Published in final edited form as:

J Mol Cell Cardiol. 2011 December ; 51(6): 885–893. doi:10.1016/j.yjmcc.2011.07.023.

Mitofusins are required for angiogenic function and modulate different signaling pathways in cultured endothelial cells¹

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Abstract

The mitofusin proteins MFN1 and MFN2 function to maintain mitochondrial networks by binding one another and initiating outer mitochondrial membrane fusion. While it has recently been recognized that vascular endothelial cells rely upon mitochondria as signaling rather than energy-producing moieties, the role of mitochondrial dynamics in endothelial cell function has not been addressed. To begin to understand what role mitochondrial dynamics play in this context, we examined the regulation of MFN1 and MFN2 and the consequences of siRNA-mediated knockdown of these proteins in cultured endothelial cells. Treatment with VEGF-A led to the upregulation of MFN2 and, to a lesser extent, MFN1. Knockdown of either MFN led to disrupted mitochondrial networks and diminished mitochondrial membrane potential. Knockdown of either MFN decreased VEGF-mediated migration and differentiation into network structures. MFN ablation also diminished endothelial cell viability and increased apoptosis under low mitogen conditions. Knockdown of MFN2 uniquely resulted in a decrease in the generation of reactive oxygen species as well as the blunting of the gene expression of components of the respiratory chain and transcription factors associated with oxidative metabolism. In contrast, ablation of MFN1 led to the selective reduction of VEGF-stimulated Akt-eNOS signaling. Taken together, our data indicate that mitochondrial dynamics, particularly those mediated by the mitofusins, play a role in endothelial cell function and viability.

Keywords

mitofusins; nitric oxide; apoptosis; energy metabolism; mitochondria

¹ **Abbreviations:** Endothelial cells (EC), reactive oxygen species (ROS), nitric oxide (NO), mitofusin-1 (Mfn1), mitofusin-2 (Mfn2), quantitative PCR (qPCR), human umbilical vein endothelial cells (HUVEC)

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Disclosures

None.

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Introduction

Endothelial cells (EC) are at the crux of nutrient supply and demand in metabolically active tissues such as muscle, brain, liver and heart. To this end, EC have critical roles in nutrient transport, environmental sensing and integrating the signaling events from surrounding tissue [1, 2]. The integrity of the vascular endothelium is also critical for proper development of the placenta in mammals to deliver nutrients to developing tissues in utero [3, 4]. These complex regulatory roles, mediated by nuclear receptors [5], microRNAs [6], and soluble factors [7], dictate the metabolic capacity of endothelium downstream of intracellular signaling events.

EC largely rely upon anaerobic glycolysis, and they utilize oxidative phosphorylation for a small fraction of total ATP production [8, 9]. Rather than function in energy production, endothelial mitochondria appear to primarily serve roles in cellular homeostasis and the activation of signaling cascades. Mitochondria of the vascular endothelium are critical for maintaining Ca^{2+} concentration [10], integrating apoptotic stimuli [11], and generating reactive oxygen species (ROS) [12]. Coupled with nitric oxide (NO), ROS have emerged as an important signaling molecule in endothelium [13, 14] and plays a critical role in environmental sensing [15, 16] and angiogenesis [17]. Though the mechanism remains unclear, it is evident that mitochondrial function is required for ROS-induced (H_2O_2 -mediated) receptor tyrosine kinase transactivation and activation of the Jnk and Akt signaling pathways [18] and the activation of antioxidants [19] in endothelial cells.

Mitochondria continuously undergo homotypic fusion and fission events that are collectively referred to as mitochondrial dynamics. The first step in fusion is the interaction of the outer membranes of mitochondria, mediated by the transmembrane GTPases referred to as mitofusin-1 and -2 (Mfn1, Mfn2) [20, 21]. It is believed that a Mfn-Mfn interaction is the first step of mitochondrial membrane fusion, followed by Opa1-mediated fusion of the mitochondrial inner membranes [22]. Loss of *Mfn1* or *Mfn2* leads to embryonic lethality in mice [23] that can be circumvented by using a conditional loss-of-function strategy whereby *Mfn1* is deleted solely in the embryo proper, yet allowing for normal *Mfn1* expression in the tissues of the placenta [24]. While there is a great deal of structural similarity between Mfn1 and Mfn2 at the primary protein level, there are critical differences that have been revealed upon further study [24–30]. Somatic deletion of *Mfn1* or *Mfn2* results in embryonic lethality but *Mfn2* is required in trophoblast giant cells whereas *Mfn1*-null animals die at midgestation [23]. Further, unlike *Mfn1*-null mice, placental rescue of *Mfn2* yields a mouse with postnatal defects including movement disorders and impaired cerebellar development, revealing an integral role for *Mfn2* in neural development and function [24].

Vascular dysfunction is coupled to obesity and insulin resistance [25], and mitochondria in endothelial cells appear to play important roles in the vascular pathology associated with metabolic stress [26, 27]. Animal and cell culture models have demonstrated that high levels of glucose cause mitochondria fragmentation, increased production of ROS and apoptotic cell death in endothelial cells [28, 29]. These studies link metabolic and vascular function with perturbations in mitochondrial networks in endothelial cells. In addition, it is recognized that metabolic dysfunction can lead to angiogenic defects associated with an abrogation in VEGF-A signaling [30]. However, the potential link between VEGF-mediated angiogenesis and mitochondrial dynamics has not been examined previously.

Here we report that VEGF-A induces MFN1 and MFN2 in cultured human umbilical vein endothelial cells (HUVEC) and that loss of either *MFN* alters mitochondrial network formation and membrane potential. These alterations also lead to diminished endothelial cell migration and differentiation into network structures in response to VEGF-A stimulation.

These data demonstrate that mitofusins are essential for endothelial cell functions that are associated with angiogenesis.

Materials and methods

Reagents

Human umbilical vein endothelial cells were purchased from Lonza. Mfn1 and Mfn2 antibodies were purchased from Abcam. Antibodies directed against JNK, Thr183/Tyr185-JNK, Akt1, Ser473-Akt1, Ser1177-Nos3, and Tubulin were from Cell Signaling. The Nos3 antibody was from Santa Cruz Biotechnology. Secondary antibodies (donkey anti-mouse HRP and donkey anti-chicken-HRP) were purchased from Santa Cruz Biotechnology. Dichlorofluorescein and JC-1 dyes were from Molecular Probes. Unless otherwise stated, all other reagents and chemicals were from Sigma.

Cell Culture

HUVEC were grown in EGM-2 media (Lonza). Cells used in experiments were from passages 2–6. Prior to all experiments, cells were serum-deprived overnight in EBM2 media supplemented with 0.5% fetal calf serum. SiRNA oligo mixes (siGENOME Smartpool) were purchased from Dharmacon and 33 nm of siRNA was transfected into individual wells of a 6-well plate. Transfection was performed with Lipofectamine 2000 in Optimem as per the manufacturer's instructions.

qPCR and MFN copy number determination

Total RNA was recovered using TRIzol reagent (Invitrogen) according to the manufacturer's instruction and cDNA was synthesized from 500 ng of RNA using the SuperScript III Reverse Transcription kit (Invitrogen). Quantitative PCR (qPCR) was performed on an Applied Biosystems Step-One Plus Real-Time PCR system using SYBR Green I (Applied Biosystems) dye to detect PCR amplicons. All transcripts were normalized to the ribosomal protein *RPLP0*. *MFN* copy number was determined by qPCR as above but utilizing linearized *MFN1* or *MFN2* expression vectors (Open Biosystems) to build standard curves based on copy number. Primer sequences are available upon request.

Western Blotting

Cells were lysed with Cell Lysis buffer (Cell Signaling) containing HALT phosphatase inhibitor cocktail (Roche) and 100 mM PMSF. Lysates were centrifuged at 14,000 rpm at 4° C and the supernatant was recovered and the protein concentration determined with BCA Protein Assay reagents (Thermo Scientific). Lysates were resolved via SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blotted with the indicated primary antibodies at a 1:1000 dilution followed by a horseradish peroxidase-conjugated secondary antibody at 1:5000. Detection was carried out with the ECL Plus kit (Amersham Biosciences).

Microscopy

Cells were grown on german borosilicate glass coverslip slides precoated with Poly L-Lysine. Cells were stained with 50 nm Mitotracker green (Molecular Probes) for 30 minutes at 37° C, washed extensively and visualized in Hank's balanced saline solution. Microscopy was performed with a Nikon TE 2000 deconvolution microscope with constant settings and stacks with a step of 200nm. ImageJ was used to deconvolute the images by iterative 3D deconvolution and analysis of the mitochondrial network was performed using the method published by Koopman [31].

Angiogenesis Assays

Migration activity was examined using a modified Boyden chamber assay wherein 2×10^4 cells were placed in the upper well of a chamber containing a fibronectin-coated 3.0 micron transwell insert. In the lower chamber, EBM-2 supplemented with 0.5% FCS and 20 ng/ml rhVEGF-A was added and cells were allowed to migrate overnight. The following day, the cells on the underside of the inserts were washed with PBS, fixed with 2% glutaraldehyde and stained with Hoechst 33342 and counted. To assess formation of vascular networks, 5×10^4 cells were differentiated on 250 μ l of growth factor-reduced Matrigel (BD Biosciences) overnight in the presence of 20 ng/ml rhVEGF. Network formation was quantified using ImageJ software to measure the surface area covered by networks in each well of a 24-well plate.

Cell viability and TUNEL Staining

Cells were grown on 4-chamber slides (Falcon) and starved overnight after siRNA knockdown. TUNEL staining was performed with the In Situ Cell Death Detection kit-Fluorescein (Roche) according to the manufacturer's instructions. Cell Viability was assessed with transfected HUVEC in 96-well plates using the Promega CellTiter 96 AQueous kit.

ATP measurement

ATP was measured in HUVECs grown in 96-well plates. After overnight serum-deprivation, ATP was measured using the Cell-Titer Glo assay kit (Promega, Madison, WI) and quantified by using a standard curve made from serial dilutions of ATP. Cells were grown in duplicate under identical conditions and used for protein determination.

Mitochondrial DNA quantitation

HUVEC were grown in 6-well dishes, transfected with siRNA oligos and DNA recovered with Qiagen DNeasy kits. DNA was utilized in a SYBR-based qPCR reaction with primers specific for intronic regions of the mitochondrial gene *MTCO1* and the nuclear gene *LPL*. Quantification was performed with a standard curve.

JC-1 Staining

Cells (2×10^5) were transfected in 6-well plates. After incubation in low mitogen media overnight, media was changed to HBSS and cells were allowed to equilibrate for 1 hour at 37° C. Cells were incubated in 2.5 μ g/ml JC-1 for 20 minutes, washed 3 times in HBSS and gently scraped into a 96-well plate and subject to fluorescence detection and ratio determination- Red: Ex 550 nm, Em 600 nm; Green: Ex 485 nm, Em 535 nm.

Flow Cytometry

After incubation in low mitogen media overnight, cells were incubated in 10 ng/ml DCF for 30 minutes at 37° C. Cells were washed 3 times with PBS, recovered with trypsin-EDTA, resuspended in starvation media, filtered through a 40 μ m cell strainer and subject to FACS analysis on a Becton Dickinson FACScan machine utilizing Cellquest analysis software. Live cells were identified using forward and side scatter measurements and this cell population was gated on for DCF fluorescence.

Statistical Analysis

All data are presented at means $\pm$ S.E.M. as indicated in figure legends. Differences were analyzed by Student's T test or one-way analysis of variance with Tukey post-hoc test. A level of $P < 0.05$ was accepted as statistically significant.

Results

MFN expression in HUVEC

We utilized quantitative PCR to determine the relative expression of the Mitofusin-encoding genes in HUVEC. The use of *MFN1* or *MFN2* specific primers demonstrated that per microgram of total RNA, there are approximately 8,000 copies of *MFN1* and 65,000 copies of *MFN2*, making *MFN2* the more abundant mitofusin at the transcript level in HUVEC (Figure 1A). To establish a link between endothelial cell function and the Mfns, serum-deprived HUVEC were stimulated with 30 ng/ml VEGF-A for 2 or 4 hours. Within 2 hours, VEGF-A treatment led to an induction of both *MFN1* and *MFN2* expression (Figure 1B). Notably, the degree of *MFN2* induction was much greater than that of *MFN1*.

To address the role of MFN-mediated mitochondrial dynamics in endothelial cells, *MFN1* and *MFN2* expression was reduced using siRNA oligonucleotides. HUVEC were transfected with targeting or control oligonucleotides and allowed to grow in high-mitogen media (EGM-2) for approximately 48 hours, at which time media was changed to low-mitogen media and cells were grown overnight. Assessment of gene expression of all five fusion-fission genes demonstrated the selective silencing of *MFN1* and *MFN2* by their cognate siRNA oligos (Figure 2A). This silencing extended to the level of protein (Figure 2B). Ablation of *MFN1* led to an $85 \pm 3.3\%$ reduction in *MFN1* protein and ablation of *MFN2* led to an $85 \pm 1.2\%$ reduction in *MFN2* protein.

Quantitative measures of MFN-knockdown mitochondria

We next sought to examine the effects of *MFN* ablation on mitochondrial morphology. By using the vital dye Mitotracker Green, computer-aided morphometric analyses were performed to measure the aspect ratio (AR), which measures the network length and form factor (FF), which assesses network branching, of individual mitochondria of live EC. For both AR and FF, a value of '1' is ascribed to highly fragmented mitochondria that are a geometric circle. Measuring the AR and FF of >50 individual mitochondria in cells treated with a non-targeting control (NTC) siRNA ($n = 33$ cells) or *MFN1* or *MFN2* siRNAs ($N = 13$ cells each) demonstrated a highly significant reduction in both AR (NTC = 2.97 ± 0.068 ; *MFN1* = 1.88 ± 0.035 ; *MFN2* = 2.21 ± 0.046) and FF (NTC = 2.86 ± 0.103 ; *MFN1* = 2.07 ± 0.094 ; *MFN2* = 2.00 ± 0.162) in *MFN*-knockdown EC, consistent with a change in mitochondrial morphology towards a more fragmented network (Figure 2C, D). Taken together, these data indicate that siRNAs against *MFNs* can be used to attenuate message and protein of mitofusins as well as significantly disrupt mitochondrial morphology.

Angiogenic activities of MFN-knockdown EC

To address the potential role of mitofusins in endothelial cell angiogenic activity, HUVEC with knockdown of *MFN1* or *MFN2* expression were subject to assays of VEGF-induced EC differentiation into network structures. In this assay, siRNA-treated HUVEC were incubated in low mitogen media overnight and subsequently placed on growth factor reduced matrigel in the presence of VEGF. In this assay, the matrigel serves as an ex-vivo extracellular matrix for the EC to navigate and align such that networks form [32]. *MFN*-knockdown HUVEC form less extensive endothelial networks when the network density was analyzed (Figure 3A, B). As network formation requires cellular migration on the matrigel, we also investigated whether attenuation of *MFNs* affects the ability for EC to migrate towards VEGF. Using a modified Boyden-chamber assay, we found that roughly half as many cells with reduced *MFN* expression, either *MFN1* or *MFN2*, migrated towards the VEGF-A chemotactic signal, as did cells with wild-type levels of *MFNs* (Figure 3C).

Mitofusins are required for endothelial survival under stress

To address whether *MFNs* are integral to the survival of EC in culture, HUVEC were treated with siRNAs, allowed to recover in full growth media and subsequently incubated in low-mitogen media overnight and used to assess cellular viability via an MTS-based assay. Upon serum deprivation, a significant loss of viability was observed with attenuation of either MFN1 or MFN2 (Figure 4A). To directly test for the serum-deprivation-induced apoptosis, HUVEC treated in this manner were also assayed via TUNEL staining. The knockdown of either MFN1 or MFN2 led to an increase in TUNEL-positive/apoptotic cells upon serum deprivation (Figure 4B).

To address whether attenuating *MFN* expression alters mitochondrial homeostasis, possibly contributing to the increase in cell death, we examined mitochondrial membrane potential ($\Delta\Psi$) by stressing cells with 100 μM H_2O_2 and measuring $\Delta\Psi$ with the voltage-sensitive mitochondrial dye TMRM. Addition of H_2O_2 led to a loss of $\Delta\Psi$ as measured by a decrease in TMRM fluorescence in NTC siRNA-treated HUVEC of ~40% after 12 minutes (Figure 4C). Ablation of *MFN1* or *MFN2* led to a more rapid and greater loss of TMRM fluorescence indicating a sensitization to oxidative stress. Under MFN-knockdown conditions, there was ~40% loss of TMRM fluorescence by 3.5 minutes.

MFN-regulation of mitochondrial function

To further characterize effects of MFN depletion on the properties of EC, quantitative PCR for mitochondrial (mt) DNA and nuclear (n) DNA using primers specific for genomic regions of *MTCO1* and *LPL1*, respectively. No changes could be detected in the ratio of mtDNA:nDNA in EC treated with any of the various siRNAs (Figure 5A), indicating that MFN ablation does not affect the quantity of the mitochondrial genome. Additionally, the ATP concentrations in cells treated with various siRNAs were not altered (Figure 5B).

Mitochondrial membrane potential was probed utilizing the ratiometric dye JC-1 to determine whether knockdown of MFNs had an impact upon mitochondrial function. Attenuation of either MFN1 or MFN2 resulted in a significant loss of membrane potential relative to NTC-treated EC (Figure 5C). These data were confirmed by co-staining live EC with the membrane potential-sensitive TMRE and the non-specific dye Mitotracker Green (data not shown). As mitochondrial respiratory activity can contribute to production of reactive oxygen species (ROS), the fluorescent dye dichlorofluorescein (DCF) was utilized to probe ROS production. Initially, MFN-depleted EC were used at a resting state to assess the basal production of ROS. A significant decrease in ROS production was seen solely with the attenuation of MFN2 (Figure 5D). To explore the effect of *MFN* attenuation on maximal ROS production, we analyzed the high levels of ROS produced during the suspension of these anchorage-dependent cells [33]. Flow cytometric analyses of EC under these conditions revealed a decrease in maximal ROS production in cells when *MFN2*, but not *MFN1*, expression was reduced by siRNA treatment (Figure 5D, E).

Altered signaling pathways with attenuation of MFN expression

Endothelial cells are highly sensitive to their environment and orchestrate signaling pathways in response to growth factor stimulation and oxidant stress. Recent studies have shown that mitochondria play an important role in mediating Akt and JNK signaling pathways in EC [18]. Thus, we examined the requirements for MFNs in H_2O_2 - and VEGF-A-mediated signaling. Treatment of cells with 100 μM H_2O_2 for 30 minutes resulted in activation of the JNK pathway as demonstrated by phosphorylation of JNK1 at Thr183/Tyr185 (Figure 6A). JNK activation was significantly attenuated in either MFN1- or MFN2-knockdown HUVEC. Notably, the reduction in JNK activation was greatest in cells treated with siRNA against *MFN2*. Additionally, cells were treated with 50 ng/ml VEGF-A for 15

minutes to assess Akt1 and Nos3 (eNOS) signaling. Strikingly, Akt1 and Nos3 activation, via phosphorylation at serine 473 and 1177 residues, was diminished in MFN1-knockdown but not MFN2-knockdown cells (Figure 6B). The functional outcome of this dysregulation was investigated by measuring the levels of cGMP, a surrogate for NO, produced by HUVECS. Consistent with the signaling data, VEGF-A failed to induce NO biogenesis when *MFN1* but not *MFN2* levels were reduced by siRNA treatment (Figure 6C).

Discussion

Previous studies on the role of mitochondrial dynamics, particularly those focused on the mitofusins, have primarily dealt with cells that rely heavily upon mitochondria as sources of energy including neurons [24, 34], skeletal muscle [35], and cardiac myocytes [36]. In contrast, EC rely primarily on glycolysis and it is proposed that mitochondria predominantly play a signaling role in these cells [8, 37]. Here, we demonstrate for the first time that the mitofusin proteins have an integral role in endothelial cell function and survival. In this context, we propose that the mitofusins are an important component of the signaling function that mitochondria play within these cells. In support of this notion, we show that VEGF-A stimulation promotes mitofusin expression in EC and that the ablation of either MFN1 or MFN2 leads to profound changes in mitochondrial network morphology as measured by reductions in mitochondrial length as well as degree of mitochondrial branching. These changes are accompanied by diminished VEGF-mediated angiogenic potential as assessed through cell migration and network formation assays and diminished cell viability in the absence of mitogens.

Consistent with reports in other cell types [22, 23, 36], the knockdown of *MFNs* resulted in a decrease of $\Delta\Psi$ as measured with the dye JC-1 and coupling Mitotracker Green staining to the voltage-sensitive dye TMRE. It is notable that the loss of $\Delta\Psi$ in individual mitochondria is a stochastic event, resulting in a small number of mitochondria within a given cell that have lost $\Delta\Psi$ [38]. This concept is consistent with our observations that cells with knockdown of *MFN1* or *MFN2* are found to have marked variation in the degree of TMRE staining, whereas cells treated with NTC siRNA are far more uniform in their staining of TMRE (data not shown), indicating that the altered mitochondrial morphology is linked to membrane potential heterogeneity. It has been demonstrated that post-fission, a daughter mitochondrion with a loss of $\Delta\Psi$ is less likely to undergo subsequent fusion and more likely to be eliminated from the mitochondrial pool via autophagy [39]. Thus, it follows that mitochondria lacking *MFN1* or *MFN2* would be more likely to be targeted for autophagy given the increased probability that the membrane potential is compromised. However, this scenario may be more complicated as it has been suggested that for an efficacious fission event, fusion must occur, to enable the exchange of mitochondrial components, followed by their segregation into daughter mitochondria that are destined for elimination [40].

HUVEC with reduced levels of *MFN1* or *MFN2* behaved the same in regard to reduced angiogenic capacity and viability. However, the knockdown of *MFN2* has a larger impact on mitochondrial ROS production, whereas the knockdown of *MFN1* specifically abrogated VEGF-A signaling. Thus both MFN1 and MFN2 are required for endothelial cell function and survival, but the mechanisms by which they exert these actions may be quite different. Studies of *Mfn* gene ablation in mice have documented that Mfn1 and Mfn2 have redundant functions in maintaining mitochondrial homeostasis [24, 35], but mechanistic studies in cultured cells have shown that Mfn1 and Mfn2 also have distinct functions. For example, Mfn1 has higher GTPase activity than Mfn2 and more efficiently promotes mitochondrial fusion [41]. Opa1-mediated fusion is dependent upon Mfn1 but not Mfn2 [22], and a Ras-binding domain located on the N-terminus of Mfn2 but not Mfn1 is thought to confer a growth-suppressive effect by sequestering Ras [42]. Additionally, Mfn2 is capable of

tethering mitochondria to ER to maintain Ca^{2+} concentrations [43] and also interacts with microtubule-associated transport systems to ensure proper location of mitochondria in neurons [34]. Lastly, *Mfn2*, but not *Mfn1*, has been linked to cellular functions including oxidative metabolism [44] and is induced by caloric restriction [45] and repressed in obese and diabetic individuals [46, 47].

Alterations in mitochondrial morphology have been associated with changes in the oxidant status of cells. For example, treatment of rat cardiomyoblasts with superphysiological concentrations of glucose results in increased ROS production and mitochondrial fragmentation, both of which can be blocked by the introduction of a dominant negative isoform of Drp1 [48]. It has also been demonstrated that there is a breakdown of mitochondrial networking, increase in superoxide generation and alterations in Opa1 and Drp1 expression in the coronary vascular endothelial cells of streptozotocin-induced diabetic mice [28]. These reports have led to speculation that mitochondrial fragmentation is linked to increased ROS production. However, our data are not consistent with this simple hypothesis. Ablation of either MFN1 or MFN2 led to a disruption of the mitochondrial network but no changes in ROS production were observed by the manipulation of MFN1. Furthermore, ablation of *Mfn2* led to mitochondrial fragmentation but a decrease in ROS production both at baseline and when cultured in cell suspension to examine maximal ROS production. Collectively, these findings suggest that the relationship between mitochondrial morphology and ROS production is more complex than previously appreciated and that MFN1 and MFN2 may have markedly different effects on the oxidant status of the cell.

Links between mitochondria and numerous survival and stress signaling pathways have been identified but the extent of this cross-regulation and how they relate to mitochondrial dynamics in vascular endothelial cells has yet to be determined. We provide evidence that the mitofusin proteins are required for endothelial cell viability, angiogenic function and to an extent, production of ROS. Moreover, we have highlighted differences between MFN1 and MFN2 in their influence on various signaling steps within endothelial cells. JNK activation by ROS was inhibited by ablation of MFN1 or MFN2, and these data extend previous observations that the mitochondrion functions as a proximal target to H_2O_2 -induced signaling and growth factor receptor transactivation [20]. Furthermore, while the signaling cascade activated by VEGF-A is complex [49], our data show that the attenuation of MFN1 expression acts apart from MFN2 production in the regulation of the Akt/eNOS signaling pathway. Thus MFN1 and MFN2 appear to modulate endothelial cell migration, differentiation and survival by influencing different aspects of angiogenic signaling.

We present the first evidence showing that signaling and angiogenic function rely upon the mitofusins in cultured cells of the vascular endothelium. At the most empirical level, knockdown of either *MFN* leads to a reduction in mitochondrial membrane potential, indicating a loss of integrity. Furthermore, knockdown of *MFN1* or *MFN2* sensitizes cells to stresses such as serum-deprivation and blunts the response of signaling pathways to external stimuli, consistent with a cellular phenotype of compromised angiogenic capacity. The loss of the mitochondrial protein Prohibitin-1 demonstrates a similar phenotype, whereby cells with *Phb1* knockdown are poorly angiogenic, display attenuated responses to signaling molecules, and have dysfunctional mitochondria [50]. Furthermore, chemical inhibition of mitochondrial function or depletion of mitochondria results in a failure of cells to respond to external stimuli such as oxidative stress and alters the level of cell death in response to the same stimuli [20]. From these studies and others, it is clear that functional mitochondria are integral to cellular signaling responses to external stimuli and are required for a myriad of physiological processes by endothelial cells. In our studies, knockdown of *MFN* results in dysfunctional mitochondria and this is manifest in altered signaling responses and angiogenic function. We have shown specific pathway changes that occur with knockdown

of *MFN1* or *MFN2*; a blunting of the downstream pathway of VEGF signaling with *MFN1* knockdown and a loss of ROS generation with *MFN2* knockdown. Because both pathways are highly relevant for signaling in endothelial cells, this study indicates that, while the mitofusins are functionally different, their ablation leads to a similar end result (i.e., diminished angiogenic function and survival under stress).

A link between VEGF-induced angiogenic signaling and mitochondrial biogenesis has previously been described [51], and it is conceivable that the induction of *MFN1* and *MFN2* represent a step in this process. Herein, we show an increase in mitofusin proteins in response to VEGF-A stimulation. It is unclear, however, whether VEGF-A signaling is unique in its ability to regulate *MFN* expression, or whether other angiogenic molecules, such as fibroblast growth factors or angiopoietins, confer the same effect or can substitute in the absence of VEGF-A.

Ultimately, it will be of interest to extend this work into animal models of vascular endothelial cell *Mfn1* and *Mfn2* ablation. To date, most studies have examined animal models of *Mfn2* ablation. Work performed in animals examining loss of *Mfn2* demonstrated an interesting phenomenon whereby conditional deletion of *Mfn2* leads to cell type-specific phenotypes. Ablation of *Mfn2* from neurons generates a neurodegenerative phenotype owing to the requirement for *Mfn2* in Purkinje cells for dendrite outgrowth, spine formation and cell survival [24]. However, ablation of *Mfn2* alone in skeletal muscle is reported to have no effect on muscle phenotype [35]. In adult cardiac myocytes, ablation of *Mfn2* leads to a modest myocardial hypertrophy at baseline and impaired function following isoproterenol infusion, yet protects cells from oxidative and ischemic stress. In contrast, knockdown of *Mfn2* in neonatal cardiac myocytes sensitizes cells to the very same oxidative stresses. This likely indicates a context-dependent function of *Mfn2* in somatic cells. In this regard, it is notable that the metabolic activity of cardiac myocytes and vascular endothelial cells can be quite different, and these cells differ dramatically in their content of mitochondria. Furthermore, endothelial cells are thought to primarily utilize mitochondria to activate survival and stress signaling pathways, whereas mitochondria have a predominant metabolic role in other cell types, such as cardiac and skeletal myocytes.

Conclusions

The importance of mitochondria in signaling in endothelial cell function has been well described [13, 26, 27]. However, whether mitochondrial dynamics contribute to mitochondria-dependent endothelial function has heretofore remained unknown. Here it is shown that, in response to VEGF, *MFN*-depleted EC demonstrated reduced migration as well as diminished network formation when differentiated on Matrigel substrate. Upon mitogen-deprivation, EC with knockdown of *MFN* expression show reduced viability and an increase in apoptosis. Collectively, these observations suggest a previously unknown role for the mitofusin proteins in EC function and survival. These findings provide a framework for future studies to assess the role of mitofusin proteins on vascular function *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Sources of Funding

This study was funded by National Institutes of Health grants R21 HL102874, R01 AG034972, R37 AG015052 and P01 HL068758 to K. Walsh, NIH grants P01 HL068758 and R37 HL104017 to M. Bachschmid and the Ruth Kirchstein Postdoctoral Fellowship to J.J. Lugus and G. A. Ngoh.

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Highlights

- Depletion of MFN1 or MFN2 leads to mitochondrial network fragmentation.
- Depletion of MFN1 or MFN2 diminished endothelial cell migration and differentiation
- Depletion of MFN1 or MFN2 reduced survival under mitogen-deprivation conditions.
- MFN1 depletion selectively attenuated VEGF-simulated Akt-eNOS signaling.
- MFN2 depletion selectively decreased ROS production.

Figure 1. MFNs are induced by VEGF

A) Copy number assessment of *MFN1* and *MFN2* in unstimulated HUVEC. B) Western blot examining the relationship between VEGF-signaling and mitofusin proteins. HUVEC were serum-starved for 16 hours and then treated with 30 ng/ml VEGF-A for the time period indicated. Graphs below blots are summary data from 4 independent experiments. * $P < 0.05$ vs. starved cells.

Figure 2. Depletion of *MFN1* or *MFN2* results in disrupted mitochondrial morphology in HUVEC

A) Expression of the *Fus-Fis* genes by qRT-PCR. All genes were normalized against the ribosomal gene *RPLP0*. The expression of a given gene under NTC siRNA was normalized as '1'. $n = 5$. B) Immunoblots on samples from A) examining protein levels of MFN1 and MFN2. Right panel is quantification of protein levels from 5 different experiments. $**P < 0.005$ vs. NTC siRNA. $n = 5$. C) Deconvolution microscopy of HUVEC treated with various siRNAs and visualized with 50 nM Mitotracker Green vital dye. D) Summary data of

mitochondrial morphology measuring the aspect ratio and form factor of individual mitochondria from >13 cells. * $P < 0.05$, ** $P < 0.005$ vs. NTC siRNA.

Figure 3. MFNs are required for proper angiogenic function of HUVEC *in vitro*

A) Representative results from *in vitro* tube formation assays plating 5×10^4 HUVEC on Matrigel-coated wells and differentiating for 16–18 hours in the presence of 20 ng/ml VEGF. B) Quantification of tube/network formation from *in vitro* tube formation assays ($n = 4$). C) Measurement of cell migration using a modified Boyden chamber assay. Number indicates cells that migrated through a fibronectin-coated transwell insert in response to 20 ng/ μ l VEGF-A ($n = 5$). * $P < 0.05$, ** $P < 0.005$ vs. NTC siRNA.

Figure 4. Attenuation of *MFN1* or *MFN2* in HUVEC compromises cell viability

A) Decreased cell viability when *MFNs* are knocked down in HUVEC as assessed by utilizing an MTS-based assay ($n = 6$). B) Detection of apoptotic cell death with TUNEL staining. Upper panels are cells stained with DAPI to label all nuclei. Lower panels are images of cells probed with an antibody against dsDNA breaks. Graph to right is quantification of percentage of TUNEL positive cells. C) Oxidative stress-induced loss of TMRM fluorescence. Graph to right is quantification of cumulative loss of TMRM fluorescence over 12 minutes. * $P < 0.05$, ** $P < 0.005$ versus NTC siRNA.

Figure 5. Attenuation of *MFN1* or *MFN2* in HUVEC results in altered mitochondrial function
 A) Quantitation of mtDNA:nDNA ratio by qPCR using primers specific for *MTCO1* and *LPL*, respectively ($n = 5$). B) Measurement of ATP concentration. ATP is normalized to total cellular protein ($n = 4$). C) Measurement of mitochondrial membrane potential using the vital dye JC-1. Measurement is a normalized ratio of red to green fluorescence ($n = 5$). D) Measurement of ROS in unstimulated (starved) cells. Y-axis indicates the relative DCF fluorescence level. E) Detection of cellular ROS species by flow cytometry. The X-axis is DCF fluorescence (log scale) and Y-axis is event count. Panel to right is summary data of flow cytometry experiments ($n = 4$, >10,000 viable cells were analyzed per condition). * $P < 0.05$, ** $P < 0.005$ versus NTC siRNA, # $P < 0.05$ vs. MFN1 knock-down.

Figure 6. Altered signaling and NO biogenesis when MFNs are knocked down in HUVEC
 A) Cells were treated with respective siRNA oligos, serum starved overnight and stimulated with 100 μ M H₂O₂ for 30 minutes and then the lysates subject to western blotting. Graph below blot summarizes data from 5 independent experiments. B) Cells were treated with respective siRNA oligos, serum starved overnight and stimulated with 50 ng/ml VEGF-A for 10 minutes and then the lysates subject to western blotting. Graph below blot summarizes data from 4 independent experiments. C) Cells were treated with respective siRNA oligos, serum starved overnight and stimulated with 50 ng/ml VEGF-A for 10 minutes and then the lysates utilized in a cGMP ELISA. Total cGMP was normalized to cellular protein ($n = 4$). * $P < 0.05$ vs. control (non-treated).