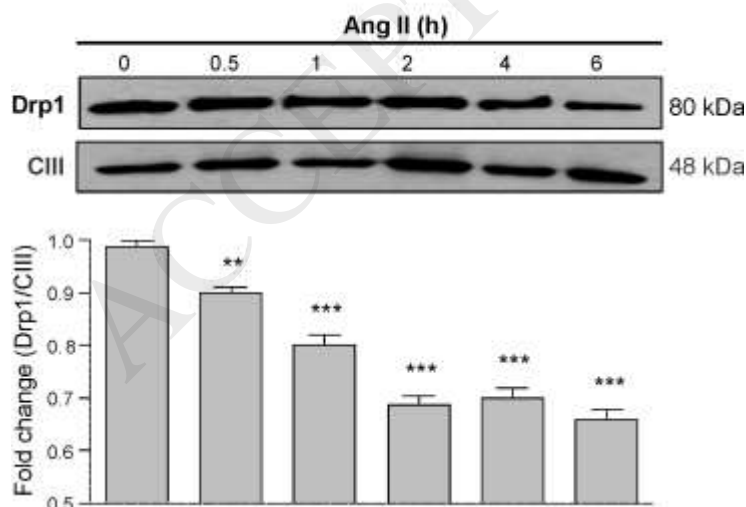


Figure 7: Ang II reduces Drp1 mitochondrial levels.

H295R cells were treated with Ang II (100 nM) for the times indicated. Mitochondrial proteins were isolated and analyzed by western blot. The membrane was sequentially developed using anti-Drp1 and anti-CIII antibodies. Representative Western blot images and fold change respect to control, taken as 1. Results are expressed as the mean \pm SD of three experiments: ** p < 0.05; *** p < 0.001 vs control.

Figure 8

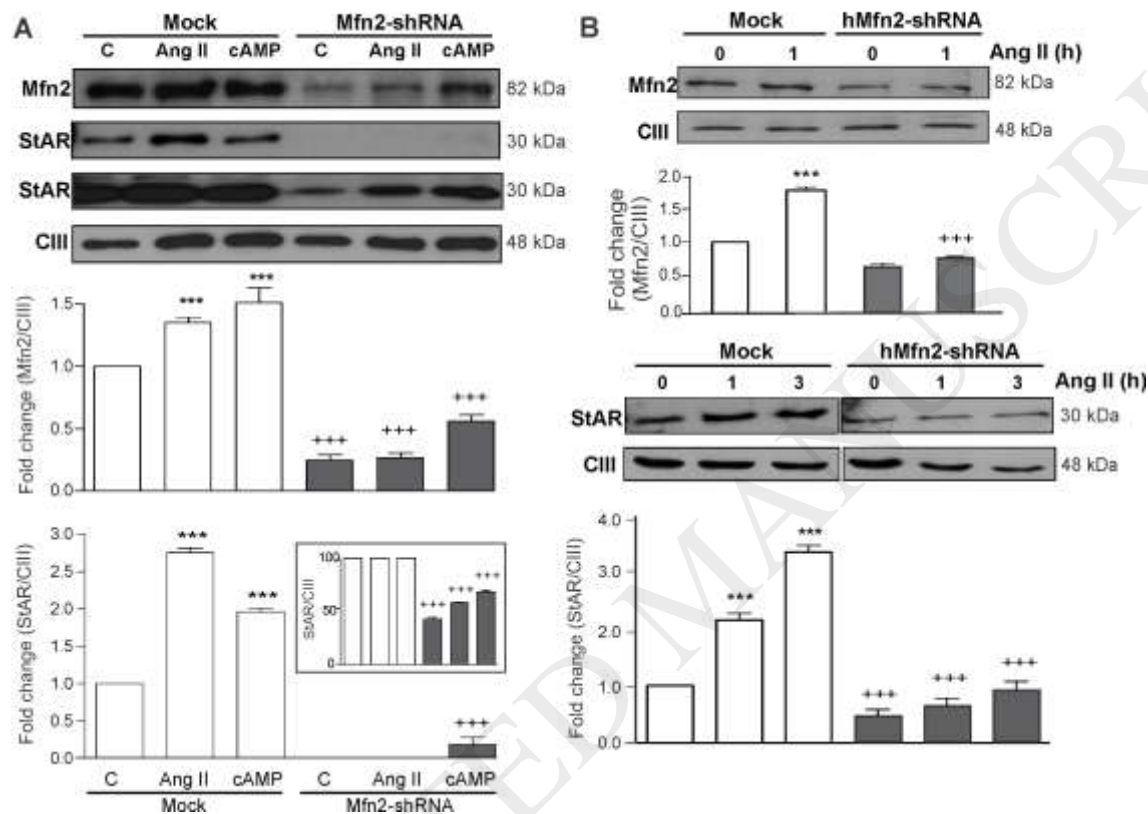


Figure 8: StAR mitochondrial localization depends on Mfn2 levels.

H295R cells were transfected with a pSUPER.retro empty vector (mock) or containing the sequence for short hairpin RNA for (A) murine Mfn2 (Mfn2-shRNA) or for (B) human Mfn2 (hMfn2-shRNA) to knockdown Mfn2. After 48 hours, cells were stimulated with Ang II (100 nM) or 8Br-cAMP (1 mM) for 1h (panel A) and with Ang II for the indicated times (panel B). Mfn2 and StAR levels in mitochondrial proteins in the presence of empty vector (mock) or shRNA were tested. Representative Western blots of three experiments are shown and anti-CIII antibody was used sequentially in the same membranes, as loading control. Inset in panel A shows StAR quantification of an overexposed autoradiography (more intense and second StAR blot), to allow the detection of remnant StAR protein in mitochondria of Mfn2-shRNA-transfected cells. Results are expressed as the mean \pm SD of three experiments: *** p < 0.001 vs mock without stimuli, +++ p < 0.001 shRNA vs mock for each treatment.

Figure 9

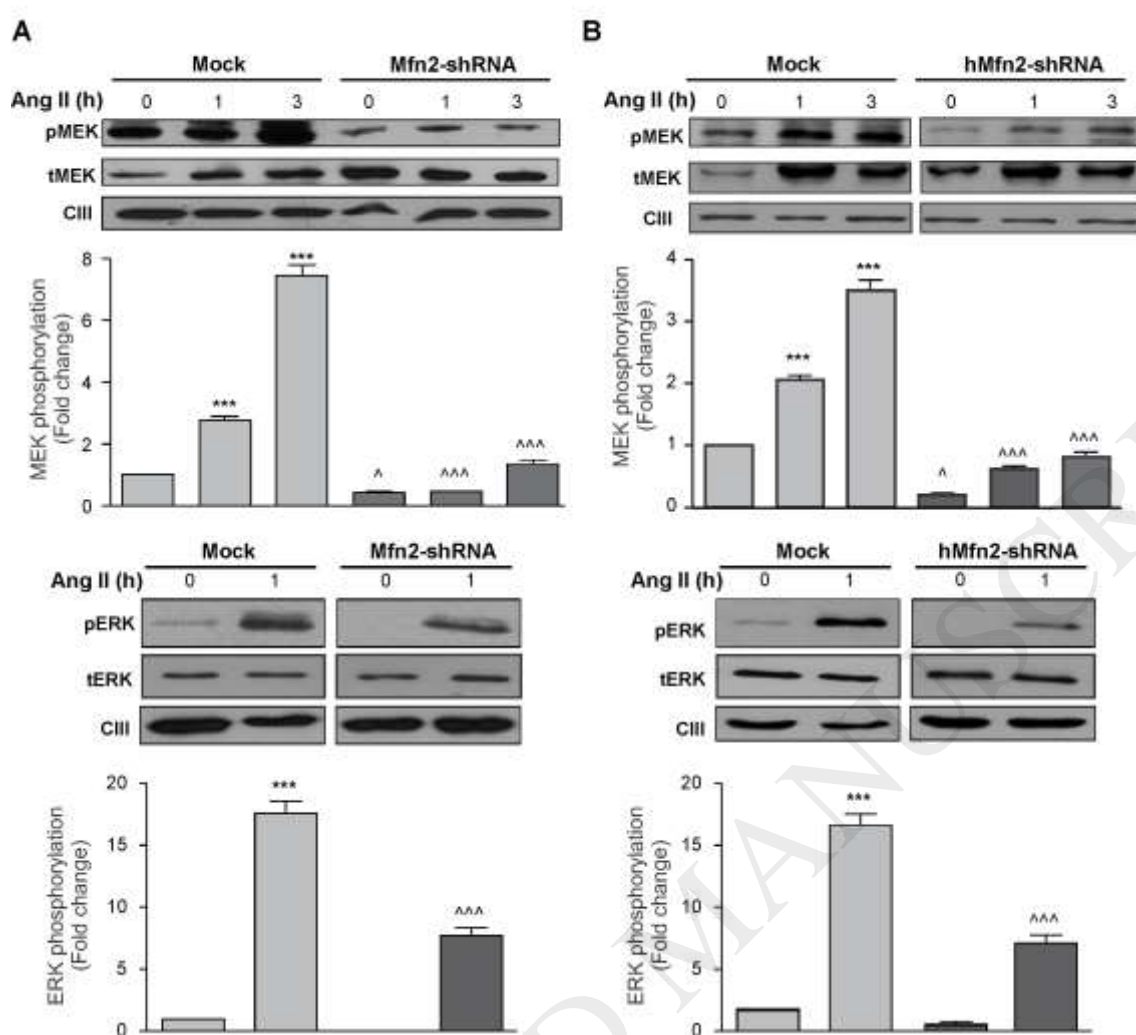


Figure 9: Mfn2 participates in mitochondrial MEK and ERK phosphorylation upon stimulation with Ang II.

H295R cells were transfected with a pSUPER.retro empty vector (mock) or shRNA for (A) murine Mfn2 (Mfn2-shRNA) or for (B) human Mfn2 (hMfn2-shRNA) to knockdown Mfn2. After 48 hours, the cells were incubated with Ang II (100 nM) for different times. Mitochondrial proteins were isolated and analyzed by Western blot. Membranes were developed using anti-pMEK and total MEK, pERK and total ERK and anti-CIII antibodies, as loading control. Representative Western blot images are shown and variations of phospho/total kinases are indicated as fold change. Results are expressed as the mean \pm SD of three experiments: **p < 0.05; ***p < 0.001 Ang II mock vs control mock; ^p < 0.05, ^^p < 0.001 murine or human Mfn2-shRNA vs mock for each treatment.

Figure 10

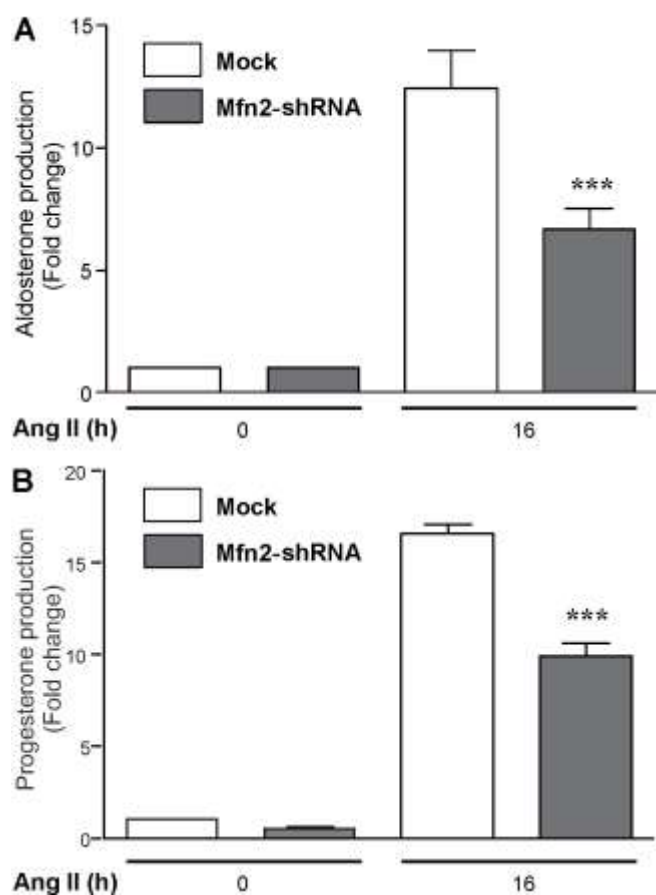


Figure 10: Mitochondrial Mfn2 is required for steroids synthesis.

H295R cells were transfected with pSUPER.retro empty vector (mock) or containing Mfn2-shRNA and then Ang II (100 nM) was added to the culture media during 16 hours. Aldosterone (A) and progesterone (B) were quantified by RIA. Results are expressed as the corresponding steroid production (pg of hormone/mg of protein) as fold change considering control values as 1. *** $p < 0.0001$ Mfn2-shRNA with Ang II vs. mock with Ang II.