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Original article

Hypoxia signaling controls postnatal changes in cardiac mitochondrial morphology and function

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ABSTRACT

Fetal cardiomyocyte adaptation to low levels of oxygen *in utero* is incompletely understood, and is of interest as hypoxia tolerance is lost after birth, leading to vulnerability of adult cardiomyocytes. It is known that cardiac mitochondrial morphology, number and function change significantly following birth, although the underlying molecular mechanisms and physiological stimuli are undefined.

Here we show that the decrease in cardiomyocyte HIF-signaling in cardiomyocytes immediately after birth acts as a physiological switch driving mitochondrial fusion and increased postnatal mitochondrial biogenesis. We also investigated mechanisms of ATP generation in embryonic cardiac mitochondria. We found that embryonic cardiac cardiomyocytes rely on both glycolysis and the tricarboxylic acid cycle to generate ATP, and that the balance between these two metabolic pathways in the heart is controlled around birth by the reduction in HIF signaling. We therefore propose that the increase in ambient oxygen encountered by the neonate at birth acts as a key physiological stimulus to cardiac mitochondrial adaptation.

1. Introduction

Fetal cardiomyocytes are adapted to function in low levels of oxygen *in utero*, whereas adult cardiomyocytes are extremely vulnerable to hypoxia, as evidenced by high rates of mortality and morbidity caused by ischemic heart disease [1]. Shortly after birth, cardiomyocytes lose their capability to adapt to hypoxic conditions. The process underlying this postnatal maturational process is not understood.

It is thought that fetal cardiomyocytes generate ATP predominantly by glycolysis and glucose oxidation [2]. At birth, ambient oxygen levels increase sharply as the neonate emerges into the terrestrial environment. Immediately after birth, cardiac output, and therefore cardiac energy requirements, increase rapidly [3]. This increase is achieved through switching from glycolysis to β-oxidation of lipids, which generates larger amounts of ATP per unit of substrate than glycolysis [2,4], and is accompanied by an increase in mitochondrial number [5].

Recently, our understanding of molecular mechanisms controlling postnatal cardiac bioenergetics and mitochondrial remodeling has advanced, but physiological stimuli controlling these processes remain to be defined. This information is of interest with respect to future strategies to protect adult cardiomyocytes in ischemic heart disease and to increase our understanding of human cardiac conditions presenting around birth.

Mitochondrial biogenesis is upregulated following birth as a consequence of increases in *Pgc1α/β* expression [5], although the physiological stimuli for this are yet to be described. Furthermore, it is recognized that mitochondrial morphology is intimately linked with function, fusion of mitochondria being associated with increased β-oxidation of lipid [6,7]. Fetal mitochondria are "fragmented" in appearance, whereas postnatal mitochondria appear elongated, consistent with the metabolic switch occurring in the heart flowing birth [2]. Mitofusin proteins (MFNs) are implicated in the control of postnatal mitochondrial fusion, although, again, the relationship to physiological changes around birth is not known [8].

We investigated the mechanisms controlling neonatal cardiac mitochondrial remodeling at birth. Immediately following birth, ambient levels of oxygen encountered by the neonate increase significantly [9]. We have recently shown that the postnatal decrease in cardiac hypoxia signaling controls the lipid metabolizing gene expression program [10] and postnatal remodeling of the cardiac conduction system [11].

Abbreviations: VHL, Von Hippel–Lindau factor; HIF, Hypoxia inducible factor; Pgc1, Peroxisome proliferator-activated receptor gamma co-activator 1; TMRM, Tetramethylrhodamine, methyl ester; MFN, Mitofusin.

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Additionally, cell culture experiments have shown that overexpression of hypoxia inducible factor (HIF) leads to diminished respiratory capacity and inhibited mitochondrial biogenesis [12,13]. We therefore hypothesized that the increase in cardiac oxygen tension acts, via HIF signaling, as a developmental "switch", driving postnatal maturation of the heart. We investigated the effects of the reduction in hypoxia signaling following birth on the structure and function of cardiac mitochondria.

2. Results

2.1. The morphology of cardiac mitochondria around birth is dependent on hypoxia signaling

In situ measurement of mitochondrial function *in utero* is not currently possible without exposing the cells to oxygen, leading to artifacts due to oxygen-induced maturational processes. We therefore studied hearts from mice born into normobaric hypoxia ($Fi0_2$ 10%) and *αMHC-Cre::VHL*$^{(fl/fl)}$ mice which maintain constitutively high levels of HIF signaling in the presence of normoxia [14] in order to inhibit postnatal maturational processes dependent on postnatal reductions in HIF signaling. We also generated stable HL1 cell lines transfected with an shRNA construct directed against VHL mRNA, which has a similar effect of stabilizing HIF expression in normoxia (Supplementary Fig. 1).

Perinatal changes in mitochondrial morphology have been observed in the mouse [15] and rabbit [16] from day 3 onwards and in the guinea pig [17] sheep [18], and mouse [8] around birth. These studies used electron microscopy, and consistently observed a shift in mitochondrial morphology from many small and ovoid to fewer long and rectangular mitochondria with an increase in proximity between mitochondria and the myofibrils with time after birth. In concordance with these previously published studies, we observed that mitochondria change shape from small and round before birth to larger and rectangular following birth (Fig. 1A and Supplementary Fig. 2). We estimated that percentage area occupied by mitochondria in the cell increases from E16.5 (10%) to P10.5 (34%), with the largest increase occurring in the first few days after birth between P0.5 and P2.5 (Fig. 1B). We observed that the mitochondrial cristae become more dense and uniform between E16.5 and P10.5, with an observable decrease in glycogen storage granules sequentially from E16.5 to P10.5 and lipid droplets observed on micrographs from the day of birth onwards (P0.5) (Fig. 1C and Supplementary Fig. 2). We noted that mitochondrial morphology changes during the perinatal period. Mitochondrial matrix in P2.5 mitochondria appears less dense, and sarcomeres more dense and defined on EMs compared with E18.5 mitochondria (Fig. 1D and Supplementary Fig. 2).

We went on to examine whether perinatal changes in cardiac mitochondrial morphology are dependent on changes in ambient oxygen levels and hypoxia signaling immediately following birth. We found that interpreting mitochondrial morphology in electron micrographs was difficult, due to plane of sectioning issues. Therefore, we used primary cultures of cardiomyocytes, which enable the use of vital dyes to assess mitochondrial morphology more sensitively than electron microscopy (Fig. 2A). Primary culture of embryonic cardiac cells involves removal from a hypoxic environment *in vivo* and exposure to potentially artifactually high levels of oxygen in culture. We therefore assessed the effect of cardiomyocyte culture time on fusion phenotype of the heart cells. We digested and plated E18.5 embryonic hearts and found that the length of time in culture increased the proportion of fused mitochondria until 8 h post-plating ($p = 0.004$) (Fig. 2B). At a culture time of 18 h the association reversed. This emphasizes the difficulty in interpretation of mitochondrial morphology after prolonged periods of cell culture. Cardiomyocyte viability was not compromised by 45-min incubation with MitoTracker dye: at 24 h after MitoTracker treatment, we observed adherent cells, often displaying synchronized beating.

Observers blinded to their origin examined primary cell cultures of cardiomyocytes, after 45-min of MitoTracker dye treatment in cells one hour after plating. We found an increase in the proportion of cardiac cells exhibiting mainly fused mitochondria from E18.5 to P10.5. We observed an increase in the numbers of fused mitochondria after birth with only 27% mitochondria being fused at E18.5 compared to 62% fused at P2.5 ($p = 0.05$) (Fig. 2C).

At birth, the mammalian embryo encounters a sharp increase in ambient oxygenation [9]. We have previously shown that cardiac levels of HIF1α protein, a key mediator of hypoxia signaling fall, but are elevated in neonatal hearts homozygous null for VHL [10]. We therefore investigated whether neonatal changes in cardiac HIF signaling drive the changes in cardiac morphology, number and function in the neonatal heart.

To assess the impact of hypoxia signaling on mitochondrial morphology we examined electron micrographs of hearts from postnatal day 10.5 (P10.5) *αMHC-Cre::VHL*$^{(fl/fl)}$ mice. Mitochondria from *αMHC-Cre::VHL*$^{(fl/fl)}$ mice appeared immature with respect to controls (Fig. 2D). The image in Fig. 2D is taken from a αMHC-Cre heart (constitutive cre expressing), which is on a C57BL/10 background as opposed to the (CBA/Ca × C57BL/10) strain used for the wild-type analysis outlined in Fig. 1 and Supplementary Fig. 2. This strain difference could explain the different mitochondrial morphology observed between these two images. Further examples of mitochondrial morphology are pictured in Supplementary Fig. 2. In primary cultures of cardiomyocytes using Mitotracker dye, we found that mitochondria from P10 *αMHC-Cre::VHL*$^{(fl/fl)}$ hearts exhibited significantly higher numbers of fragmented-appearing mitochondria compared with controls ($p < 0.001$) (Fig. 2E).

2.2. Expression of mitochondrial fusion proteins is controlled in the heart by hypoxia signaling

Mitochondrial morphology is regulated by a family of mitochondrial fusion and fission proteins [8,19]. We therefore investigated expression of the fusion proteins, *MFN1*, *MFN2* and *OPA1*, and fission proteins, *DRP1* and *FIS1* at regular intervals from E16.5 to P10.5. Western blotting of protein extracts of ventricular tissue revealed an increase in fusion protein expression from E16.5 in a stepwise manner through to P10.5 with the largest increase taking place between P0.5 and P2.5 in *MFN1* and *MFN2* ($p = 0.05$) and between P2.5 and P10.5 in *OPA1* ($p = 0.05$) (Figs. 3A–C, representative blots in Supplementary Fig. 3). There were no changes detectable in fission protein *DRP1* and *FIS1* levels over this time period (Supplementary Fig. 4). We found that in *αMHC-Cre::VHL*$^{(fl/fl)}$ hearts, expression of fusion proteins *MFN1* and *OPA1* was significantly reduced compared with controls ($P < 0.001$ and $p = 0.04$ respectively, 2 tailed t tests $n = 4$ hearts each group) (Fig. 3D). We analyzed cardiac mRNA expression of MFN1, MFN2 and OPA1. We found that levels of all three mRNAs increase significantly in the heart at birth, and this rise is significantly attenuated in hearts from *αMHC-Cre::VHL*$^{(fl/fl)}$ neonates (Figs. 3E–G). These data suggest that postnatal HIF-dependent mitochondrial fusion could be mediated by increases in *MFN1, MFN2* and *OPA1* expression. Interestingly, no significant change in mRNA levels of MFN1, MFN2 and OPA1 was seen in adult inducible, cardiac specific VHL deleting mercremer::*VHL*$^{(fl/fl)}$ hearts, following 5 days of tamoxifen treatment.

We went on to examine whether HIF1α binds to the 5' promoters of MFN1, MFN2 and OPA1. We analyzed published 5' promoter sequences for these 5 genes, and found several canonical (A/G)CGTG HIF1α binding sites in each case (Fig. 4A). We assayed whether HIF1α directly binds these promoters using anti HIF1α serum to perform chromatin immunoprecipitation. We found that in each case, chromatin containing at least one HIF1α-binding site promoter fragment was enriched 20 fold over non-amplified sequences in chromatin from embryonic αMHC-Cre control hearts (Figs. 4B-D), implying that HIF1α binds to these sites *in vivo*. Chromatin Immunoprecipitation with anti-HIF1α serum in chromatin from P2.5 control hearts revealed differences in binding of HIF1α to several sites in these three promoters (Figs. 4E-F). These changes were not seen in their entirety in chromatin from P2.5