Mitochondrial Motility and Vascular Smooth Muscle Proliferation

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Objective—Mitochondria are widely described as being highly dynamic and adaptable organelles, and their movement is thought to be vital for cell function. Yet, in various native cells, including those of heart and smooth muscle, mitochondria are stationary and rigidly structured. The significance of the differences in mitochondrial behavior to the physiological function of cells is unclear and was studied in single myocytes and intact resistance-sized cerebral arteries. We hypothesized that mitochondrial dynamics is controlled by the proliferative status of the cells.

Methods and Results—High-speed fluorescence imaging of mitochondria in live vascular smooth muscle cells shows that the organelle undergoes significant reorganization as cells become proliferative. In nonproliferative cells, mitochondria are individual (≈2 μm by 0.5 μm), stationary, randomly dispersed, fixed structures. However, on entering the proliferative state, mitochondria take on a more diverse architecture and become small spheres, short rod-shaped structures, long filamentous entities, and networks. When cells proliferate, mitochondria also continuously move and change shape. In the intact pressurized resistance artery, mitochondria are largely immobile structures, except in a small number of cells in which motility occurred. When proliferation of smooth muscle was encouraged in the intact resistance artery, in organ culture, the majority of mitochondria became motile and the majority of smooth muscle cells contained moving mitochondria. Significantly, restriction of mitochondrial motility using the fission blocker mitochondrial division inhibitor prevented vascular smooth muscle proliferation in both single cells and the intact resistance artery.

Conclusion—These results show that mitochondria are adaptable and exist in intact tissue as both stationary and highly dynamic entities. This mitochondrial plasticity is an essential mechanism for the development of smooth muscle proliferation and therefore presents a novel therapeutic target against vascular disease. (Arterioscler Thromb Vasc Biol. 2012;32:3000-3011.)

Key Words: mitochondria ■ proliferation ■ remodeling ■ resistance arteries ■ smooth muscle

Smooth muscle proliferation is central to vascular hyperplasia and neointima formation in diseases including atherosclerosis and hypertension. Proliferation is also a significant problem that limits the success of cardiovascular disease treatments, including failures of coronary bypass vein grafts and the restenosis that follows balloon angioplasty or stent insertions.1–4 New targets and treatments to inhibit vascular smooth muscle proliferation are required.

Mitochondria control virtually every aspect of cell function via changes in ATP concentration, by contributing to Ca²⁺ signaling, influencing redox potential, and controlling levels of reactive oxygen species.5,6 The morphology of mitochondria is thought to be essential for the function of the organelle. In some cells, the morphology is complex, and mitochondria exist in a variety of forms in large part because the organelle shows dynamic behavior and may almost constantly change shape and move via Brownian motion, stochastically determined directed motion, long-range motor-driven displacement, fission, and fusion.7–9 However, in other cells, the appearance of the organelle is rather simple and exists as ovoid (prolate spheroid) entities that show relatively little movement and are largely unchanging.10–14

The significance of the differences in behavior of mitochondria to the physiological function of cells is unclear, but some explanation may come from the various cell types examined. Most studies describing mammalian mitochondrial dynamics have been in cultured cells, particularly in cancerous and immortalized cell lines and cultured neurons, although some have also been carried out in cultured primary or neonatal cell lines, including those of heart, liver, pancreas, and smooth muscle.13,15–17 As a result of the emphasis on cultured cells, the role of mitochondrial dynamics in native, differentiated cells and intact tissue is largely unknown. In the cardiovascular system, particularly little is known about mitochondrial movement. In adult heart cells and the intact heart where all the necessary proteins for mitochondrial dynamics to occur are

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exhibited, current research has been unsuccessful in measuring mitochondrial dynamics, that is, there are no reports showing significant mitochondrial movements. The only change detected in adult heart cells has been a restricted Brownian motion, which was attributed to morphological changes of the organelle arising from the contraction and expansion of the mitochondrial matrix (condensed/orthodox transitions). Interestingly, in the various cultured cells in which mitochondrial dynamics are common, while significant advances have been made on the identity of proteins mediating and regulating mitochondrial movement, an understanding of the physiological purpose and cellular function of mitochondrial dynamics is much more preliminary. The physiological function of one type of mitochondrial dynamics, motor-driven displacement, is probably best appreciated in neurons. Mitochondria are synthesized in the neuronal cell body and then transported down the axon, which provides a clear purpose for the movement in these rather unusually structured but highly organized cells. Motor-driven displacement in neurons may also help position mitochondria at locations where energy demand is high. Increases in [Ca\textsuperscript{2+}] inhibit motor-driven mitochondrial movement\textsuperscript{25-27} to contribute to positioning the organelles where ATP or Ca\textsuperscript{2+} buffering is required. Yet in other cell types, the physiological function served by motor-driven displacement of mitochondria is poorly understood and mitochondria may not move at all.\textsuperscript{16} Indeed, other than in neurons, mitochondrial movement has not been directly observed in any intact tissue.

Other major forms of mitochondrial dynamics are fission and fusion events which have been linked to apoptosis, maintenance of cellular homeostasis, and mitochondrial quality control via autophagy, but each is unresolved. For example, mitochondrial fission may be involved in the segregation of dysfunctional mitochondria for the organelles’ removal by autophagy,\textsuperscript{28} but a lack of mitochondrial fission may lead to increased mitochondrial autophagy.\textsuperscript{29} Mitochondrial fragmentation caused by loss of fusion proteins leads to defects in mitochondrial respiration and oxygen consumption,\textsuperscript{30} yet forced fusion of mitochondria also results in a reduction in respiration and oxygen consumption.\textsuperscript{10} Mitochondrial fission may precede apoptosis,\textsuperscript{31,32} although this proposal has also been challenged\textsuperscript{33,34} and enhanced mitochondrial fission does not induce but may protect against apoptosis.\textsuperscript{35} Although contradictory, the results do nonetheless suggest that disturbing dynamics reduces mitochondrial and cell function, although the precise consequences are unclear.

The questions arise as to why mitochondria move in only some cells, whether mitochondrial dynamics are a feature of native intact tissue, and what role the dynamics play in native cell physiology. The present study was undertaken to clarify these issues in native single smooth muscle cells from cerebral resistance arteries and in the intact resistance artery. The data shows that mitochondria are immobile structures in native nonproliferative cells. However, when cells enter a proliferative state, mitochondria become highly dynamic structures. When mitochondrial dynamics were prevented, smooth muscle proliferation was inhibited. We propose that the change in behavior of mitochondria from being rigidly immobile to being highly dynamic is an essential element of vascular smooth muscle proliferation.

### Materials and Methods

An expanded Methods section is provided in the online-only Data Supplement.

### Cell Isolation and Culture

Smooth muscle cells from resistance-sized (~50–200 μm) cerebral and posterior cerebral arteries from male guinea pigs were enzymatically isolated. Cells were stored at 4°C for use within 6 hours or were diluted 1:9 in 50:50 Ham F-12 and Waymouth MB752 media supplemented with 10% fetal bovine serum, 0.1 U/mL penicillin, and 100 μg/mL streptomycin and grown on 13-mm coverslips in 24-well plates maintained at 37°C in humidified air (5% CO\textsubscript{2}).

### Intact Artery Preparation and Culture

To visualize mitochondria, arteries were cannulated and pressurized in an arteriograph and then loaded with 100 nmol/L tetramethylrhodamine ethyl ester perchlorate (at 36°C). For myogenic tone and intracellular [Ca\textsuperscript{2+}] measurement, sections of arteries were loaded with 10 μmol/L Fura-2-AM.

### Fluorescence Imaging of Cells and Intact Arteries

Freshly isolated (native) cells or cells grown in culture conditions were loaded with 100 nmol/L tetramethylrhodamine ethyl ester, as described previously,\textsuperscript{11,36} or MitoTracker green (100 nmol/L for 15 minutes) and imaged on an inverted epifluorescence microscope (Nikon TE2000U) with a x100 1.3 NA S-Fluor oil objective plus internal x1.5 converter lens. Emitted light was imaged by a Photometrics Cascade 512B camera (Roper Scientific).\textsuperscript{30,35}

### Image Analysis

Images were analyzed using Metamorph 7.1.3 (Molecular Devices), ImagePro 7.0 (MediaCybernetics) or the MotionStudio\textsuperscript{7} package. MotionStudio was used to track individual mitochondria to sub-pixel accuracy (typically ±10 nm)\textsuperscript{39} using an image correlation-based approach. Briefly, in the first frame of a sequence, each mitochondrion was used by the software to create a template. Image correlation was then applied to locate the template within the next frame, and the template image was then updated to be that of the object in the second frame. The template finding and updating process is repeated for all frames to build up a track of mitochondria positions. Refreshing the template image in each frame accommodates gradual changes in the image of the mitochondria, caused by morphological changes, changes in pointing direction of noncircular objects, and photobleaching. Further analysis was performed, including measuring the instantaneous speed and the time-variant displacement of the mitochondria. Displacement was defined as the absolute distance between a mitochondrion’s position at a given time and its location in the first frame. The instantaneous speed of the mitochondria was computed by measuring the rate of change of position during 1-second intervals. This interval was chosen as the one long enough to smooth the effects of measurement noise but short enough to accurately determine the speed without averaging out brief, directed motion events.\textsuperscript{7}

### Measurement of Mitochondrial Area

Cerebral artery smooth muscle cells were maintained in culture for 4 days in 10 μmol/L mitochondrial division inhibitor (Mdivi-1) or vehicle control (0.1% dimethyl sulfoxide). Contiguous mitochondria were defined as areas that displayed synchronous increases or decreases in tetramethylrhodamine ethyl ester fluorescence highlighted by subtracting a duplicate image stack that was offset by 5 frames (and hence 0.5 seconds) and applying either an inclusive threshold (to detect increases) or exclusive threshold (to detect decreases).

### Western Blotting

Six vessels for each time point were homogenized in 50 μL CellLytic M solution (Sigma) using Eppendorf tube pestles. Homogenates were
centrifuged and supernatants immediately frozen at −20°C or mixed with 4× reducing sample buffer and separated by standard SDS-PAGE on a 12% gel, transferred to nitrocellulose membrane, blocked with 5% BSA for 1 hour, and then incubated with primary antibodies (monoclonal mouse anti–mitofusin-2, polyclonal rabbit anti–proliferative cell nuclear antigen, monoclonal mouse anti–mitochondrial cytochrome oxidase IV, or polyclonal rabbit anti–GAPDH). Horseradish peroxidase–conjugated anti-mouse or anti-rabbit secondary antibodies were used for visualization using enhanced chemiluminescence.

Immunocytochemistry
Pressurized (40 mm Hg) arteries were fixed (10% formalin), permeabilized (0.5% Triton X-100), and blocked (2% BSA) before incubating first with rabbit anti–proliferative cell nuclear antigen and then with anti–rabbit-Alexa 488 plus mouse anti–α-actin-Cy3 conjugate before mounting in Vectashield containing 4',6-diamidino-2-phenylindole. Fluorescence images were captured on a Leica SP5 confocal microscope (×40 objective) and analyzed (Metamorph 7.1.3).

[3H]-Thymidine Incorporation Assay
Cells were grown to ~70% confluence in 12-well plates, quiesced for 24 hours in media containing 0.1% fetal bovine serum, and then returned to media containing 10% fetal bovine serum for 18 hours. [3H]-Thymidine (9.25 kBq) was added to each well for the final 6 hours of the 24-hour treatment period. Cells were washed with ice-cold PBS followed by 10% trichloroacetic acid and then by 10% sodium dodecyl sulfate in 0.2 mol/L sodium hydroxide, and radioactivity was quantified by liquid scintillation (Packard 1500 Tri-Carb or ScintSafe3).

Fluorescence-Activated Cell Sorter Analysis
Cells were grown to ~70% confluence in 6-well plates, harvested, resuspended in PBS, and fixed in ice-cold 70% ethanol at 4°C overnight. Cells were then washed with PBS, incubated with 50 μg/mL RNase A for 1 hour at 37°C, and stained with 40 μg/mL propidium iodide. Cell cycle profiles were acquired by flow cytometry (BD FACSDiva software; BD Biosciences) and analyzed using the software FlowJo.

Statistical Analyses
Other than Figures 5A and 6, a parametric distribution of raw data around mean values was confirmed before statistical testing. Mean values±SE are shown (unless otherwise indicated) and were analyzed using unpaired Student t tests or, for >2 groups, ANOVA with Games-Howell post hoc comparison of variance (for samples with unequal variance). α was set to 0.05, and P<0.05 was considered significant. The median mitochondrial areas with and without Mito-1 treatment were compared using a Mann-Whitney U test. The normalized data in Figure 6 were analyzed using Kruskal-Wallis tests, with P values adjusted for multiple testing. t test was carried out in Microcal Origin v6.0, with Bonferroni considerations applied for cell cycle data; all other tests were carried out in SPSS 19 for Windows.

Results
In native vascular smooth muscle cells, mitochondria were ovoid in appearance and randomly dispersed (Figure 1A); that is, mitochondria showed no pattern or symmetry to their arrangement through the cytosol. There was little evidence for movement arising from motor-driven events (Figure 1A–1E; online-only Data Supplement Information and Video I in the online-only Data Supplement). Indeed, the instantaneous speed of the mitochondria, which was estimated by comparing the organelles’ position during 1-second intervals, approached zero (Figure 1F). Over the time course of these experiments, there was no evidence for mitochondria merging or dividing, which suggests that neither fission nor fusion occurred. However, a small restricted mitochondrial movement did occur (Figure 1E). That movement comprised mitochondrial Brownian diffusion and global motion of the host cell but failed to significantly displace the organelle from its center location (Figure 1D) and the overall movement of the organelle (Figure 1C) was insignificant (<1%) compared with the size of the mitochondrion (Figure 1D). Thus, mitochondria of native smooth muscle appear rigidly immobile, constrained possibly by cytoskeletal structures (eg, microtubules).39–44 However, disruption of microtubules (nocodazole, 10 μmol/L) did not increase mitochondrial mobility in native cells (n=7; P>0.05; Figure 2A). Together, in native vascular smooth muscle cells, mitochondria appear to be ovoid, stationary, randomly dispersed organelles that are largely immobile.

Interestingly, within 24 hours of maintenance under cell culture conditions in media that contained serum (to promote proliferation), mitochondria in these same cells began to take on a more diverse architecture and range from being small spheres, short rod-shaped structures, long filamentous entities, and networks (Figure 1A and 1B; online-only Data Supplement Information and Video II in the online-only Data Supplement). The majority of mitochondria also became highly dynamic structures and continuously moved and changed shape (Figure 1A–1C, 1E, and 1F; online-only Data Supplement Information and Video II in the online-only Data Supplement). The movement of the organelle (Figure 1C and 1E) was now a significant fraction of the size of the mitochondrion (Figure 1D). Several types of motility occurred, which included bursts of motion that cover large distances, trafficked-like directed motions (in which mitochondria shuttle back and forth along the same path), as well as wiggling and looping movements (Figure 1C; online-only Data Supplement Information and Video III in the online-only Data Supplement). The bursts of movement (≈160 nm/s; Figure 1C and 1F; online-only Data Supplement Information and Videos II and III in the online-only Data Supplement) correspond to fast, linear travel apparently similar to stochastically determined (molecular motor) driven motion events.7 Mitochondrial movements in cells in culture were inhibited by treatment with the microtubule-disrupting agent nocodazole (10 μmol/L; n=8; P<0.05) but not the actin-disrupting agent latrunculin B (10 μmol/L; n=8; P>0.05; Figure 2B), suggesting that microtubules are required for the motor-driven events.

The transport of mitochondria along microtubules is mediated by the kinesin and dynein families of motor proteins.26,43 Kinesins move with a regular step size of 8 nm,46 whereas dynein has a more complex behavior and covers a range of step sizes between 8 and 32 nm, tending toward 8 nm under load.47 These step sizes are significantly smaller than the bursts of motor-driven events observed in the present study. For example, 2 mitochondria in cultured cells move ≈100 nm in 4 seconds (Figure 1E), corresponding to ≈13 motor steps. The largest displacement observed in this example (500 nm) corresponds to over 60 motor steps. Multiple motors are required to move larger organelles, such as mitochondria.48,49 so the bursts of motion imply a significant degree of coordination among the motors, and the velocity of ≈160 nm/s (Figure 1C and 1F) is equivalent to ≈20 kinesin steps per second.
Increases in [Ca^{2+}]_{c} arrest mitochondrial motility in H9c2 myoblasts and cultured primary neurons. Perhaps the absence of mitochondrial motility in native cells arose from an elevated [Ca^{2+}]_{c}; however, lowering [Ca^{2+}]_{c} by removing extracellular Ca^{2+} did not initiate mitochondrial motility in native smooth muscle cells (n=5; Figure 2A). Neither did replacing the extracellular HEPES-buffered salt solution with culture media containing serum induce motility in native cells (without maintenance in culture, n=5; Figure 2A) even at 37°C (Figure 2C). Interestingly, the velocities and duration of movement bursts in the cultured cells at 37°C increased substantially compared with the movement measured at room temperature (Figure 2; online-only Data Supplement Information and Video IV in the online-only Data Supplement). At 37°C, the maximum velocities of mitochondria in cultured cells were of the order of 1000 nm/s, which equates to ≈135 kinesin steps per second. Interestingly, the maximum reported stepping rates for kinesin in the absence of load is ≈250 to 300 steps/second.51

The results suggest that native single cells that had been isolated by harsh enzymatic treatment do not show mitochondrial dynamics, whereas smooth muscle cells...
maintained in conditions (culture) that differ significantly from the normal physiological environment do show mitochondrial dynamics. The question arises as to whether the absence or presence of mitochondrial dynamics is a physiological feature of the organelle. To clarify this issue, we examined mitochondria within the smooth muscle of intact cerebral resistance arteries ($\approx 200 \mu m$ diameter). The arteries were maintained at physiological pressure and temperature (40 mm Hg; 36°C) and in their in vivo geometry (Figure 3A). These resistance arteries are important for the maintenance of constant cerebral blood flow via the process of myogenic reactivity, in which elevation of intralumenal pressure causes an increase in $[\text{Ca}^{2+}]_c$ and a decrease in vessel diameter that is maintained until the pressure decreases (Figure 3B).52 In the

![Figure 2](image)

Figure 2. Mitochondrial motility in native and cultured smooth muscle cells. A, In native cerebral resistance artery smooth muscle cells, neither nocodazole (10 $\mu$mol/L) nor replacement of the normal cellular bathing solution with either Ca$^{2+}$-free solution or cell culture media induced mitochondrial movement. The total number of mitochondria moving per minute were counted and corrected for mitochondrial area within each cell; the histogram shows mean±SE for n=25 cells for each treatment ($P>0.05$, nocodazole; $P>0.05$, Ca$^{2+}$-free; $P>0.05$, media). B, Cerebral resistance artery cells were maintained in culture for 4 days and then incubated with nocodazole (10 $\mu$mol/L) or latrunculin B (10 $\mu$mol/L). The total number of mitochondria moving per minute were counted and corrected for mitochondrial area within each cell; the histogram shows mean±SE for n=8 cells for each treatment ($P<0.05$, nocodazole; $P>0.05$, latrunculin B each cf. control). *$P<0.05$ was considered significant; note the increase in scale compared with Figure 2A. C, At 37°C, the total number of mitochondria moving per minute were counted and corrected for mitochondrial area within each cell; the histogram shows mean±SE for n=14 cells from at least 3 independent cell isolations. D, At 37°C, the motion tracks from mitochondria from the native cells (top) show essentially no movement (like those at room temperature). Cultured cells (bottom) show wide ranging movement with increased displacement and velocities compared with those measured at room temperature (Figure 1C–1F). The plot shows the $x$–$y$ displacement and velocities of each mitochondrion. Scale bars for speed and displacement are shown. E, At 37°C, the instantaneous speed of the mitochondria was measured by comparing the organelles’ position during 1-second intervals. The speeds for the mitochondria tracked within the native (red) and cultured (blue) cells are plotted. The speeds have been separated in the vertical axis for clarity. The mitochondria of native cells are inactive. In the cultured cell, many bursts of high-speed motion occurred with a maximum speed that was approaching 10× that measured at room temperature (1000 nm/s). F, At 37°C, the displacement of the tracked mitochondria was plotted as a function of time. Displacement is defined as the distance between the position of an organelle at time $t$ and at time 0. Mitochondria in cultured cells (blue) undergo bursts of motion that cover large distances compared with the displacement of mitochondria within the native cells (red). Images were acquired at 7.3 Hz.
cerebral resistance arteries, the large majority of mitochondria did not move (Figure 3C; online-only Data Supplement Information and Video V in the online-only Data Supplement), however, a small number of mitochondria displayed directed motion like that characteristic of cultured cells.

A major difference between cultured and freshly isolated native smooth muscle is that the former are dividing rapidly, whereas native cells are thought to be largely noncycling and in a quiescent state (although not terminally differentiated and may reenter the cell cycle and proliferate).53,54 In the intact artery, there is a constant replacement of old with newly generated cells; perhaps those cells with motile mitochondria are proliferative. To test this possibility, proliferation was encouraged in the intact artery by maintaining segments of intact artery in culture conditions (up to 4 days) with growth factors. In these conditions, smooth muscle proliferation increased as measured by 2 separate proliferative markers. Proliferative cell nuclear antigen increased and mitofusin-2 decreased as measured by immunoblotting (Figure 4C), with no change in mitochondrial numbers (as measured by COX IV, data not shown). Significantly, the increased proliferation was associated with a considerable increase in mitochondrial motility in the intact pressurized arteries. Indeed, the majority of mitochondria in the arteries became motile and the majority of smooth muscle cells contained motile mitochondria (Figure 4A and 4B; online-only Data Supplement Information and Video VI in the online-only Data Supplement). Therefore, it would seem that in intact arteries, like the isolated single cell, there is only a small amount of mitochondrial movement within native adult smooth muscle cells and that proliferation increases both the number of cells that contain motile mitochondria and the number of motile mitochondria themselves.

Perhaps the increase in mitochondrial dynamics is a requirement for proliferation to occur. To test this possibility, we examined whether or not inhibition of mitochondrial motility could prevent vascular smooth muscle proliferation. Mitochondrial motility can be reduced by the small-molecule inhibitor Mdivi-1,55 which inhibits dynamin-related protein oligomerization to prevent mitochondrial fission.56 Mdivi-1 increases mitochondrial network connectivity and thus decreases mobility. When Mdivi-1 was included in the culture media, the extent of connectivity increased as assessed by the rise in electrical continuity among mitochondria. The increased continuity was measured by transient changes in the mitochondrial membrane potential (flickers)12,57 using the membrane potential-sensitive dye tetramethylrhodamine ethyl ester. The median area of individual mitochondrial membrane potential flickers increased in Mdivi-1 (from 1.91±0.34 to 3.74±1.40 μm², median±SD of the median; P<0.001 by Mann-Whitney U test), and the fraction of mitochondria with an area >10 μm² increased from 1.3% to 15% (Figure 5A). These results suggest that larger areas of mitochondria were in electrical continuity because of Mdivi-1–induced fusion.

Mdivi-1 also inhibited mitochondrial dynamics in both isolated single smooth muscle cells (Figure 5B) and intact cerebral resistance artery segments (Figure 5C) that had been maintained in culture for 4 days. A higher concentration of Mdivi-1 was required to inhibit mitochondrial motility in intact arteries than individual cells (50 μmol/L as opposed to 10 μmol/L, data not shown), in agreement with that required to inhibit myocardial infarct size in a mouse model of ischemia–reperfusion injury.58 Mdivi-1 inhibited proliferation of cultured resistance artery smooth muscle cells. Three separate experiments confirmed this conclusion. First, Mdivi-1 (10 μmol/L) decreased proliferation as measured by decreased incorporation of 3H-thymidine (Figure 6A). Second, Mdivi-1 (10 μmol/L; 48 hours) produced a shift in cell cycle from S to enrich the G0/G1 phase (Figure 6B and 6C). As a control, an enrichment of the G2/M phase by
nocodazole (50 ng/mL; 476 nmol/L; 16 hours) was confirmed (Figure 6B and 6C). Third, Mdivi-1 also inhibited the increase in proliferation in intact segments of cerebral resistance arteries that were maintained in culture, as measured by immunofluorescence staining of proliferative cell nuclear antigen within the smooth muscle layer (Figure 6C and 6D). Together, mitochondrial dynamics are required for proliferation to occur.

An off-target effect of Mdivi-1 is a possible block of rapidly activating delayed rectifier and acetylcholine-activated K⁺ channels. Blocking the activity of some types of K⁺ channels may regulate proliferation, and there is interaction between mitochondrial activity and plasma membrane ion channel expression. In the present experiments, 4-aminopyridine (10 mmol/L) at a concentration that produces at least an equivalent block of the rapidly activating delayed rectifier K⁺ channels failed to inhibit proliferation as measured by 3H-thymidine incorporation (Figure 6F). Block of other K⁺ channels (Ca²⁺-activated and ATP-sensitive) with tetraethylammonium (10 mmol/L) and glibenclamide (10 μmol/L) did reduce proliferation (measured by 3H-thymidine incorporation; data not shown), although these channels are not reported to be blocked by Mdivi-1. Because acetylcholine exerts little or no effect directly on vascular smooth muscle, the acetylcholine-activated K⁺ channel is unlikely to be expressed.

Rapamycin is used commonly as an antiproliferative agent. In other experiments, intact segments of cerebral resistance artery were maintained in culture for 4 days in the presence of rapamycin (10 μmol/L). However, rapamycin did not inhibit the onset of extensive mitochondrial motility in these vessels (n=19; data not shown). These results suggest that disruption of mitochondrial motility may inhibit the transition of smooth muscle cells to a proliferative phenotype at a stage earlier than rapamycin, potentially preventing their reentry into the cell cycle.

**Discussion**

Native, adult smooth muscle cells are largely nonproliferative and in a quiescent state. However, smooth muscle is not terminally differentiated as cells may reenter the cell cycle and proliferate in response to various stimuli, both beneficial (such as angiogenesis) and pathological (such as damage-induced vascular remodeling). In the present study, mitochondria in...
adult vascular myocytes were ovoid structures and dispersed randomly through the cytosol. The organelle showed only a restricted Brownian motion, which failed to move the mitochondria from its center position, and there was no evidence for motor-driven events or fission or fusion. The experiments suggest that mitochondria are rigidly immobile and relatively uniform in appearance in adult vascular myocytes. However, in proliferative vascular cells mitochondria existed as an assortment of shapes, which included small spheres, short rod-shaped structures, long filamentous entities, loops, and networks. There was also extensive mitochondrial mobility, which included large-directed movement and Brownian-like displacement, each of which substantially displaced the mitochondria. We believe this to be the first demonstration of a transition in mitochondrial dynamics as cells change from a native to a proliferative form.

The almost continuous movements and rearrangement of mitochondria are thought to be important for cell function, although the precise role played by mitochondrial dynamics is unclear. Among proposals for the function served by mitochondrial dynamics are that the movement enables recruitment of the organelle to particular subcellular compartments, contributes to the content exchange between mitochondria, or facilitates mitochondrial communication with the cytosol. However, many studies have generated apparently contradictory results (see Introduction section), and because mitochondrial imaging investigations have been carried out frequently in tumor-derived cells with abnormal bioenergetic properties (eg, HeLa and HL-1 cells), the very significance of the dynamics for normal mitochondrial and cell function has been questioned. Here, mitochondrial dynamics were imaged successfully in real time in intact arteries under physiological artery configurations and pressures. These observations suggest that dynamics are a physiological feature of mitochondria. In the intact artery, there is constant replacement of old with newly generated cells; it may be that cells in which mitochondrial motility occurred are proliferative. Support for this proposal was found in the observation that when proliferation was encouraged in the intact arteries, the number of cells showing mitochondrial movement and the extent of movement in each cell substantially increased. Furthermore, the results suggest that mitochondrial mobility is not only a feature observed in proliferative cells but is required for cells to proliferate. To examine this, mitochondrial mobility was reduced using the fission inhibitor Mdivi-1, which, by inhibiting fission, resulted in larger mitochondria and networks with decreased mobility. Mdivi-1 inhibited vascular smooth muscle proliferation. We believe this to be the first direct demonstration of mitochondrial mobility in an intact organ in real time in the cardiovascular system and to our knowledge in any tissue other than neurons. It is tempting to speculate that the mitochondrial movements are required to facilitate distribution of mitochondria to daughter cells during division.

Figure 5. Inhibiting mitochondrial fission prevents mitochondrial motility in cultured smooth muscle cells or artery segments. A, Cerebral resistance artery cells were maintained in culture for 4 days in the absence or presence of 10 μmol/L Mdivi-1. Cells were then incubated with tetramethylrhodamine ethyl ester (TMRE) and imaged to observe spontaneous flickers of mitochondrial membrane potential, indicating areas of electric (and hence inner mitochondrial membrane) continuity. The area of each mitochondrial flicker was measured for ≥60 mitochondria from ≥8 cells for each of control and Mdivi-1–treated (black bars) cells. B, After 4 days in culture in the presence of 10 μmol/L Mdivi-1, mitochondrial movement was restricted to regions of mitochondria at the periphery of large mitochondrial networks. The histogram shows quantification of the numbers of mitochondria observed to move per minute, corrected for mitochondrial area, for n≥12 cells (mean±SE; P<0.001). C, Intact resistance artery segments (∼1 mm length) were maintained in culture conditions for 4 days in the absence or presence (black bars) of 50 μmol/L Mdivi-1. Arteries were then cannulated, pressurized (40 mm Hg), and incubated with TMRE to image mitochondrial motility. The histograms show quantification of the number of mitochondria observed to move and the number of cells containing motile mitochondria per minute, corrected for in-focus mitochondrial area, for n≥6 regions of artery (mean±SE; P<0.001 for both comparisons). Scale bars, 10 μm; *P<0.05 was considered significant.
Although changes in mitochondrial mobility have not been previously reported during cell proliferation, changes in mitochondrial architecture during the cell cycle have been observed as a result of fission and fusion events. The mito-chondria fission inhibitor Mdivi-1 was used in the present study to reduce mitochondrial dynamics rather than to study the role of fission and dynamin-related protein per se. Nonetheless, the results with Mdivi-1 (ie, reduced proliferation) may also suggest that mitochondrial fission (as well as mitochondrial dynamics) is required for cell division to occur. Mitochondrial fission has been linked to preventing and promoting each of autophagy and apoptosis, and the role of mitochondrial fission in cell division is also uncertain. For example, in HeLa cells mitochondria existed as elongated structures and interconnected networks, and a dynamin-related protein–mediated fission of the organelle occurred in early mitosis. In yeast, filamentous mitochondria are distributed to the daughter buds at mitosis. However prevention of mitochondrial fission by inhibiting the function of dynamin-related protein (HeLa cells) or homolog (Dnm1, yeast) did not prevent mitosis from occurring. On the other hand, in a recent study, inhibiting dynamin-related protein function (using Mdivi-1 or small interfering RNA) blocked cell cycle progression and reduced cell proliferation rates in cells cultured from human pulmonary artery, suggesting that mitochondrial fission is required for proliferation to occur.

Figure 6. Inhibiting mitochondrial motility reduces proliferation in single smooth muscle cells and intact resistance artery. A, Proliferation of cultured smooth muscle cells, as measured by 3H-thymidine incorporation, is inhibited when mitochondrial motility is reduced by Mdivi-1 (n=6; mean±SE; P>0.05 [1 μmol/L], P<0.05 [10 μmol/L], and P<0.05 [50 μmol/L] by Kruskal-Wallis independent ANOVA, significance adjusted to account for multiple testing). B, Representative flow-cytometry histograms obtained from control cells and cells in either Mdivi-1 (10 μmol/L) or nocodazole (50 ng/mL; 476 nmol/L). The plots show the distribution of the cells as a function of propidium iodide fluorescence intensity. The histograms identify 3 distinct populations: cells in G0/G1 (green), S (yellow), or G2/M (blue). Mdivi-1 increased the fraction of cells in G0/G1, and nocodazole increased the fraction of cells in G2/M. C, The percentage of cells in each cell cycle stage was quantified. Mdivi-1 significantly enriched the G0/G1 phase and decreased the S phase. Nocodazole increased the fraction of cells in the G2/M phase and decreased those in the G0/G1 phase (n=3; mean±SE; *P<0.05). D, Proliferation of resistance artery smooth muscle in the absence and presence of Mdivi-1 as measured by immunocytochemical proliferative cell nuclear antigen (PCNA) staining in smooth muscle α-actin–positive cells of artery segments (≈1 mm length) pressure-fixed after organ culture for 0 or 4 days. Images show the intact artery labeling of PCNA, smooth muscle actin, nuclei (4’,6-diamidino-2-phenylindole), and an overlay of all 3. E, Quantified PCNA immunofluorescence staining from the intact resistance arteries (from experiments like those of D) shows that smooth muscle proliferation is inhibited by Mdivi-1 (n=6; scale bar, 20 μm; mean±SE; *P<0.01 [day 4 cf. day 0], P<0.05 [day 4+Mdivi-1 cf. day 4]). F, Proliferation of cultured smooth muscle cells (as measured by 3H-thymidine incorporation) in the absence and presence of the K+ channel blocker 4-aminopyridine (4-AP; 10 mmol/L; n=3).
There are several phases to cell cycle progression during proliferation. Progression may include a quiescent phase in which the cells are not dividing (G0), a long growth phase (G1), a DNA replicating phase (S), a short growth phase (G2), and cell division (M). The transition in cell cycle from G1 to S phases can be modulated by the metabolic status of the cell, and mitochondrial dysfunction activates at least 2 signals that impose a G1 to S transition checkpoint. In keeping with these observations, in the present study, inhibiting dynamin-related protein oligomerization with Mdivi-1 significantly decreased the fraction of cells in the S and increased those in the G0/G1 phase. These results suggest that altering mitochondrial dynamics prevents cell cycle progression to the S phase. However, in a previous study, Mdivi-1 and small interfering RNA directed against dynamin-related protein resulted in G2/M phase enrichment. Differences in the time course of the experiments and concentrations of Mdivi-1 may explain differences in results. Short (4 hours) inhibition of dynamin-related protein drives cells from G0 to S in the absence of growth factors. However, prolonged (48 hours) inhibition (as in the present study) inhibits S-phase entry. Differences in the concentration of Mdivi-1 used in the present (10 μmol/L) and previous (25 μmol/L) study may also contribute to the differences in results. Although Mdivi-1 (25 μmol/L) increased the fraction of cells in the G2/M phase, in the same study, Mdivi-1 (10 μmol/L, used in the present study) did not increase the fraction of cells in the G2/M stage.

The present study has highlighted the adaptability of mitochondria and the occurrence of mitochondrial dynamics under physiological conditions. Hitherto, in the cardiovascular system, and especially in native cells, relatively little was known about the function mitochondrial movement performs. In various cardiac immortalized cell lines and neonatal cells, mitochondria are highly dynamic organelles that continuously move and change their shape. However, in native cell types, the significance and very occurrence of mitochondrial movement are questioned. For example, in cardiac myocytes only a restricted Brownian motion but no large-scale movement was detected. Several studies have shown that mitochondria in adult cardiomyocytes behave as distinct entities, are arranged in a longitudinal lattice between the myofilbrils, do not form reticular networks, and show no evidence for fusion or fission. Although not directly visualized, it is nonetheless believed that some type of mitochondrial dynamics may occur in adult heart, smooth muscle, and endothelial cells. This conclusion is derived from observations that various treatments (eg, coronary ligation, ischemia–reperfusion injury, diabetes mellitus) may produce unusually large or small mitochondria. These results suggest that mitochondria, at least in cases of extreme stress, are not entirely static and can undergo dynamics in the form of fission or fusion. There are physiological consequences to these fission and fusion events. Treatment with the mitochondrial fission inhibitor Mdivi-1 inhibited opening of the permeability transition pore to reduce cardiomyocyte cell death after ischemia–reperfusion and reduced myocardial infarct size in vivo in mice subjected to coronary artery occlusion. Mdivi-1 also reduced tubular cell apoptosis and kidney damage during renal ischemia–reperfusion injury. In pulmonary hypertension, electron microscopy measurements revealed that mitochondria appeared smaller than those in control pulmonary artery smooth muscle, suggesting the organelle undergoes fission in disease. Significantly, inhibiting dynamin-related protein (with Mdivi-1) restored mitochondrial size and exhibited antiproliferative effects by reducing the muscularization of small pulmonary arteries (consistent with an antiproliferative effect) in pulmonary hypertensive animals. This effect decreased pulmonary vascular resistance and right ventricular hypertrophy and increased exercise capacity. Together, these results suggest that reducing mitochondrial fission can limit cardiovascular damage and raise the possibility that manipulating mitochondrial dynamics may be protective in cardiovascular disease.

The organization of mitochondria in native smooth muscle is ill-defined. In the present study, there was no obvious repeating pattern to the organization as occurs in cardiac myocytes, and mitochondria appeared approximately randomly and uniformly distributed through the cytoplasm. Electron microscopy measurements revealed that mitochondria appeared smaller than those in control pulmonary artery smooth muscle, suggesting the organelle undergoes fission in disease. Significantly, inhibiting dynamin-related protein (with Mdivi-1) restored mitochondrial size and exhibited antiproliferative effects by reducing the muscularization of small pulmonary arteries (consistent with an antiproliferative effect) in pulmonary hypertensive animals. This effect decreased pulmonary vascular resistance and right ventricular hypertrophy and increased exercise capacity. Together, these results suggest that reducing mitochondrial fission can limit cardiovascular damage and raise the possibility that manipulating mitochondrial dynamics may be protective in cardiovascular disease.

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Disclosures

None.

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Mitochondrial Mobility and Vascular Proliferation


Mitochondrial Motility and Vascular Smooth Muscle Proliferation
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SUPPLEMENT MATERIAL I

METHODS

Cell isolation and culture

Smooth muscle cells from the superior cerebellar and posterior cerebral arteries of a male guinea pig (DH, ~500 g, euthanised by sodium pentobarbital overdose in accordance with the Animal (Scientific Procedures) Act UK 1986) were enzymatically isolated by incubation in isolation buffer (55 mM NaCl, 80 mM Na-glutamate, 6 mM KCl, 1 mM MgCl2, 10 mM glucose, 10 mM HEPES, 0.2 mM EDTA and 0.1 mM CaCl2, pH 7.3, 34.5°C) plus 1.24 mg ml⁻¹ BSA, 2.2 mg ml⁻¹ type F collagenase and 1 mg ml⁻¹ hyaluronidase for 14 min, followed by a second incubation in isolation buffer plus 1.24 mg ml⁻¹ BSA, 1.7 mg ml⁻¹ papain and 0.7 mg ml⁻¹ dithioerythritol for 14 min. Tissue was washed 3 times in isolation buffer and then 3 times in the same solution without BSA. Individual myocytes were released by gentle trituration. Cells were stored at 4°C for use within 6 h or were diluted 1:9 in 50:50 Ham F-12 and Waymouth MB752 media supplemented with 10% FBS, 0.1 U/ml penicillin and 100 μg/ml streptomycin and grown on 13 mm coverslips in 24-well plates maintained at 37°C in humidified air (5% CO₂).

Intact artery preparation and culture

Sections of arteries were cannulated in an arteriograph (Living Systems Instrumentation, St. Albans, VT, USA) containing oxygenated Krebs solution (118.4 mM NaCl, 25 mM NaHCO3, 1.13 mM NaH2PO4, 4.7 mM KCl, 11.2 mM glucose, 2.7 mM CaCl2, 1.2 mM MgCl2, bubbled with 95% O₂/5% CO₂) and then allowed to equilibrate in the dark in oxygenated Krebs solution plus 100 nM tetramethylrhodamine ethyl ester perchlorate (TMRE) at 36°C for 30 min at an intraluminal pressure of 20 mmHg. Intraluminal pressure was then raised to 40 mmHg for the duration of the experiment. All intact artery experiments were at 36°C and Krebs bubbled with 95% O₂/5% CO₂ throughout. In other experiments, sections of arteries 1-2 mm in length were placed in culture media as above and maintained at 37°C in humidified air (5% CO₂) for up to 4 days prior to cannulation. Pressurisation, loading with TMRE and imaging as for fresh arteries. For myogenic tone measurement, sections of arteries were allowed to equilibrate with 10 μM Fura2-AM for 2-3 hr at room temperature in incubation buffer (without BSA or enzymes) prior to
cannulation and pressurisation in oxygenated Krebs solution in a custom-made arteriograph.

**Fluorescence imaging of cells and intact arteries**

Freshly-isolated cells were loaded with 100 nM TMRE in isolation buffer (20 min, 22°C) or mitotracker green (100 nM for 15 min) then settled onto a coverslip before being washed into extracellular solution (80 mM Na glutamate, 40 mM NaCl, 20 mM TEA-Cl, 1.1 mM MgCl₂, 3 mM CaCl₂, 10 mM HEPES, 30 mM glucose, 100 nM TMRE, pH 7.4). Prior to imaging cells grown in culture conditions on 13 mm diameter coverslips, media was replaced with isolation buffer plus 100 nM TMRE (20 min, 37°C). A coverslip was then removed and placed cell-side uppermost on top of a 24 x 50 mm No. 0 coverslip, which was then attached to an open imaging chamber filled with extracellular solution.

Cells and arteries were imaged on an inverted epifluorescence microscope (Nikon TE2000U) with a 100x 1.3 NA S-Fluor oil objective plus internal 1.5x convertor lens. The output of a xenon arc lamp monochromator system (PTI Inc, 560 ± 5 nm) guided via an optical light guide, through a field stop diaphragm and a ND4 filter before being reflected off a long-pass dichroic mirror (Chroma) reflective from 550-570 nm and transmissive >580 nm to illuminate the field. Emitted light was collected through the objective, dichroic and a barrier filter and imaged by a Photometrics Cascade 512B camera (Roper Scientific) controlled by EasyRatio pro software (PTI Inc)¹,². Images were acquired at 10 or 30 Hz (100 or 30 ms exposure) for 90 s only (to minimise photo-toxicity) unless otherwise indicated. Imaging experiments on cells were carried out at room temperature and 37°C (using a microscope stage top incubator (TokaiHit INUB-ONICS-F1)).

**Image Analysis**

Images were analyzed using either Metamorph 7.1.3 (Molecular Devices), ImagePro 7.0 (MediaCybernetics) or the MotionStudio³ package. In the case of analysis with Metamorph, images were background subtracted and smoothed using a 9 frame rolling average. The number of moving mitochondria observed over 60 s within a full-frame image stack was manually counted and corrected for mitochondrial area or percentage of intact artery image that was in focus. For intact arteries, an estimate of the number of cells containing mobile mitochondria was also
counted. MotionStudio\(^3\) measures the motion tracks of mitochondria to sub-pixel accuracy (typically \(~10\) nm\(^4\)) using an image correlation based approach. Briefly, individual mitochondria are selected in the first frame of a video sequence and the organelle image is used by the software to create a template. Image correlation is then applied to find the location of this template within the next frame\(^3,5\) and the template image then updated to be that of the object in the second frame. The template finding and updating process is repeated for all frames to build up a track of particle positions. Refreshing the template image in each frame accommodates gradual changes in the image of the mitochondria, caused by morphological changes, changes in pointing direction of non circular objects and photobleaching. Further analysis was performed including measuring the instantaneous speed and the time-variant displacement of the mitochondria. Displacement was defined as the absolute distance between a mitochondria's position at a given time and its location in the first frame. The instantaneous speed of the mitochondria was computed by measuring the rate of change of position over 1 second intervals. This interval was chosen as one long enough to smooth the effects of measurement noise but short enough to accurately determine the speed without averaging out brief, directed motion events\(^3\).

**Measurement of mitochondrial area**

Cerebral artery smooth muscle cells maintained in culture for 4 days in the presence of \(10\) \(\mu\)M Mdivi-1 or vehicle control (0.1% DMSO) were loaded with \(100\) nM TMRE (20 min, \(37^\circ\)C) and imaged as above. Regions of contiguous mitochondria were defined as regions displaying synchronous increases or decreases in TMRE fluorescence. Images were analyzed using Metamorph, background was subtracted and images smoothed using a 9 frame rolling average. Transient localised differences were highlighted by subtracting a duplicate image stack that was offset by 5 frames (and hence \(0.5\) s), applying either an inclusive threshold (to detect increases) or exclusive threshold (to detect decreases) and measuring the area of localised change.

**Western blotting**

Intact arteries were frozen in liquid \(N_2\) immediately following dissection or after maintenance in culture. 6 vessels were pooled for each timepoint, homogenised in \(50\) \(\mu\)l CellLyticM solution (Sigma) using Eppendorf-tube pestles, which were then rinsed with an additional \(30\) \(\mu\)l solution. Homogenates were incubated at room
temperature for 15 min before centrifugation at 13,000 rpm at 4 ºC for 20 min. Supernatants were immediately frozen at -20 ºC or mixed with 4x reducing sample buffer and separated by standard SDS-PAGE on a 12% gel, transferred to nitrocellulose membrane then blocked with 5% BSA for 1 hr, incubated with primary antibodies: monoclonal mouse-anti-mitofusin-2 (ab56889, Abcam, 1:500), polyclonal rabbit-anti-PCNA (ab2426, Abcam, 1:500), monoclonal mouse-anti-mitochondrial cytochrome oxidase IV (ab, Abcam, 1:1000), or polyclonal rabbit-anti-GAPDH (ab9485, Abcam, 1:500) overnight at 4 ºC or at room temperature for 1 hr; incubated with 1:80,000 HRP-conjugated anti-mouse or anti-rabbit secondary antibody for 1 hr at room temperature then visualized using ECL.

**Immunocytochemistry**

Arteries were cannulated, pressurised to 40 mmHg and fixed in 10% formal basic solution for at least 30 min prior to removal to individual 1.5 ml tubes. Whilst in the 1.5 ml tubes the arteries were permeabilised with 0.5% Triton X-100 and blocked with 2% BSA for 1 hr before incubation with rabbit anti-PCNA (ab2426, Abcam, 1:50) overnight at 4 ºC and then anti-rabbit-Alexa 488 (Invitrogen, 1:100) plus mouse-anti- α-actin-Cy3 conjugate (Sigma, 1:100) for 1 hr prior to mounting the arteries in Vectashield containing DAPI. Fluorescence images were captured on a Leica SP5 confocal inverted microscope with 40x oil objective, no digital zoom applied and constant illumination and gain settings throughout. Images were analyzed using Metamorph 7.1.3 to select smooth muscle α-actin positive regions and measure total PCNA staining within these regions.

**[^3]H]-Thymidine incorporation assay**

Cells were grown to ~70% confluency in 12 well plates, quiesced for 24 h in media containing 0.1% FBS then returned to media containing 10% FBS for 24 h.[^3]H]-Thymidine (9.25 kBq) was added to each well for the final 6 h of this 24 h treatment period. Cells were washed with ice cold PBS followed by 10% trichloroacetic acid then by 10% sodium dodecyl sulfate in 0.2 M sodium hydroxide and radioactivity was quantified by liquid scintillation (Packard 1500 Tri-carb or ScintSafe3).
FACS analysis

Cells were grown to ~70% confluency in 6 well plates, harvested, resuspended in PBS and fixed in ice-cold 70% ethanol (added dropwise while vortexing to ensure proper fixation of cells and prevent clumping) at 4°C overnight. Cells were then washed with PBS, incubated with 50 μg/ml RNase A for 1 h at 37°C and stained with 40 μg/ml propidium iodide. Cell cycle profiles were acquired by flow cytometry (BD FACSDiva software; BD Biosciences) and analyzed using the software FlowJo.

Statistical analyses

Other than Figures 5a and 6, a parametric distribution of raw data around mean values was confirmed prior to statistical testing. Mean values ± standard error (s.e.) are shown (unless otherwise indicated) and were analysed using unpaired Student’s t-tests or, for more than 2 groups, ANOVA with Games-Howell post-hoc comparison of variance (for samples with unequal variance). α was set to 0.05, P values < 0.05 were considered significant. The median mitochondrial areas with and without Mdivi-1 treatment were compared using a Mann-Whitney U-test. The normalised data in Figure 6 was analysed using Kruskal-Wallis tests with P values adjusted for multiple testing. t-test were carried out in Microcal Origin v6.0, with Bonferroni considerations applied for cell cycle data, all other tests in SPSS 19 for Windows.

Chemicals, drugs and enzymes

Papain was from Worthington; TMRE, mitotracker green, F-12 and Waymouths’ media and FBS from Invitrogen; 100x Pen/Strep from PAA Cell Culture Company; Mdivi-1 was from Enzo Life Sciences. Collagenase, hyaluronidase, BSA and all other chemicals were from Sigma.

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