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Accessibility
Actin-dependent mitochondrial internalization in cardiomyocytes: evidence for rescue of mitochondrial function

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ABSTRACT
Previously, we have demonstrated that the transplantation of viable, structurally intact, respiration competent mitochondria into the ischemic myocardium during early reperfusion significantly enhanced cardioprotection by decreasing myocellular damage and enhancing functional recovery. Our in vitro and in vivo studies established that autologous mitochondria are internalized into cardiomyocytes following transplantation; however, the mechanism(s) modulating internalization of these organelles were unknown. Here, we show that internalization of mitochondria occurs through actin-dependent endocytosis and rescues cell function by increasing ATP content and oxygen consumption rates. We also show that internalized mitochondria replace depleted mitochondrial (mt)DNA. These results describe the mechanism for internalization of mitochondria within host cells and provide a basis for novel therapeutic interventions allowing for the rescue and replacement of damaged or impaired mitochondria.

KEY WORDS: Mitochondria, Transplantation, Cardioprotection, Mitochondrial DNA, Endocytosis

INTRODUCTION
Myocardial ischemia/reperfusion injury is associated with mitochondrial damage and dysfunction that detrimentally alters oxygen consumption and energy synthesis (Lesniewsky et al., 2004; McCully et al., 2007). These events occur during ischemia and extend into reperfusion to severely compromise myocardial functional recovery and cellular viability. In previous studies, we demonstrated that the transplantation of viable, structurally intact, respiration competent, mitochondria, into the ischemic zone of the myocardium during early reperfusion significantly decreases myocardial ischemia/reperfusion injury (McCully et al., 2009; Masuzawa et al., 2013). These studies also established that transplanted mitochondria are internalized into cardiomyocytes and increase oxygen consumption rates and ATP production (Masuzawa et al., 2013).

The cellular mechanism(s) that enable the internalization of transplanted mitochondria were unknown. In this report, we investigate the potential mechanisms modulating mitochondrial internalization into cardiomyocytes using pharmacological blockers of clathrin mediated endocytosis, actin mediated endocytosis, macro-pinocytosis, and tunneling nanotubes (Bereiter-Hahn et al., 2008; Cowan et al., 2001; Huang et al., 2013; Islam et al., 2012; Kitani et al., 2014; Le et al., 2000; Lou et al., 2012; Mahammad and Parmryd, 2015; Masters et al., 2013; Nakase et al., 2004; Spees et al., 2006). We also investigate the effects of mitochondrial transplantation on the rescue of cell function and replacement of mtDNA in host cells with depleted mtDNA. Our results indicate that transplanted mitochondria are internalized by an actin dependent pathway. Once internalized the mitochondria rescue cell function and replace damaged mtDNA in host cells.

RESULTS AND DISCUSSION
Mitochondrial internalization into cardiomyocytes
Previously, we have demonstrated the uptake of autologous mitochondria in vitro and in vivo (Masuzawa et al., 2013). Our results demonstrated that transplanted mitochondria were internalized in host cardiomyocytes. In this report, we have extended these earlier studies using the pH sensitive pHrodo Red SE label as an indicator of endocytosis (Miksa et al., 2009; Neaga et al., 2013). pHrodo Red SE label enables fluorescent measurement of internalization based on the increase in fluorescence as the mitochondria are internalized within the cells (Miksa et al., 2009; Neaga et al., 2013).

Our results recapitulate our previous observations and demonstrate that cardiomyocytes co-incubated with mitochondria are internalized in a time-dependent manner. Mitochondrial internalization into cardiomyocytes is evident following 1 hour co-incubation (26±8.2 mitochondrial per nucleus) and is increased following 4 hours co-incubation (186±24.6 mitochondrial per nucleus) and following 24 hours co-incubation (257±69.1 mitochondrial per nucleus; Fig. 1A). The percentage of mitochondria internalized at each time point is shown in Fig. 1C. The cellular mechanism(s) that enable the internalization of transplanted mitochondria were unknown. In this report, we investigate the potential mechanisms modulating mitochondrial internalization into cardiomyocytes using pharmacological blockers of clathrin mediated endocytosis, actin mediated endocytosis, macro-pinocytosis, and tunneling nanotubes (Bereiter-Hahn et al., 2008; Cowan et al., 2001; Huang et al., 2013; Islam et al., 2012; Kitani et al., 2014; Le et al., 2000; Lou et al., 2012; Mahammad and Parmryd, 2015; Masters et al., 2013; Nakase et al., 2004; Spees et al., 2006). We also investigate the effects of mitochondrial transplantation on the rescue of cell function and replacement of mtDNA in host cells. Our results indicate that transplanted mitochondria are internalized by an actin dependent pathway. Once internalized the mitochondria rescue cell function and replace damaged mtDNA in host cells.
caveola-dependent-clathrin dependent endocytosis (Le et al., 2000; Mahammad and Parmryd, 2015; Pfeiffer et al., 2014) nocodazole (Noco, 4 ng/mL) was used to block tunneling nano tubes (Huang et al., 2013; Islam et al., 2012; Lou et al., 2012; Spees et al., 2006), and 5-(N-Ethyl-N-isopropyl)amiloride (EIPA) at 10 mM, 50 mM or 100 mM was used to block macro-pinocytosis (Kitani et al., 2014; Nakase et al., 2004) (Fig. 2A,B).

Our studies show that mitochondrial internalization by cardiomyocytes following 4 hours co-incubation is unaffected following pre-incubation with methyl-β-cycloexextrin, nocodazole, or with 5-(N-Ethyl-N-isopropyl)amiloride suggesting that tunneling nanotubes, caveola-dependent-clathrin dependent endocytosis and macro-pinocytosis are not involved in mitochondrial internalization into cardiomyocytes (Fig. 2A–C). Only pre-incubation with cytochalasin D significantly decreased (p<0.05) the internalization of mitochondria into cardiomyocytes and decreased ATP content as compared to no inhibitor (Fig. 2A–C).

The ability of cytochalasin D to inhibit actin-dependent endocytosis and phagocytosis has been well characterized and this mechanism would agree with the proposed endosymbiosis origin of mitochondria (Ivanov, 2008; Margulis, 1975). Other mechanisms are less likely to be involved based on the method of...
mitochondrial presentation and mitochondrial size. In all of our studies, mitochondria are presented externally and subsequently become internalized by host cells. This is in contrast to intercellular mitochondrial transfer where it has been proposed that mitochondria are passed from one individual cell to another via tunneling nanotubes (Huang et al., 2013; Islam et al., 2012; Lou et al., 2012; Spees et al., 2006). Although it is possible that intercellular mitochondrial transfer occurs following internalization, this mechanism does not modulate initial mitochondrial internalization.

The role of macro-pinocytosis while recently suggested to play a role in mitochondrial internalization (Kitani et al., 2014) also does not appear to be involved in mitochondrial internalization into cardiomyocytes. Kitani et al., 2014, used 25 or 50 μM EIPA to block mitochondrial internalization in human uterine endometrial cancer cells. In our studies we have used three concentrations (10, 50 and 100 μM) of EIPA and found no decrease in the internalization of mitochondria into cardiomyocytes suggesting that in cardiomyocytes, macro-pinocytosis does not play a role in mitochondrial internalization.

The mechanism by which the internalized mitochondria escape endosomes remains to be elucidated and is beyond the scope of this report. Our results agree with our previous observations where no cellular extensions in either cardiomyocytes (in vitro) or myocardial sections (following in vivo mitochondrial transplantation experiments) were detected and no colocalization of internalized mitochondria with any lysosomal, caveolar or autophagosomal markers was observed (Masuzawa et al., 2013). Overall, our results establish that internalization of mitochondria into cardiomyocytes is time dependent and occurs by actin-dependent endocytosis.

**Rescue of mitochondrial function and replacement of mtDNA**

To characterize the functional contribution of internalized mitochondria, we performed studies using HeLa p0 cells depleted of mtDNA (Kukat et al., 2008). HeLa p0 cells are capable of energy generation through fermentation but lack oxygen consumption capacity due to depletion of electron transport chain proteins encoded by mtDNA. Our studies demonstrate that co-incubation of HeLa p0 cells with mitochondria isolated from HeLa cells containing intact mtDNA rescues HeLa p0 cell function by significantly increasing ATP content and oxygen consumption rates. ATP content was significantly increased in HeLa p0 cells following co-incubation with mitochondria at 24, 48, 72 hours and 1 and 2 weeks. The enhanced intracellular ATP content corresponds to significant increases in oxygen consumption rates of HeLa p0 cells after mitochondrial internalization (Fig. 3B).

The effects of mitochondrial transplantation on the rescue of mitochondrial function in HeLa p0 cells were not absolute as ATP content and oxygen consumption rate were significantly decreased as compared to that in HeLa cells (Fig. 3A,B). This is likely due to the culture conditions used in our studies that required media containing high glucose (4.5 g/l D-glucose) and 50 mg/ml uridine (Hashiguchi and Zhang-Akiyama, 2009). We observed that HeLa cells grown in this medium grew more slowly than those grown with 1 g/l D-glucose and no uridine (results not shown). Thus, the number of control HeLa p0 cells as compared to HeLa p0 cells containing internalized HeLa mitochondria may have been disproportionally overrepresented due to the medium being optimized specifically for their growth. We did not change the media to low glucose media following co-incubation with mitochondria as this would have limited the survival of HeLa p0 cells that did not internalize mitochondria from HeLa cells. HeLa p0 cells cannot survive in low glucose media due to the complete reliance on glucose fermentation for ATP synthesis. HeLa p0 cells also require media containing uridine due to a deficiency of pyrimidine biosynthesis (Hashiguchi and Zhang-Akiyama, 2009).
Of significance, PCR analysis clearly demonstrated replacement of mtDNA in HeLa p0 cells following mitochondrial transplantation. We were unable to quantify the absolute levels of mtDNA in the rescued HeLa p0 cells as HeLa cells contain an unbalanced number of chromosomes (Adey et al., 2013). While our results show that the absolute quantity of mtDNA in HeLa p0 cells co-incubated with HeLa cell mitochondria (containing intact mtDNA) is significantly less than that observed in HeLa cells, the mtDNA present is sufficient to significantly enhance intracellular ATP content and oxygen consumption rate as compared to untreated HeLa p0 cells. We did not investigate if the mtDNA in the mitochondria transplanted into HeLa p0 cells replicated or if the transplanted mitochondrial population expanded as the rescued HeLa cells divided as this was beyond the scope of the current paper and thus remain to be demonstrated.

Although the rescue of cardiomyocytes following ischemia and reperfusion injury is the primary goal of our research, the potential of mitochondrial transplantation is not limited to cardioprotection alone. Our methodology has recently been utilized by others to show protection from ischemia-reperfusion injury in the liver (Lin et al., 2013) and to enhance drug sensitivity in human cancer cells (Elliott et al., 2012). In summary, our data suggest that mitochondrial transplantation has potential to rescue cell function and replace damaged mitochondrial DNA in many cell types and disease states.

In conclusion, we show that the internalization of mitochondria in cardiomyocytes occurs through actin-dependent endocytosis and rescues cell function through enhanced ATP content and oxygen consumption rate. We also show that internalized mitochondria replace depleted mtDNA. These results provide a mechanism for the internalization of mitochondria within host cells and a basis for novel therapeutic interventions allowing for the rescue and replacement of damaged or impaired mitochondria.

**MATERIALS AND METHODS**

**Cardiomyocytes isolation and culture**
Neonatal rat cardiomyocytes were isolated from 1-day-old Lewis rat pups the Neonatal Cardiomyocyte Isolation System (Worthington Biochemicals, Lakewood, NJ, USA) (Masuzawa et al., 2013).

**Mitochondria isolation and labeling**
Mitochondria were isolated and viability was determined as previously described (Masuzawa et al., 2013). The isolated mitochondria were labeled with pHrodo red particle label (Life Technologies, Grand Island, NY, USA) (Miksa et al., 2009; Neaga et al., 2013) for 10 minutes at 4°C and then washed four times in respiration buffer (250 mmol/l sucrose, 2 mmol/l KH2PO4, 10 mmol/l MgCl2, 20 mmol/l K+-HEPES buffer, pH 7.2, 0.5 mmol/l K+-EGTA, pH 8.0, 5 mmol/l glutamate, 5 mmol/l KH2PO4, 10 mmol/l MgCl2, 20 mmol/l K+-HEPES buffer, pH 7.2, 0.5 mmol/l K+-EGTA, pH 8.0, 5 mmol/l glutamate, 5 mmol/l malate, 8 mmol/l succinate, 1 mmol/l ADP). The labeled mitochondria were resuspended in fresh respiration buffer and the last wash supernatant was saved. The labeled mitochondrial (1×106/well) were co-incubated with cardiomyocytes (50,000/well). At the conclusion of each time point, the media was removed and the cells were washed four times with 1× PBS and 200 μl of fresh medium was added to each well. Control cardiomyocytes were co-incubated with the last pHrodo wash. Mitochondrial internalization was determined using ImageJ 1.48 software (imagej.nih.gov/ij/download/).

**Blockers of mitochondrial internalization**
Following two days of culture, cardiomyocytes were pre-treated for 30 minutes with either cytochalasin D (CytoD, 10 μM) (Bereiter-Hahn et al., 2008; Cowan et al., 2001; Kastl et al., 2013), methyl-[β]-cyclodextrin (MβCD, 1 mM) (Le et al., 2000; Mahammad and Parmryd, 2015; Pfeiffer et al., 2014), nocardazole (Noco, 4 μg/mL) (Huang et al., 2013; Islam et al., 2012; Lou et al., 2012; Spees et al., 2006) or 5-(N-Ethyl-N-isopropyl) amiloride (EIPA, 10 μM, 50 μM or 100 μM) (Kitani et al., 2014; Nakase et al., 2004) ; all from Sigma-Aldrich, St. Louis, MO, USA. All blockers were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA) and resuspended in media (≥1:10,000 dilution). Control cardiomyocytes were pre-treated by co-incubation with an equal molar concentration of DMSO in media for 30 minutes. Following pre-treatment, the media was removed and the cardiomyocytes were washed four times with 1× PBS, and 200 μl of fresh media was added to each well. The cardiomyocytes were then co-incubated with pHrodo labeled mitochondria (1×106/well) or with the final pHrodo wash. Internalization of mitochondria was determined by as described above. All results were compared to control cardiomyocytes co-incubated with pHrodo-labeled mitochondria.

**ATP determination**
ATP content was determined using the ATPlite Luminescence ATP Detection Assay System (Perkin Elmer, Waltham, MA, USA) (Diepart et al., 2010; Lanza and Nair, 2009; Manfredi et al., 2002). All assays were performed in the absence of fluorescent dyes as it has been previously reported that these dyes may interfere with mitochondrial function (Poot et al., 1996).

**Rescue of mitochondrial function and replacement of mitochondrial DNA in HeLa p0 cells**
HeLa p0 cells containing deleted mtDNA (Kukat et al., 2008) were co-incubated with isolated mitochondria from HeLa cells containing intact mtDNA. mtDNA deletion in HeLa p0 cells was confirmed by PCR analysis and by oxygen consumption rate. HeLa cells were cultured as described (Masuzawa et al., 2013). HeLa p0 cells were cultured in DMEM-high glucose-GLUTAmx (4.5 g/l) with 10% fetal bovine serum, 100 mg/ml sodium pyruvate, 50 mg/ml uridine and Antibiotic-Antimycotic (all from Life Technologies, Grand Island, NY, USA) (Hashiguchi and Zhang-Akiyama, 2009; Kukat et al., 2008). HeLa p0 cells were cultured in 24 well plates (5,000/well) for 24 hours. Mitochondria were isolated from HeLa cells and co-incubated (1×106/ well) with HeLa p0. Control HeLa p0 cells were co-incubated with vehicle (respiration buffer) only. Following 72 hours, the cells were washed four times in 1× PBS, collected by trypsination and then replated on 100 mm culture plates for one, two and three weeks. Cell cultures were split at 80% confluence. At specified end points, the cells were washed four times in 1× PBS and then collected by trypsination. The cells were then used for determination of ATP content, mtDNA and oxygen consumption rate.

**Oxygen consumption rate**
Oxygen consumption rate was determined by Oxytherm (Hansatech Instruments Ltd, Norfolk, UK) (Papandreou et al., 2006). In brief, 3×106 cells were suspended in fresh DMEM-high glucose-GLUTAmx with 10% fetal bovine serum, 100 mg/ml sodium pyruvate, 50 mg/ml uridine and Antibiotic-Antimycotic (all from Life Technologies, Grand Island, NY, USA) at 37°C. The cell suspension was transferred to the oxytherm chamber and oxygen consumption was determined for 5 minutes and then cyanide (0.2 M) was added to terminate the respiration.

**PCR analysis**
DNA was isolated and PCR was performed as described (Levitsky et al., 2003). The primers used for mtDNA detection were (5’-CAAATAT-CATTCTGAGG-3’) and (5’-GGTTTTGGGTTTGGCAG-3’) (Levitsky et al., 2003). The human B-globin gene was used as a control; GH20 (5’-GAAGAGCCAAGACAGGTAC-3’) and GH21 (5’-GGAAAATAGA-CCAATAGGCACG-3’) (TaKaRa Shuzo Co. Ltd, Otsu, Japan).

**Fluorescent microscopy**
Fluorescent microscopy studies were performed as previously described (Masuzawa et al., 2013). Some samples were stained with MitoTracker Red CMX Ros (100 nmol/l), Alexa 488-phalloidin (1:50 dilution), and DAPI (500 nmol/l) (all from Invitrogen, Grand Island, NY, USA). Primary antibodies were detected with highly cross-absorbed goat anti-mouse or
anti-rabbit Alexa-conjugated secondary antibodies (Invitrogen, Grand Island, NY, USA).

Statistical analysis
Statistical analysis was performed using SAS (version 6.12) software package (SAS Institute, Cary, NC, USA). The mean±SE for all data was calculated for all variables. Statistical significance was assessed using repeated measures analysis of variance (ANOVA) with group as a between subjects factor and time as a within subjects factor. Tukey honestly significant difference test was used for comparisons between control and other groups to adjust for the multiplicity of tests. Statistical significance was claimed at P<0.05.

Competing interests
The authors declare no competing or financial interests.

Author contributions
JDM, CAP, DBC designed the experiments and wrote the manuscript. PDN and SL provided clinical input and editorial aid.

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Results
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