Extramitochondrial OPA1 and adrenocortical function

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A R T I C L E  I N F O

Abstract

We have previously described that silencing of the mitochondrial protein OPA1 enhances mitochondrial Ca\(^{2+}\) signaling and aldosterone production in H295R adrenocortical cells. Since extramitochondrial OPA1 (emOPA1) was reported to facilitate cAMP-induced lipolysis, we hypothesized that emOPA1, via the enhanced hydrolysis of cholesterol esters, augments aldosterone production in H295R cells. A few OPA1 immunopositive spots were detected in ~40% of the cells. In cell fractionation studies OPA1/COX IV (mitochondrial marker) ratio in the post-mitochondrial fractions was an order of magnitude higher than that in the mitochondrial fraction. The ratio of long to short OPA1 isoforms was lower in post-mitochondrial than in mitochondrial fractions. Knockdown of OPA1 failed to reduce db-cAMP-induced phosphorylation of hormone-sensitive lipase (HSL), Ca\(^{2+}\) signaling and aldosterone secretion. In conclusion, OPA1 could be detected in the post-mitochondrial fractions, nevertheless, OPA1 did not interfere with the cAMP – PKA – HSL mediated activation of aldosterone secretion.

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Q1 The precursor of corticosteroids is cholesterol. Cholesterol may be synthetized within the endoplasmic reticulum or taken up from plasma lipoproteins. Cholesterol esters, taken up by endocytosis of receptor-bound LDL particles, are hydrolyzed in the endoplasmic reticulum. More important for steroid biosynthesis is HDL-transferred esterified cholesterol, taken up by scavenger receptor B1 and hydrolyzed by cholesterol esterase (Rone et al., 2009). At least two components of this complex, StAR and the translocator protein TSPO (previously named as peripheral benzodiazepine receptor), are phosphorylated and induced by PKA (Cherradi et al., 2003) to increase the expression of HSL.

Transfer of the released cholesterol to the side-chain cleaving enzyme cytochrome P450scc (CYP11A1), located in the IMM, is carried out by a complex of cytosolic and mitochondrial proteins (Rone et al., 2009). At least two components of this complex, STAR and the translocator protein TSPO (previously named as peripheral benzodiazepine receptor) are phosphorylated and induced by PKA (Dyson et al., 2008; Fleury et al., 2004; Manna et al., 2002; Midzak et al., 2011). Although Ca\(^{2+}\) - mobilizing agonists (through Ca\(^{2+}\) and protein kinase C) were also reported to phosphorylate StAR (Betancourt-Calle et al., 2001; Cherradi et al., 1998) their major effect is the induction of StAR expression (Clark et al., 1995; Lucki et al., 2012; Martin et al., 2008).

In adipocytes activated PKA phosphorylates and brings about translocation of the cytosolic HSL to the membrane fraction (Hirsch and Rosen, 1984). This translocation requires the 62-kDa protein perilipin 1 (Plin 1) located on the surface of lipid droplets (Greenberg et al., 1991; Miyoshi et al., 2006). It is assumed that non-phosphorylated Plin 1 inhibits the access of phosphorylated HSL to its substrate (Brasaemle et al., 2009; Szalay et al., 2003; Tansey et al., 2001).

The dual action of HSL is under hormonal control. ACTH, through cAMP-PKA, phosphorylates and thus activates the enzyme (Hirsch and Rosen, 1984; Holm et al., 2000; Kraemer et al., 2004; Trzecki and Boyd, 1974) and also induces its expression (Granneman and Moore, 2008; Holysz et al., 2011). Calcium ion, the second messenger of angiotensin II, acts via CaMKII to activate the translocator protein (Rone et al., 2009). At least two components of this complex, STAR and the translocator protein TSPO (previously named as peripheral benzodiazepine receptor) are phosphorylated and induced by PKA (Dyson et al., 2008; Fleury et al., 2004; Manna et al., 2002; Midzak et al., 2011). Although Ca\(^{2+}\) - mobilizing agonists (through Ca\(^{2+}\) and protein kinase C) were also reported to phosphorylate StAR (Betancourt-Calle et al., 2001; Cherradi et al., 1998) their major effect is the induction of StAR expression (Clark et al., 1995; Lucki et al., 2012; Martin et al., 2008).

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1. Introduction

The precursor of corticosteroids is cholesterol. Cholesterol may be synthetized within the endoplasmic reticulum or taken up from plasma lipoproteins. Cholesterol esters, taken up by endocytosis of receptor-bound LDL particles, are hydrolyzed in the endoplasmic reticulum. More important for steroid biosynthesis is HDL-transferred esterified cholesterol, taken up by scavenger receptor B1 (Rone et al., 2009) and hydrolyzed by cholesterol esterase (Rodrigueza et al., 1999). The esterase was recently found to be identical with the HSL of lipocytes (Kraemer et al., 2004). Following reesterrification cholesterol accumulates in special, phospholipid layer bounded droplets. Rapid increase of cortisol secretion during stress or increased aldosterone secretion during acute fluid loss requires the rapid mobilization of cholesterol stored in these lipid droplets (Hattangady et al., 2011; Vinson et al., 1992). Deesterification is performed again by HSL (Kraemer et al., 2004).

Abbreviations: [Ca\(^{2+}\)]\(_{\text{im}}\), mitochondrial Ca\(^{2+}\) concentration; AKAP, A-kinase anchoring protein; CaMKII, Ca\(^{2+}\)/calmodulin-dependent kinase II; COX IV, cytochrome c oxidase IV; db-cAMP, dibutiryl-cAMP; emOPA1, extramitochondrial OPA1; HSL, hormone-sensitive lipase; IMM, inner mitochondrial membrane; IMS, mitochondrial intermembrane space; Mfn 1, mitofusin 1; OMM, outer mitochondrial membrane; OPA1, Optic Atrophy 1; PDI, protein disulfide isomerase; PKA, protein kinase A; Plin, perilipin; StAR, Steroidogenic Acute Regulating Protein; TSPO, (mitochondrial) Translocation Protein (previously peripheral benzodiazepine receptor).

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et al., 2001; Zhang et al., 2003). Albeit PKA-mediated phosphorylation of Plin is not required for the translocation, phosphorylation is essential for hormone-induced lipolysis (Miyoshi et al., 2006). It is assumed that phosphorylation of Plin 1 permits the activation of adipocyte triglyceride lipase (Grahneman et al., 2011; Subramanian et al., 2004; Yamaguchi et al., 2007) which provides diacylglycerol for further hydrolysis by HSL (Zimmermann et al., 2004).

A recently described factor participating in hormonally-induced lipolysis is Optic Atrophy 1 (OPA1). OPA1 (and its ortholog Mgmp1 in yeast) has been known as a dynamin-related mitochondrial GTPase protein. In cooperation with Mitofusin 1 (Mfn 1) OPA1 induces mitochondrial fusion and its mutation is the most common cause of type 1 autosomal dominant optic atrophy (Belenguer and Pellegrini, 2012; Cipolat et al., 2004). Unexpectedly, OPA1 has been detected in lipocytes on the surface of Plin-coated lipid droplets (Pidoux et al., 2011). Immunocytochemical and immunoprecipitation studies showed that PKA binds to the Plin-associated OPA1 and the formation of this complex results in Plin phosphorylation and lipolysis. OPA1 contains an A-kinase binding domain and thus it may function as an AKAP. In fractionation studies the amount of OPA1 in the lipid droplet fraction far exceeded that in the mitochondrial fraction. Based on these observations a significant role has been attributed to OPA1 in the hormonal control of lipolysis, assuming that OPA1 potentiates the phosphorylation of Plin 1 by PKA and thus makes further steps possible (presumably the activation of adipocyte triglyceride lipase) (Greenberg et al., 2011; Pidoux et al., 2011). We are not aware of studies on emOPA1 in cell types other than adipocyte.

In humans OPA1 has eight mRNA isoforms and the expressed proteins can be separated in five bands between 96 and 84 kDa in Western blots. The two higher-molecular mass bands, the so-called long isoforms are mixture of isoforms 1, 2, 4 and 7 whereas the three short isoforms contain proteolytic products of the long ones and also isoforms 3, 5, 6 and 8. The long isoforms are attached to the IMM, the soluble short ones were found in the IMS partly associated to the OMM (Deletrè et al., 2000; Lenaers et al., 2009; Liesa et al., 2009). OPA1 controls the diameter of the junction of cristae (Scorrano et al., 2002) and thus modifies the molecular diffusion between the lumen of the cristae and the IMS (Frey et al., 2002) and/or between the boundary (inner) and cristal membrane (Sukhorukov and Bereiter-Hahn, 2009). Knockdown of OPA1 in H295R human adrenocortical cells, probably due to the altered diffusion conditions, facilitates the transfer of cytosolic Ca2+ signal into the mitochondrial matrix (Fülöp et al., 2011) resulting in enhanced aldosterone production (Spät et al., 2012).

In addition to HSL another factor participating in lipolysis, Plin1a (formerly Plin A) has also been detected in Y-1 murine adrenocortical cells (Servetnick et al., 1995). Other Plin isoforms, predominantly Plin1c (formerly Plin C), could also be detected following incubation with cholesterol (Hsieh et al., 2012). Importantly, Plin1a was phosphorylated in a cAMP-dependent manner (Servetnick et al., 1995). The data showing that identical molecules participate in the control of lipid metabolism in adipocytes and adrenocortical cells prompted us to examine whether OPA1, functioning as an AKAP, is a feasible candidate for regulating cAMP-induced steroid secretion. The verified and hypothetical mechanisms supplying cholesterol to mitochondria are shown in Fig. 1. We presumed that silencing of OPA1, if functioning as an AKAP in the extramitochondrial space, would reduce PKA-mediated steroid production to a greater extent than PKA-independent response. Our observations indicate that OPA1 is present in the extramitochondrial compartment in H295R cells but the role of emOPA1 in the control of steroid secretion could not be demonstrated.

2. Materials and methods

2.1. Materials

NIH-H295R cells (ATCC, CRL-2128) were purchased from LGC Standards Gmbh, Wesel, Germany. siRNA and silencing RNA products as well as OPTI-MEM, Lipofectamine 2000, Fluo-4 and MitoTracker Deep Red were purchased from Life Technologies (Paisley, UK).

Cholesteryl-Ester-BODIPY FL C12 (C-3927MP) was from Life Technologies (San Diego, CA, USA). UlterosG was from Bio Sepra (Cergy-Saint-Christophe, France). 2mt-eGFP (eGFP fused with a doublet of human cytochrome c oxidase target sequence) was a gift from Dr. B. Enyedi (Budapest, Hungary). Coat-A-Count RIA kit was purchased from Siemens Health Care Diagnostics (Los Angeles, CA).

Primary antibodies were purchased as follows: anti-OPA1 monoclonal antibody (612606): BD Bioscience (Franklin Lakes, NJ). Anti-Plin1a antibody (sc-4040): Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Plin1c antibody (sc-15362): Santa Cruz Biotechnology (Santa Cruz, CA). Anti-PLIN1 antibody (ab111083): Abcam (Cambridge, MA). Anti-HSL antibody (h130) and anti-CaMKII antibody (9402) were purchased from Cell Signaling Technology (Beverly, MA). Anti-actin antibody (A2228) was purchased from Sigma (St. Louis, MO). Anti-PLIN2 antibody (ab109659) and Anti-PLIN3 antibody (ab111078) were purchased from Abcam (Cambridge, MA). Anti-PLIN4 antibody (ab111079) was purchased from Abcam (Cambridge, MA). Anti-PLIN5 antibody (ab111080) was purchased from Abcam (Cambridge, MA). Anti-PLIN6 antibody (ab111081) was purchased from Abcam (Cambridge, MA). Anti-PLIN7 antibody (ab111082) was purchased from Abcam (Cambridge, MA). Anti-PLIN8 antibody (ab111083) was purchased from Abcam (Cambridge, MA). Anti-OPA1 antibody (612606) was purchased from BD Bioscience (Franklin Lakes, NJ). Anti-HSL ant
2.2. Cell culture and transfection

Microscopic examination was undertaken with a Zeiss LSM710 incubator. The cells were H295R cells were grown in DMEM/Ham’s F12 (1:1 v/v) containing 1% ITS, 2% UltraSer G, 100 U/ml penicillin and 100 μg/ml streptomycin. Passage numbers 4–20 were used. For transfection with 2mt-eGFP (for confocal microscopic examination) we used 0.4 μg plasmid DNA with 0.4 μl Lipofectamine 2000 in 220 μl OPTI-MEM medium. The transfection was performed on day 2 and repeated on day 3. The cells were fixed one day later. For transfection with scrambled RNA or siRNA (for the examination of HSL phosphorylation and in aldosterone experiments) the cells were electroporated before plating, using the Neon electroporator and kit (MPK10025) of Life Technologies. For measuring aldosterone production the cells were stimulated with appropriate agonists on day 4 (2-h stimulation) or on days 3 and 4 (24-h stimulation). For confocal measurement of [Ca^{2+}] the cells were transfected with siRNA on day 2, applying RNAiMax.

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Mfn1 (1:500), OPA1 (1:500), PDI (1:3000). Anti-mouse and anti-rabbit secondary antibodies were applied at a dilution of 1:5000 and 1:2500, respectively. For quantitative estimation of OPA1 and COX IV a dilution series of the mitochondrial samples were run parallel with samples of the two supramitochondrial fractions. For quantitative estimation of OPA1 and COX IV a dilution series of the mitochondrial samples were run parallel with samples of the two supramitochondrial fractions. Cytosolic and fat cake samples were compared with mitochondrial samples of comparable optical density on the radiograms. Integral density of regions of interest (ROIs), measured with Image J 1.43u, was corrected for background. The resulting value was regarded as indicator of the amount of separated protein.

2.6. Aldosterone production

For aldosterone experiments the cells were transfected by means of electroporation on day 1 using either equal amounts of three siRNA preparations for OPA1 (HSS107431, 107432 and 107433) or siRNA for Mfn1 (5141600) or a 1:1 mixture of non-silencing RNA species with appropriate GC content (12935400 and 129305200). Following electroporation about 2.5 × 10^5 transfected cells per sample were plated on a 24-well culture dish. The cells were incubated in the tissue culture medium until the 2nd (for 24-h stimulation) or 3rd day (for 2-h stimulation) when the medium was replaced with 0.1% UltroSer G. Next day a 1-h preincubation in serum-free medium was followed by a 2-h or 24-h incubation in the serum-free medium, in the presence of appropriate drugs. Aldosterone content of the incubation medium was measured with Coat-A-Count RIA kit, for calibration synthetic aldosterone was dissolved in cell-free incubation medium.

2.7. Measurement of cytosolic Ca^{2+} concentration

Cells transfected with Mfn1 or OPA1 siRNA were examined for [Ca^{2+}], three days after transfection. Fluorescence of cells preloaded with Fluo 4 and MitoTracker Deep Red was monitored at room temperature in multitrack mode with Zeiss LSM710 confocal laser scanning microscope, operated with ZEN 11.0 software. The cells were examined with a 63×/1.4 oil immersion objective (Plan-Apochromat, Zeiss). Transfection was regarded successful if mitochondria showed fragmented pattern.

2.8. Protein determination

Protein content of cell samples was estimated with Bradford assay for aldosterone measurements and with BCA assay before SDS-PAGE separations.

2.9. Statistics

Means ± SEM are shown. For estimating significance of differences unpaired t-test, factorial ANOVA and Tukey HSD test (Statistica 11) were used.

3. Results

3.1. Location of OPA1 in H295R cells

The intracellular location of OPA1 was first examined in cells transfected with mitochondrially targeted eGFP (2mt-eGFP). As shown in Fig. 2, mitochondria were immunopositive for OPA1 and a few OPA1 spots could be found also out of the eGFP-labeled...
particles. Altogether, emOPA1 could be detected in 10 out of 21 cells. In order to localize the presumed emOPA1 more precisely, we applied triple staining. Cholesterol storing lipid droplets were stained with the fluorescent cholesterol-ester mimetic CholEsteraryl-BODIPY FL C₁₂, OPA1 was immunostained and IMS was labeled with cytochrome c antibody. In average two emOPA1 immunopositive spots have been detected in 34 cells. These spots were mostly located in the vicinity of lipid droplets although OPA1 immunopositivity around the droplets predominantly colocalized with mitochondria (Fig. 3). emOPA1 could not be detected in another 62 cells. No OPA1 immunoreactivity was found after omitting the primary antibody (not shown). Examination with lambda scan mode of confocal microscopy also revealed extramitochondrial immunopositive spots for OPA1 (Supplementary Fig. 1).

3.2. OPA1 in subcellular fractions

The homogenized cells were separated into three fractions by differential centrifugation. Mitochondria were pelleted at 12,000 $g_{\text{max}} \times 15$ min. To obtain cytosol the supernatant was centrifuged at 31,000 $g_{\text{max}} \times 25$ min. A cloudy aggregate floating in the uppermost layer of the cytosolic fraction was withdrawn and termed (in analogy to fat cells) fat cake (Greenberg et al., 1991). The mitochondrial, cytosolic and fat cake fractions were analyzed for OPA1 and the IMM-marker COX IV by Western blotting.

All three fractions contained OPA1 as well as COX IV (Fig. 4 and Supplementary Table 2). In order to estimate mitochondrial contamination, the ratio of OPA1 to COX IV was estimated for all three fractions, taking this ratio in the mitochondrial fraction as unit. The ratio increased in the cytosol over mitochondria by a factor of 11.5 ± 3.5 units ($n = 3$). Due to insufficient amount of protein in one experiment the ratio in the fat cake could be estimated only in two fractionation studies where it attained 26.5 and 33.5 units. These data indicate that the amount of OPA1 in the extramitochondrial fractions was more than attributable to mitochondrial contamination (Supplementary Table 2).

The balance of the long (L) and short (S) isoforms of OPA1 in the cytosolic fraction was shifted towards the short ones as compared to that in the mitochondrial fraction. Whereas the S isoforms may have originated from the IMS of mitochondria with damaged OMM, L isoforms, which are anchored to the IMM (Delettre et al., 2000; Lenaers et al., 2009; Liesa et al., 2009), may not have such an origin. The contamination of the post-mitochondrial fractions with cytochrome c (Supplementary Table 2) supports the assumption that S isoforms may originate from damaged mitochondria.

3.3. Effect of OPA1 silencing on HSL phosphorylation

In order to test the presumed AKAP function of OPA1 we examined the knock-down of OPA1 on phosphorylation of HSL, a major target of PKA. Exposing the cells to db-cAMP for 2 h brought about a concentration dependent increase in the phosphorylated fraction of the enzyme ($p = 0.0009$). Although the mean extent of phosphorylation was somewhat lower in cells transfected with OPA1 siRNA...
Next we examined whether angiotensin II-induced phosphorylation of hormone-sensitive lipase (HSL) by 2-h stimulation with db-cAMP (0.05 or 0.5 mM) or 1 nM angiotensin II (angio). (A) The Western blot shows phosphorylated (upper panel) and total HSL (lower panel) on the same membrane. Statistics are shown for the effect of db-cAMP (n = 4) (B) and angiotensin II (n = 4) (C). The ordinates show the quotients of phosphorylated and total HSL densities. The effect of OPA1 siRNA was not significant (ANOVA, p = 0.19).

(Fig. 5 and Supplementary Fig. 2), the effect of silencing was not statistically significant (p = 0.19).

Next we examined whether angiotensin II-induced phosphorylation of HSL was dependent on OPA1. The hormone (1 nM) doubled the formation of phospho-HSL. The knock-down of OPA1 was without effect (Fig. 5).

3.4. Effect of OPA1 silencing on cAMP-induced Ca2+ signaling

Eighty-five Mfn1-silenced cells and 52 OPA1-silenced cells were stimulated with 0.05 mM db-cAMP. Most of the cells failed to respond within 10 min. The evaluable response in 7 control cells was almost thrice as high as that in 3 OPA1 – silenced cells; however, the number of responsive cells was too low for obtaining any conclusion. When the cells were stimulated with 0.5 mM db-cAMP, 8 out of 42 Mfn1-silenced cells and 9 out of 39 OPA1-silenced cells exhibited characteristic Ca2+ signal, with a lag-time between 4 and 10 min. Silencing failed to modify the amplitude of Ca2+ peaks (Supplementary Fig. 3).

3.5. Effect of OPA1 silencing on aldosterone production

In order to ascertain whether emOPA1 exerts any effect on cholesterol supply of steroid production we examined the effect of OPA1 silencing on the PKA-dependent aldosterone production. Considering that siRNA for OPA1 induces fragmentation of mitochondria (Olichon et al., 2003) and the consecutive changes in mitochondrial volume/surface ratio might influence signaling processes, comparison with maintained OPA1 expression but fragmented mitochondria were also required. Knock-down of Mfn1 also induces fragmentation without changes in the structure of IMM (Arnoult et al., 2005; Eura et al., 2003). Transfection with siRNA for OPA1 in H295R cells efficiently reduced the expression of OPA1 protein and did not influence that of Mfn1 protein (Fig. 1 in Fülöp et al., 2011) whereas transfection with siRNA for Mfn1 exerted reciprocal effect (not shown). Knock-down of OPA1 and Mfn1 induce fragmentation with indistinguishable morphometric changes (Figs. S1 and S3 in Fülöp et al., 2011) therefore, in addition to the effect of transfection with scrambled RNA the effect of siRNA against Mfn1 on hormone production has also been examined.

Aldosterone production has been studied under two different conditions. During acute (2-h) stimulation with db-cAMP or angiotensin II hormone production depends on the activation of the mechanism transporting cholesterol to the IMM. In contrast, during long-term stimulation several proteins participating in hormone production are also synthesized (Coulombe et al., 1996; Hattangady et al., 2011; Spät and Hunyady, 2004). The greatly enhanced steroid production will then be much more dependent on available free cholesterol.

Aldosterone response to db-cAMP or angiotensin II was examined in cells transfected with either control RNA or Mfn1 siRNA or OPA1 siRNA. Each group was stimulated with either 0.05 or 0.5 mM db-cAMP or 1 nM angiotensin II for 2 h or with 0.5 mM db-cAMP or 10 nM angiotensin II for 24 h. Qualitatively similar aldosterone responses were measured in the short and long-term stimulation series. db-cAMP stimulated aldosterone production in each group in a concentration-dependent way. Comparison of aldosterone production rates after transfection with various RNA species showed that highest responses were attained in the OPA1-silenced cells (for the effect of OPA1 siRNA: p = 10^-4) (Fig. 6 and 7). Importantly, knock-down of OPA1 equally affected the response to db-cAMP and angiotensin II, related to the effect of either type of control transfection (Table 1). These measurements indicate that the effect of OPA1 siRNA does not depend on the involvement of PKA in the stimulation of aldosterone production.

4. Discussion

Mitochondrial protein OPA1 located in close proximity with the lipid droplets has recently been described in lipocytes (Pidoux et al., 2011). Functioning as an AKAP it amplifies the PKA-induced phosphorylation of Plin 1, thus permitting enhanced lipolytic ac-
Fig. 6. Effect of knock-down of OPA1 on short-term aldosterone production. The cells were transfected by means of electroporation with scrambled RNA or siRNA against OPA1 or Mfn1 before plating. (Knock-down of Mfn1 evokes mitochondrial fragmentation similar to the effect of siRNA for OPA1.) Three days later the cells were stimulated with db-cAMP (0.05 or 0.5 mM) or angiotensin II (1 nM) for 2 h. Mean ± SEM of 6 experiments derived from 4 separate cell passages are shown. The effect of agonists and that of RNA were highly significant (ANOVA, \( p < 10^{-5} \)).

Fig. 7. Effect of knock-down of OPA1 on long-term aldosterone production. The cells were transfected as described for Fig. 6, however, two days after plating the cells were stimulated with db-cAMP (0.5 mM) or angiotensin II (10 nM) for 24 h. Mean ± SEM of 6 experiments derived from 3 separate cell passages are shown. The effect of agonists and that of RNA were highly significant (ANOVA, \( p < 10^{-5} \)).

Table 1

<table>
<thead>
<tr>
<th>Agonist</th>
<th>24 h Control RNA</th>
<th>Mfn1 siRNA</th>
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<th>Mfn1 siRNA</th>
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<tr>
<td>0.05 mM db-cAMP</td>
<td>1.400 ± 0.133</td>
<td>1.340 ± 0.131</td>
<td>1.367 ± 0.072</td>
<td>1.356 ± 0.076</td>
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<tr>
<td>0.5 mM db-cAMP</td>
<td>1.580 ± 0.150</td>
<td>1.450 ± 0.114</td>
<td>1.514 ± 0.094</td>
<td>1.289 ± 0.012</td>
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<tr>
<td>1 nM angiotensin II</td>
<td>1.555 ± 0.128</td>
<td>1.514 ± 0.094</td>
<td>1.292 ± 0.063</td>
<td></td>
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<tr>
<td>10 nM angiotensin II</td>
<td>1.555 ± 0.128</td>
<td>1.514 ± 0.094</td>
<td>1.292 ± 0.063</td>
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assumption that mitochondrial contamination is a major factor accounting for the presence of OPA1 in the post-mitochondrial fractions. Unexpectedly, the pattern of OPA1 isoforms in the mitochondrial fraction differed from that in the post-mitochondrial fractions, being relatively enriched in short isoforms in the latter ones. Since long isoforms are strongly attached to the IMM by their transmembrane domain whereas the short isoforms, lacking transmembrane domain 1 (Song JCB 2007) are only weakly attached to membranes (Satoh et al., 2003), the damage of the OMM may lead to the leakage of short isoforms only. (Note that only a small fraction of cytochrome c is bound to membranes (Cortese et al., 1998), therefore its leakage may exceed that of short OPA1 isoforms.) The presence of cytochrome c in the cytoso1 supports the assumption that OMM damage of variable extent may have occurred during cell homogenization.

Since immunocytochemical and cell fractionation studies failed to provide unambiguous indication for the significance of emOPA1 we examined the effect of OPA1 siRNA on three PKA targets, namely HSL, the cAMP-evoked Ca2+ signaling and aldosterone production.

Two-hour stimulation with db-cAMP significantly enhanced the phosphorylation of HSL. Transfection with OPA1 siRNA was followed by a slight, statistically insignificant decrease in the mean value of phosphorylation. Angiotensin II-induced phosphorylation of HSL, accounted for by Ca2+ dependent activation of CaMKII (Cherradi et al., 1998) was not sensitive to OPA1 siRNA. AC, as well as 8-bromo-cAMP induce sustained Ca2+ response after a lag-time of a few minutes in rat (Tremblay et al., 1991) and human glomerulosa cells (Gal1o-Payet et al., 1996). Similarly to myocardial cells (Sperelakis and Schneider, 1976) this effect of cAMP in glomerulosa cells is brought about by the phosphorylation of L-type Ca2+ channels by PKA (Derroux et al., 1991). It follows that cAMP-induced Ca2+ signal could be regarded as an indicator of OPA1 functioning as an extramitochondrial AKAP. db-cAMP-induced Ca2+ signal in small fraction of the cells only. The signal developed after a lag-time of several minutes and in quite a few cases it was not substantial enough for reliable measurement. For these reasons the effect of OPA1 silencing on the effect of 0.05 mM db-cAMP could not be conclusively evaluated. At a higher concentration (0.5 mM) of the agonist, one third of the cells displayed Ca2+ signal and it was resistant to silencing of OPA1.

Irrespective of any effect of OPA1 on HSL in c-AMP activated cells, its presumed action on PKA should facilitate phosphorylation and induction of STAR that in turn should lead to increased steroid production. Moreover, the biological significance of OPA1 in H295R cells depends should be indicated on by its effect on aldosterone secretion. Although aldosterone is one of several steroids produced by H295R cells, all these steroids derive from the same cholesterol pool and therefore aldosterone can be regarded as an appropriate indicator of mitochondrial access to cholesterol. At designing the experiments it had to be recalled that siRNA for OPA1 evokes fragmentation of mitochondria. In H295R cells the mitochondrion fragmenting effect of Mfn1 siRNA is comparable with that of OPA1 siRNA (Fülöp et al., 2011), therefore cells transfected with siRNA against Mfn1 were considered the appropriate control.

In principle, silencing of OPA1 might exert a dual action on aldosterone production, depending on the mode of action (cAMP or Ca2+) of the physiological agonist. Although angiotensin II is a Ca2+ mobilizing agonist and the action of ACTH is mediated by cAMP, there is some overlap between their mode of action. Angiotensin II, acting via the heterotrimeric G-protein Gi (Gutowski et al., 1991), induces acute aldosterone secretion by IP3-mediated Ca2+ release (Enyedi et al., 1985), followed by Ca2+ influx (Hunyady et al., 1994; Kramer, 1988). The ensuing Ca2+ signal is a prerequisite for increased aldosterone secretion (Spät and Hunyady, 2004). ACTH or cAMP may also induce Ca2+ influx and this Ca2+ influx contributes to the stimulation of aldosterone (Ball et al., 1982). Ca2+ influx may be attributed to PKA-enhanced activation of L-type Ca2+ channels (Gal10-Payet et al., 1996; Lenglet et al., 2002). db-cAMP-induced Ca2+ signals were observed also in the present experiments. It follows that Ca2+ signaling may be a common element in the action of angiotensin II and cAMP. In contrast to Ca2+ signaling, cAMP is not a common mediator of the two agonists. In addition to Gi, angiotensin II also activates the adenylyl cyclase inhibitory G protein Gi (Enyedi et al., 1986; Maturana et al., 1999). This may be the reason why the peptide does not increase the generation of cAMP (Bell et al., 1981; Hausdorff et al., 1987) despite the probable expression (Tait and Tait, 1999) of Ca2+ sensitive isoforms of adenylyl cyclase (Hanoune and Defer, 2001).

Cytosolic Ca2+ signal is transferred into the mitochondrial matrix (Hajnóczky et al., 1995; Pralong et al., 1992; Rizzuto et al., 1992) and the elevated mitochondrial [Ca2+] contributes to the enhancement of steroid production (Spät et al., 2012; Wiederkehr et al., 2011). Therefore changes in mitochondrial Ca2+ metabolism should be considered at evaluating the effect of OPA1 knockdown on aldosterone production. Applying the mitochondrionally targeted Ca2+-sensitive proteins 4tm-D2 and mt-inverse Pericam we observed knockdown of OPA1 significantly enhanced mitochondrial Ca2+ uptake both in intact and permeabilized H295R and HeLa cells (Fülöp et al., 2011). This enhancement was associated with increased aldosterone production (Spät et al., 2012). Our data on HeLa cells have not been confirmed by Kushnareva et al. (2012) who estimated [Ca2+]i with Rhod-2, a dye less specific for mitochondria than the mitochondrially targeted proteins applied in our experiments. Allowing to the increased circularity (a parameter indicating fragmentation) of mitochondria in the OPA1-silenced cells (Fülöp et al., 2011) Kushnareva et al. attributed our results to swelling of mitochondria. It should, however, be recalled that we observed enhanced Ca2+ uptake also in comparison with Mfn1-silenced cells, displaying circularity identical with that in OPA1-silenced cells. Kushnareva et al. also reported that the number of Ca2+ pulses required to activate mitochondrial permeability transition pore is less in OPA1-silenced cells than in sham-transfected controls. This observation may indicate enhanced rather than attenuated Ca2+ uptake (cf. e.g. (Joiner et al., 2012; Mallikar4aman et al., 2012)). In addition to increased mitochondrial Ca2+ uptake knock-down of OPA1 may enhance aldosterone production also by enhanced cholesterol transport through the IMM into the matrix, as observed in trophoblast cells (Wasilewski et al., 2012).

Again, in contrast to Ca2+ signaling, cAMP is not a common intracellular mediator of ACTH and All. Therefore, in OPA1-silenced cells, due to the elimination of the presumed AKAP function, the amplification of secretory response to db-cAMP should be smaller than in angiotensin II-stimulated cell. Nevertheless, this was not the case. In fact, silencing of OPA1 the fold-response to all the stimuli increased in each group to the same extent, irrespective whether PKA participated in the activation of steroid production. This indicates that AKAP action of OPA1 has no role in the activation of cAMP-PKA mediated hormone production.

A special member of the AKAP family, AKAP121 can be an-chored to mitochondria and may compartmentalize PKA as well as other proteins on the OMM (Wong and Scott, 2004). In Leydig cells, cAMP-induced STAR expression and steroidogenesis were found to correlate with the extent of AKAP 121 expression (Dyson et al., 2008). Expression and role of AKAP121 in H295R cells de-serve elucidation.

In a recent review on OPA1 Belenguer and Pellegrini (Belenguer and Pellegrini, 2012) emphasized the possibility that OPA1 reported to be present in the lipid droplet fraction of adipocytes (Pi- doux et al., 2011) may have been due to contamination with mitochondria. These authors raised several questions concerning the proposed role of OPA1 in lipolysis. Our observations support
the presence of OPA1 in the extramitochondrial space, for the first time in a cell type other than adipocyte. At the same time we obtained no evidence for the role of this fraction of OPA1 in cAMP-mediated steroid hormone production, the specific biological function of adenocortical cells.

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Appendix A. Supplementary material

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References


