Increased hypoxia-inducible factor-1α in striated muscle of tumor-bearing mice

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Am J Physiol Heart Circ Physiol 312: H1154–H1162, 2017. First published March 24, 2017; doi:10.1152/ajpheart.00090.2016.—Cancer cachexia is a progressive wasting disease resulting in significant effects on the quality of life and high mortality. Most studies on cancer cachexia have focused on skeletal muscle; however, the heart is now recognized as a major site of cachexia-related effects. To elucidate possible mechanisms, a proteomic study was performed on the left ventricles of colon-26 (C26) adenocarcinoma tumor-bearing mice. The results revealed several changes in proteins involved in metabolism. An integrated pathway analysis of the results revealed a common mediator in hypoxia-inducible factor-1α (HIF-1α). Work by other laboratories has shown that extensive metabolic restructuring in the C26 mouse model causes changes in gene expression that may be affected directly by HIF-1α, such as glucose metabolic genes. M-mode echocardiography showed progressive decline in heart function by day 19, exhibited by significantly decreased ejection fraction and fractional shortening, along with posterior wall thickness. Using Western blot analysis, we confirmed that HIF-1α is significantly upregulated in the heart, whereas there were no changes in its regulatory proteins, prolyl hydroxylase domain-containing protein 2 (PHD2) and von Hippel-Lindau protein (VHL). PHD2 requires both oxygen and iron as cofactors for the hydroxylation of HIF-1α, marking it for ubiquination via VHL and subsequent destruction by the proteasome complex. We examined venous blood gas values in the tumor-bearing mice and found significantly lower oxygen concentration compared with control animals in the third week after tumor inoculation. We also examined select skeletal muscles to determine whether they are similarly affected. In the diaphragm, extensor digitorum longus, and soleus, we found significantly increased HIF-1α in tumor-bearing mice, indicating a hypoxic response, not only in the heart, but also in skeletal muscle. These results indicate that HIF-1α may contribute, in part, to the metabolic changes that occur during cancer cachexia.

NEW & NOTEWORTHY We used proteomics and metadata analysis software to identify contributors to metabolic changes in striated muscle during cancer cachexia. We found increased expression of hypoxia-inducible factor-1α in the heart and skeletal muscle, suggesting a potential target for the therapeutic treatment of cancer cachexia.

IN THE UNITED STATES, cancer is the second leading cause of death behind cardiovascular disease, whereas in the United Kingdom, cancer is the leading cause of death (38, 47). As the quality of life index and longevity continue to increase, cancer, with a higher incidence after midlife, will likely become a more prevalent problem in developing and third world countries (19). In the United States, ~40% of men and women will be diagnosed with cancer at some point in their lifetime (14). Cancer, a heavy burden on its own, can also lead to the development of cachexia (43, 44), a wasting syndrome in which muscle and adipose tissue experience marked loss and breakdown, which is not nutritionally reversible. In addition to the fat and muscle wasting, there are significant appetite and metabolic complications in cachectic patients. Cachectic patients also suffer a reduced quality of life, experiencing greater fatigue, exercise intolerance, and depression (45, 46). Cachexia is inherently difficult to treat because of the lack of Federal Drug Administration-approved drugs, and cachexia causes patients to be refractory to conventional cancer treatments. For patients with cancer, there is a 30–80% chance that they will develop cachexia, with certain cancers such as gastric, prostate, colon, and lung cancer having higher incidences of cachexia (13). Among the patients who develop cachexia, it is the primary cause of death in 20–40% of them (44). The extensive burden and complications of cachexia warrant further investigation into the mechanisms of progression to allow development of new therapeutic strategies.

The heart was not typically considered at risk for wasting during the development of cancer-induced cachexia because of normalized heart weight showing no significant loss. However, recent research has shown that cardiac function is compromised in a tumor-burdened setting. Heart samples from patients who died as a direct result of cachexia showed significant fibrotic remodeling, indicative of heart failure (39). With the use of an experimental model of cancer cachexia, the colon-26 (C26) adenocarcinoma cell line, it has also been shown that tumor burden can significantly impact cardiac function (52). CD2F1 mice subcutaneously injected with this cell line show reduced ejection fraction and fractional shortening with a reduction in posterior wall thickness during systole (52). Studies have implicated the ubiquitin proteasome system, autophagy, ANG II, and matrix metalloproteinases in this cardiac dysfunction (5, 40, 42). However, other possible contributors to cardiac and skeletal muscle dysfunction remain unknown.

To uncover possible mechanisms of cardiac and skeletal muscle dysfunction, a proteomics approach was used. The
proteomics approach, however, creates several problems that must be overcome, namely, sorting and analyzing the data. A data analyzer from Qiagen (Valencia, CA) was used [Ingenuity Pathway Analysis (IPA)] to sort the data and determine connections. Both metabolic and cytoskeletal differences were linked to hypoxia-inducible factor-1α (HIF-1α). Until this study, HIF-1α has not been extensively studied outside of the native tumor environment. A study by Tian et al. (42), using the C26 model of cachexia, demonstrated that cardiac tissue expresses several RNA markers consistent with increased reliance on glucose metabolism and decreased fatty acid oxidation. Increased reliance on glucose is a hallmark of anaerobic metabolism because of the inability to perform fatty acid oxidation. These effects have not been limited to animal models, as metabolism is also significantly altered in human patients suffering from cancer. A cause of cachexia is thought to be increased reliance on the Cori cycle. Tumors secrete inflammatory factors and, thereby, cause the breakdown of fat stores and increased gluconeogenesis by the liver in an anaerobic fashion (48). Because this process is highly inefficient, it creates wasted energy and futile cycling, possibly contributing to the cachectic state (9). HIF-1α is capable of causing these metabolic reprogramming events, but the degree to which it is involved in cancer-induced cachexia is unknown.

HIF-1α is a transcription factor that responds to the amount of oxygen available in the environment, increasing in expression during hypoxia (50). HIF-1α is a transcription factor for more than 60 genes (50). In normoxic conditions, HIF-1α is hydroxylated by prolyl hydroxylase-2 (PHD2); this posttranslational modification is then recognized by the ubiquitin ligase von Hippel-Lindau tumor suppressor (VHL). VHL ubiquinates the hydroxylated HIF-1α, marking it for destruction by the proteasome complex (18). PHD2 itself requires several cofactors to perform the hydroxylation reaction, namely, oxygen and iron (8). In hypoxic conditions, HIF-1α expression increases as PHD2 is no longer able to mark HIF-1α for destruction. HIF-1α is known to be upregulated in human tumors, contributing to the development of large tumors that have a hypoxic core (35, 53). Increased hypoxia in other tissues during cancer progression has been understudied, and it is possibly confounded with the normal restriction of peripheral blood supply during the end stages of cancer. Because of the ability of HIF-1α to cause substantial metabolic reprogramming, there may be a threshold in which even a minor amount of increased HIF-1α could cause significant effects. Further study will need to be performed to understand when HIF-1α becomes relevant in this biological system.

In this study, we demonstrated, through a proteomics approach, coupled with data analysis, that HIF-1α is a possible contributor to heart and skeletal muscle pathology of tumor-bearing mice. We also demonstrated possible mechanisms contributing to HIF-1α increases and discuss possible consequences of HIF-1α upregulation.

MATERIALS AND METHODS

Animal husbandry and care. Adult female mice (~10 wk old), weighing 20–22 g, were obtained from Charles River Laboratories (Charles River, Wilmington, MA). Mice were housed one to three per cage and were maintained on a 12:12-h light-dark cycle at 25°C. Mice were provided ad libitum access to water and standard rodent chow throughout the duration of the study. All animal care and procedures were approved by the Ohio State University Institutional Animal Care and Use Committee.

Animal study and exclusion criteria. Animal studies were carefully monitored during the study to avoid complications arising from a moribund state, which may complicate hypoxia-related findings. Despite losing ~10–20% body mass, mice remained active and alert.
Mice showing signs of tumor ulceration, impaired mobility, hunched posture, or guarding behavior were removed from the study and were not used for any further biochemical studies, and their final weight values were not reported for statistical analysis.

**Tumor model.** C26 cells were maintained as previously described (52). Approximately 1 wk after arrival, mice were randomly assigned to receive an injection of tumor cells or saline vehicle. Cells \((5 \times 10^5)\) were injected subcutaneously between the scapulae so that tumor growth would not disrupt locomotion. Tumor growth was evident as early as day 7 post injection. Mice became moribund at approximately day 21. After blood collection, a cardiectomy was performed, and the left ventricle (LV) was dissected, snap frozen in liquid nitrogen, and stored at \(-80^\circ\text{C}\) until further biochemical analyses.

**Proteomics and bioinformatics.** Proteomics analysis was performed by the OSU Proteomics Core, as previously described (25). Briefly, samples were marked with dye and subjected to two-dimensional electrophoretic separation (Fig. 1). The gels were then scanned using a Typhoon 9400 variable mode scanner (GE Healthcare, Aurora, OH) to visualize the dye-labeled proteins. After identification of differences in samples between control and tumor-bearing mice, the proteins were extracted from the gel and prepared for mass spectrometry. Capillary liquid chromatography-tandem mass spectrometry was used to identify peptide sequences. These peptide sequences were compared with the National Center for Biotechnology Information NR database and then checked to ensure that only proteins with a Mascot score of 100 or higher were accepted.

**Proteomics data analysis.** IPA software (Qiagen) was used to sort and analyze the proteomics data. An IPA compatible data set from the proteomics results was uploaded into the IPA Knowledge Database, and an interactive report was generated. To determine connections, Pathway Builder software, provided as part of the IPA analysis software, was used to grow connections from the identified proteins.

**Table 1. Effects of HIF-1α on transferrin, albumin, thrombin, and transglutaminase in tumor-bearing mice**

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Fold Change</th>
<th>Effect on HIF-1α</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin</td>
<td>+10.5</td>
<td>Increases HIF-1α</td>
<td>27</td>
</tr>
<tr>
<td>Thrombin</td>
<td>+3.7</td>
<td>Increases HIF-1α</td>
<td>12</td>
</tr>
<tr>
<td>Transglutaminase</td>
<td>+4.2</td>
<td>Increases HIF-1α</td>
<td>21</td>
</tr>
<tr>
<td>Transferrin</td>
<td>+3.8</td>
<td>HIF-1α increases expression</td>
<td>33</td>
</tr>
</tbody>
</table>

HIF-1α, hypoxia-inducible factor-1α.

![Fig. 2. Time course measurements showing the weight of the mouse (body weight), weight of the mouse minus the tumor weight (i.e., carcass weight), soleus weight, extensor digitorum longus (EDL) weight, heart weight, tumor weight, and spleen weight. *P < 0.05.](http://ajpheart.physiology.org/)
These networks were continually expanded and combined until common targets were identified for further analysis.

**Echocardiography.** Cardiac function was determined using echocardiography with a VEVO 2100 Ultrasound System (Visual Sonics, Toronto, ON, Canada). Mice were anesthetized using isoflurane: 3% in 100% oxygen for induction and 1% in 100% oxygen for maintenance. During echocardiography, animals were monitored for heart rate measurements to ensure that their rate was ~500 beats/min during maintenance, and the percentage of isoflurane was changed to maintain this rate without waking the animal. Animals were placed on a warm table, fur around the chest was removed using a depilatory agent, and temperature was measured via a rectal probe. Prewarmed ultrasound gel was applied to a 15-MHz transducer probe optimized for mouse echocardiography. The transducer was placed on the parasternal short axis to obtain a view of the LV at the midpapillary level for image capture and measurements. LV dimensions [LV end-diastolic dimension and LV end-systolic dimension (LVEDd and LVEDs)] as well as posterior wall thickness during systole and diastole (PWTs and PWTd) were acquired using the leading edge method, as recommended by the American Society for Echocardiography. Fractional shortening (in %) was calculated using the following formula: fractional shortening \( \% \) was calculated using the following formula: fractional shortening \( \% \) = \( \left( \frac{\text{LVED} \text{d} - \text{LVED} \text{d}}{\text{LVED} \text{d}} \right) \times 100 \). Ejection fraction was also measured and calculated using the following formula: ejection fraction = \( \left( \frac{\text{stroke volume}}{\text{end-diastolic volume}} \right) \times 100 \). For serial measurements, the same mice were used consecutively for each time point using the same conditions. Mice whose fur had grown back had their fur removed using a depilatory agent as previously stated, and animals were checked during each session in case of adverse reactions that could affect echocardiographic measurements. For echocardiography results, \( n = 6 \) was used for each experimental group.

**SDS-PAGE and Western blot analysis.** Proteins were isolated from sample tissue and run as previously described (5, 49). Briefly, gels were run at a constant voltage until the tracking dye ran off the gel. Protein was then transferred onto a nitrocellulose membrane, which was blocked with BSA for an hour at room temperature. Blots were incubated with primary antibody [HIF-1α (MGC3) (ThermoScientific, Grand Island, NY), PHD2 (ab109088) (Abcam, Cambridge, MA), VHL (ab284343) (Abcam), and c-Kit (C-19) (Santa Cruz Biotechnology, Dallas, TX)] for an hour at room temperature or at 4°C overnight. Blots were washed multiple times with Tris-buffered saline-Tween 20 before the addition of secondary antibody for incubation for an hour at room temperature. After being washed with Tris-buffered saline-Tween 20, blots were visualized using the 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium color reaction. Digital images of the blots were quantitated using ImageJ (National Institutes of Health (NIH), Bethesda, MD) software.

For loading control, separate gels were run under the same conditions and were stained using Coomassie blue instead of being transferred. The region containing \( \alpha/\beta\)-actin was used as the loading control. Digital images of the gels were quantitated using ImageJ, which was used to calculate the protein fold change.

**Blood gas measurements.** To measure blood gas values, an iStat blood analyzer (Abbott, Princeton, NJ) was used along with the CG8+ Cartridge (Abbott). Blood was drawn each week from the submandibular vein, and ~100 \( \mu \)l were dispensed into the cartridge sample port. Blood draws were performed on conscious, unanesthetized mice to avoid complications that anesthesia could have on respiratory rate. Mice were gripped at the scruff and held so that the submandibular vein was easily accessible. A needle was then used to pierce the skin over the submandibular vein, and blood was collected in a dropwise fashion into an Eppendorf tube. Blood was immediately transferred to the iSTAT biochip in a process that took no more than a few seconds to keep blood gases as close to physiological values as possible. Values were recorded and then analyzed for the significance of differences between groups.

Venous oxygen content (\( \text{CV}_0 \)) was calculated using the following equation: \( \text{CV}_0 = \left[ \frac{\text{Hb} \times 1.34 \times (\text{SO}_2/100)}{+ \left( \frac{\text{PO}_2 \times 0.031}{}} \right) \right] \), where \( \text{Hb} \) is hemoglobin, \( \text{SO}_2 \) is the percentage of hemoglobin saturated with oxygen, and \( \text{PO}_2 \) is the partial pressure of oxygen. \( \text{Hb}, \text{SO}_2, \) and \( \text{PO}_2 \) were measured directly by the iSTAT, whereas the other values are constants representing the amount of oxygen carried by hemoglobin and the amount of oxygen dissolved in plasma, respectively.

**Oil red O staining and quantitation.** Hearts from control and tumor-bearing mice were preserved in cryo-embedding media (OCT), frozen in liquid nitrogen, and stored at −80°C until sectioned. The OCT blocks were sectioned using a cryostat and fixed onto glass slides. Sections were stained in oil red O stain (Abcam) for 45 min. Sections were rinsed in distilled water three times. Slides were stained in Harris hematoxylin, washed in tap water, and washed in ammonia water to contrast the nuclei followed by another wash in tap water. Slides were then mounted with aqueous mounting media.

Slide images were captured using an Olympus IX73 microscope (Olympus, Pittsburgh, PA) at \( \times 20 \) magnification. The stained slides...
Identification of downstream targets and upstream effectors of HIF-1α levels. IPA analysis of the proteomics results indicated connections to HIF-1α. The HIF-1α-suppressive protein HSP68 was decreased in tumor-bearing mice, whereas several downstream HIF-1α proteins, such as transferrin, albumin, thrombin, and transglutaminase, were increased in tumor-bearing mice (Table 1).

**Body and tissue weights.** Time course measurements showing the weight of the mouse (body weight), weight of the mouse minus the tumor weight (i.e., carcass weight), soleus weight, extensor digitorum longus (EDL) weight, heart weight, tumor weight, and spleen weight are shown in Fig. 2.

**Depressed myocardial function in late tumor development.** Short-axis M-mode echocardiography of the hearts of control and tumor-burdened mice revealed significant myocardial function depression at 19 days post tumor inoculation. In addition to depressed fractional shortening and ejection fraction, there was a significant decrease in PWTs at 19 days postinjection without a change in PWTd (Fig. 3).

**Increase in HIF-1α and c-Kit protein levels in the myocardium.** Densitometry scans of the immunoblots showed that HIF-1α was significantly increased in the myocardium of tumor-bear- ing mice. Regulators of HIF-1α, PHD2, and VHL were unchanged in the myocardium between control and tumor-bearing mice. Additionally, c-Kit, a marker of differentiation and downstream of HIF-1α, was slightly but significantly increased in the myocardium (Fig. 4).

**Decreased oxygen levels in venous blood.** Submandibular venous blood from control and tumor-burdened mice showed a reduction of oxygen concentration at the third week after tumor inoculation. No changes were observed in Pco2 or total CO2 in either control or tumor-burdened mice (Fig. 5).
Increased lipid deposition in the myocardium and fatty acid-binding protein-4 RNA expression. Stained sections revealed significant lipid deposition in the myocardium of tumor-bearing mice compared with control mice (Fig. 6). To determine whether the lipid deposition represented immature adipocytes, the RNA expression of fatty acid-binding protein-4 (FABP4) was measured and was significantly increased in tumor-bearing mice compared with control mice (Fig. 6).

Increase in HIF-1α protein levels in skeletal muscle. HIF-1α was significantly increased in the soleus, EDL, and diaphragm of tumor-bearing mice compared with control mice, as determined by densitometry of immunoblots (Fig. 7).

**DISCUSSION**

Our findings demonstrate increased HIF-1α in the heart, EDL, soleus, and diaphragm of tumor-bearing mice. Although proteomics was originally performed only on the heart, leading to the focus on HIF-1α, it became evident that other tissues might be affected as well. Finding increased amounts of HIF-1α, without a compensatory increase in PHD2 or VHL, necessitated looking for a plausible mechanism to explain the increased HIF-1α. Because PHD2 requires both oxygen and iron as a cofactor to hydroxylate HIF-1α so that it can be recognized by VHL for ubiquination, we determined the amount of oxygen through blood gas measurements as well as the amount of iron present in the myocardium. There was a significantly lower oxygen concentration in the venous blood of tumor-bearing mice, indicating a hypoxic environment. We also examined the EDL, soleus, and diaphragm, and all were found to have significantly increased expression of HIF-1α. The effect of hypoxia and subsequent increase in HIF-1α in the heart has both beneficial and detrimental effects. Transient hypoxia and induction of HIF-1α have been shown to be cardioprotective in high-altitude training and in myocardial...
A plausible mechanism for this is that HIF-1α regulates the oxygen consumption of mitochondria, providing protection during hypoxic events and decreased oxygen availability (36). In the heart, c-Kit has been implicated as a progenitor marker leading to cardiomyocyte differentiation (24, 34). Previous studies have shown that hypoxia and the action of HIF-1α can induce c-Kit expression, possibly as a protective mechanism to replenish apoptotic cells as a response to hypoxia (22). Our findings show an increased amount of c-Kit in the myocardium of tumor-bearing mice, indicating that HIF-1α may be involved in progenitor cell activation. Sustained hypoxia and a subsequent increase in HIF-1α are associated with detrimental effects on the heart (11). The lack of available oxygen impacts the β-oxidation of fatty acids, preventing lipids from being used as an energy source; this, in turn, leads to lipid accumulation in the myocardium (4, 30, 32).

In agreement with previous findings, we found depressed myocardial function in the hearts of tumor-bearing mice that reached significance at 19 days postinjection (7, 41). This is most likely due to the LV wall being compromised, evidenced by the reduction in PWTs. There were no changes to PWTd, however, indicating that the compromised function of the heart is not solely due to atrophy as a dilated cardiomyopathy phenotype, in which both walls would have reduced thickness. Although previous findings have looked at only day 21 posttumor cell injection, we examined the mice at three time points. By doing this, we detected that there is a progressive decline in the tumor-burdened heart, beginning at approximately day 14. Speckle tracking is a relatively new echocardiographic method that examines wall movement in a short-axis view and can reveal deficiencies before overt functional depression is detected. Speckle tracking could be used in future studies to reveal structural changes in the heart that occur before significantly decreased fractional shortening and ejection fraction. This may also resolve some questions concerning the reduction in PWTs without the corresponding decrease in PWTd, as speckle tracking may show changes in the structure of the LV wall that would otherwise be missed by conventional M-mode and B-mode measurements.

Our results confirm that there is lipid accumulation in the hearts of tumor-bearing mice, which could contribute to cardiac dysfunction through lipid toxicity. Lipid accumulation typically results in an increase in peroxisome proliferator-activated receptor (PPAR)-α; however, hypoxia has been shown to decrease PPAR-α expression with a consequent shift from lipid to glucose utilization (28). PPAR-α has been shown to be decreased in the C26 model of cancer cachexia (41). Studies have shown that HIF-1α acts as an adipocyte mobilization factor. Increases in HIF-1α cause the release of preadipocyte factors into the bloodstream (3, 15). The extensive lipid deposition in the myocardium, along with the increase in HIF-1α, raised the question of whether adipocyte cells had infiltrated the myocardium. FABP4 is a marker of adipocyte progenitor cells, and RNA expression is associated with ter-

Fig. 7. Protein fold changes in the diaphragm, EDL, and soleus of control and tumor-bearing mice as determined by densitometry using the actin-stained gel as a loading control. *P < 0.05.
calcium moving from the intracellular to the extracellular environment has been associated with increased hypoxia as a result of decreased mitochondrial content and function in the hearts of tumor-bearing mice, but it appears that HIF-1α may play a prominent role in these changes.

Because of the decreased oxygen concentrations in the arterial blood of tumor-bearing mice, we determined whether HIF-1α was also increased in skeletal muscle. Our findings show that HIF-1α is significantly increased in the EDL, soleus, and diaphragm. This indicates that there might be a broad-based hypoxic response in skeletal muscle. The role of HIF-1α in skeletal muscle is not as well understood compared with its role in the myocardium. Most of the available knowledge is based on endurance and exercise training studies, in which transient increases in HIF-1α promote increased oxidative capacity, angiogenesis, and muscle repair (1, 17). However, HIF-1α expression quickly returns to normal after the induction of repair and regeneration. Chronic cardiac hypoxia is associated with decreased mitochondrial capacity and content (16). In the C26 model, skeletal muscle has distorted mitochondria, although no studies have been reported on the oxidative capacity of these mitochondria (37). The diaphragm in mice is primarily composed of fast fibers and also shows increased expression of HIF-1α, similar to the EDL and soleus. This represents a paradox in which the diaphragm would need to be needed to increase respiration to restore oxygen levels to normal. It was thought that the diaphragm might have protective mechanisms to maintain function during hypoxia. However, a study involving exposure of mice to a hypoxic environment showed that the diaphragm has reduced mitochondrial capacity and integrity (10). The most common cause of death of cachectic patients is respiratory failure. Muscle weakness is related to hypoxia and provides new mechanistic/therapeutic insight. Our findings demonstrate a novel pathway in the setting of cancer cachexia.

DISCUSSIONS

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES


