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Hypoxia-induced Suppression of c-Myc by HIF-2 α in Human Pulmonary Endothelial Cells Attenuates TFAM Expression

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Abstract

The adaptive response to hypoxia is mediated in large part by stabilization of the hypoxia-inducible factors, HIF-1 α and HIF-2 α . A hallmark of this response is the metabolic shift to decreased oxidative phosphorylation and increased glycolysis. We hypothesized that hypoxic responses would include a suppression of mitochondrial gene expression. We determined the effects of hypoxia on TFAM, a key mitochondrial transcription factor, in normal pulmonary artery endothelial cells. Hypoxia decreased gene expression of *TFAM* and that of its upstream regulator, the transcriptional co-activator *PGC1 β* . Although HIF-1 α and HIF-2 α pathways both contributed to hypoxia-mediated *PGC1 β* suppression, *TFAM* suppression was regulated solely by HIF-2 α -dependent mechanisms. We found that HIF-2 α suppresses TFAM by decreasing c-Myc expression. In addition, we show a role for c-Jun in this pathway, linking HIF-2 α with attenuation of c-Jun activation. Taken together, these findings establish a new link between HIF-2 α and MAPK-signaling that mediates the adaptive regulation of mitochondrial gene expression under low oxygen tension.

Keywords

Hypoxia inducible factors; endothelial cells; TFAM; c-Myc; c-Jun

1. Introduction

Hypoxia inducible factors, HIF-1 α and HIF-2 α , mediate the cellular response to reduced oxygen tension. Under normal oxygen tension, HIF-1 α and HIF-2 α are targeted for proteasomal degradation following their hydroxylation by O₂-dependent prolyl hydroxylases [1,2]. Under hypoxic conditions, the stable HIF- α isoforms translocate to the nucleus where they heterodimerize with HIF- β (also called the aryl hydrocarbon receptor nuclear translocator) to promote transcription of genes with HIF-response-elements (HRE) [3]. The

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transcriptional program regulated by active HIF factors mediates a metabolic shift characterized by decreased oxygen consumption and increased glycolytic capacity. HIF-dependent actions, such as the regulation of specific miRNAs (hypoxamirs), coordinate the hypoxia-mediated decrease in electron transport with the reduced availability of molecular oxygen [4,5]. The suppression of mitochondrial function has been associated with a protective effect, possibly due to the decreased production of reactive oxygen species in cells with decreased mitochondrial content [5,6]. Thus, we were interested in understanding additional pathways by which HIF factors regulate mitochondrial homeostasis in endothelial cells.

TFAM is a mitochondrial transcription factor that regulates expression of genes encoded by mitochondrial DNA [7]. Its expression has been shown to be essential for mitochondrial biogenesis and mitochondrial function [8,9,10]. Transcription of TFAM, which is encoded by the nuclear genome, is driven by NRF-nuclear factors and facilitated by NRF interactions with peroxisome proliferator-activated receptor γ coactivator family members (PGC1 α , PGC1 β , and PRC1) [11]. Among their actions, PGC1/nuclear factor complexes orchestrate the coordinate expression of genes in the nuclear and mitochondrial genome to regulate mitochondrial biogenesis and metabolic responses to physiological stimuli [12,13]. In a renal carcinoma cell line (RCC), decreased mitochondrial content and ATP production were attributed to HIF-1 α -mediated inhibition of c-Myc, a nuclear transcription factor that has been found to regulate *PGC1 β* transcription [14]. The c-Myc protein plays an important role in regulating the cell cycle, and coordinates increased cellular proliferation and mitochondrial biogenesis independent of PGC1 cofactors [15,16]. In addition, c-Myc can drive tumorigenesis, and c-Myc was initially referred to as the V-Myc, avian myelocytomatosis viral oncogene homolog, due to its oncogenic properties. Nonetheless, there is a complex relationship among HIF factors and c-Myc. Thus, RCC cells with normoxic upregulation of HIF-2 α rather than HIF-1 α have increased *MYC* transcription [17,18], suggesting that HIF-1 α and HIF-2 α have opposing effects on c-Myc expression, and, consequently, may differentially regulate *PGC1 β* expression. Notably, the relationship among HIF factors and c-Myc in endothelial cells and how they contribute to TFAM expression are unclear.

Here, we explored the pathways involved in the hypoxia-mediated suppression of TFAM. Our findings demonstrate a role for PGC1-dependent and -independent pathways in modulating endothelial *TFAM* gene expression. Furthermore, our findings suggest a predominant role for HIF-2 α in the hypoxia-mediated suppression of *TFAM* via its role in modulating c-Jun activation to regulate c-Myc expression.

2. Materials and methods

2.1. Cell culture, treatments and transfections

Human pulmonary artery endothelial cells (HPAEC) were cultured in EBM-2 media (Lonza) supplemented with EGM-2 (Lonza) without antibiotics at 37°C in 5% CO₂. Experiments were performed between passage five and eight with cells 70–80% confluent. For hypoxia studies, cells were treated for 24 or 42 h with 0.2 % O₂, 5 % CO₂, with N₂ balance at 37°C

using a modular hypoxia chamber. SP600125 was purchased from Cell Signaling and resuspended in dimethyl sulfoxide as recommended by the manufacturer.

Transfections with small interference RNA (siRNA) were performed using Lipofectamine® 2000 (Life Technologies) and custom stealth siRNAs for PGC1 α (sense: 5' - GAACGACUUGGAUACAGACAGCUUU); PGC1 β (sense: 5' - GGAGCAACUCUAUGCUGACUUUCCA); HIF-1 α (CACCAAAGUUGAAUCAGAAGAUACA); HIF-2 α (sense: 5' - CAGGUGGAGCUAACAGGACAUAGUA); c-Myc (sense: 5' - CCAACAGGAACUAUGACCUCGACUA); or control siRNAs (5' - GAAUCAGGUUAUAGACACACGCGUUU for PGC1 α experiments; 5' - GGAUCAAUAUCGUCGUUCAUCGCCA for PGC1 β experiments; or 5' - CACGAAGUUUAAGACGAUAACAACA for all other experiments). Cells were transfected, then 48 h later media was exchanged and plates were exposed to hypoxia or maintained under normal O₂ for an additional 24 or 42 h prior to harvesting, as indicated in the figure legends.

To overexpress c-Myc, cells were infected with the Ad-c-myc adenovirus (Vector Biolabs) for 24 h prior to hypoxia. An empty adenovirus (Ad-CMV-null, Vector Biolabs) was used as a vector control.

2.3. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated using RNeasy Mini kit (Qiagen), and the Advantage RT-for-PCR kit (Clontech) was used for cDNA synthesis. The PRISM 7900 HT Sequence Detector (Applied Biosystems) was used to perform real time TaqMan Assays with the TaqMan Universal PCR Master Mix (Invitrogen). The Ct method of relative quantification was used for data analysis, with *ACTB* (β -actin) expression as the endogenous control. To compare relative mRNA levels between *PGC1 α* and *PGC1 β* transcripts, the comparative Ct method was used [19], normalizing data to *ACTB* gene expression. For the Ct method, 0.5 μ g mRNA was used in cDNA synthesis; for the Ct method 12 μ g mRNA was used in cDNA synthesis.

2.4. Western-blot analysis

Whole cell lysates were prepared in cell lysis buffer with protease inhibitors, or lysates were prepared using a nuclear and cytoplasmic extraction kit (Active Motif, Carlsbad, CA). Proteins were separated by SDS-PAGE-electrophoresis and transferred to PVDF membranes using a semi-dry transfer apparatus. Membranes were exposed to primary antibodies overnight. Detection was accomplished using HRP-linked secondary antibodies and the ECL detection system (Amersham Biosciences). Band density on X-ray films was quantitated with the VersaDoc scanning system and accompanying software (BioRad).

2.4. Statistical analysis

Means were determined from 3 or more independent experiments. Data were tested for the normal distribution as determined by the Shapiro-Wilk normality test followed by analysis by parametric tests. Significant changes were determined by t-test when only 2 groups were present. Otherwise, significant changes were determined by ANOVA or two-way ANOVA,

followed by post-hoc Student-Newman-Keuls analysis where $p < 0.05$ was accepted as significant. GraphPad Prism 7 and StatPlus software packages were used for statistical analysis.

3. Results

3.1. Hypoxia suppresses the expression of TFAM and PGC1 β in human pulmonary artery endothelial cells

Hypoxia significantly decreased *TFAM* gene expression by 30 % after 24 h ($p < 0.001$) (Fig. 1A). At 42 h hypoxia *TFAM* RNA levels remained significantly lower (44% of normoxic levels, $p < 0.005$), and *TFAM* protein was decreased by 34 % ($p < 0.005$) (Fig. 1B). Previous findings suggest that *TFAM* expression is regulated by the nuclear coactivators PGC1 α and PGC1 β . We found that hypoxia suppressed *PGC1 β* gene expression by approximately 70 % ($p < 0.0005$) (Fig. 1A) and that *PGC1 β* transcripts were more abundant than *PGC1 α* transcripts in HPAEC. The Ct values for *PGC1 α* were > 40 using 0.5 μ g of RNA as starting material in qRT-PCR. To detect *PGC1 α* transcripts reproducibly, we prepared cDNA from 12 μ g of RNA. Hypoxia increased *PGC1 α* gene expression approximately 2-fold ($p < 0.02$) (Fig. 1C). We next compared the relative Ct for *PGC1 β* and *PGC1 α* in qRT-PCR performed on the concentrated cDNAs (Fig. 1C). The Ct for *PGC1 β* transcripts was $7.2 \times 10^{-3} \pm 9.7 \times 10^{-4}$ and was $1.2 \times 10^{-6} \pm 3.3 \times 10^{-7}$ for *PGC1 α* ; suggesting *PGC1 β* transcripts are approximately 3-orders of magnitude more abundant than *PGC1 α* transcripts. Targeted knockdown of PGC1 β alone significantly decreased *TFAM* expression by 41.6 %, and the effect of hypoxia on this suppression was additive (Fig. 1D). In contrast, knockdown of *PGC1 α* decreased *TFAM* expression by 10.6% under normoxic conditions but did not augment hypoxia-mediated suppression of *TFAM* (Fig. 1D). Taken together, these findings suggest that PGC1-independent pathways may contribute to the suppression of *TFAM* under hypoxia.

3.2. HIF-1 α and HIF-2 α differentially regulate TFAM and PGC1 β gene expression

In endothelial cells, 24 h of hypoxia promoted the stability of HIF-1 α as well as HIF-2 α (Fig. 2A). To determine whether HIF-1 α and/or HIF-2 α regulate hypoxia-mediated suppression of *TFAM*, we used siRNA to knockdown their expression (Fig. 2B). Knockdown of HIF-1 α or HIF-2 α individually failed to eliminate hypoxia-mediated suppression of *PGC1 β* , whereas the simultaneous knockdown of both HIF-1 α and HIF-2 α blocked hypoxia-mediated suppression of *PGC1 β* . In contrast, loss of HIF-2 α alone was sufficient to block hypoxia-mediated *TFAM* gene suppression.

3.3. c-Myc expression and TFAM

c-Myc is another biogenic factor that has been shown to regulate mitochondrial gene expression in some cells. We found that hypoxia decreased nuclear c-Myc protein levels by 27 % ($p < 0.001$) (Fig. 3A) in HPAEC; therefore, we considered whether c-Myc regulated *TFAM* expression in these cells. Knockdown of c-Myc significantly decreased *TFAM* gene expression by 77 %, under normoxia, and hypoxia had no additional effect on the suppression of *TFAM* gene expression (Fig. 3B). Overexpression of c-Myc significantly increased *TFAM* gene expression by 50% and 75%, respectively, ($p < 0.05$) in normoxia and

hypoxia, compared to AdCtrl-treated cells grown under these conditions (Fig. 3C). Consistent with these findings, knockdown of c-Myc significantly reduced and overexpression of c-Myc significantly increased TFAM protein levels (Fig. 3D & E).

3.4. HIF-2 α , c-Jun activation, and c-Myc

We next examined whether HIF-2 α regulated c-Myc expression. At the gene expression level, knockdown of HIF-2 α increased c-Myc mRNA, whereas the lack of HIF-1 α had no effect on c-Myc gene expression (Fig. 4A). In nuclear extracts, absence of HIF-1 α increased HIF-2 α expression and significantly reduced c-Myc expression whereas the knockdown of HIF-2 α restored c-Myc expression even in the absence of HIF-1 α (Fig. 4B & C).

We next examined how HIF-2 α regulates *MYC* gene expression. Previous studies suggest that AP-1, a nuclear factor that consists of Fos/Jun and Jun/Jun family dimers, promotes c-Myc gene expression [20]. Furthermore, the phosphorylation of c-Jun at ser63 (phJun) is known to enhance AP-1 transactivation. We found that after 24 h of hypoxia, the levels of phJun in nuclear extracts are significantly increased in cells lacking HIF-2 α compared to cells treated with siCtrl or those with a knockdown of HIF-1 α alone (Fig. 5A). Under these conditions, levels of c-Jun were not significantly changed. To test whether c-Jun activation contributes to the apparent upregulation of c-Myc in the absence of HIF-2 α , cells were treated with 25 μ M SP600125, an inhibitor of the upstream c-Jun N-terminal kinase (JNK), in order to block Jun phosphorylation. The JNK inhibitor significantly decreased phJun in hypoxic cells (Fig. 5B) and attenuated the up-regulation of c-Myc in siHIF-2 α transfected cells, suggesting that HIF-2 α mitigates *MYC* expression by suppressing Jun activation.

4. Discussion

Hypoxia plays an important role in the pathobiology of heart disease, stroke, chronic lung disease, and cancer, and is an important stressor to which cells have evolved a range of adaptive responses. *In vivo*, the response of endothelial cells to hypoxia are essential for survival following ischemia as these cells mediate angiogenic responses that restore blood flow, as has been shown in myocardial ischemia models [21,22,23]. Thus, conditions that attenuate normal HIF-upregulation following cardiac ischemia are associated with enhanced tissue damage, whereas those that promote HIF induction have a better prognosis, in part, due to the preservation of myocardial capillary density [22,24]. Similarly in a recent study, hypoxic treatment following myocardial infarction stimulated cardiac regeneration and preserved endothelial function, in part, due to enhanced capillary growth [25]. Interestingly, in this model system, hypoxia-induced cardiac regeneration correlated with HIF stabilization and decreased cardiomyocyte mitochondrial content and function, as well as decreased reactive oxygen species production. Recent studies suggest that HIF-2 α -mediated pathways in endothelial cells may contribute to the pathogenesis of pulmonary hypertension [26], a condition where prolonged chronic hypoxia promotes increased vascular resistance, pulmonary vascular remodeling, and, ultimately, right heart failure, in part, due to the stabilization of HIF. Thus, understanding how endothelial cells respond to hypoxia may provide insights into adaptive responses as well as disease pathology.

Many of the effects of hypoxia are mediated via activation of HIF-dependent programs that contribute to a metabolic shift during hypoxia [4,5]. Regulation of specific miRNAs (hypoxamirs) has been shown to contribute to these hypoxic responses [4,5]. In particular, miR210 suppresses the expression of iron-sulfur cluster assembly proteins, decreasing the activity of iron-sulfur-dependent redox-active proteins, including that of mitochondrial complex I [5]. We were interested in other pathways that coordinately regulate mitochondria in noncancerous cells, and found that hypoxia significantly decreased the expression of TFAM. Therefore, we focused the current study on how the HIF factors control the expression of TFAM, a major regulator of mitochondrial function [8,9,10].

PGC1 co-activators synchronize the responses of nuclear and mitochondrial genes. In our cell system, in contrast to *PGC1 β* , *PGC1 α* gene expression was barely detectable (Ct values >40) when we used our standard qRT-PCR method with 0.5 μ g RNA for cDNA production and undiluted cDNA in TaqMan assays with several different gene-specific probes. To improve the detection of this transcript, we used 12 μ g of RNA in cDNA synthesis and then used these concentrated cDNAs undiluted for quantitation. This strategy decreased within-sample variability and generated Ct values between 36–40 for *PGC1 α* . Under the same conditions, *PGC1 β* probes had Ct values between 24–26 and the mean 2^{-Ct} for *PGC1 β* was 3-orders of magnitude greater than that for *PGC1 α* transcripts.

PGC1 α is thought to play a major role in mediating mitochondrial biogenesis in many cell types [27,28,29,30,31]. In peripheral blood mononuclear cells from chronic kidney disease patients, *PGC1 α* gene expression was found to be decreased along with that of TFAM and other nuclear encoded mitochondrial genes [30]. Interestingly, the gene expression of nuclear respiratory factor-1 (*NRF1*), a transcription factor that works together with the cofactors PGC1 α and PGC1 β [32] to regulate mitochondrial biogenesis, was also downregulated in these cells, and the expression of PGC1 β was not determined in this study. In retinal tissue from rats, diabetes increased *PGC1 α* gene expression concurrently with an upregulation of *TFAM* gene expression, and similar changes were found in bovine retinal endothelial cells exposed to high glucose [33,34]. In diabetic mouse models, *PGC1 α* gene expression was upregulated in endothelial cells isolated from other vascular beds and, in culture, high glucose upregulated endothelial cell expression of PGC1 α by more than 5-fold [35]. Consequently, upregulation of PGC1 α caused by the diabetic phenotype or by forced transgenic overexpression inhibited endothelial cell migration in cultured cells and attenuated angiogenesis *in vivo* [35], whereas the presence of the angiogenic factor VEGF significantly augmented migration while suppressing the expression of *PGC1 α* . Although it is unclear why levels of *PGC1 α* are low compared to *PGC1 β* in our cell system, our studies were carried out in the presence of media that contains supplemental VEGF as well as other growth factors. It is also worth noting that the expression and function of PGC1 β was not assessed in the earlier studies of PGC1 α in diabetes cited above. Regardless, PGC1 β and PGC1 α regulate similar, but not identical, functions and their roles may depend on the system studied. Thus, in *PGC1 β* knockout mice there was a significant decrease in the mitochondrial content of heart and muscle, and alterations in fat metabolism in liver in mice lacking PGC1 β [27]; however, in adipose tissue, the change in mitochondrial mass caused by PGC1 β deficiency was minor, possibly due to the upregulation of *PGC1 α* expression in PGC1 β -deficient fat cells. Consistent with the relatively high level of *PGC1 β* expression in

our cells, *PGC1 β* knockdown decreased TFAM gene expression by over 40%, whereas knockdown of *PGC1 α* decreased TFAM gene expression (modestly) by approximately 10%. Interestingly, hypoxia had opposite effects on *PGC1 α* and *PGC1 β* gene expression in our cell system, upregulating *PGC1 α* and decreasing *PGC1 β* .

Pathways for HIF- α -mediated suppression of *PGC1 β* have been shown in cancer cells [14]; however, to date, little is known about the effects of hypoxia on *PGC1 β* and *TFAM* expression in normal cells. We found that hypoxia suppressed gene expression of both *PGC1 β* and *TFAM* in endothelial cells. Knockdown of *PGC1 β* failed to eliminate hypoxia-mediated suppression of TFAM, suggesting that other factors contribute to the hypoxia-mediated suppression of TFAM. Manipulation of HIF-1 α and HIF-2 α expression provided further proof of the *PGC1 β* -independent mechanism that contributes to TFAM expression: hypoxia-mediated *PGC1 β* gene suppression required both HIF-1 α and HIF-2 α , whereas *TFAM* gene suppression was predominantly determined by HIF-2 α . c-Myc has been shown to regulate mitochondrial biogenesis independent of PGC-1 cofactors [6,15,16] in cancer cells, in the myocardium and in myoblasts. Recent studies show that a c-Myc consensus binding site in the TFAM promoter regulates transcription independent of the effects of PGC1; c-Myc overexpression increases TFAM transcription but not in the absence of this binding site [6]. Our findings suggest a role for c-Myc in modulating these pathways in primary endothelial cells as well.

c-Myc is a helix-loop-helix leucine zipper protein that binds to E-boxes (CACGTG) as a heterodimer with MAX [16,36,37]. MAX binding to c-Myc is regulated by competition with MAX dimerization proteins (MAD) [36], including the HIF-1 α -induced Mxi1 [14,38]. c-Myc-dependent gene transcription can also be regulated by transcriptional and post-transcriptional mechanisms, including post-translational protein modifications such as acetylation, phosphorylation, and ubiquitination [39]. In some cancer cells, both HIF-1 α and HIF-2 α contribute to hypoxia-mediated degradation of c-Myc [40], whereas in renal carcinoma cells, HIF-1 α and HIF-2 α were reported to have opposing actions on c-Myc expression [18]. In particular, in tumors that express only HIF-2 α , c-Myc was reported to be upregulated [17,18].

In contrast to the effects reported in various cancer cells, our findings suggest that HIF-2 α primarily suppresses c-Myc levels in primary endothelial cells. Thus, in our experiments, knockdown of HIF-2 α increased *MYC* gene expression, whereas knockdown of HIF-1 α had no effect on *MYC* gene expression. Also, similar to the effects of HIF-2 α knockdown on *TFAM* gene expression, knockdown of c-Myc suppressed *TFAM* gene expression and eliminated any further hypoxia-mediated suppression of TFAM, suggesting that c-Myc mediates the effects of HIF-2 α on TFAM suppression.

Our findings also show a role for c-Jun in the HIF-2 α mediated suppression of c-Myc (see the diagram in Fig. 5C). Although other studies have reported increased expression of c-Jun following short-term hypoxia [41], we did not find evidence for an increase in c-Jun expression following 24 h hypoxia. Instead, we found that knockdown of HIF-2 α increased the phosphorylation of c-Jun in hypoxia, and that blocking c-Jun activation attenuated c-Myc expression. Double knockdown of HIF-1 α and HIF-2 α suggests that the presence of HIF-1 α

is not necessary to activate c-Jun. Rather, absence of HIF-1 α alone increases HIF-2 α protein approximately 2-fold (Fig. 4) to augment the hypoxia-mediated suppression of c-Myc. Thus, although HIF-1 α may contribute to c-Myc expression, c-Myc suppression under hypoxia involves HIF-2 α -mediated mechanisms that inhibit the activation of c-Jun to attenuate c-Myc expression, thereby limiting mitochondrial gene expression. It has been suggested that decreased expression of c-Myc would limit mitochondrial biogenesis and cellular proliferation [42], thereby preventing the rampant growth found in some tumors that have simultaneous upregulation of c-Myc and HIF-factors [43]. In renal cell carcinomas (RCC) that are caused by a loss of the von Hippel-Lindau tumor suppressor gene, JNK pathways are upregulated leading to an increase in phospho-Jun. Although a link between c-Myc and phospho-Jun was not explored in the context of RCC, excess Jun activation was linked to growth and tumorigenesis in this carcinoma [44].

Recent studies confirm the essential function of c-Myc in normal endothelial cell proliferation, as c-Myc-deficient cells show decreased DNA replication and an increase in senescence [45]. Thus, in endothelial cells, HIF-mediated suppression of c-Myc may decrease the expression of TFAM concurrently with the hypoxia-mediated suppression of mitochondrial oxidative phosphorylation in order to reduce transcription of the mitochondrial genome. Interestingly, in a model of systemic hypoxia in which inspired oxygen was maintained at 7% for periods of 1–2 weeks, cardiac tissue showed a decrease in mitochondrial cristae, mitochondrial DNA, and mitochondrial metabolism, suggesting that the stabilization of HIF factors leads to a coordinate suppression of mitochondrial content along with the suppression of mitochondrial electron transport chain activity to decrease the production of damaging reactive oxygen species [25]. These cellular responses to chronic hypoxia promoted cell regeneration, especially following ischemic injury, where a substantial increase in vascular tissue was measured. Hypoxia is well known to stimulate angiogenesis via HIF-mediated upregulation of VEGF and other pro-angiogenic factors [46,47,48], suggesting that these pathways can compensate for a hypoxia-induced suppression of Myc. It is well established that activation of HIF-1 α promotes the expression of glycolytic genes that are usually regulated by c-Myc under normoxia [42]. Thus, although the upregulation of HIF factors compensates in some ways for the decrease in c-Myc under hypoxia, it appears that the stabilization of HIF-2 α in endothelial cells may suppress c-Myc-mediated expression of mitochondrial genes, such as TFAM.

In contrast to the findings that mitochondrial gene expression and biogenesis are suppressed when HIF factors are stabilized [14,25], reoxygenation has been shown to activate the biogenic program. Thus, in a hypoxic-ischemic brain injury model 6 h after a 2 h transient exposure to hypoxic conditions, the expression level of NRF-1, a biogenic factor, was upregulated, followed by increases in TFAM gene expression at 9–14 h following the end of the hypoxic treatment [49]. Concurrently, expression of the mitochondrial gene *cytochrome c oxidase IV (COX IV)* was upregulated, and citrate synthase activity was augmented, indicating an increase in mitochondrial function. Similar results were obtained in a model of transient (2 h) cerebral ischemia/reperfusion [50]. In this model, 24 h after the start of the reperfusion expression of *PGC1 α* , *NRF-1*, and *TFAM* genes was increased along with the upregulation of COX IV and COX I proteins, and citrate synthase activity. In both examples, reperfusion/reoxygenation stimulated the biogenic response; however, it is unclear whether

hypoxic endothelial cells will respond similarly to the restoration of normal oxygen tension. Nonetheless, these *in vivo* findings support the concept that mitochondrial gene expression and function are regulated by oxygen tension.

HIF-1 α and HIF-2 α have overlapping but not identical functions in promoting hypoxia-mediated responses. Their unique actions may be a consequence of their preferential association with different nuclear factors and cofactors [51,52,53,54]. In addition, HIF-1 α is ubiquitously expressed, whereas HIF-2 α has a limited cell distribution in cells such as fibroblasts, pneumocytes, macrophages, and endothelial cells [55]. Thus, our findings provide insights into the role of the HIF factors in regulating the response to hypoxia in endothelial cells. We show that HIF-2 α suppresses the expression of c-Myc and that c-Myc regulates *TFAM* gene expression. Furthermore, our data suggest that HIF-2 α attenuates c-Jun activation under hypoxic conditions to suppress c-Myc expression. These findings establish a new link between HIF-2 α and c-Jun pathways that are part of the adaptive response to hypoxia.

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Highlights

- Hypoxia causes reprogramming of mitochondria in pulmonary artery endothelial cells.
- The mitochondrial transcription factor, TFAM, is downregulated by HIF-2 α pathways.
- HIF-2 α decreases TFAM by suppressing c-Myc expression.
- HIF-2 α -mediated mechanisms attenuate c-Jun activation to regulate c-Myc.

Fig. 1.

Hypoxia suppresses TFAM expression. (A) Quantitative real-time PCR (qRT-PCR) with 0.5 μ g RNA, using actin as an endogenous control, was performed to monitor *TFAM* (n=5) and *PGC1β* (n=3) gene expression after 24 h hypoxia. Shown are mean $\pm$ SD. (B) Western blot for TFAM and actin following 42 h hypoxia or normoxia. Graphs show the relative densitometries corrected for actin; mean $\pm$ SD (n=4) are plotted. (C) qRT-PCR with 12 μ g RNA was used to monitor *PGC1α* gene expression in normoxia and hypoxia and to compare the ΔC_T of *PGC1α* and *PGC1β* transcripts in normoxic conditions (normalized to actin) (n=4). *, significantly different (p<0.00001). (D) Cells were transfected with siRNA to *PGC1β*, *PGC1α*, or a scrambled control (siCtrl) for 48 h prior to an additional 24 h normoxia or hypoxia treatment. *TFAM* gene expression was quantitated as in (A), and the data represent mean $\pm$ SD (n=3–4). *, significantly different from siCtrl under normoxia; #, significantly different (p<0.05) from all other conditions by post-hoc analysis following ANOVA; &, significantly different (p<0.05) from all normoxic conditions.

Fig. 2.

The role of HIF-1α and HIF-2α in the suppression of *PGC-1β* and *TFAM* gene expression.

(A) Nuclear extracts were prepared from cells cultured in normoxia or hypoxia for 24 h and analyzed for the expression of HIF-1α and HIF-2α. Representative Western blots are shown; graphs show the relative densitometries corrected for actin (mean ± SD, n=4). (B) Cells were transfected with control siRNA (siCtrl), siHIF1α, siHIF2α, or siHIF1α and siHIF2α simultaneously for 48 h prior to 24 h hypoxia or normoxia. Gene expression was analyzed by quantitative real-time PCR using actin as an endogenous control. The data represent mean ± SD (n=3–6 for each condition). *, significantly (p<0.05) different from the corresponding normoxic condition by post-hoc analysis following ANOVA.

Fig. 3.

c-Myc regulates TFAM expression. (A) Nuclear extracts were prepared from cells cultured under 24 h normoxia or hypoxia and analyzed for the expression of c-Myc. Representative Western blots are shown; graphs show the relative densitometry corrected for actin (mean $\pm$ SD, n=4). (B–E) Cells were transfected with control siRNA or siMyc or infected with control or c-Myc adenovirus prior to normoxia or hypoxia treatment. (B, C) *TFAM* gene expression was analyzed by quantitative real-time PCR using actin as an endogenous control. The data represent mean $\pm$ SD (n=4–5). *, significantly different from corresponding normoxic condition; #, significantly different from all Ctrl conditions. Note that the AdMyc hypoxia is significantly different from the AdCtrl hypoxia, as well. (D,E) Whole cell extracts were analyzed by Western blot for the expression of TFAM and actin. Graphs show the relative densitometries corrected for actin (mean $\pm$ SD, n=3–4). *, significantly different than corresponding Ctrl normoxia; #, significantly different from all Ctrl conditions.

Fig. 4.

c-Myc is suppressed by HIF-2α. Cells were transfected with control siRNA (siCtrl), siHIF2α, or siHIF1α and siHIF2α simultaneously for 48 h prior to 24 h hypoxia or normoxia. (A) *MYC* gene expression was determined by quantitative real-time PCR using actin as an endogenous control (n=3). &, significantly different from siCtrl and siHIF1α conditions. (B) Nuclear extracts were analyzed by Western blot. (C) The relative band densities for c-Myc and HIF-2α compared to actin (mean ± SD, n=5–7) are shown in the graphs. * Significantly different from siCtrl normoxia; &, significantly different from all other conditions; # significantly different from all hypoxic conditions.

Fig. 5.

Role of c-Jun in HIF-2α-mediated suppression of c-Myc. (A) Cells were transfected with control siRNA (siCtrl), siHIF2α, or siHIF1α and siHIF2α simultaneously for 48 h prior to 24 h hypoxia or normoxia. Nuclear extracts were analyzed by Western blot. Graphs show the relative band densities corrected for actin (mean ± SD, n=3–4). Significance (p<0.05) was determined by *post-hoc* analysis following ANOVA. * Significantly different from siCtrl and siHIF1α conditions. (B) Cells were transfected with siCtrl or si siHIF2α. 25 μM SP600125 (SP6), a c-Jun kinase inhibitor, or vehicle (veh) was added prior to the final 24 h incubation in hypoxic or normoxic conditions (n=5). Nuclear extracts were analyzed by Western blot, band densities were normalized for actin and are presented relative to the siCtrl normoxia treated with vehicle, which was set to 100. Two-way ANOVA was performed on hypoxic samples followed by post-hoc analysis. *, significantly different from all other conditions. (C) Hypoxia stabilizes HIF-1α and HIF-2α expression and decreases c-Myc, and TFAM expression. HIF-2α mechanisms are responsible for the suppression of c-Myc via the attenuation of c-Jun activation (ph ser 63). This can be mimicked by the JNK inhibitor, SP6000125. HIF-1α suppresses HIF-2α expression, as its absence more than doubles the levels of HIF-2α to enhance c-Myc suppression. HIF-1α and other mechanisms may contribute to c-Myc expression in other ways.