Influence of the availability of iron during hypoxia on the genes associated with apoptotic activity and local iron metabolism in rat H9C2 cardiomyocytes and L6G8C5 skeletal myocytes

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Abstract. The differential availability of iron during hypoxia is presumed to affect the functioning of cardiac and skeletal myocytes. Rat H9C2 cardiomyocytes and L6G8C5 myocytes were cultured for 48 h in normoxic or hypoxic conditions at the optimal, reduced or increased iron concentration. The mRNA expression levels of markers of apoptosis [B-cell lymphoma-2 (Bcl2; inhibition) and Bcl-2-activated X protein (Bax; induction)], atrophy (Atrogin), glycolysis (pyruvate kinase 2; PKM2) and iron metabolism [transferrin receptor 1 (TfR1; iron importer), ferroportin 1 (FPN1; iron exporter), ferritin heavy chain (FTI; iron storage protein) and hepcidin (HAMP; iron regulator)] were determined using reverse transcription-quantitative polymerase chain reaction, and cell viability was measured using an tetrazolium reduction assay. Cardiomyocytes and myocytes, when exposed to hypoxia, demonstrated an increased Bax/Bcl-2 gene expression ratio (P<0.05). Additional deferoxamine (DFO) treatment resulted in further increases in Bax/Bcl-2 in each cell type (P<0.001 each) and this was associated with the 15% loss in viability. The analogous alterations were observed in both cell types upon ammonium ferric citrate (AFC) treatment during hypoxia; however, the increased Bax/Bcl-2 ratio and associated viability loss was lower compared with that in case of DFO treatment (P<0.05 each). Under hypoxic conditions, myocytes demonstrated an increased expression of PKM2 (P<0.01). Additional DFO treatment caused an increase in the mRNA expression levels of PKM2 and Atrogin-1 (P<0.001 and P<0.05, respectively), whereas AFC treatment caused an increased mRNA expression of PKM2 (P<0.01) and accompanied decreased mRNA expression of Atrogin-1 (P<0.05). The expression augmentation of PKM2 during hypoxia was greater upon low iron compared with that of ferric salt treatment (P<0.01). Both cell types upon DFO during hypoxia demonstrated the increased expression of TfR1 and HAMP (all P<0.05), which was associated with the increased Bax/Bcl-2 ratio (all R>0.6 and P<0.05). In conclusion, during hypoxia iron deficiency impairs the viability of cardiomyocytes and myocytes more severely compared with iron excess. In myocytes, during hypoxia iron may act in a protective manner, since the level of atrophy is decreased in the iron-salt-treated cells.

Introduction

Optimal oxygen supply is of the utmost importance for the effective functioning of cells with high energy demand, including cardiomyocytes and myocytes, since their metabolism relies on the oxidative pathway (1-3). Nonetheless, their productive performance is critically contributed to by another agent, namely iron (4). As an essential component of co-factors present both in oxygen carriers and in numerous enzymes of oxidative metabolism, iron constitutes the cornerstone of tissue oxygen supply and energy generation (4-7). Notably, the experimental data demonstrates that hypoxia and an iron deficient state may have to some extent, similar consequences in both cardiac and skeletal myocytes at the molecular level, including impairment of the mitochondrial electron transport...
chain and triggering a shift from oxidative to glycolytic metabolism (8-13).

Previous evidence suggests that the optimal iron homeostasis is crucial for patients with various cardiovascular disorders, including coronary artery disease (14) and heart failure (15-17), in which hypoxia is an important causative factor. Notably, data from previous clinical studies confirmed the beneficial effects of iron therapy in non-anemic iron-deficient patients with heart failure accompanied by skeletal myopathy (18-23). To date, the majority of studies have investigated the molecular links between hypoxia and iron metabolism in different cells involved in systemic iron homeostasis, including macrophages, enterocytes, hepatocytes or erythroid precursor cells (24-28); however, very little attention has been given to gain an insight into molecular associations between oxygen and iron availability in the cells of cardiac or skeletal muscle tissues. Robach et al (29) investigated the influence of hypoxia on the expression of selected genes of iron metabolism, namely transferrin receptor and L-ferritin (FTH), in skeletal muscle; however, the studies involved human subjects exposed to high-altitude hypoxia where it was difficult to obtain changing experimental conditions.

Since hypoxic conditions are difficult to introduce and manipulate in humans and animals, the present study used cell culture models. The aim of the present study was to investigate the associations between selected genes involved in iron metabolism and cellular apoptotic activity assessed in diverse oxygen and iron availabilities in two rat cell lines: Cardiomyocytes (H9C2) and skeletal myocytes (L6G8C5).

Materials and methods

Cell culture. Rat H9C2 cardiomyocytes and rat L6G9C2 (L6) skeletal myocytes (Sigma-Aldrich, St. Louis, MO, USA) were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich), supplemented with 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 2 mmol/l glutamine, 10^5 diluted 10,000 U/ml penicillin and 10 mg/ml streptomycin (all from Sigma-Aldrich). For passaging, the cells were washed with phosphate-buffered saline (PBS; without Ca^{2+} and Mg^{2+}) and were released by trypsinization (Sigma-Aldrich). The cells were maintained according to manufacturer's protocols.

Experimental schedule. Rat H9C2 cardiomyocytes and rat L6G9C2 skeletal myocytes were cultured with an addition of deferoxamine (DFO; Sigma-Aldrich) or ammonium ferric citrate (AFC; Sigma-Aldrich) in order to change iron accessibility in hypoxia for those cells in the cultured medium during hypoxia treatment for 48 h. DFO is a selective iron chelator commonly used in the cell culture studies. It has been reported that addition of DFO into the culture medium reduces iron concentration both in the cellular environment and inside the cell, since DFO can be taken up by fluid phase endocytosis (30). AFC was applied in cell culture studies in order to induce intracellular iron accumulation (31,32).

Modulation of iron concentration in the cellular environment. H9C2 and L6 cells were seeded at 0.3x10^4 cells/well in a volume of 2 ml in 6-well plates. The cells were cultured under hypoxic conditions (1% O_{2}, 5% CO_{2}, 94% N_{2}), which were generated in a standard cell culture incubator by displacing O_{2} with infusion of N_{2}, which was supplied by an external high-pressure liquid nitrogen tank, in differential iron availability in the growth media for 48 h. The cells were separated into three groups: i) Optimal iron concentration (standard iron concentration in DMEM with 10% FBS); ii) reduced iron concentration (iron chelation using 100 µM DFO); iii) increased iron concentration (supplementation with 200 µM AFC). The compounds were added to the cells from 1,000X concentrated stocks diluted in culture medium.

Assessed parameters. Multiple parameters were assessed during the present study. These parameters reflected the cellular condition and iron status assessed in the states of different iron availability in culture medium of H9C2 and L6 lines. These parameters were looking at the expression levels of various genes in the following processes: i) Apoptosis, including B-cell lymphoma (Bcl)-2-associated X protein (Bax; inducer) (33), Bcl-2 (inhibitor) (34) and the Bax/Bcl-2 gene expression ratio (35); ii) proteolysis, including Atrogin-1, which is a marker of protein degradation in myocytes and a marker of muscle atrophy (36); iii) glycolysis, including pyruvate kinase (PKM2; marker of non-oxidative metabolism) (37); iv) intracellular iron metabolism, including transferrin receptor type 1 (TfR1; cellular iron importer) (38), ferroportin (FPN1; cellular iron exporter) (39), FTH heavy chain (iron storage protein) (40) and hepcidin (HAMP; iron metabolism regulator protein) (41).

Cell viability tetrazolium reduction assay (MTS). The MTS assay is a colorimetric method used to determine the number of viable and metabolically active cells in proliferation and cytotoxicity assays, as previously described (42). MTS assays were performed, according to manufacturer's protocol (CellTiter 96® AQueous One Solution Cell Proliferation Assay; Promega Corporation, Madison, WI, USA). Briefly, 2x10^4 H9C2 or L6 cells were seeded into each well of 96-well plates and were treated for 48 h with DFO, AFC or PBS (control) in hypoxia or in normoxia, as described above. A total of 20 µl CellTiter 96® AQueous One Solution reagent was added to each well and the absorbance at 490 nm was measured after 2 h incubation in 37°C (KClonJUNIOR™; BioTek Instruments, Inc., Winooski, VT, USA). The viability of the control cells was treated as 100%.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The total RNA was prepared from H9C2 or L6 cells harvested from 6-well tissue culture plates using the RNeasy Fibrous Tissue Mini kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. The protocol included an on-column DNase digestion to remove the genomic DNA. First-strand cDNA was synthesized using a SuperScript III First-Strand Synthesis system with oligo(dT)20 primer (Invitrogen; Thermo Fisher Scientific, Inc.).

Based on the rat genomic and cDNA sequences the following primers were used: Bcl-2, forward: 5'-AGCATG CGACCTCTGTGTTGA-3' and reverse 5'-TCACCTGTGTGG GCCCGAGGTATG-3'; Bax, forward: 5'-TGGCGATGAAACT GGACAACA-3' and reverse 5'-CAGCAGAGAGACCT CTGGG-3'; Atrogin-1 forward: 5'AGCTTGTGGCATGTT ACCCA-3' and reverse 5'-GAGCAGCTCTGTGGTTTGT-3'.
Effects of hypoxia with concomitant optimal, reduced or increased iron availability on tissue-specific expression of genes involved in energy metabolism and proteinolysis in myocytes. Hypoxic conditions of L6 cell culture caused an increase in the expression of PKM2, which responsible for the rate-limiting step of glycolysis, when compared with cells cultured in normoxia (P<0.01). Myocytes exposed to DFO during hypoxia demonstrated increased mRNA expression levels of PKM2 and Atrogin-1 (P<0.001 and P<0.05, respectively) as compared with the cells cultured in normal iron concentration, suggesting the further enhancement of non-oxidative glycolytic pathway and protein degradation in the cells. The different changes were observed in myocytes treated with AFC, since the increase in the expression of PKM2 (P<0.01) was accompanied by a decrease in the mRNA expression of Atrogin-1 (P<0.05) as compared with the cells cultured in optimal iron concentration. The augmented expression of PKM2 in myocytes during hypoxia was greater upon low iron availability compared with during ferric salt treatment (P<0.01). Notably, the expression of Atrogin-1 during hypoxia and concomitant elevated iron availability was decreased as compared with cells cultured in hypoxia and normal or decreased iron concentration (P<0.05 and P<0.01, respectively; Fig. 2).

Effects of hypoxia with concomitant optimal, reduced or increased iron availability on the expression of genes involved in iron influx and efflux in cardiomyocytes and myocytes. Both L6 and H9C2 cell lines exposed to hypoxia demonstrated, as compared with the cells cultured in normoxia, a decrease in the expression of TfR1 (P<0.05 each) and FPN1 (P<0.05 and P<0.01, respectively). The addition of DFO to the culture medium of cardiomyocytes and myocytes during hypoxia caused an increased expression of TfR1 (P<0.01 and P<0.001,respectively), reflecting iron demand, thus facilitated entrance of iron to the cells. However, this did not affect the expression of FPN1 compared with the cells cultured in normal iron concentration.

The AFC treatment of cardiomyocytes and myocytes during hypoxia resulted in a decreased and increased, respectively, expression of TfR1 (P<0.05 each). Additionally, this resulted in an increased expression of FPN1 (P<0.05 each) in each cell line. Under hypoxic conditions with concomitant changing iron availability, the myocytes demonstrated low expression of FPN1 when compared with the cells cultured in normoxia (P<0.01) (Fig. 3A and B).

Effects of hypoxia with concomitant optimal, reduced or increased iron availability on the expression of the iron storage gene in cardiomyocytes and myocytes. Low oxygen treatment of L6 cells caused, as compared with cells cultured...
in normoxia, an increased expression of the iron storage gene, \textit{FTH} (P<0.05). Exposure to DFO of cardiomyocytes during hypoxia resulted, as compared with hypoxia-treated controls, in unchanged mRNA expression of \textit{FTH}, while hypoxic culture of L6 cells exposed to low iron demonstrated an increase in the expression of the iron storage gene (P<0.01).

The different pattern of changes was observed upon ferric salt treatment during hypoxia. An increased expression of \textit{FTH} in both cardiomyocytes and myocytes was observed compared with the cells cultured in normal iron concentration (P<0.05 and P<0.01, respectively) (Fig. 3C).

Effects of hypoxia with concomitant optimal, reduced or increased iron availability on the expression of iron regulator \textit{HAMP} in cardiomycocytes and myocytes. Low oxygen treatment of H9C2 and L6 cells caused an increment in the expression of iron regulator, \textit{HAMP} (P<0.05 and P<0.01, respectively). An addition of DFO to the culture medium of both studied cell lines during hypoxia caused a further increase in the expression of \textit{HAMP} (P<0.05 and P<0.001) as compared with the cells cultured in a normal iron concentration. The exposition of cardiomycocytes and myocytes to AFC treatment in hypoxia resulted in an unchanged or a decreased expression of \textit{HAMP} (P<0.01) (Fig. 3D).

An increased expression of iron-related genes in a low iron state during hypoxia is associated with an increased apoptotic activity of cardiomycocytes and myocytes. In both
cardiomyocytes and myocytes, the expression of genes involved in iron influx (TfR1) and regulation (HAMP) was associated with the Bax/Bcl-2 gene expression ratio in the whole spectrum of iron status during hypoxia (all R>0.6, P<0.05). In particular, in a low iron state, the observed upregulation of TfR1 and HAMP genes was associated with increased apoptotic activity of each of the cell lines. In addition, L6 cells demonstrated positive correlation between the expression of FTH and the Bax/Bcl-2 gene expression ratio (R=0.64, P<0.05) (Fig. 4).

Discussion

The present study compared the influence of increased or reduced iron availability during hypoxia on the viability and apoptotic activity of the cardiac and skeletal myocytes. It was discovered that during hypoxia, the reduced iron concentration had a more negative impact on the viability and functioning of the studied myocytes compared with the elevated iron availability. Furthermore, in these cells cultured during hypoxia and with differential iron availability, the present study was able to investigate changes in the expression of iron turnover genes and to establish associations between the upregulated expression of genes involved in iron influx (TfR1) and regulation (HAMP), and the enhanced susceptibility to cellular suicide.

Firstly, the present study demonstrated that hypoxic conditions diminished the viability of cardiomyocytes and myocytes. The accompanying changes in the expression of genes involved in the regulation of apoptosis supported the results from the viability assay, since the gene expression ratio of pro-apoptotic Bax to anti-apoptotic Bcl-2 increased upon low oxygen. Furthermore, it was demonstrated that the exposure of the studied cells to either iron depletion or iron excess during hypoxia hampered the cellular viability and increased Bax/Bcl-2 gene expression ratio more severely compared with hypoxia treatment alone. This finding was consistent with the study of Walter et al (44) who demonstrated that both iron overload and iron deficiency are detrimental to the functioning of liver mitochondria (44). With this work, besides highlighting the presence of analogous U-shaped association between iron availability and vitality of the studied cells during hypoxia, the present study identified that upon low oxygen conditions, iron deficiency has a more negative impact on the cellular viability and with a greater extent favors apoptosis rather than iron excess.

To the best of our knowledge, the local iron metabolism has only been partially assessed in the cell culture models of
myocardium and skeletal muscles (43,44). It remains insufficiently investigated upon hypoxia conditions; to date it was explored in skeletal muscle on the model of tissue samples from patients subjected to high-altitude hypoxia (29). The present results are consistent with the study of Robach et al (29), as we observed the hypoxia-driven decrease in the mRNA expression of \( \text{TfR1} \) and increase in the mRNA expression of \( \text{FTH} \). However, the present model of cell cultures allowed manipulations within the oxygen and iron availabilities, which were not amenable in humans. Therefore, the present study has provided the first evidence, to the best of our knowledge, of the direct response of iron turnover genes to hypoxia and changing iron availability within the isolated model of cardiomyocytes and skeletal myocytes cultured in vitro.

It was shown that the exposure of cardiomyocytes and myocytes to low oxygen altered the expression of iron turnover genes in each of the cell lines. Notably, these changes vary from the previously reported data, since to date, the studies on the expression of iron-related genes during hypoxia have been performed predominantly in the cells involved in systemic iron homeostasis, including hepatocytes, macrophages, enterocytes or erythroid precursor cells (24-28). For example, the responses of the aforementioned cells to low oxygen involved a hypoxia-driven upregulation of both \( \text{TfR1} \), in order to enable an enhanced iron uptake by erythrocytes, and \( \text{FPN1} \), resulting in the enhanced release of iron into the bloodstream from iron storing and distributing cells (47-49). Furthermore, hypoxia was demonstrated to downregulate the expression of \( \text{HAMP} \) and to increase the expression of \( \text{FTH} \) in hepatocytes (50-52).

As mentioned previously, the expression changes that occurred during hypoxia within cardiomyocytes and skeletal myocytes, which both represent tissues of minor importance in respect to systemic iron homeostasis, are very often inconsistent with data previously obtained from the other cell types. In hypoxic conditions, both studied cell lines have demonstrated a decrease in the expression of transferrin receptor and this change was accompanied with a decreased expression of ferroportin gene. Notably, unlike the previous data reporting hypoxia-driven decrement of hepatic hepcidin expression, the present finding demonstrated in both cardiomyocytes and myocytes during hypoxia a markedly increased expression of hepcidin. It is worthwhile mentioning that the function of this iron regulatory hormone locally expressed in the heart and skeletal muscle remains to be fully understood (53-55) and in depth research in this field is required. Furthermore, in skeletal myocytes, the alteration of the expression of iron storage protein, \( \text{FTH} \), which was increased in low oxygen was observed. This change may be associated with the potential antioxidant role of \( \text{FTH} \) in the protective mechanism of myocytes against the hypoxia-mediated generation of reactive oxygen species (51,52,56).
In addition, the present study has demonstrated that the treatment with an iron chelator upon hypoxia resulted in an increased expression of transferrin receptor gene in both cardiomyocytes and myocytes. This finding indicated that the environmental iron depletion, along with low oxygen, may induce an adaptive reaction of the cells, which enables iron resorption. Notably, increased expression of $TfR$ was also demonstrated, reflecting a cellular iron starvation, suggesting an association with the elevated $Bax/Bcl-2$ gene expression ratio, thus increased apoptosis. Both cell lines, when exposed to DFO in hypoxia, demonstrated a markedly increased level of $HAMP$ and this change was associated with the elevated $Bax/Bcl-2$ gene expression ratio. Therefore, it may be hypothesized that locally expressed hepcidin may account for an

Figure 4. Correlations between the $Bax/Bcl-2$ ratio in H9C2 (open circle) and L6G8C5 (black triangle) cells, and the expression levels of (A) $TfR$, (B) $HAMP$ and (C) $FTH$. TfR1, transferrin receptor type 1; FTH, ferritin heavy chain; HAMP, hepcidin; Bcl, B-cell lymphoma; Bax, Bcl-2-associated X protein.
important agent in stress conditions, including hypoxia and/or iron deficiency.

In the case of skeletal myocytes, several issues are worthy of consideration. Firstly, hypoxia and concomitant either reduced or increase iron availability caused an increased expression of the PKM2 gene, thus, the enhancement of anaerobic glycolysis. This finding suggested that myocytes underwent an oxidative-to-glycolytic shift in the experimental conditions. These observations are consistent with the previously reported data on the influence of hypoxia or iron deficiency on skeletal muscle metabolism (8,9). However, the present study has confirmed that the elevation of iron concentration during hypoxia favors glycolysis to a lesser extent compared with iron limitation. Furthermore, it was demonstrated that in hypoxic conditions the increased iron concentration may be to a certain extent protective since the expression of the marker of muscle atrophy, Atrogin-1, was decreased upon elevated iron availability in comparison with hypoxia alone or hypoxia and iron chelator treatment. Notably, Atrogin-1 expression indicated the imbalance between anabolic and catabolic processes in the myocytes and favored protein breakdown. It appears that during hypoxia the elevated iron availability may suppress muscle atrophy, which results in the loss of muscle mass and leads to muscle weakness, inactivity and increased mortality (36).

Taking together, the present preliminary data suggested that during hypoxia, iron excess is less detrimental for the vitality of cardiomyocytes and skeletal myocytes, compared with an iron deficiency situation. In addition, it may be hypothesized that the increased availability of iron during hypoxia may be beneficial to a certain extent for skeletal myocytes in the context of preventing muscle atrophy. This premise may stand for a molecular substantiation of the efficacy of iron therapy for the improvement of muscle functional capacity (e.g. in patients with heart failure and concomitant iron deficiency).

Notably, it may be interesting to further investigate the role of the locally expressed hepcidin in cardiomyocytes and skeletal myocytes since the pattern of its expression differs from what has already been explored in hepatocytes.

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References

Transcriptional and post-transcriptional regulation by:


