The Effect of OPA1 on Mitochondrial Ca\(^{2+}\) Signaling

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Abstract

The dynamin-related GTPase protein OPA1, localized in the intermembrane space and tethered to the inner membrane of mitochondria, participates in the fusion of these organelles. Its mutation is the most prevalent cause of Autosomal Dominant Optic Atrophy. OPA1 controls the diameter of the junctions between the boundary part of the inner membrane and the membrane of cristae and reduces the diffusibility of cytochrome c through these junctions. We postulated that if significant Ca\(^{2+}\) uptake into the matrix occurs from the lumen of the cristae, reduced expression of OPA1 would increase the access of Ca\(^{2+}\) to the transporters in the crista membrane and thus would enhance Ca\(^{2+}\) uptake. In intact H295R adrenocortical and HeLa cells cytosolic Ca\(^{2+}\) signals evoked with K\(^+\) and histamine, respectively, were transferred into the mitochondria. The rate and amplitude of mitochondrial [Ca\(^{2+}\)]\(_{\text{im}}\) rise (followed with confocal laser scanning microscopy and FRET measurements with fluorescent wide-field microscopy) were increased after knockdown of OPA1, as compared with cells transfected with control RNA or mitofusin1 siRNA. Ca\(^{2+}\) uptake was enhanced despite reduced mitochondrial membrane potential. In permeabilized cells the rate of Ca\(^{2+}\) uptake by depolarized mitochondria was also increased in OPA1-silenced cells. The participation of Na\(^+/\)Ca\(^{2+}\) and Ca\(^{2+}/\)H\(^+\) antiporters in this transport process is indicated by pharmacological data. Altogether, our observations reveal the significance of OPA1 in the control of mitochondrial Ca\(^{2+}\) metabolism.

Introduction

Recent observations obtained in imaging and electron tomographic studies revealed a dynamically changing structure [1,2] and led to a revised concept of the structure and function of mitochondria. The changes in the number and size of mitochondria involve alterations in the inner mitochondrial membrane (IMM). The invaginations of the IMM, termed cristae, display various conformations under changing energetic conditions. The cristae are connected to the inner boundary membrane (i.e. the part of IMM between two neighboring cristae) by narrow tubular junctions which have a diameter of 15–40 nm [3–6]. These junctions may impede free diffusion and thus may induce the formation of a gradient of ions, molecules and macromolecules between the intermembrane space (IMS) and the lumen of the cristae [5,7].

The fusion of mitochondria is regulated by the transmembrane GTPase proteins mitofusin (Mfn) 1 and 2 and OPA1 [1]. Mutation of the OPA1 gene is the most prevalent cause of the type 1 Autosomal Dominant Optic Atrophy [8,9]. Although the fundamental pathological phenomenon is the degeneration of retinal ganglion cells with subsequent atrophy of the optic nerve [1,10,11], the protein is expressed in all examined human tissues, explaining the accidental association of blindness with external ophthalmoplegia and various neuromuscular lesions [12–14]. Reduced expression of OPA1 was also reported in ischaemic heart failure [15] showing that insufficient expression of the protein may have far-reaching consequences.

OPA1 (and Mgm1, its ortholog in the yeast) is a dynamin-related GTPase protein. Due to alternative splicing, its gene is transcribed into 8 mRNA isoforms [16]. The protein is tethered to the IMM [17–19] and localized in the IMS [18,20]. In Western blot analysis 5 separate bands (designated a to e) of molecular weight, ranging from 94 to 86 kDa, can be found. Two long isoforms are anchored to the IMM and three soluble short forms are located in the IMS. These latter bands are the proteolytic products of the long forms [21,22]. Heteromultimeric complex formation of Opa1 was suggested on the basis of the relatively constant stoichiometry of the long and short isoforms [23]. On their own long and short isoforms have little impact on the fusion of mitochondria, but when coexpressed they functionally complement one another [24].

OPA1 regulates the diameter of the crista junction. The tightness of junctions correlates with the oligomerization of the membrane-bound and the soluble forms in the IMS [25]. Knockdown of either the Mfn1 [26–28] or the OPA1 gene brings about the fragmentation of the mitochondria [19,29,30], moreover, knocking down of OPA1 (or Mgm1) gene also evokes drastic desorganisation of the cristae [29,31–34]. An essential component of the structural change is the dilatation of the junctions [4] (but see [6]). It has been proposed several years ago that the size of the junction may modify the diffusion of molecules like adenine nucleotides [3].
The role of the junction in the control of mitochondrial metabolism has been suggested by numerous observations. The majority of cytochrome $c$ reductase, $F_{1}F_{0}$ ATPase [35] and of cytochrome $c$ oxidase [36], as well as the uncoupling protein 1 (in brown adipocytes) [37] are found within the cristae membrane. Only 10–15% of cytochrome $c$ is found free in the IMS, while the major fraction can be found in the cristae [4,30]. The proapoptotic agent truncated Bid (t-Bid) known to evoke disassembly of OPA1 oligomers [6,25] brought about a drastic increase in the junction diameter [4]. This increase was associated with enhanced cytochrome $c$ release from dixigonin-permeabilized mitochondria [4,6]. The effect of t-Bid could be potentiated with silencing of OPA1 gene [33] and overcome with overexpression of OPA1 [25], indicating that the clearance of the junction is controlled by OPA1. These observations strongly suggest that the state of OPA1 may modify substrate-dependent enzymatic and transport processes occurring in the crista membrane.

Calcium mobilising agonists generate cytosolic Ca$^{2+}$ signal that is rapidly transferred into the mitochondrial matrix. This sequestration of Ca$^{2+}$ shapes the cytosolic Ca$^{2+}$ signal and thereby modifies all the Ca$^{2+}$ transport processes. The mitochondrial Ca$^{2+}$ signal enhances the reduction of mitochondrial pyridine nucleotides and thus potentiates ATP formation (reviewed e.g. in [39]) and hormone secretion [40]. Out of various mechanisms responsible for Ca$^{2+}$ transport from the IMS into the mitochondrial matrix (reviewed in [41–43]) the most important one is the Ruthenium red-sensitive mitochondrial Ca$^{2+}$/H$^{+}$ uniporter (MCU), the driving force of the transport is the mitochondrial membrane potential (–180 mV, inside negative) (reviewed in [41]). Patch-clamp studies revealed that the uniporter is an inwardly rectifying cation channel [44,45] and a 40 kDa membrane protein has recently been shown to exhibit the conductive function [46,47]. A recently characterized protein, MiCu1 seems to be its Ca$^{2+}$-sensing subunit [48]. The electrogenic mitochondrial Na$^{+}$/Ca$^{2+}$ antiporter was also reported to transport Ca$^{2+}$ into the mitochondria [49,50]. Similarly, the electrogenic Ca$^{2+}$/H$^{+}$ antiporter, identified as Letm1 [51] may also be responsible for Ca$^{2+}$ uptake by depolarized mitochondria. In spite of the progress in the elucidation of the structure and function of the transporters there are no data available on their location within the IMM. The only available exception is the demonstration of mitochondrial Na$^{+}$/Ca$^{2+}$ exchanger (NCLX) in the crista membrane with immunoelectron microscopy [52]. Nevertheless, schematic drawings in several recently published reviews locate the transporters in the boundary membrane and no Ca$^{2+}$ transport is indicated within the cristae [42,43,53–56]. Here we report that the expression of OPA1 modifies mitochondrial Ca$^{2+}$ uptake, suggesting the significance of the crista membrane in Ca$^{2+}$ uptake. Our results imply that OPA1 may be a target of factors controlling mitochondrial Ca$^{2+}$ metabolism.

**Results**

**Mitochondrial morphology in OPA1 siRNA-transfected cells**

In order to evaluate the effect of OPA1 on mitochondrial Ca$^{2+}$ uptake we depressed its expression with siRNA. Since silencing of OPA1 has been known to evoke fragmentation of the mitochondria (see Introduction) and the ensuing increase in surface/volume ratio might accelerate the increase in [Ca$^{2+}$]$_{m}$, a group of cells was treated with Mfn1 siRNA with the intention of evoking similar mitochondrial fragmentation. Western blot analysis confirmed the specificity of the OPA1 siRNA on the expression of OPA1 (Figure 1). (The moderate reduction of protein expression in H295R cells may be accounted for by the poor transfectability of this cell line.) To test whether Mfn1-silenced cells are more appropriate controls for OPA1-silenced cells than those transfected with control (non-silencing) RNA, the morphology of mitochondria was compared in the three groups. As shown in Figures S1 and S2 for H295R and HeLa cells, the filamentous mitochondrial pattern in cells exposed to control RNA changed into fragmented, round-shaped mitochondria in OPA1 and Mfn1-silenced cells. In H295R cells the median value of the length of single mitochondria diminished from 2.30 µm in control RNA-treated cells to 1.06 and 0.98 µm in cells exposed to Mfn1 and OPA1 siRNA, respectively (Figure S3). In HeLa cells the median length changed from 2.73 to 0.40 and 0.24 µm, respectively (Figure S3). Another conventional indicator of fragmentation, the circularity (for a circle its value is 1) increased from a median of 0.199 to 0.447 and 0.452 in H295R cells and from 0.217 to 0.743 and 0.803 in HeLa cells (Figure S3). The comparable values obtained in OPA1 and Mfn1 siRNA-treated cells show that the latter one is an appropriate control for studying the effect of OPA1 on Ca$^{2+}$ metabolism.

**Effect of OPA1 silencing on mitochondrial Ca$^{2+}$ uptake in intact cells**

For monitoring cytosolic Ca$^{2+}$ responses the fluorescent dye Fura-2 or Fura-FF was used. For following changes in mitochondrial [Ca$^{2+}$]$_{m}$ ([Ca$^{2+}$]$_{m}$) in intact cells the FRET-based, mitochondrial targeted, Cameleon-derived fluorescent protein 4mt-D1-cpV [57] or 4mt-D2-cpV [58], also targeted into the mitochondria, was applied. Their $K_{0.5}$ for Ca$^{2+}$ was about 10 µM and 85 nM, respectively. (Using null-point titration we measured pH = 8.0 in the mitochondria of resting HeLa and H295R cells, therefore the $K_{0.5}$ values were determined at pH 8.0.) The FRET ratio of the fluorescent proteins was insensitive to pH in the 7.6–8.2 range (data not shown). Fura-2 and 4mt-D2-cpV proved to be the appropriate sensors in H295R cells whereas Fura-FF and 4mt-D1-cpV were applied in HeLa cells.

In intact H295R cells we examined the transfer of cytosolic Ca$^{2+}$ signal, elicited with 25 mM K$^{+}$, into the mitochondrial matrix. Whereas the cytosolic signals were almost identical in the Mfn1 and OPA1 siRNA-treated cells, the FRET ratio of 4mt-D2-cpV, reflecting [Ca$^{2+}$]$_{m}$, showed a significant increase in the latter

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**Figure 1. Effect of OPA1 and Mfn1 siRNA on the expression of OPA1.** The cells were transfected with OPA1 siRNA, Mfn1 siRNA or a non-silencing RNA on the day following plating (day 2). The samples were lysed on day 5, run on SDS-PAGE, transferred onto nitrocellulose membrane, incubated with anti-OPA1 mouse monoclonal antibody then with anti-mouse immunoglobulin-horseradish-peroxidase conjugate. Protein disulphide isomerase was used as loading control. doi:10.1371/journal.pone.0025199.g001
Mitochondrial membrane potential after gene silencing

Enhanced mitochondrial Ca\(^{2+}\) uptake can be attributed to increased mitochondrial membrane potential (Ψ\(_{m}\)), increased activity of the Ca\(^{2+}\) transporting system as well as enhanced access of Ca\(^{2+}\) to the transporter. Assessment of Ψ\(_{m}\) with tetramethyl rhodamine methylester (TMRE) in H295R cells revealed that mitochondria were depolarized in OPA1 siRNA-transfected cells as opposed to the effect of Mfn1 siRNA (p = 5×10\(^{-7}\), Figure S4), a change attenuating rather than enhancing Ca\(^{2+}\) uptake. OPA1 siRNA reduced Ψ\(_{m}\) examined with tetramethyl rhodamine ethylester (TMRE) also in HeLa cells (p = 0.0001). The reduction of Ψ\(_{m}\) has been confirmed with JC-1 (5,5'-dihexyl-2,2'-thiatricrolo-1,1',3'-tetraethyl-benzimidazolyl-carbocyanine iodide) (p = 0.0001, Figure S4).

Effect of OPA1 silencing on mitochondrial Ca\(^{2+}\) uptake in permeabilized cells

In order to exclude extramitochondrial sites of OPA1 action and mostly to ensure identical driving force in each experimental group we next examined the effect of gene silencing in digitonin-permeabilized cells. The cells had been transfected with mitochondrially targeted inverse Pericam (mt-inv-Pericam). Its high Ca\(^{2+}\) affinity (K\(_{i}\) = 0.2 µM at pH 7.4 [59] and ~80 nM at pH 8.0; not shown) rendered it an appropriate sensor for measuring initial Ca\(^{2+}\) uptake rate but no data could be obtained for the amplitude of the Ca\(^{2+}\) response. Following the permeabilization the cells were depolarized with a Ca\(^{2+}\)-free cytosol-like medium lacking mitochondrial substrates but completed with 10 µM rotenone, 8 µg/ml oligomycin, 10 µM FCCP and 50 ng/ml valinomycin for 2 minutes. To induce mitochondrial Ca\(^{2+}\) uptake [Ca\(^{2+}\)]\(_{i}\) in the superfusion medium was raised from 0 to 5 µM, still in the presence of the drugs. In OPA1 silenced H295R cells Ca\(^{2+}\) uptake rate increased by a mean 30% (p = 0.034) as compared with the effect of control RNA and amounted nearly to the double of that measured in Mfn1 silenced cells (p = 0.010, Figure 5). Applying the same protocol in HeLa cells, no difference in Ca\(^{2+}\) uptake rate was detected when [Ca\(^{2+}\)]\(_{i}\) was raised to 2 µM but upon adding 5 µM Ca\(^{2+}\) a mean 55% increase in uptake rate was observed in OPA1-silenced cells as compared with the effect of Mfn1 silencing (p = 0.00008). Moreover, OPA1-knockdown augmented Ca\(^{2+}\) uptake rate when compared to control RNA treatment (p = 0.0008, Figure 6). The 2 min depolarizing treatment did not change the immunoblot pattern of OPA1 (Figure 6).

Pharmacological characterization of the Ca\(^{2+}\) transport mechanism in depolarized mitochondria

Mitochondrial Ca\(^{2+}\) uptake by MCU (in polarized mitochondria) and by the H\(^{+}\)/Ca\(^{2+}\) antiporter (Letm1) is inhibited by Ruthenium Red. In permeabilized, OPA1-silenced H295R cells the drug (15 µM) strongly inhibited Ca\(^{2+}\) uptake rate (p = 0.00016), CGP-37157 (25 µM), an inhibitor of the mitochondrial Na\(^{+}\)/Ca\(^{2+}\) exchanger exerted a similar effect (p = 0.00016). No inhibition was observed with cyclosporin A (10 µM), an inhibitor of the mitochondrial permeability transition pore (mPTP) (Figure S5 panel A and Table S1). Ruthenium Red reduced Ca\(^{2+}\) uptake rate to one fifth of the control (p = 0.00001) in permeabilized HeLa cells transfected with OPA1 siRNA. CGP-37157 and cyclosporin A failed to exert any effect on Ca\(^{2+}\) uptake (Figure S5 Panel B and Table S1).

Discussion

Mitochondrial Ca\(^{2+}\) accumulation occurs predominantly through the recently identified MCU protein [46,47], an inwardly rectifying Ca\(^{2+}\) channel [44]. The unporter does not function in depolarized mitochondria [60], under such conditions the mitochondrial Na\(^{+}\)/Ca\(^{2+}\) exchanger (NCLX) and/or the Ca\(^{2+}\)/H\(^{+}\) exchanger (Letm1) may be responsible for mitochondrial Ca\(^{2+}\) accumulation [49,51,61,62]. An electronmicroscopic study detected the Na\(^{+}\)/Ca\(^{2+}\) exchanger predominantly in the crista membrane [52] but no information is available for the localization of MCU or Letm1 within the IMM. The major purpose of the present study was to elucidate whether Ca\(^{2+}\) uptake occurs exclusively on the inner boundary membrane as suggested in the schemes of several recently published reviews (see Introduction) or it also takes place in the crista membrane [4,6,33]. Recalling that OPA1-knockdown increases the diffusibility of cytochrome c through the crista junctions [4,6,33] we presumed that if Ca\(^{2+}\) is sequestered through the crista membrane, in OPA1-silenced cells the transport should be enhanced.

In accordance with previous reports [19,29,30] silencing of OPA1 induced fragmentation of mitochondria. It had to be considered that the fragmentation results in increased surface/volume ratio that in turn may lead to amplified increases in [Ca\(^{2+}\)]\(_{i}\). Therefore cells transfected with Mfn1 siRNA, also displaying mitochondrial fragmentation [26–28], rather than control RNA-treated cells were regarded as appropriate control.Significantly, shortening and circularity of mitochondria were comparable in the OPA1 and Mfn1 silenced groups.

In K\(^{+}\)-stimulated H295R cells mitochondrial Ca\(^{2+}\) uptake was enhanced by OPA1 knockdown as compared to control RNA or Mfn1 siRNA-transfected cells. Stimulation of HeLa cells with histamine elicited cytosolic Ca\(^{2+}\) signal which was rapidly transferred into the mitochondrial matrix. Whereas OPA1 siRNA had no obvious effect on the generation of mitochondrial Ca\(^{2+}\) signal in cells showing low cytosolic Ca\(^{2+}\) response, significantly bigger mitochondrial Ca\(^{2+}\) response and Ca\(^{2+}\) uptake rate were attained after OPA1 knockdown in cells showing high cytosolic response. We presume that at higher Ca\(^{2+}\) load the access of Ca\(^{2+}\) to those transporters that are localized in the crista membrane becomes the rate-limiting factor of the transport. Confirming previous reports [11,24,63], knockdown of OPA1 but not that of Mfn1 resulted in mitochondrial depolarization implying that in OPA1 silenced cells Ca\(^{2+}\) uptake rate increased in spite of smaller...
Figure 2. Effect of OPA1 or Mfn1 silencing on Ca^{2+} signaling in intact H295R cells. The cells were transfected with control RNA or siRNA and 4mt-D2-cpV on the day following plating (day 2) and once again with control RNA or siRNA on day 3. On day 5, after preloading with Fura-2 AM, the cells were stimulated with 25 mM K^+. Changes in cytosolic [Ca^{2+}] ([Ca^{2+}]_c) were monitored by measuring Fura-2 excitation ratio while [Ca^{2+}]_m was indicated by the FRET ratio of 4mt-D2-cpV. Both ratios are normalized to those obtained in the control period. Representative cytosolic Ca^{2+} signals (A) and mitochondrial Ca^{2+} uptake curves (B) are shown for cells transfected with control RNA, Mfn1 or OPA1 siRNA. C: peak [Ca^{2+}]_c, D: peak [Ca^{2+}]_m E: [Ca^{2+}]_c, response normalized to peak [Ca^{2+}]_c, (Δ[Ca^{2+}]_c/Δt)/[Ca^{2+}]_c indicating mitochondrial responsiveness; F: the slope of [Ca^{2+}]_m rise related to peak [Ca^{2+}]_m (cells not displaying a mitochondrial Ca^{2+} response were omitted from this statistics). Data are shown for control (C), Mfn1 siRNA (M) or OPA1 siRNA-transfected (O) groups. Results represent mean ± SEM, the number of observations is shown within the columns.

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driving force. The above data indicate that OPA1 attenuates mitochondrial Ca$^{2+}$ signaling in intact cells.

In order to ensure stable and identical driving force in each group $\Psi_m$ was dissipated in permeabilized cells. Permeabilization of control RNA-transfected cells resulted in the formation of toroids, probably due to a partial dissociation of the mitochondria from the microtubules [64]. The permeabilization did not cause any detectable morphological change of the fragmented mitochondria in siRNA-transfected cells (not shown). After complete depolarization [Ca$^{2+}$]was raised to 2 or 5 µM, ensuring that the concentration gradient of Ca$^{2+}$ should be the only driving force of the transport [65]. ATP depletion [66] as well as protonophores may evoke proteolysis of OPA1 [21,22,67], however, this did not occur within 2 minutes in the depolarizing medium. Elevation of [Ca$^{2+}$] to 5 µM induced an immediate mitochondrial Ca$^{2+}$ influx in permeabilized H295R cells and Ca$^{2+}$ uptake rate was significantly increased after silencing OPA1. Silencing also augmented mitochondrial Ca$^{2+}$ accumulation in permeabilized HeLa cells exposed to 5 rather than to 2 µM Ca$^{2+}$. This phenomenon resembles the observation in intact cell where the signal transfer was accelerated by OPA1 siRNA only in cells displaying high cytosolic Ca$^{2+}$ signal.

Ca$^{2+}$ uptake may have been enhanced in OPA1-silenced cells due to hyperpolarization, increased expression or higher Ca$^{2+}$ affinity of the transporter or increased access of Ca$^{2+}$ to the transporter. OPA1 siRNA reduces $\Psi_m$ and therefore the driving force of Ca$^{2+}$ uptake. (As to the effect of mitochondrial pH, it should be recalled that the protonophore FCCP was present in all the experiments on permeabilized cells.) Had the density or affinity of the transporter increased, enhanced Ca$^{2+}$ accumulation could be expected even in the presence of lower [Ca$^{2+}$], but this was not the case in HeLa cells. Therefore it can be considered that, when [Ca$^{2+}$] is high, Ca$^{2+}$ supply of the transporters located in the cristal membrane is a limiting factor. Enhanced access of Ca$^{2+}$ to these transporters in OPA1-silenced cells then augments the efficiency of the Ca$^{2+}$ uptake process. Nevertheless, the possibility should be kept in mind that if ablation of OPA1 evokes the relocation of the transporter molecules from the crista into the boundary membrane, mitochondrial Ca$^{2+}$ metabolism would alter similarly to the present observations. Unfortunately, this is difficult to prove or disprove at this stage.

Which transport mechanism is located in the crista membrane? The predominant mechanism of Ca$^{2+}$ uptake in polarized mitochondria is the MCU [41–43]. The Ca$^{2+}$ transporting capacity of the supposedly electrogenic H$^+$/Ca$^{2+}$ antiporter [51] as compared to that of MCU, is negligible [68]. The kinetics of Ca$^{2+}$ uptake in our cells argues against any role of the rapid mode of Ca$^{2+}$ uptake [69]. The enhancement of mitochondrial Ca$^{2+}$ uptake in intact, OPA1-silenced cells is compatible with the presence of MCU in the cristal membrane.

The transport mechanism was further analyzed in OPA1 siRNA-transfected, permeabilized cells after depolarizing the mitochondria. MCU is inactive in depolarized mitochondria [60]. Ca$^{2+}$ uptake by depolarized mitochondria in permeabilized HeLa cells was reduced by Ruthenium Red. The Ca$^{2+}$/H$^+$ exchanger Letm1 is expressed in HeLa cells and is inhibited by Ruthenium Red [51]. Ca$^{2+}$ uptake was not influenced by CGP-37157, an inhibitor of the Na$^+/Ca^{2+}$ antiporter. In the excitable
cell type H295R Ruthenium Red almost completely abolished Ca\(^{2+}\) uptake and CGP-37157 also exerted a strong inhibition [70]. The pharmacological data suggest that, in addition to MCU, both the Ca\(^{2+}\)/H\(^{+}\) and Na\(^{+}/Ca\(^{2+}\) exchanger in H295R cells and the former one in HeLa cells participate in the enhanced Ca\(^{2+}\) uptake after knockdown of OPA1. In this respect it is worthwhile to recall that both antiporters are present in the adrenal cortex [70]. Cyclosporin A, an inhibitor of mPTP had no effect in either cell type. Indeed, in lack of contact between the crista and outer mitochondrial membranes mPTP may not be directly involved in Ca\(^{2+}\) uptake through the crista membrane. In a study on murine retinal ganglion cells, transiently stimulated with K\(^{+}\), the subsequently added protons induced bigger cytosolic Ca\(^{2+}\) signals in OPA1-silenced than in control cells [11]. The data which show the resultant of mitochondrial Ca\(^{2+}\) release and elimination of cytosolic Ca\(^{2+}\) by Ca\(^{2+}\)/ATPases, can be attributed to changes in mitochondrial Ca\(^{2+}\) accumulation. However, in lack of inappropriate control with fragmented mitochondria, the role of changes in various transports processes (e.g. rate of mitochondrial depolarization, rate of Ca\(^{2+}\) pumping) should also be considered. Our direct measurements of mitochondrial Ca\(^{2+}\) metabolism not only demonstrated the increased rate of Ca\(^{2+}\) uptake in OPA1-silenced cells but also excluded the role of mitochondrial membrane potential in this action of the protein and suggested the presence of Ruthenium Red-sensitive transport mechanisms in the crista membrane.

Summarizing, the reduction of OPA1 expression results in enhanced mitochondrial Ca\(^{2+}\) uptake rate and augments the mitochondrial Ca\(^{2+}\) signal. These results unambiguously demonstrate that OPA1 restrains mitochondrial Ca\(^{2+}\) uptake. On the basis of published data we attribute the uptake attenuating effect of OPA1 to decreased permeation of Ca\(^{2+}\) through the junction of the cristae. It follows that enhanced Ca\(^{2+}\) uptake in the absence of normal OPA1 function may be an aggravating component of OPA1-related diseases. Therefore OPA1 may be a target of factors modifying mitochondrial Ca\(^{2+}\) handling.

**Materials and Methods**

**Cell culture and transfection**

H295R cells (CRL-2128, ATCC, Manassas, VA) were grown in DMEM/Ham’s F12 (1:1 v/v) containing 1% ITS\(^{+}\), 2% UltroSer G, 100 U/ml penicillin and 100 μg/ml streptomycin. HeLa cells (CCL-2, ATCC, Manassas, VA) were grown in DMEM containing 10% heat-inactivated FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. Passage numbers 3–20 were used.

Cells (about 4\(^{10^{3}}\) H295R or 10\(^{4}\) HeLa) were plated onto 24-mm diameter circular glass coverslips on day 1. For plasmid transfection we used 1 μg DNA (or 2 μg in case of H295R transfection with 4mt-D2-cpV) with transfection reagent (2 μl Lipofectamine 2000 for H295R or 2–3 μl FuGENE-HD for HeLa cells) in 1.1 ml OPTI-MEM medium. The transfection was performed on day 2 or 3. For silencing OPA1 a mixture of three siRNA species (1299003) was applied, for that of Mfn1 the product 5141600 and for control a non-silencing RNA with

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**Figure 4.** Mitochondrial Ca\(^{2+}\) signaling in intact, OPA1 or Mfn1 siRNA-treated HeLa cells displaying large [Ca\(^{2+}\)]\(_{m}\) peak. (displaying normalized Fura-FF ratios greater than 1.25), shown in Figure 3 right to the broken vertical line. Representative curves are shown for [Ca\(^{2+}\)]\(_{m}\) measured with 4mt-D1-cpV (A) and for [Ca\(^{2+}\)]\(_{m}\) measured with 4mt-D2-cpV (B) after stimulation with 5 μM histamine in cells transfected with control RNA, Mfn1 siRNA or OPA1 siRNA. Statistics for peak [Ca\(^{2+}\)]\(_{m}\) (C), peak [Ca\(^{2+}\)]\(_{m}\) (D), Δ[Ca\(^{2+}\)]\(_{m}\)/[Ca\(^{2+}\)]\(_{m}\) (indicating the slope of initial increase in [Ca\(^{2+}\)]\(_{m}\)), indicating mitochondrial responsiveness (E) and the slope of [Ca\(^{2+}\)]\(_{m}\) rise related to peak [Ca\(^{2+}\)]\(_{m}\) (F) are shown for control (C), Mfn1 siRNA (M) or OPA1 siRNA-transfected (O) groups. Results represent mean ± SEM, the number of observations is shown within the columns.

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**Figure 5.** Mitochondrial Ca\(^{2+}\) uptake in permeabilized H295R cells transfected with OPA1 or Mfn1 siRNA. The cells were transfected with control RNA or siRNA on the day following plating (day 2) and with inverse Pericam targeted into the mitochondria (mt-inv-Pericam) on day 2 or 3. On day 5 the cells were permeabilized, superfused with a cytosol-like medium, V\(_{m}\) was dissipated with 10 μM rotenone, 8 μg/ml oligomycin, 10 μM FCCP and 50 ng/ml valinomycin for 2 minutes. Then, in the presence of the drugs, [Ca\(^{2+}\)]\(_{m}\) was raised from 0 to 5 μM. [Ca\(^{2+}\)]\(_{m}\) was monitored by means of confocal microscopy, applying mt-inv-Pericam, the fluorescence of which exhibits inverse correlation with [Ca\(^{2+}\)]\(_{m}\). Fluorescence measured at saturating [Ca\(^{2+}\)]\(_{m}\) (F\(_{m}\)) was subtracted from each fluorescence value. The data were normalized for the control period. Representative mitochondrial Ca\(^{2+}\) uptake curves are shown for cells transfected with Mfn1 or OPA1 siRNA (note that decreasing F/F\(_{0}\) values indicate increasing [Ca\(^{2+}\)]\(_{m}\)) (A); effect of OPA1 siRNA as compared with that of control RNA (B) or Mfn1 siRNA (C) on the slope of initial decrease of normalized mt-inv-Pericam fluorescence (indicating the slope of initial increase in [Ca\(^{2+}\)]\(_{m}\)). Results represent mean ± SEM, the number of observations is shown within the columns.

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appropriate GC content (12935400 or 129305200), all from Invitrogen (Paisley, UK). Transfection took place on day 2, 40 pmol siRNA were added with 1 ml Lipofectamine RNAiMAX in 1.1 ml OPTI-MEM medium. When RNA was co-transfected with the plasmid coding for the Ca\textsuperscript{2+} sensitive protein, the protocol for plasmid transfection was applied. In experiments on intact H295R cells RNA transfection was repeated on day 3. The transfection reagents were purchased from Invitrogen, with the exception of FuGENE-HD (Roche, Mannheim, Germany). The experiments were conducted 56–76 hours after the first transfection.

**Immunoblotting**

10\textsuperscript{5} cells cultured in 24-mm diameter dishes were suspended in ice-cold lysis buffer (100 mM NaCl, 30 mM HEPES pH 7.4, 0.2% Triton X-100, 20 mM NaF, 2.5 mM Na-EGTA, 2.5 mM Na-EDTA, 10 mM benzamidine, 0.075 U/ml Aprotinin, 1:100 Sigma Mammalian Protease Inhibitor Cocktail, 1 mM sodium-vanadate, 10 mM PMSF). Insoluble fraction was removed with centrifugation. Protein concentration was measured with Bradford or BCA assay. The supernatant was completed with 1/3 volume reducing buffer (125 mM TrisCl pH 6.8, 40% glycerol, 20% mercaptoethanol, 0.02% bromophenolblue, 280 mM SDS). Samples were run on 8% SDS-PAGE and transferred onto nitrocellulose membrane (pore size: 0.45 μm).

The membrane was incubated with 3% milk powder + 0.1% Tween 20 in PBS to block nonspecific binding sites. Detection of OPA1 was performed with anti-OPA1 mouse monoclonal antibody (Cat. No 612606, BD Bioscience, Franklin Lakes, NJ) followed by incubation with anti-mouse immunoglobulin-horseradish-peroxidase conjugate (1:2000) (GE Healthcare, Amersham, UK). Protein disulphide isomerase was used as loading control (anti-PDI antibody: ab2792, Abcam, Cambridge, UK).

**Figure 6. Effect of OPA1 or Mfn1 silencing on mitochondrial Ca\textsuperscript{2+} uptake in permeabilized HeLa cells.** For the transfection protocol and measurement of fluorescence see legend of Figure 5. On day 5, after the dissipation of \( \Psi_m \) (see legend of Figure 5), [Ca\textsuperscript{2+}] of the superfusion medium was raised from 0 to 2 or 5 μM. A: mitochondrial response to 5 μM Ca\textsuperscript{2+}. Representative curves are shown for cells transfected with Mfn or OPA1 siRNA (note that decreasing F/F\textsubscript{0} values indicating increasing [Ca\textsuperscript{2+}]m). B: Western blot shows that 2-min exposure to the depolarizing medium did not change the pattern of immunoreactive OPA1. P: permeabilisation, D: dissipation of \( \Psi_m \), C: control. The slope of initial increase in [Ca\textsuperscript{2+}]m in permeabilized cells superfused with 2 μM (C) or 5 μM Ca\textsuperscript{2+} (D and E) is shown in cells transfected with control RNA (D), Mfn1 siRNA (C and E) and OPA1 siRNA (C, D, E). C: control RNA, M: Mfn1 siRNA, O: OPA1 siRNA. Results represent mean ± SEM, the number of observations is shown within the columns.

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Confocal microscopy

The rate of mitochondrial Ca$^{2+}$ uptake and mitochondrial membrane potential were examined with confocal microscopy. Cells plated onto glass coverslips were placed on the stage of Zeiss LSM510 confocal laser scanning microscope equipped with a 40×/1.3 oil immersion objective (Plan-Neofluar Zeiss). Mc-inv-Pericam (gifted by Prof. A. Miyawaki, Saitama, Japan) was excited at 490 nm; emitted light was filtered using BP 500–550 nm emission filter. Rhod-2 (3 μM for 15 min) was excited at 543 nm; the emitted light was filtered using LP 560 filter. The optical slice was 5 μm, image acquisition frequency in the Ca$^{2+}$ uptake experiments was 1–0.2 Hz. The experiments were performed at room temperature. The solutions were applied with a solenoid valve-equipped, gravity-driven superfusion system, terminating at 31°C. Solutions

Incubation of intact cells was performed in a modified Krebs-Ringer solution containing 3.6 mM K+ (1.2 mM Ca$^{2+}$, 0.5 mM Mg$^{2+}$, 10 mM Hepes and 2 mM HCO$_3^-$; pH 7.4). In experiments studying the effect of K+, NaCl was partly replaced with N-methyl-d-glucamine or K+ in order to maintain Na+ and osmotic concentrations constant. Permeabilization was carried out in a cytosol-like medium (117 mM KCl, 6 mM NaCl, 1 mM KH$_2$PO$_4$, 2 mM Na+ pyruvate, 2 mM Na+ succinate (for HeLa) or 2 mM Na+ succinate (for H295R), 2 mM K+ADP, 2 mM EGTA, 10 mM K+HEPES or 10 mM K+MOPS) with 25 μg/ml digitonin at room temperature for 10 minutes. To adjust the [Ca$^{2+}$]i and [Mg$^{2+}$] of the cytosol-like media, EGTA, HEDTA, CaCl$_2$, MgCl$_2$ and ADP were used as calculated by the Chelator software [72]. The [Ca$^{2+}$]i of the applied solutions was checked with a Ca$^{2+}$ selective electrode (Orion, Cambridge, UK). Calculated [Mg$^{2+}$] was 0.5 mM.

Statistics

Means ± S.E.M. are shown. All the experiments were performed on at least two different cell passages. For estimating significance of differences, Student’s unpaired t-test (with or without Welch correction), one-way ANOVA, Tukey’s or unequal N HSD post hoc tests or Kruskal-Wallis test were used, as appropriate. Data were analyzed with Statistica 9.

Supporting Information

Figure S1 Morphology of the mitochondria of RNA-transfected H295R cells. The cells were transfected with control RNA (A), Mfn1 siRNA (B) or OPA1 siRNA (C) on the day following plating (day 2) and with mitochondrially targeted GFP on day 3. Confocal microscopy was performed on day 4. The framed areas are shown in the right-hand column; zoom: 4×. Optical slice thickness was 1 μm. Bars, 10 μm. (TIF)

Figure S2 Morphology of the mitochondria of RNA-transfected HeLa cells. The cells were transfected with control RNA (A), Mfn1 siRNA (B) or OPA1 siRNA (C) on the day following plating (day 2) and with mitochondrially targeted GFP on day 3. Confocal microscopy was performed on day 5. The framed areas are shown in the right-hand column; zoom: 4×. Optical slice thickness was 1 μm. Bars, 10 μm. (TIF)

Figure S3 Morphometry of the mitochondria of RNA-transfected cells. For the experimental protocol see the legend of Figure S1 (H295R) or 2 (HeLa). The histograms show the length and circularity of mitochondria in H295R cells (A and B, resp.) and in HeLa cells (C and D, resp.). Analysis was performed with ImageJ 1.6.0, as suggested [71]. (TIF)
Figure S4 Mitochondrial membrane potential of RNA-transfected HeLa cells. Transfection with control RNA, Mfn1 or OPA1 siRNA was performed on the day following plating. Three days after plating, cells were stained applying TMRE in HeLa cells, respectively. TMRE fluorescence or JC-1 ratio (red/green) over the mitochondrial region were normalized to that measured after depolarisation with FCCP. Means + SEM are shown, the number of observations is shown within the columns. (TIF)

Table S1 Significance of differences for the experiments shown in Figure S5. C: control, RR: Ruthenium Red, CG: CGP-37157, Cy: cyclopiazonic A. (DOC)

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Author Contributions
Conceived and designed the experiments: AS. Performed the experiments: LF GS AS. Analyzed the data: LF GS. Contributed reagents/materials/analysis tools: BE PV. Wrote the paper: AS.
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