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Inhibition of mitochondrial genome expression triggers the activation of CHOP-10 by a cell signaling dependent on the integrated stress response but not the mitochondrial unfolded protein response

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ABSTRACT

Mitochondria-to-nucleus communication, known as retrograde signaling, is important to adjust the nuclear gene expression in response to organelle dysfunction. Among the transcription factors described to respond to mitochondrial stress, CHOP-10 is activated by respiratory chain inhibition, mitochondrial accumulation of unfolded proteins and mtDNA mutations. In this study, we show that altered/impaired expression of mtDNA induces CHOP-10 expression in a signaling pathway that depends on the eIF2α/ATF4 axis of the integrated stress response rather than on the mitochondrial unfolded protein response.

1. Introduction

Vestige of its α-proteobacterial origin, mitochondria keep DNA (mtDNA) encoding 13 polypeptides, all subunits of the electron transport chain (ETC), as well as 2 ribosomal RNAs (rRNAs) and 22 transfer RNAs (tRNAs) that are required for their translation. Still, this only represents 1% of the mitochondrial proteome, estimated at up to 1500 proteins that are therefore predominantly encoded by the nuclear DNA (Calvo and Mootha, 2010). Altered expression of mitochondrial proteins is increasingly associated with many and various human diseases (Boczonadi and Horvath, 2014). If mutations within genes encoding components or assembly factors of the ETC are the most obvious cause of mitochondrial dysfunction and pathology, the better understanding of mtDNA maintenance and expression has shed light on new mutations associated with mitochondrial diseases. Indeed, those not only arise from alterations in the mtDNA but also in nuclear genes encoding proteins required for regulation of mtDNA expression. On one hand, mutations or deletions within one of the 2 rRNAs or 22 tRNAs encoding genes will affect the translation of the 13 mtDNA-encoded subunits of the ETC. Such alterations lead to mitochondrial diseases such as MELAS (Mitochondrial Encephalopathy, Lactic Acidosis, and Stroke-like episodes), MERRF (Myoclonic Epilepsy with Ragged Red Fibers) and KSS (Kearns–Sayre Syndrome) mainly associated with mutations within $tRNA^{leu}$ (A3243G), $tRNA^{lys}$ (A8344G) or large-scale mtDNA deletion, respectively (Goto et al., 1990; Maceluch and Niedziela, 2006; Shoffner et al., 1990). On the other hand, the proteins required to support mtDNA expression are encoded by the nuclear genome (Vafai and Mootha, 2012). Therefore, it is not surprising that an increasing number of human mitochondrial diseases have been linked with nuclear genes encoding proteins involved in mitochondrial protein synthesis such as tRNA-modifying enzymes, aminoacyl-tRNA synthetases, ribosomal proteins, elongation, mRNA stability and termination factors as well as translation activators (Pearce et al., 2013). Altered expression of mtDNA results in mitochondrial dysfunction and perturbs cellular homeostasis as mitochondria is important not only for ATP production by oxidative phosphorylation but also for lipid

Abbreviations: AARE, amino acid responsive element; ATF4, activating transcription factor 4; CHOP-10, C/EBP homologous protein 10; CK2, casein kinase 2; CREB, cAMP-responsive element binding protein; COX, cytochrome C oxidase; eIF2α, eukaryotic translation initiation factor 2A; ERAD, endoplasmic-reticulum-associated protein degradation; ERSE, endoplasmic reticulum stress element; erUPR, unfolded protein response of the endoplasmic reticulum; ETC, electron transport chain; HERP, homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1; EtBr, ethidium bromide; FCS, fetal calf serum; GCN2, general control non-derepressible-2; HRI, heme-regulated inhibitor; HSPD1, heat shock 60 kDa protein 1; ISR, integrated stress response; JNK, c-Jun N-terminal Kinase; KSS, Kearns-Sayre Syndrome; MELAS, Mitochondrial Encephalopathy, Lactic Acidosis, and Stroke-like episodes; MERRF, Myoclonic Epilepsy with Ragged Red Fibers; mtUPR, mitochondrial unfolded protein response; mtDNA, mitochondrial DNA; NFAT, nuclear factor of activated T cells; NFκB, Nuclear Factor kappa B; OTC, ornithine transcarbamylase; PERK, PKR-like ER-kinase; PKR, protein kinase double-stranded RNA-dependent; TOM40, translocase of the outer membrane; Trib3, tribble 3.

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metabolism, synthesis of iron–sulfur cluster, calcium and ROS signaling as well as regulation of programmed cell death. Hence, general feature of mitochondrial diseases include reduced ATP production, decreased membrane potential, altered calcium homeostasis and ROS production (James et al., 1996; Lenaz et al., 2004; Moudy et al., 1995; Vives-Bauza et al., 2006).

Coordinated expression of both genomes and communication between mitochondria and nucleus is thus critical to maintain proper cell function. Transcriptional regulators are key players in mitochondria-to-nucleus communication as they orchestrate the expression of nuclear genes in response to both external challenges and organelle dysfunction to regulate processes such as mitochondrial biogenesis, cell proliferation, metabolism and apoptosis, as reviewed in Ryan and Hoogenraad, (2007). Some of them have very well described signaling pathways activated in response to mitochondrial stress such as among other NFAT (nuclear factor of activated T cells), NFκB (nuclear factor kappa B) and CREB (cAMP-responsive element binding protein) triggered by increased cytosolic calcium concentration because of altered mitochondrial buffering function (Arnould et al., 2002; Biswas et al., 1999, 2003). First and mainly reported in the context of endoplasmic reticulum stress, the transcription factor CHOP-10 (C/EBP homologous protein 10, also known as DDIT3 or GADD153) has also been reported to respond to mitochondrial dysfunction. Indeed, increased expression of the transcription factor has been demonstrated in the context of disrupted mitochondrial protein homeostasis (proteostasis) (Moisoi et al., 2009; Zhao et al., 2002), inhibition of mitochondrial ETC (Ishikawa et al., 2009; Vankoningsloo et al., 2006) and mutation within mtDNA (Cortopassi et al., 2006; Fujita et al., 2007). However, events and stress signaling pathways associated with mitochondrial dysfunction-induced CHOP-10 overexpression are not clear. Hoogenraad and colleagues have demonstrated that CHOP-10 is regulated by AP-1 in the context of the mitochondrial unfolded protein response (mtUPR), a specific quality control process that aims to resolve proteotoxic stress by coordinating expression of mitochondrial chaperones and proteases such as HSPD1 (also known as chaperonin 60 or HSP60) and ClpP, respectively (Horibe and Hoogenraad, 2007). Briefly, in this model, overexpression of a truncated version of the enzyme ornithine transcarbamylase (OTCΔ) results in its accumulation as an unfolded proteins within mitochondrial matrix that leads to JNK (c-Jun N-terminal Kinase) activation and phosphorylation of the AP-1 family member c-Jun. Once translocated into the nucleus, this transcription factor increases CHOP-10 expression that in turn triggers expression of stress responsive genes (Horibe and Hoogenraad, 2007). Moreover, the impairment of the stoichiometric equilibrium between nuclear and mtDNA-encoded proteins is another condition known to induce mtUPR as suggested in rho^0 cells and demonstrated for murine cells incubated in the presence of doxycycline, an antibiotic of the tetracycline family that inhibits mitochondrial translation (Houtkooper et al., 2013; Martinus et al., 1996). So far, the global mechanism of mtUPR is still missing in mammals as cell signaling activated by accumulation of unfolded proteins has been mainly described in *Caenorhabditis elegans* (Pellegrino et al., 2013).

However, others have shown that increased expression of CHOP-10 associated with mitochondrial respiration defect relies more on the integrated stress response (ISR) than mtUPR (Cortopassi et al., 2006; Fujita et al., 2007; Ishikawa et al., 2009; Moisoi et al., 2009). ISR is a signaling pathway converging to eIF2α phosphorylation and characterized by a global reduction in protein synthesis, together with a selective overexpression of stress-responsive genes (Fujita et al., 2007; Silva et al., 2009). ISR sensors consist of a set of four different kinases: PERK (PKR-like ER-kinase), GCN2 (general control non-derepressible-2), HRI (heme-regulated inhibitor) and PKR (protein kinase double-stranded RNA-dependent). These four kinases are activated in response to various cues such as endoplasmic reticulum stress, amino acid depletion, viral infection, oxidative stress, heme deprivation, UV irradiation and proteasome inhibition (Donnelly et al., 2013; Harding et al., 2000, 2003). Even if eIF2α phosphorylation reduces cytosolic protein synthesis, mRNAs containing short upstream open reading frames are preferentially translated, such as ATF4, ATF3 and CHOP-10 (Wek et al., 2006). The latter is a pleiotropic transcription factor that is associated with cell death/survival under ER stress as it can either trigger or prevent apoptosis (Fujihara et al., 2009; Zinszner et al., 1998). In addition to its expression level, CHOP-10 can be regulated by post-transcriptional modifications such as phosphorylation by p38 or CK2 (casein kinase 2), resulting in an enhanced or attenuated transcriptional activity, respectively (Ubeda and Habener, 2003; Wang and Ron, 1996). Together with other transcription factors of the bZIP family, ATF4 and CHOP-10 regulate the expression of specific stress genes such as the ERAD (endoplasmic-reticulum-associated protein degradation)-associated protein HERP (homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1) and the pseudokinase Trib3 (tribble 3) (Harding et al., 2003; Kilberg et al., 2009).

In this study, as CHOP-10 can be activated by both mtUPR and ISR, we analyzed the regulation of CHOP-10 expression in different cell models in which mtDNA expression is impaired, such as mitochondrial genome depletion and translation inhibition, to better understand signaling pathways triggered by mitochondrial dysfunction that could converge to the activation of this transcription factor. The investigation of these stress signaling pathways might help to better characterize mitochondrial diseases associated with mtDNA depletion or impaired organelle translation.

2. Materials and methods

2.1. Cell culture, transfection and reagents

The HeLa adenocarcinoma and Hep3B hepatocellular carcinoma cell lines were purchased from ATCC (CCL-2 and HB-8064, respectively). HeLa rho^0 cells were a kind gift of Professor R. Wiesner (University of Cologne, Germany). HeLa cells were grown in Minimum Essential Media (MEM, Gibco) supplemented with pyruvate (11360-039, Gibco), non-essential amino acid (11140-035, Gibco) and 10% of fetal calf serum (FCS) (Gibco). HeLa rho^0 cells were grown in 4.5 g/l glucose-containing Dulbecco's modified Eagle Medium (DMEM, Gibco) supplemented with pyruvate, non-essential amino acid, 10% of fetal calf serum and 50 μg/ml uridine. Hep3B cells were cultured in Roswell Park Memorial Institute medium (RPMI 1640 Invitrogen, 21875) containing 10% of FCS. Fresh human hepatocytes (HEP220) were obtained from Biopredic international and kept in medium for long-term culture (MIL600C) supplemented with additive (ADD27111C). All cell lines were kept at 37 °C under a humidified atmosphere containing 5% CO_2. Stock solution of doxycycline (Sigma, D9891-1G) was prepared in deionized water at a concentration of 15 mg/ml, filter sterilized and used at a final concentration of 15 or 30 μg/ml as indicated.

For reporter gene experiments, HeLa cells have been transfected with SuperFect transfection reagent from Qiagen (301305). Briefly, cells were seeded one day before transfection in 12-well plates (50,000 cells/well). Cells were transiently transfected with 0.6 μg of a Firefly luciferase reporter plasmid and 0.4 μg of a plasmid containing a cDNA encoding Renilla luciferase under the control of the TK promoter (pRenillaLuc-TK), used for normalization. The DNA/SuperFect ratio was 1:2 and cells were incubated with complexes for 3 h in the absence of serum before refreshing with new medium, containing doxycycline or not, for 48 h.

For silencing experiments, On-target plus siRNA smartpools purchased from Dharmacon were used: ATF4 (L-005125-00-0005), PERK (L-004883-00-0005), GCN2 (L-005314-00-0005), HRI (L-005007-00-0005), PKR (L-003527-00-0005). Non-targeting On-target plus siRNA (D-001810-10-05) have been used as negative control. 200,000 cells were seeded in 6-well plates in complete growth medium. The next day, cells were transfected with 50 nM siRNA diluted in opti-MEM (Gibco, 31985-070) and using Dharmafect 1 (T-2001-01) as transfection reagent, following the manufacturer's recommendation. After 24 h of