There were errors published in J. Cell Sci. 124, 2143-2152.

In the section given below, PtdIns(3,4,5)P3 was on four occasions incorrectly printed instead of the correct Ins(1,4,5)P3.

We apologise for this mistake.

Increased mitochondrial Ca2+ drives the adaptive metabolic boost observed during early phases of ER stress

Increases in mitochondrial respiration and ATP production are often consequences of increases in mitochondrial Ca2+ (Green and Wang, 2010). In order to determine whether early phases of ER stress induced by tunicamycin increased mitochondrial Ca2+, we treated cells expressing cytosolic or mitochondrial aequorins with histamine [which evokes Ins(1,4,5)P3-dependent Ca2+ release] and compared their mitochondrial Ca2+ uptake. We observed that histamine led to a mitochondrial Ca2+ uptake that was significantly higher in tunicamycin-pretreated cells (P<0.05; 4 hours) than in untreated cells (Fig. 6A). Cytosolic Ca2+ increased similarly in tunicamycin-treated and untreated cells (Fig. 6B). These results indicate that the differences in mitochondrial Ca2+ levels are not due to altered Ca2+ release mediated by the Ins(1,4,5)P3 receptor but to an enhanced mitochondrial Ca2+ uptake, presumably due to the increased apposition of ER and mitochondrial Ca2+ channels. By using a different dye, Fura-2, we monitored the peak cytosolic Ca2+ levels after thapsigargin addition, reflecting the kinetics of Ca2+ release after sarcoplasmic/endoplasmic reticulum Ca2+-ATPase (SERCA) inhibition. After 4 hours of tunicamycin treatment, the thapsigargin-induced Ca2+ peak was increased, and it was further elevated by inhibition of mitochondrial Ca2+ uptake using Ru360 (Fig. 6C). These results suggest that, besides the Ins(1,4,5)P3-receptor-mediated direct Ca2+ transfer from the ER to neighboring mitochondria, an additional phenomenon associated with the early phases of ER stress involves Ca2+ leak from the ER, also resulting in mitochondrial Ca2+ uptake. Indeed, no mitochondrial Ca2+ uptake following the thapsigargin-induced Ca2+ leak was observed in Mfn2-knockout cells (Fig. 6D), which is reflected by the lack of effect of Ru360. This result further indicates that juxtaposition of mitochondria with the ER is necessary for the mitochondrial Ca2+ uptake evoked by Ca2+ leak during early phases of ER stress.

Finally, to test whether mitochondrial Ca2+ levels control the metabolic mitochondrial boost, we measured oxygen consumption rates resulting from OXPHOS in the presence of the Ins(1,4,5)P3 receptor inhibitor xestospongin B or the mitochondrial Ca2+ uptake inhibitor RuRed. We observed that both xestospongin B and RuRed decreased the rate of oxygen consumption after tunicamycin treatment (Fig. 7A,B), which confirms that increased mitochondrial Ca2+ uptake, resulting from ER–mitochondrial coupling, is necessary for the metabolic response observed during early phases of ER stress. Therefore, in order to evaluate whether the early metabolic boost forms part of an adaptive response triggered by ER stress, we inhibited mitochondrial Ca2+ uptake and measured cell viability [through propidium iodide (PI) incorporation] and ΔΨm. We observed that the inhibition of mitochondrial Ca2+ uptake during the early phase of ER stress increased cell death (PI-positive cells) and also decreased ΔΨm at 48 hours (Fig. 7C).

In total, the results presented in this study suggest strongly that Ca2+ transfer resulting from enhanced ER–mitochondrial coupling leads to a localized increase in mitochondrial metabolism, thus providing energetic substrates key for a cellular adaptive response in face of ER stress. Further experiments will determine whether this bioenergetic response is necessary for improving the energetic state of the ER, and therefore its folding capacity, or, as it is restricted to perinuclear zones, for the activation of a specific nuclear transcriptional program that participates in the cellular adaptation to stress.
Increased ER–mitochondrial coupling promotes mitochondrial respiration and bioenergetics during early phases of ER stress

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Summary

Increasing evidence indicates that endoplasmic reticulum (ER) stress activates the adaptive unfolded protein response (UPR), but that beyond a certain degree of ER damage, this response triggers apoptotic pathways. The general mechanisms of the UPR and its apoptotic pathways are well characterized. However, the metabolic events that occur during the adaptive phase of ER stress, before the cell death response, remain unknown. Here, we show that, during the onset of ER stress, the reticular and mitochondrial networks are redistributed towards the perinuclear area and their points of connection are increased in a microtubule-dependent fashion. A localized increase in mitochondrial transmembrane potential is observed only in redistributed mitochondria, whereas mitochondria that remain in other subcellular zones display no significant changes. Spatial re-organization of these organelles correlates with an increase in ATP levels, oxygen consumption, reductive power and increased mitochondrial Ca2+ uptake. Accordingly, uncoupling of the organelles or blocking Ca2+ transfer impaired the metabolic response, rendering cells more vulnerable to ER stress. Overall, these data indicate that ER stress induces an early increase in mitochondrial metabolism that depends crucially upon organelle coupling and Ca2+ transfer, which, by enhancing cellular bioenergetics, establishes the metabolic basis for the adaptation to this response.

Key words: Ca2+, Metabolism, Mitochondria, Mitofusin 2 (Mfn2), Unfolded protein response (UPR), Endoplasmic reticulum stress

Introduction

Endoplasmic reticulum (ER) stress is a cellular state in which the folding capacity of the ER is overwhelmed owing to an increase in protein load or disruption in the folding environment (Berridge, 2002). The accumulation of unfolded proteins is detected by transmembrane sensors at the ER surface, which initiate a transduction cascade known as the unfolded protein response (UPR). During this response, the induction of a specific set of nuclear genes is observed, as well as a general arrest of translation, in order to restore the folding capacity of the ER (Ron and Walter, 2007). If protein homeostasis is not re-established, the initially adaptive UPR becomes an inducer of cell death, leading to apoptosis (Nakagawa et al., 2000). The chaperones, foldases and folding quality-control proteins that are induced during ER stress are well characterized (Hetz and Glimcher, 2009), as are the general mechanisms that transduce this response into apoptosis (Rasheva and Domingos, 2009). However, the metabolic adjustments necessary for cell survival during ER stress are poorly understood. From an energetic point of view, the requirements of the cell for metabolic substrates become enhanced in order to adapt to different stress conditions (Liu et al., 2005; Ikeshige et al., 2006; Haga et al., 2010). On the basis of these premises, it is likely that mitochondria participate in the cellular adaptive response to ER stress, possibly determining cell fate after activation of the UPR.

Interactions between the ER and mitochondria occur throughout their networks, both physically and functionally (Lebiedzinska et al., 2009). The molecular foundations of this crosstalk are diverse, and Ca2+ is one of the most important signals that these organelles use for communication (Szabadkai and Duchen, 2008). Ca2+ allosterically increases the activity of matrix dehydrogenases required for mitochondrial respiration and promotes ATP production by disinhibition of the ATP synthase (Brown, 1992; Balaban, 2009). By contrast, mitochondrial Ca2+ overload can result in permeability transition and activation of intrinsic apoptosis (Szabadkai and Rizzuto, 2004; Hajnoczky et al., 2006). Because the channels through which Ca2+ enters mitochondria are of low affinity, it has been proposed that regions of close proximity between mitochondria and the ER are necessary for Ca2+ entry into the mitochondrial matrix (Rizzuto et al., 1998). A major determinant of the ER–mitochondria interface is the distance between their...
surfaces, controlled by the movement of these organelles along the cytoskeleton (Hollenbeck and Saxton, 2005; Boldogh and Pon, 2006). Ca^{2+} modulates this distance, as its release from ER channels is a signal that locally arrests mitochondrial motility and promotes their docking at the ER surface, enhancing Ca^{2+} transfer and energy supply (Yi et al., 2004). Similarly, late phases of ER stress promote mitochondrial immobilization and coupling to the ER surface, leading to mitochondria-dependent apoptosis (Chami et al., 2008). Constitutive Ca^{2+} transfer from the ER to mitochondria is essential for the maintenance of baseline bioenergetics (Green and Wang, 2010), but whether Ca^{2+} participates during ER stress as a signal to promote an adaptive mitochondrial response remains unknown.

Here, we focused on the metabolic events that occur during the initial stages of ER stress, before lethal events. We provide evidence that mitochondrial and reticular networks approach and interact in a microtubule-dependent manner during the onset of ER stress. This increased coupling is restricted to the perinuclear region and results in augmented Ca^{2+} transfer, leading to a localized enhancement in mitochondrial respiration that increases the reductive capacity and ATP production. These results suggest that mitochondria mobilized close to the ER confer a stress-activated bioenergetic response that ultimately contributes favorably to the cellular adaptation to ER stress.

**Results**

**Mitochondria approach perinuclear ER during early phases of ER stress**

The antibiotic tunicamycin, derived from *Streptomyces lyosuperficus*, blocks the synthesis of N-linked glycoproteins (N-glycans), therefore it is widely used in cell biology to induce the UPR (Price and Tsvetanova, 2007). We subjected HeLa cells to tunicamycin treatment and checked for ER stress markers by immunoblotting. After 6 hours of treatment with 0.1 and 1 μg/ml tunicamycin, we observed an increase in the expression of the ER chaperone and UPR effector CHOP (also known as DDIT-3), as well as an increase in the phosphorylation of both the translation initiation factor eIF2α and the UPR transducer JNK (Fig. 1A). Although at the early stage (4–6 hours) the ER stress signaling pathways are active, an increase in cell death was only detectable at 24 hours and became significant after 48 hours ($P<0.05$; Fig. 1B), suggesting that before triggering cell death, ER stress activates different processes. In order to detect changes in the morphology of the mitochondrial network during tunicamycin-induced ER stress, we quantified the area of the Mitotracker-stained mitochondrial network in relation to the whole-cell area on two-dimensional confocal stack images. After 4 hours of treatment with tunicamycin, a reduction in the cellular area occupied by the

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Fig. 1. Mitochondria translocate to perinuclear ER during early phases of ER stress. (A) Western blot analysis of HeLa cells treated for 4 hours with tunicamycin (Tun) as indicated. (B) The quantification of dead cells (PI positive) and cells with a low ΔΨm [low DiOC6(3) staining] after treatment with 1 μg/ml tunicamycin was determined by flow cytometry. (C) Confocal images of Mitotracker-Green-stained mitochondria in control cells or cells treated with 0.5 μg/ml tunicamycin for 4 hours. Quantification of the percentage ratio of mitochondrial area:whole cell area is shown for HeLa cells treated with 0.5 μg/ml tunicamycin as indicated. Co, control (untreated). (D) Example of the radial fluorescence analysis of subcellular zones. (E,F) Quantification of the radial fluorescence of control cells or cells treated with 0.5 μg/ml tunicamycin for 4 hours. Data are means ± s.e.m. *$P<0.05$ compared with controls within the same subcellular zone or as indicated. Scale bars: 10 μm.
mitochondrial network was observed, which returned to control levels after 20 hours (Fig. 1C). This suggested either a rapid decrease in whole mitochondrial volume or, more likely, a subcellular redistribution and/or condensation of the organelle network. Thus, we next performed a study of the distribution of the mitochondrial network in cells subjected to 4 hours of treatment with tunicamycin. An algorithm for the ImageJ software was created to scan the radial fluorescence, measured from the center of the nucleus towards the plasma membrane in a full-angle mode (0–360°), as illustrated in Fig. 1D. Using this tool, we compared changes in the subcellular distribution of the mitochondrial network between untreated controls and tunicamycin-treated cells. The 4-hour tunicamycin treatment led to a significant increase in mitochondrial abundance in the perinuclear zone as compared with that in controls, with a parallel decrease detected in the central and peripheral subcellular areas (Fig. 1E). In order to determine whether the ER behaved in a similar manner, we studied the radial subcellular distribution of ER-targeted red fluorescent protein (ER-RFP) after 4 hours of tunicamycin treatment. A redistribution of the ER towards the perinuclear area was also evident (Fig. 1F), suggesting that the interaction between the mitochondrial network and the ER increases during early ER stress conditions.

To elucidate further whether the approaching of the organelles correlates with increased coupling between them, we performed colocalization studies on confocal planes and monitored the points of interaction between the ER and mitochondria by quantifying the Manders’ coefficient, which is a measure of the fraction of one structure in contact with the other (Manders et al., 1993; Costes et al., 2004). HeLa cells were transfected with ER-RFP and mitochondria were stained with Mitotracker Green (Fig. 2A). The Manders’ coefficient M1 indicates the fraction of the ER that colocalizes with mitochondria (Fig. 2B), whereas the Manders’ coefficient M2 indicates the fraction of mitochondria that colocalizes with the ER (Fig. 2C). In both cases, we observed that tunicamycin treatment led to increased interaction between the organelles, which was more pronounced in the fraction of mitochondria interacting with the ER. In order to define further the subcellular location where these interactions were enhanced, we...
quantified the Manders’ coefficients within the predefined subcellular regions. We observed that the fraction of ER that colocalizes with mitochondria was enriched in the perinuclear zone (Fig. 2D), and notably the fraction of mitochondria that colocalizes with the ER was most enhanced in the same compartment (Fig. 2E). We also used thapsigargin as an alternative ER stress inducer, in order to validate the data obtained with tunicamycin. Thapsigargin-treated HeLa cells displayed a redistribution of the ER and mitochondria towards the nucleus and also increased their reticular and mitochondrial connection points, as quantified by the Manders’ coefficients M1 and M2 within the redistributed networks (supplementary material Fig. S1A–D). To verify further our findings with a different technique we performed electron microscopy (EM) experiments, which showed that there was a significant increase in the number of mitochondria displaying close ER contacts after 4 hours of tunicamycin treatment ($P<0.001$) and a clear redistribution of both organelles towards the perinuclear zone (Fig. 2F,G). Although the whole mitochondrial network was condensed (Fig. 1C), we additionally observed that, in comparison with control cells, tunicamycin-treated cells displayed an increased area per mitochondrion [controls: $0.27\pm0.09 \mu m^2$, $n=13$; tunicamycin-treated: $0.37\pm0.10 \mu m^2$, $n=13$ (means±s.e.m)], suggesting an increase in mitochondrial volume during ER stress. Altogether, these data indicate that, during the early stages of ER stress, the mitochondrial network approaches the ER in regions of close proximity to the nucleus, increasing the coupling points between both organelles.

**Microtubules coordinate ER–mitochondrial interactions**

Interactions between mitochondria and the cytoskeleton are necessary for mitochondrial morphology, motility and distribution (Yi et al., 2004; Hollenbeck and Saxton, 2005; Frederick and Shaw, 2007). Indeed, microtubules have been described as the hub for mitochondrial and ER movements (Friedman et al., 2010). On this basis, we evaluated the contribution of the microtubular network to the organelle rearrangement by employing nocodazole, a blocker of microtubule polymerization (Samson et al., 1979; Modriansky and Dvorak, 2005). By monitoring the mitochondrial and ER distribution within the predefined cellular regions, we observed that microtubule disruption significantly altered the organelle rearrangement profile observed with tunicamycin alone, leading to the retention of mitochondria and ER in the central zone (Fig. 3A,B). In addition, the increase in the coupling points between both organelles was observed primarily in the central and radial zones, rather than in perinuclear area (Fig. 3C). In addition to microtubules, the actin cytoskeleton also contributes to mitochondrial movements (Boldogh and Pon, 2006). Therefore, we studied the effects of tunicamycin in combination with cytochalasin D, which is a potent inhibitor of actin polymerization (Brenner and Korn, 1980; Casella et al., 1981). By monitoring the predefined cellular regions (and principally the perinuclear region), we detected no significant differences in the ER and mitochondrial profiles between cells treated with tunicamycin alone and cells treated with tunicamycin and cytochalasin D (supplementary material Fig. S2A,B). To study further the effect of actin microfibers in this organelle-coupling response, we quantified the Manders’ coefficient M2, which again indicates that the fraction of mitochondria that couples with the ER is not affected by actin disassembly (supplementary material Fig. S2C). Taken together, these results suggest that the dynamic microtubular network, and not the actin cytoskeleton, is necessary for the mitochondrial–ER coupling observed during early phases of ER stress.

**Localized perinuclear enhancement of mitochondrial function during early phases of ER stress**

An increase in mitochondrial function is an adaptive response to various types of stress (Duchen, 2004; Gautier et al., 2008; Addabbo et al., 2009). Therefore, we decided to monitor changes in cellular ATP production upon tunicamycin-induced ER stress. The level of total cellular ATP started to increase as early as 1 hour after tunicamycin treatment and was augmented for 4 hours; however, it displayed a substantial decrease with respect to basal levels after prolonged ER stress (20 hours; Fig. 4A). Increases in ATP levels can result from mitochondrial oxidative phosphorylation (OXPHOS) or cytosolic glycolysis coupled to lactate production and release. 2-Deoxyglucose (2-DG) is a cell-permeant glucose...
the increased apposition of ER and mitochondrial Ca2+ channels. Accordingly, knockdown of mitofusin 2 (Mfn2), which is an essential component of the mitochondrial–ER coupling molecular complex (de Brito and Scorrano, 2008), impaired the increase in oxygen consumption observed after 4 hours of tunicamycin treatment (Fig. 5C). Taken together, these results indicate that early after the onset of ER stress, a localized enhancement in mitochondrial function provides an increased amount of ATP and reductive power at the perinuclear ER–mitochondrial interface in a microtubule-dependent fashion.

**Increased mitochondrial Ca2+ drives the adaptive metabolic boost observed during early phases of ER stress**

Increases in mitochondrial respiration and ATP production are often consequences of increases in mitochondrial Ca2+ (Green and Wang, 2010). In order to determine whether early phases of ER stress induced by tunicamycin increased mitochondrial Ca2+, we treated cells expressing cytosolic or mitochondrial aequorins with histamine [which evokes PtdIns(3,4,5)3-dependent Ca2+ release] and compared their mitochondrial Ca2+ uptake. We observed that histamine led to a mitochondrial Ca2+ uptake that was significantly higher in tunicamycin-treated cells (P<0.05; 4 hours) than in untreated cells (Fig. 6A). Cytosolic Ca2+ increased early and reached a maximum at 4 hours of treatment, returning to basal levels after 20 hours (Fig. 4D). On the basis of the observation that the mitochondrial network is mobilized towards the perinuclear zone during ER stress, we next studied changes in ∆Ψm within the redistributed network. We observed that the mitochondrial network localized to the perinuclear area displayed an increased ∆Ψm, whereas in other subcellular areas ∆Ψm did not change significantly (Fig. 4E). By performing an MTT assay, we monitored the amount of reductive power that results from the mitochondrial tricarboxylic acid cycle. Tunicamycin treatment led to a significant increase (P<0.05) in reductive power at 4 hours, falling below baseline after 20 hours (Fig. 4F). These results also indicate that the metabolic responses occur before the decrease in cell viability observed at later times.

Given the necessary role of microtubules for the movement and coupling between reticular and mitochondrial networks, we wondered whether ER-induced metabolic enhancement depended upon the physical association of mitochondria with the ER. To test this, we subjected cells to tunicamycin treatment in combination with nocodazole, in order to impair the contact between both organelles. Under these conditions, the increases in ATP production, as well as in oxygen consumption, were abrogated (Fig. 5A,B), further highlighting microtubule-dependent organelle rearrangement as a necessary step in the functional coupling between the ER and mitochondria. Accordingly, knockdown of mitofusin 2 (Mfn2), which is an essential component of the mitochondrial–ER coupling molecular complex (de Brito and Scorrano, 2008), impaired the increase in oxygen consumption observed after 4 hours of tunicamycin treatment (Fig. 5C). Taken together, these results indicate that early after the onset of ER stress, a localized enhancement in mitochondrial function provides an increased amount of ATP and reductive power at the perinuclear ER–mitochondrial interface in a microtubule-dependent fashion.

**Fig. 4. Increase in cellular bioenergetics during early phases of ER stress.**

(A) Quantification of intracellular ATP levels in HeLa cells treated with 0.5 μg/ml tunicamycin (Tun) for the times indicated. (B) Quantification of intracellular ATP levels in cells treated for 4 hours with 0.5 μg/ml tunicamycin, in combination with 20 mM 2-DG, 20 μM CCCP or 1 μM oligomycin. (C) Determination of oxygen consumption in control cells, or cells treated with 0.5 μg/ml tunicamycin for 4 hours. Co, control (untreated). (D) Determination of ∆Ψm in TMRM-stained HeLa cells treated with 0.5 μg/ml tunicamycin as indicated. (E) Analysis of the radial fluorescence of TMRM-stained HeLa cells treated with 0.5 μg/ml tunicamycin for 4 hours. (F) Determination of reductive power and cell viability, through an MTT reductase activity assay, in HeLa cells treated with 0.5 μg/ml tunicamycin for the indicated times. Data are means±s.e.m. *P<0.05 compared with respective controls or as indicated.
Fig. 5. Metabolic enhancement requires an intact microtubular network. (A) Intracellular ATP levels were measured in control (untreated) HeLa cells, or cells treated with 0.5 μg/ml tunicamycin (Tun) in combination with 10 μM nocodazole as indicated. (B) Oxygen consumption was determined in control HeLa cells or cells treated with 0.5 μg/ml tunicamycin in combination with 10 μM nocodazole as indicated. (C) Oxygen consumption was determined in HeLa cells transduced with adenovirus encoding antisense mitofusin 2 (AsMfn2) or transduced with the empty vector (EV), and treated with 0.5 μg/ml tunicamycin in combination with 10 μM nocodazole as indicated. Data are means±s.e.m. *P<0.05 compared with untreated controls; †P<0.05 compared with tunicamycin alone (B) or tunicamycin plus EV (C).

Discussion

The results from this and previous studies (Rizzuto et al., 1998; Szabadkai et al., 2006) indicate that an intimate molecular interchange exists between the ER and mitochondria, and that these events become increased under stress conditions. Most of the previous studies have concentrated on the role of the ER, and its Ca2+ transfer from the ER to mitochondria drives the metabolic boost, and when these events become increased under stress conditions. Most of the previous studies have concentrated on the role of the ER, and its Ca2+ transfer from the ER to neighboring mitochondria, an additional phenomenon associated with the early phases of ER stress involves Ca2+ leak from the ER, also resulting in mitochondrial Ca2+ uptake. Indeed, no mitochondrial Ca2+ uptake following the thapsigargin-induced Ca2+ leak was observed in Mfn2-knockout cells (Fig. 6D), which is reflected by the lack of effect of Ru360. This result further indicates that juxtaposition of mitochondria with the ER is necessary for the mitochondrial Ca2+ uptake evoked by Ca2+ leak during early phases of ER stress.

Finally, to test whether mitochondrial Ca2+ levels control the metabolic mitochondrial boost, we measured oxygen consumption rates resulting from OXPHOS in the presence of the PtdIns(3,4,5)P3 receptor inhibitor xestospongin B or the mitochondrial Ca2+ uptake inhibitor RuRed. We observed that both xestospongin B and RuRed decreased the rate of oxygen consumption after tunicamycin treatment (Fig. 7A,B), which confirms that increased mitochondrial Ca2+ uptake, resulting from ER–mitochondrial coupling, is necessary for the metabolic response observed during early phases of ER stress. Therefore, in order to evaluate whether the early metabolic boost forms part of an adaptive response triggered by ER stress, we inhibited mitochondrial Ca2+ uptake and measured cell viability [through propidium iodide (PI) incorporation] and ∆Ψm. We observed that the inhibition of mitochondrial Ca2+ uptake during the early phase of ER stress increased cell death (PI-positive cells) and also decreased ∆Ψm at 48 hours (Fig. 7C).

In total, the results presented in this study suggest strongly that Ca2+ transfer resulting from enhanced ER–mitochondrial coupling leads to a localized production of mitochondrial metabolism, thus providing energetic substrates key for a cellular adaptive response in face of ER stress. Further experiments will determine whether this bioenergetic response is necessary for improving the energetic state of the ER, and therefore its folding capacity, or, as it is restricted to perinuclear zones, for the activation of a specific nuclear transcriptional program that participates in the cellular adaptation to stress.
Fig. 6. Augmented mitochondrial Ca\(^{2+}\) uptake during early phases of ER stress. (A) Representative traces of mitochondrial [Ca\(^{2+}\)]\(_{m}\) obtained from HeLa cells expressing mitochondrial aequorin, either untreated (Co, control) or treated with 1 \(\mu\)g/ml tunicamycin (Tun) for 4 hours prior to histamine addition. Statistical analysis of the peak [Ca\(^{2+}\)]\(_{m}\) is presented in the bar graph. (B) Representative traces of cytosolic [Ca\(^{2+}\)]\(_{c}\) ([Ca\(^{2+}\)]\(_{c}\)) obtained from HeLa cells expressing cytosolic aequorin, either untreated or treated with 1 \(\mu\)g/ml tunicamycin for 4 hours prior to histamine addition. Statistical analysis of the peak [Ca\(^{2+}\)]\(_{c}\) is presented in the bar graph. (C) HeLa cells were treated for 4 hours with 1 \(\mu\)g/ml tunicamycin and loaded with Fura-2 for cytosolic Ca\(^{2+}\) measurements. ER Ca\(^{2+}\) depletion was induced by addition of 1 \(\mu\)M thapsigargin, in the presence of 10 \(\mu\)M Ru360 as indicated. Peak values reflecting the kinetics after ER Ca\(^{2+}\) depletion are presented, normalized to those in control cells. (D) The same protocol as in C was used in wild-type (wt) MEFs or mitofusin2-knockout (Mfn2 ko) MEFs. Peak values reflecting the kinetics after ER Ca\(^{2+}\) depletion are presented, normalized to those in control wild-type MEFs. Data are means±s.e.m. (A and B) and means±s.d. (C and D). *P<0.05 compared with untreated controls or as indicated. **P<0.01 as indicated.

Mitochondria and the ER become more confluent when they are located at the perinuclear region, around the centrosome, whereas in peripheral regions their proximity diminishes (Darios et al., 2005). Similarly, studies on the regulation of the ER–mitochondria interface also show that the death stimulus C2-ceramide leads to organelle clustering at the perinuclear region, concomitant with an increase in the Ca\(^{2+}\) transfer between these organelles (Darios et al., 2005). In the context of ER stress, it has been described that later phases of ER stress lead to tightening of the ER–mitochondria interface, with increased mitochondrial Ca\(^{2+}\) and activation of apoptosis (Csordas et al., 2006; Chami et al., 2008). Here, we observed that ER stress leads to a redistribution of mitochondrial and reticular networks at the perinuclear zone, with increased connection points and Ca\(^{2+}\) transfer; therefore, our results agree with these precedents, but differ in the sense that the observed effects are not associated with cell death mechanisms. As these effects were obtained early during the onset of ER stress, they rather play a role in a metabolic adaptive response. In concordance with this, it has been described that cells with dysfunctional mitochondria display increased toxicity to glucose deprivation and that this sensitivity is associated with an impaired UPR (Haga et al., 2010).

In terms of bioenergetics, ER homeostasis depends upon a permanent supply of ATP and energy substrates, which are necessary for optimal protein folding (Bertridge, 2002; Gorlach et al., 2006), as well as for the clearance of aggregated proteins (Hoseki et al., 2010). Therefore, an increase in energy substrates seems essential for the adaptation to ER stress; however, this hypothesis has not been extensively studied. Previous work has demonstrated that, in response to diverse stress conditions, an increase in the cellular metabolic demands is observed (Duchen, 2004; Gautier et al., 2008; Addabbo et al., 2009). For instance, nutritional stress, induced by glucose deprivation, promotes an elevation in intracellular ATP levels (Liu et al., 2005). Glucose deprivation has been described as a condition causing ER stress and apoptosis (Ikeseji et al., 2006), and can be prevented by overexpression of the chaperone Grp75 (Heal and McGivan, 1997; Yang et al., 2008). Interestingly, this chaperone has been described as mediating the functional association of reticular and mitochondrial Ca\(^{2+}\) channels (Szabadkai et al., 2006). Therefore, it is likely that chaperone complexes containing Grp75, and perhaps other proteins such as Rab32 (Bui et al., 2010) or PACS-2 (Simmen et al., 2005), are involved in the adaptive response to ER stress. Similarly, cells lacking Mfn2 display impaired ER–mitochondrial Ca\(^{2+}\) transfer and are more prone to apoptosis (Jahani-Asl et al., 2007; Brooks et al., 2010). Accordingly, we observed that in the absence of Mfn2, both mitochondrial Ca\(^{2+}\) uptake and oxygen consumption were impaired, leading to increased cell death.

The present study demonstrates an important link between cellular stress and mitochondrial responses, establishing the metabolic basis for the adaptation to ER stress. Moreover, it defines the temporal window of the ER stress response to a wider range of events. During chronic ER stress, an increased Ca\(^{2+}\) transfer promoted by the truncated SERCA variant S1T leads to mitochondrial Ca\(^{2+}\) overload, with activation of mitochondrial apoptosis (Chami et al., 2008; Hayashi et al., 2009). Here, we focused on acute ER stress and observed that, during this phase, an increase in ER–mitochondrial Ca\(^{2+}\) transfer enhances the
mitochondrial metabolic state of mitochondria. Our results suggest a sequential series of events, in which Ca\textsuperscript{2+} leak influences the microtubule-dependent physical association between mitochondria and the ER, leading to Ins(1,4,5)P\textsubscript{3} dependent physical association between mitochondria and the ER, our results also suggest that the metabolic needs are increased in specific zones of the ER more affected by stress, whereas mitochondrial metabolism remains at baseline conditions in peripheral zones. These responses could be the consequence of a specialized and compartmentalized mechanism to segregate damage constituting an initial attempt to generate energy substrates for the peripheral tubules (Park and Blackstone, 2010). The rough envelope sheets of the ER are perinuclear, much more dense than the smooth peripheral tubules and, in addition, they are the zones receiving recently synthesized mRNA for translation. The ER close to the nucleus is enriched in translocons, which participate in protein import to the ER (Shibata et al., 2006), whereas peripheral zones are specialized in lipid biosynthesis (Park and Blackstone, 2010), and are enriched in different proteins (Shibata et al., 2008). It therefore seems probable that ER stress caused by an accumulation of misfolded proteins should manifest close to the nucleus, at least at earlier phases, which justifies the localized needs of a bioenergetic supply. We hypothesize that, above a certain threshold of ER damage, mitochondrial responses are no longer controlled, and Ca\textsuperscript{2+} overload results in permeability transition and the activation of mitochondrial apoptosis, as previously reported (Csordas et al., 2006; Chami et al., 2008).

On the basis of the results shown here, and the previous work of other groups, we propose that ER–mitochondrial coupling, and the consequent increase in mitochondrial Ca\textsuperscript{2+} uptake, belong to a dual response to ER stress conditions: on one hand, promoting an enhancement of mitochondrial metabolism as an adaptive response and, yet, on the other hand, leading to mitochondrial dysfunction as a cell death mechanism when stress is not resolved. Therefore, rather than proposing a point at which mitochondrial Ca\textsuperscript{2+} stops being pro-survival, and steps into a pro-death molecular pattern, we propose that earlier events occurring in the ER lead to tightening of the ER–mitochondrial interface and are responsible for promoting either adaptive or deleterious mitochondrial responses.

**Materials and Methods**

**ATP measurements**

Cells were plated in 96-well plates and ATP content was determined using a luciferin and luciferase assay (Cell-Titer Glo Kit; Promega), as described previously (Villena et al., 2008).

**Cell culture**

HeLa cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% fetal bovine serum (FBS). Cells were plated at 50–70% confluence on 60-mm diameter, six-, 24- or 96-well plates, according to the experiment. Cells were plated 24 hours prior to the exposition to tunicamycin for the indicated times, in the presence or absence of the different inhibitors. For transfection experiments, cells were treated with Lipofectamine 2000 at 24 hours after plating, according to manufacturer’s specifications. The different treatments were performed 24 hours after transfections. Mfn\textsubscript{2}-knockout and wild-type mouse embryonic fibroblasts (MEFs) were a gift from David Chan (Caltech, Pasadena, CA) and were maintained in DMEM supplemented with 10% FBS, 2 mM L-glutamine and 0.1mM non-essential amino acids.

**Dynamic in vivo (Ca\textsuperscript{2+}) measurements**

Basal and histamine-induced cytosolic Ca\textsuperscript{2+} signals were measured using either the cytosolic version of the recombinant Ca\textsuperscript{2+} sensor aequorin (cytAEQ) or the mutant variant restricted to the mitochondria (mitAEQ). All measurements were carried out in Krebs–Ringer modified buffer (KRb) (135 mM NaCl, 5 mM KCl, 1 mM MgSO\textsubscript{4}, 0.4 mM K\textsubscript{2}HPO\textsubscript{4}, 5.5 mM glucose and 20 mM HEPES pH 7.4), supplemented with 1 mM CaCl\textsubscript{2}. HeLa cells transiently expressing cytAEQ or mitAEQ were administrated with coelenterazine and transferred into a perfusion chamber. The light signal was collected in a purpose-built luminometer and calibrated into [Ca\textsuperscript{2+}] values. For the analysis of ER Ca\textsuperscript{2+} depletion kinetics, HeLa cells, and Mfn\textsubscript{2}-knockout and wild-type MEFs were loaded with 2 \textmu M Fura-2 (Invitrogen). 2\times10\textsuperscript{5} cells were trypsinized and resuspended in DMEM with 10% FBS. Cells were pelleted at 100 g and resuspended in 2 ml Ca\textsuperscript{2+}-free buffer (20 mM HEPES pH 7.4,
Flow cytometry analysis of ΔΨm and cell viability

The frequency of adherent and nonadherent cells with a low ΔΨm was determined by flow cytometry. Cells on 24-well plates were trypsinized and resuspended in culture medium with 40 mM 3,3-dihexyloxacarbocyanine iodide (DiOC6(3)) for 15 minutes at 37°C. The viable dye propidium iodide (PI, 1 μg/ml) was added 1 minute before experiments. Cell fluorescence was determined using a FACS Scan system (Becton Dickinson, San Jose, CA), as previously reported (Munoz et al., 2009). Living cells (PI negative) with low ΔΨm [low DiOC6(3) staining] were classified as ‘PI positive’, whereas dead cells (PI positive) with low ΔΨm were classified as ‘PI positive’, as previously described (Tajeddine et al., 2008).

Western blot analysis

Equal amounts of protein were separated by SDS-PAGE (10% polyacrylamide gels) and electrotransferred onto nitrocellulose. Membranes were blocked with 5% milk in Tris-buffered saline, pH 7.6, containing 0.1% Tween 20 (TBST). Membranes were incubated with primary antibodies at 4°C and re-blotted with horseradish-peroxidase-linked secondary antibody [1:5000 in 1% (w/v) milk in TBST]. The bands were detected using ECL, with exposure to Kodak film, and quantified by scanning densitometry.

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References


Supplementary Figure 2

A

Manders’ Coefficient M2 (Mito / ER colocalization)

Mitotracker Fluorescence Intensity (%)

ER-targeted RFP Fluorescence Intensity (%)

B

Control  Tun  Cytochalasin D  Tun + Cytochalasin D

Radial zone

Nuclear zone  Perinuclear zone  Central zone

C

Manders’ Coefficient M2 (Mito / ER colocalization)

Radial zone  Nuclear zone  Perinuclear zone  Central zone

Tun  Cytochalasin D  Tun + Cytochalasin D

Supplementary Figure 2