eNOS Correlates with Mitochondrial Biogenesis in Hearts of Congenital Heart Disease with Cyanosis

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Abstract

Background: Mitochondrial biogenesis program in heart appears to exhibit adaptive remodeling following biomechanical and oxidative stress. The adaptive mechanisms that protect myocardium metabolism during hypoxia are coordinated in part by nitric oxide (NO).

Objective: To observe mitochondrial biogenesis and nitric oxide synthase (NOS) expression in hearts of congenital heart disease with cyanosis, discuss mitochondrial response to chronic hypoxia in myocardium.

Methods: 20 patients with cyanotic (n=10) or acyanotic cardiac defects (n=10) were investigated. Samples from the right ventricular outflow tract myocardium taken during operation were studied. Morphometric analysis of mitochondria was performed with transmission electron microscope. Relative mtDNA/nDNA ratio was determined with real-time PCR. Cytochrome c oxidase subunit I (COXI), peroxisome-proliferator-activated receptor γ coactivator-1α (PGC-1α), nuclear respiratory factor 1 (NRF1), and mitochondrial transcription factor A (Tfam) transcript levels were detected by real-time fluorescent RT-PCR. COXI and nNOS, iNOS and eNOS protein levels were measured with western blot.

Results: Mitochondrial volume density (Vv) and numerical density (Nv) were significantly elevated in patients with cyanotic compared to acyanotic congenital heart disease. Elevated mtDNA and up-regulated COXI, PGC-1α, NRF1 and Tfam mRNA levels were observed in cyanotic patients. Protein levels of COXI and eNOS were significantly higher in the myocardium of cyanotic than of acyanotic patients. PGC-1α transcript levels correlated with the levels of eNOS.

Conclusion: Mitochondrial biogenesis is activated in right ventricular outflow tract myocardium in congenital heart disease with cyanosis, which could be the adaptive response to chronic hypoxia and possibly involves eNOS up-regulation. (Arq Bras Cardiol 2012;99(3):780-788)

Keywords: Biogenesis; mitochondria; heart; heart defects; congenital; nitric oxide Synthase.

Introduction

Mitochondria are the site of oxidative energy production in eukaryotic cells. Mitochondrial biogenesis is defined as the control of mitochondrial turnover, content, and number required to maintain diverse homeostatic demands across tissue types[1]. Mitochondrial biogenesis involves the coordinated action of both nuclear and mitochondrial encoded genomes. Peroxisome profilerator activated receptor gamma coactivator alpha (PGC-1α), an inducible transcriptional coactivator, has been implicated as a major regulator of the mitochondrial biogenic program. PGC-1α interacts with nuclear respiratory factor 1 (NRF1), stimulating transcription of many mitochondrial genes as well as mitochondrial transcription factor A (Tfam), a direct regulator of mitochondrial DNA replication and transcription[2]. In the heart, mitochondria are highly abundant, constitute approximately 40% of total cardiomyocyte volume, and produce more than 90% of the cell's energy[3]. Mitochondrial biogenesis program in heart appears to exhibit adaptive remodeling following biomechanical and oxidative stress, for instance, following hypoxia, and is supposed to be an explicit target for cardiac therapeutic interventions[1].

Nitric oxide (NO) is a gaseous molecule with a wide range of physiological and pathological activities, including the regulation of vessel tone and angiogenesis in ischemic cardiovascular diseases, wound healing, inflammation, and malignant diseases. NO also has complex effect on mitochondrion, including upregulating the transcriptional machinery driving biogenesis via soluble guanylate cyclase-sensitive (sGC) cGMP dependent induction of PGC-1α and reducing oxidative phosphorylation flux via competitive inhibition of respiratory chain electron transfer[4]. Cardiomyocytes show increased NO production during hypoxia[5], and some of the adaptive hypoxic responses are regulated by NO in the heart[6]. Endothelial nitric oxide synthase (eNOS) has been shown to be induced by hypoxia[7] and to be activated by hypoxia-induced transcription factor HIF[8].

Since data are lacking about the myocardial mitochondrial response to chronic hypoxemia due to congenital heart disease (CHD), we performed a detailed morphologic and molecular analysis on hearts from patients with cyanotic CHD, comparing

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gene expression profiles of key regulators of mitochondrial biogenesis and eNOS protein expression to hearts of CHD patients without cyanosis.

Materials and methods

Patients

After approval by the Human Ethical Committee of the Xinqiao Hospital, 20 patients aged 2 to 22 years (mean 9.2 years) undergoing primary cardiac surgery were enrolled in this prospective study. Ten patients had a cyanotic and ten had an acyanotic cardiac defect. This study was carried out among patients who were diagnosed with congenital heart disease with right ventricular outflow tract obstruction and had cardiac operations in Department of cardiovascular surgery of Xinqiao Hospital between 2008.01 and 2008.12. Table 1 shows the patients' clinical data. The study confirms with the principles outlined in the Declaration of Helsinki.

Cardiac operation and sampling of myocardial biopsies

Drugs given for premedication and conventional general anesthesia consisted in all cases of midazolam, fentanyl sulfate and pancuronium bromide. After institution of cardiopulmonary bypass (CPB), the aorta was cross-clamped and cardiac arrest instituted by intra-aortal injection of a 4°C cold cardioplegic solution. A biopsy was taken from the right ventricular outflow tract immediately after aortic clamping. Myocardial Samples taken for ultra-structural evaluation were immediately minced into 1–2 mm size pieces, fixed for 12 h in cold 2.5% glutaraldehyde. Samples taken for RT-PCR, PCR and Western blotting were immediately snap-frozen in liquid nitrogen and stored at –70°C until analysis.

Electron microscopic study

The glutaraldehyde fixed samples were post-fixed in osmium tetroxide; they were then embedded in epoxy resin. Ultrathin sections were stained with saturated uranyl acetate and lead citrate. Ten electron micrographs of randomly chosen fields were obtained from each sample. Mitochondrial volume density (Vv), numerical density (Nv) and mean volume (V) were determined at ×89.00 magnification according to the method of Nouette-Gaulain et al.[9] as follows. Vv is the relative volume fraction of the mitochondria determined as the relative surface fraction of the unit area comprised by all mitochondrion slices. Nv depends on mitochondria sections (NA) counted per standard measuring surface, on the size distribution (K) and, finally on the shape of the mitochondria, writing $Nv=K.NA^{3/2}/\beta.Vv^{1/2}$. The form coefficient b for ellipsoidal structures is a function of the longitudinal and transverse diameter. On the basis of previous studies it was assumed to be $\beta=2$ in the heart[10]. K is determined by the size distribution of the objects. According to previous studies in chronic hypoxic heart, it was assumed to be $K=1.1$ in the present study[10]. V is the mean volume per mitochondria obtained by simple division of Vv by Nv.

Relative mtDNA/nDNA ratio

Total genomic DNA was isolated from Myocardial Samples with the TIANamp Genomic DNA kit (Tiangen, China) according to the instructions of the manufacturer. MtDNA content relative to nuclear DNA was determined utilizing a quantitative real-time PCR performed in an ABI PRISM7900 Sequence Detection System. Specific primers were used to amplify an 86bp fragment of mitochondrial cytochrome-b or a 207bp fragment of nuclear genomic Ribosomal protein L10A gene. PCR reactions were performed as following: 95°C for 60s; 45 cycles at 95°C 15s, 60°C 15s, 72°C 45s. PCR assays were performed in triplicate for each DNA sample. The expression of mtDNA copy number relative to nuclear DNA (relative mtDNA/nDNA ratio) was determined using the $2^{-\Delta Ct}$ method[11].

Quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was extracted from Myocardial Samples using Trizol reagent (Invitrogen, USA) following the manufacturer's instructions. Reverse transcription was performed using 2 μg of RNA. First-strand cDNA synthesis was accomplished with BioRT cDNA First Strand Synthesis Kit (Bioer, China). Real-time RT-PCR amplification was performed in 20 μl of reaction mixture containing 1μl of cDNA sample, 10μl of SYBR Green Realtime PCR Master Mix (Toyobo, Japan), and 0.25μM of specific primer sets by the use of an ABI PRISM 7900 Sequence Detection System (PE Applied Biosystems, USA). Real-time RT-PCR was carried out starting with 60s at 95°C followed by a

Table 1 – Patients' data

	Acyanotic group (*n*= 10)	Cyanotic group (*n*= 10)	p-value
Gender (male/female)	6/4	4/6	NS
Age (years)	8.8 ± 5.9	9.5 ± 5.7	NS
Diagnosis	VSD with RVOT obstruction: *n*= 10	TOF: *n*= 9 DORV: *n*= 1	
Preoperative SaO_2(%)	95.2 ± 2.3	85.2 ± 3.4	p<0.01

Data are presented as mean±SD. SaO_2: transcutaneous arterial oxygen saturation; Acyanotic group: acyanotic congenital cardiac defects; Cyanotic group: cyanotic congenital cardiac defects; VSD: ventricular septum defect; RVOT: right ventricular outflow tract; TOF: tetralogy of Fallot; DORV: double outlet right ventricle; NS not significant.

denaturation step at 95°C for 15 s, an annealing step at 60°C for 15 s, and an extension step at 72°C for 45s for 45 cycles. Dissociation curve analysis was performed at the end of 45 cycles to verify PCR product identity. We drew the standard curve of each gene between a relative concentration and the threshold cycle of a 10-fold dilution series of specific PCR product. Each sample was measured by triplicates of cDNA templates and the expression level was calculated as the ratio of the mean value of the triplicate relative expression of each sample to the relative expression of Ribosomal protein L10A (RPL10A) as an internal control. Sequence Detector Systems Version 1.3.1 software (Applied Biosystems) was used for data analysis.

Western Blot analysis

Myocardial Samples were lysed in a buffer containing 10mM Tris (pH 7.5), 150mM NaCl, 1mM EDTA, 1mM EGTA, 50mM NaF, 0.5mM phenylmethylsulfonyl fluoride, 1mM sodium vanadate, 1% Triton X-100, 0.5% nonidet P-40, and 1μg/mL of leupeptin and aprotinin. The homogenate was centrifuged for 5min, and the supernatant was frozen and stored at 80°C. A BCA protein assay (Sangon, China) was used to determine protein content. Tissue lysate (100–200μg) was subjected to SDS–polyacrylamide gel electrophoresis, and protein was subsequently transferred to a PVDF membrane (Whatman Westran, UK). The membrane was pre-incubated in a buffer containing 10mM Tris (pH 7.4), 150mM NaCl, and 0.1% Tween supplemented with 5% skim milk for 1h at room temperature. Following pre-incubation, the membrane was incubated in the Tris–NaCl–Tween buffer supplemented with 5% bovine serum albumin and antibody for 1–2h at room temperature. Antibodies employed were a mouse monoclonal antibody against cytochrome c oxidase I, a rabbit polyclonal antibody against nNOS, a rabbit polyclonal antibody against iNOS, a rabbit polyclonal antibody against eNOS(all of them 1:1000, Santa Cruz, USA) and a rabbit polyclonal antibody against β-actin(1:1,000; Biosynthesis Biotechnology, Beijing, China). Following incubation, the membrane was washed with buffer for 1h, and re-probed with the appropriate secondary antibody conjugated to horseradish peroxidase (1:1,000; Biosynthesis Biotechnology, Beijing, China) in Tris–NaCl–Tween buffer supplemented with 5% skim milk for 1h at room temperature. Following incubation, the membrane was washed three times with the Tris–NaCl–Tween buffer and bands visualized by an ECL detection kit (ShineGene, Shanghai, China). Films were scanned with a laser densitometer utilizing the program Quantity One (Bio-Rad Laboratories, Canada). Relative levels of protein were standardized using β-actin as the internal control.

Statistical analysis

Results are expressed as the mean value ± SD. Normality tests were performed. The independent samples T test was used to analyze differences between patients with cyanotic or acyanotic cardiac defects. Correlation of independent parameters was assessed by the Spearmen correlation test. P-values<0.05 were considered significant. Data were analyzed with the Statistical Package for Social Sciences version 10.0.

Results

Table 1 summarizes the patients' epidemiological data. Patients with cyanotic cardiac defects had a significantly lower preoperative arterial oxygen saturation (SaO_2) than patients with acyanotic cardiac defects ($p<0.01$).

Morphometric analysis

Myocardial specimens were found to be histologically normal in most acyanotic patients. These specimens exhibited an orderly arrangement of myofibril and sarcomeres, well-distributed mitochondrion and normal Z-bands. In cyanotic patients, myofibrillar disorganization, shortening of sarcomeres, focal accumulation of mitochondrion with inequality of size and shape, intramitochondrial deposits and abnormal Z-bands and intercalated discs were observed (Figures 1 and 2). Mitochondrial volume density Vv was significantly higher in cyanotic patients than in acyanotic patients (46.47±9.73% *vs.* 34.80±10.42%, $p<0.05$). Mitochondrial numerical density Nv was also significantly higher in cyanotic patients than in acyanotic patients ($1.08\pm0.17\mu m^{-3}$ vs. $0.73\pm0.11\mu m^{-3}$, $p<0.05$).

Gene expression profile

The relative mtDNA/nDNA ratio of cyanotic patients was higher than the ratio of asyanotic patients (1.56±0.18 *vs.* 0.97±0.17, $p<0.05$). After normalization with the Rpl10a gene, mRNA expression levels in each sample were estimated as a relative expression level in comparison with the reference sample. The mRNA level of COXI was significantly increased, while the transcript levels of several key regulators of mitochondrial biogenesis (PGC-1α, NRF1 and Tfam) were coordinately up-regulated in cyanotic patients comparing with asyanotic patients($p<0.01$, Figure 3). Considering all patients, myocardial levels of PGC-1α mRNA correlated inversely with SaO_2 (Spearman correlation coefficient:–0.75, $p<0.01$; Figure 4), suggesting that hypoxemia is an important trigger for PGC-1α expression in patients with cyanotic congenital heart disease.

Protein levels

Western blot analysis revealed that COXI levels were significantly higher in the right ventricular myocardium of patients with cyanotic than in those with acyanotic cardiac defects ($p<0.05$). Very low amount of nNOS protein was detected in all myocardial samples and no difference was observed between two groups. There was no difference between the iNOS protein levels of cyanotic and acyanotic patients. Levels of eNOS were increased in cyanotic patients comparing with acyanotic patients ($p<0.05$, Figure 5). Considering all patients, myocardial concentrations of eNOS correlated inversely with SaO_2 (Spearman correlation coefficient: -0.64, $p<0.01$, Figure 6), and the PGC-1α transcript levels correlated with the levels of eNOS (Spearman coefficient: 0.58, $p<0.01$, Figure 7).

Discussion

The capacity to modulate mitochondrial number and the density of electron transfer chain complexes per unit mitochondria has been demonstrated at ultrastructural,

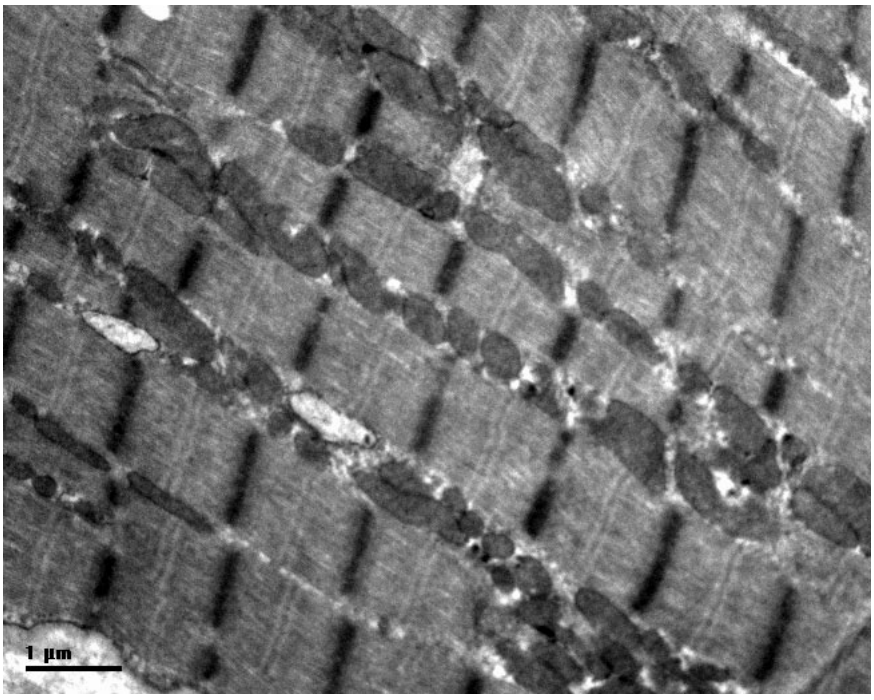

Figure 1 - *Transmission electron micrograph of mitochondria in RVOT of an acyanotic patient (×8900, bar 1 µm).*

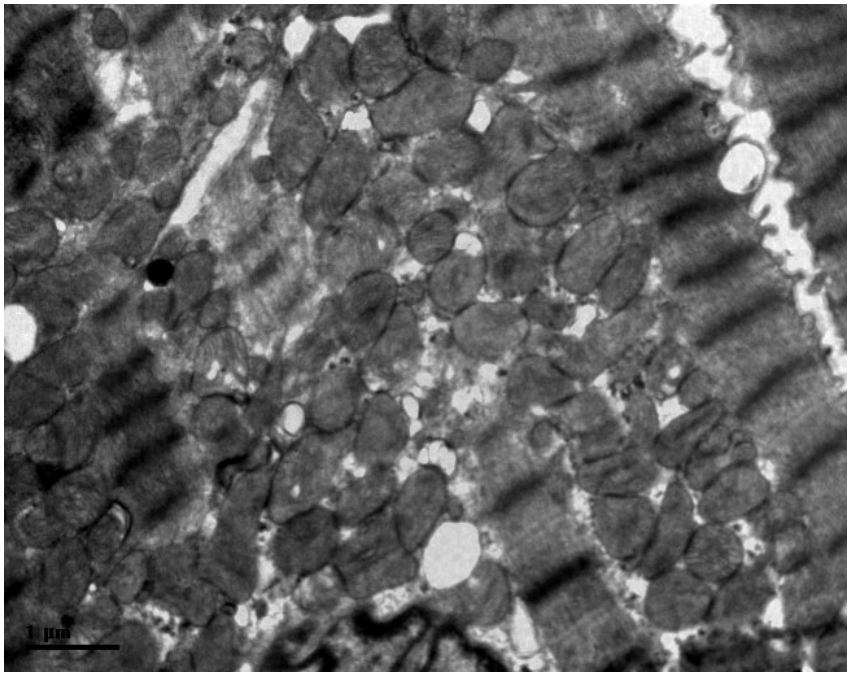

Figure 2 - *Transmission electron micrograph of mitochondria in RVOT of a cyanotic patient (×8900, bar 1 µm). Mitochondrial volume density Vv and numerical density Nv were significantly higher in cyanotic patients than in acyanotic patients ($p<0.05$).*

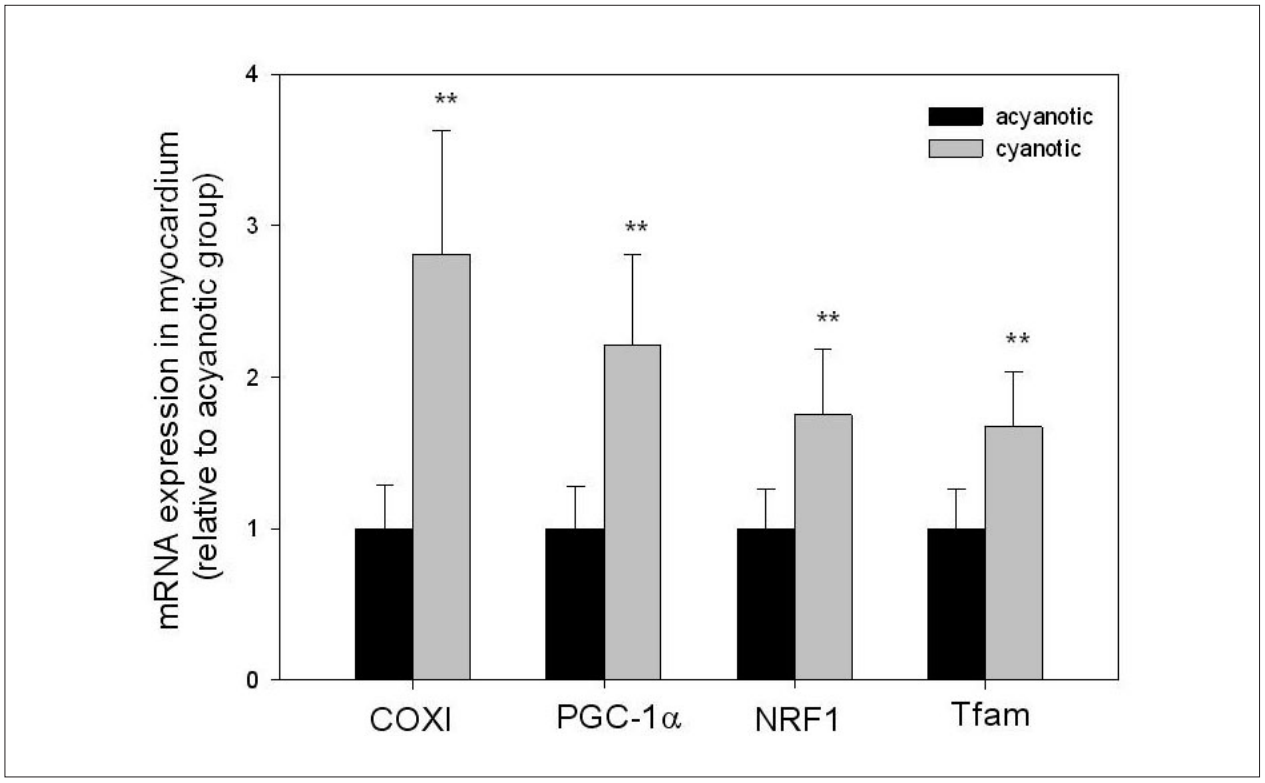

Figure 3 - *Comparisons of relative expression of myocardium mRNA between the acyanotic group and cyanotic group (**p<0.01 versus acyanotic) group).*

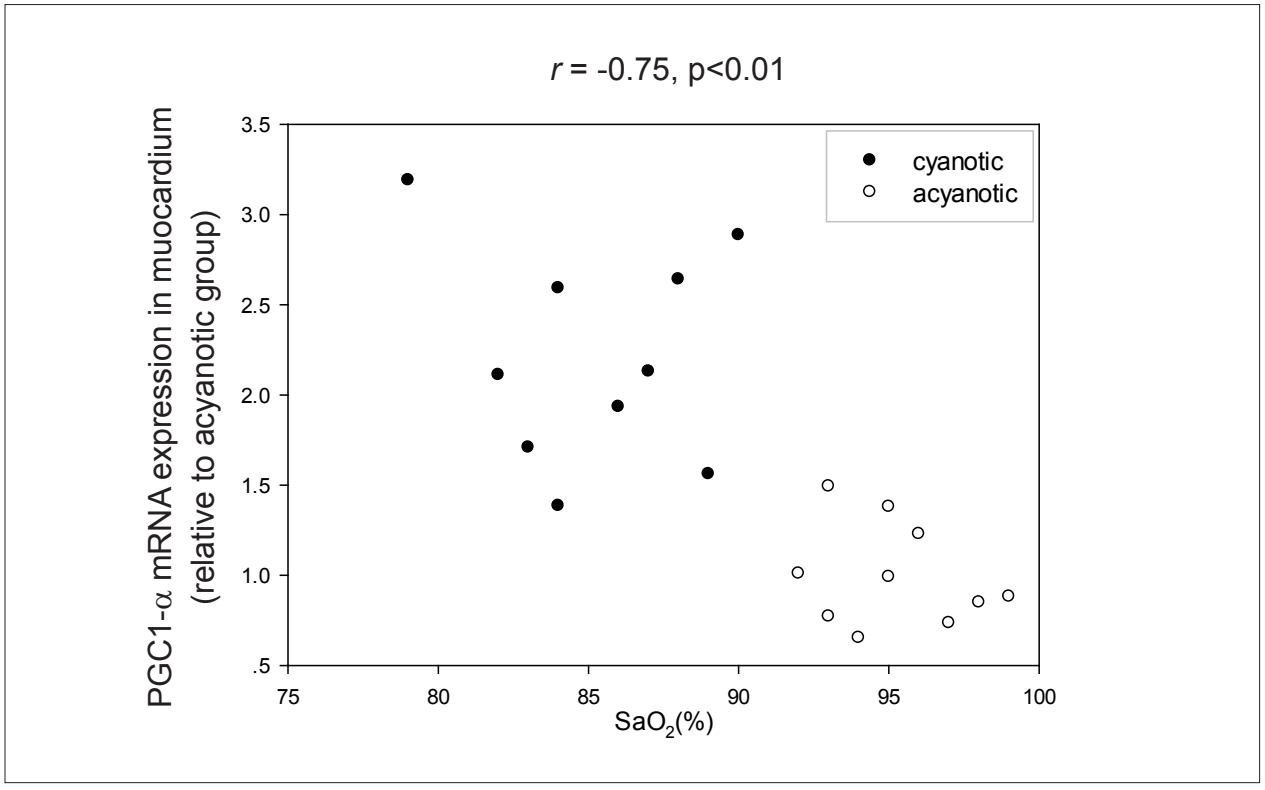

Figure 4 - *Correlation between PGC1-α mRNA expression in myocardium and SaO_2.*

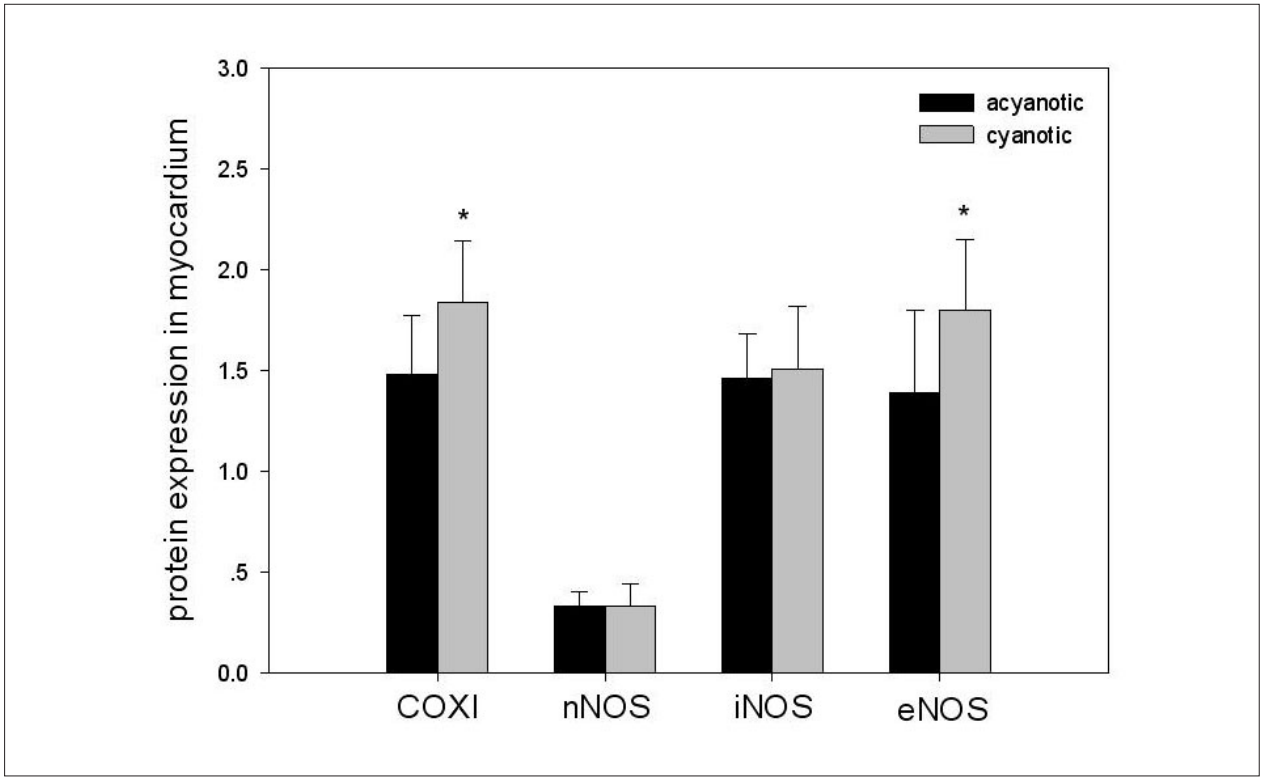

Figure 5 - *Comparisons of protein expression in myocardium between the acyanotic group and cyanotic group (*p<0.05 versus acyanotic group).*

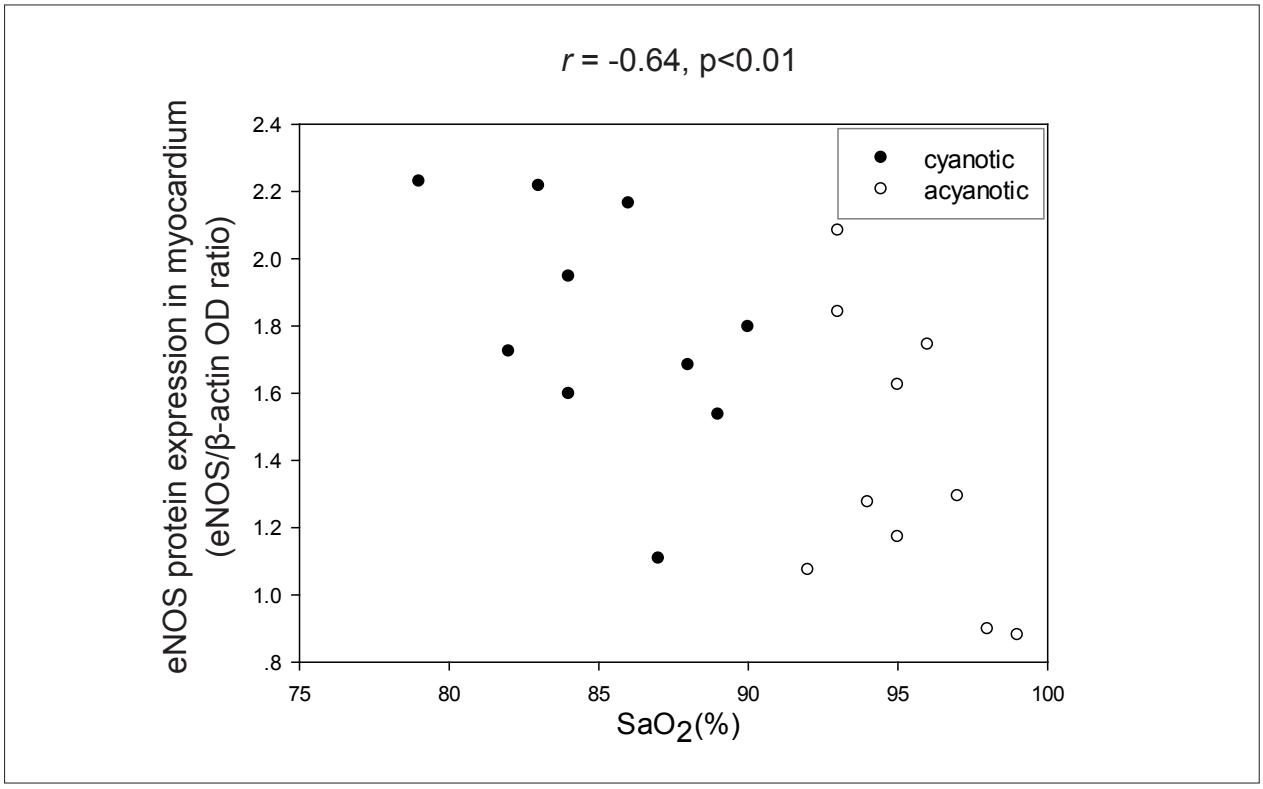

Figure 6 - *Correlation between eNOS protein expression in myocardium and SaO_2.*

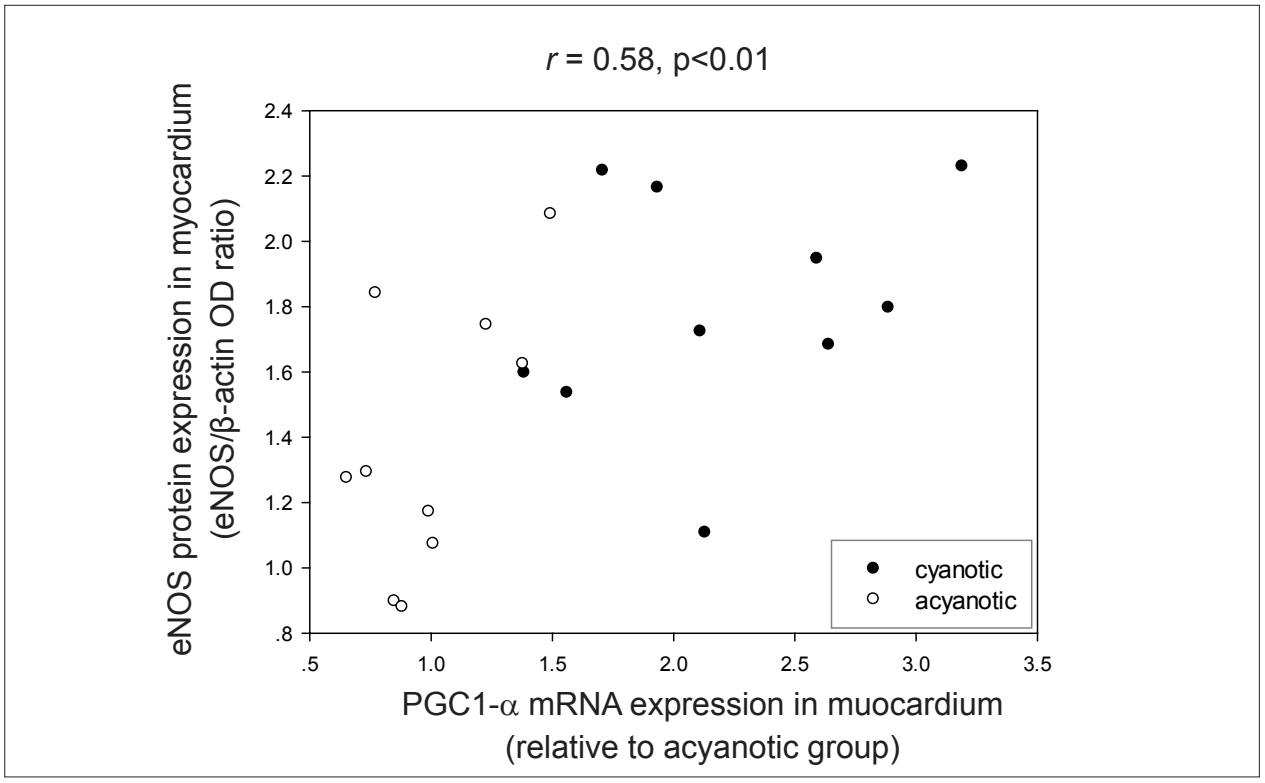

Figure 7 - *Correlation between PGC1-α mRNA and eNOS protein expression in myocardium.*

biochemical, and molecular regulatory levels. In the heart, in parallel with developmental changes in oxygen tension, hemodynamic load, and substrate preference, mitochondrial biogenesis is accelerated in the prenatal period with a peak in young adulthood with a subsequent decline during senescence[12]. Adaptive plasticity has been demonstrated by increased cardiac mitochondrial mass with a parallel induction in energy-generating capability following hypobaric hypoxia[13]. In this study, we demonstrated that mitochondrial volume density (Vv) and numerical density (Nv) were elevated in patients with cyanotic compared to acyanotic congenital heart disease, suggesting there were more mitochondria in myocardium of patients with cyanotic congenital heart disease. This response was accompanied by significantly increased mtDNA copy number and up-regulated COXI mRNA and protein expression, which was encode by mtDNA. Induction of genes involved in mitochondrial biogenesis was also observed in cyanotic group. The increase in mitochondrial mass, mtDNA, mRNA and protein demonstrated that the mitochondrial biogenesis program is up-regulated in cyanotic patients' hearts. Furthermore, mRNA of PGC-1α, the key regulator of the mitochondrial biogenic program, correlated inversely with SaO_2, suggesting that hypoxemia is an important trigger for mitochondrial biogenesis in patients with cyanotic congenital heart disease.

In skeletal muscle, mitochondrial proliferation has been shown to partly compensate for the respiratory dysfunction by maintaining overall ATP production[14]; the histopathologic hallmark of this phenomenon is the presence of an increased number of mitochondria, mostly in the subsarcolemmal region. However, in cardiac muscle, induction of mitochondrial biogenesis has been proposed as a maladaptive response in mitochondrial cardiomyopathies[15]. In fact, cardiac-specific induction of PGC-1α in mice results in cardiac dysfunction with morphologic features of myocyte mitochondrial proliferation and myofibrillar disorganization and loss. Intriguingly, both mitochondrial proliferation and cardiomyopathy are reversible upon cessation of transgene expression[16]. Our findings that mitochondrial biogenesis was up-regulated in cyanotic patients' hearts may be a compensatory mechanism of mitochondria in myocardium. Proliferated mitochondria may improve diffusion and utilization of limited oxygen with enlarged surface area. However, proliferated mitochondria may also affect reactive oxygen species (ROS) derived from mitochondria and apoptosis pathways. Therefore, whether this adaptation is beneficial to cardiac function remains unexplained and additional work is required to fully address this issue.

In the present study, we found that intramyocardial levels of eNOS were significantly elevated in patients with cyanotic congenital heart disease and correlated inversely with SaO_2. Although in a previous study eNOS-mRNA levels were reduced and iNOS-mRNA levels were increased in right atrial tissue from children with cyanotic compared to those with acyanotic congenital heart disease[17], several studies have now confirmed that hypoxia can activate eNOS and increase eNOS expression in the heart[18]. Recently, a hypoxia-responsive element has been identified in the human eNOS promoter[8] thus linking eNOS expression to HIF, the major effect of mediating hypoxic adaptive responses.

Another interesting result of the present study is the observation of an association between PGC-1α transcript levels, which present the mitochondrial biogenesis level, and myocardial levels of eNOS in patients with congenital heart disease. Chronic hypoxia-induced myocardial protection against ischemia reperfusion injury has been confirmed by several groups[19]. Consistent with augmented bioenergetic capacity as a consequence of mitochondrial biogenesis, mitochondria extracted from hypoxic hearts show increased rates of ATP synthesis[20]. Moreover, hypoxia-induced cardioprotection is associated with the upregulation of nitric oxide synthase (NOS) and is abolished by NOS inhibition[21]. Since the capacity of NO to activate the transcriptional machinery driving mitochondrial biogenesis has been established, our observation of the association between PGC-1α transcript levels and myocardial levels of eNOS may imply that eNOS and mitochondrial biogenesis play important roles in chronic hypoxia-induced myocardial protection against ischemia reperfusion injury[22].

Conclusions

The present results suggest that mitochondrial biogenesis is activated in right ventricular outflow tract myocardium in congenital heart disease with cyanosis, which could be the adaptive response to chronic hypoxia and possibly involves eNOS up-regulation.

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Potential Conflict of Interest

No potential conflict of interest relevant to this article was reported.

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Study Association

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