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RESEARCH ARTICLE

In the face of hypoxia: myoglobin increases in response to hypoxic conditions and lipid supplementation in cultured Weddell seal skeletal muscle cells

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SUMMARY

A key cellular adaptation to diving in Weddell seals is enhanced myoglobin concentrations in their skeletal muscles, which serve to store oxygen to sustain a lipid-based aerobic metabolism. The aim of this study was to determine whether seal muscle cells are inherently adapted to possess the unique skeletal muscle adaptations to diving seen in the whole animal. We hypothesized that the seal skeletal muscle cells would have enhanced concentrations of myoglobin *de novo* that would be greater than those from a C_2C_{12} skeletal muscle cell line and reflect the concentrations of myoglobin observed in previous studies. In addition we hypothesized that the seal cells would respond to environmental hypoxia similarly to the C_2C_{12} cells in that citrate synthase activity and myoglobin would remain the same or decrease under hypoxia and lactate dehydrogenase activity would increase under hypoxia as previously reported. We further hypothesized that β-hydroxyacyl CoA dehydrogenase activity would increase in response to the increasing amounts of lipid supplemented to the culture medium. Our results show that myoglobin significantly increases in response to environmental hypoxia and lipids in the Weddell seal cells, while appearing similar metabolically to the C_2C_{12} cells. The results of this study suggest the regulation of myoglobin expression is fundamentally different in Weddell seal skeletal muscle cells when compared with a terrestrial mammalian cell line in that hypoxia and lipids initially prime the skeletal muscles for enhanced myoglobin expression. However, the cells need a secondary stimulus to further increase myoglobin to levels seen in the whole animal.

Key words: Weddell seal, myoglobin, cell culture, hypoxia, skeletal muscle, C_2C_{12}, lipid.

INTRODUCTION

Myoglobin is a cytoplasmic hemoprotein, predominantly found in skeletal and cardiac muscle, that is able to reversibly bind oxygen and facilitate the diffusion of oxygen (Garry et al., 2003; Wittenberg and Wittenberg, 2003; Wittenberg et al., 1975). Studies examining the molecular regulation of myoglobin in mouse (*Mus musculus*) skeletal muscle tissue and C_2C_{12} mouse cell culture models have shown myoglobin to be predominantly regulated *via* the calcineurin/nuclear factor of activated T-cells (NFAT) pathway (Chin et al., 1998; Kanatous and Mammen, 2010; Kanatous et al., 2009). Calcium release from the sarcoplasmic reticulum during skeletal muscle activity (contraction) is the essential stimulus to activate the calcineurin enzyme. Activated calcineurin enzyme acts by dephosphorylating NFAT, which translocates into the nucleus to activate target gene programs to promote myoglobin protein expression (Chin et al., 1998; Kanatous and Mammen, 2010; Kanatous et al., 2009; Rao et al., 1997). These studies examining myoglobin regulation have demonstrated the importance of skeletal muscle contraction and stimulation in the activation of myoglobin gene expression and regulation in mammalian models.

Myoglobin has been extensively studied in hypoxia-adapted humans and animals to understand its regulation and expression in skeletal muscle tissue under low oxygen conditions. Historically, it was observed that hypoxia-adapted humans and animals possess elevated concentrations of myoglobin in skeletal and cardiac muscle, presumably because of the low oxygen environment in which they live (Hoppeler et al., 2003; Reynafarje, 1962). Kanatous and colleagues reported that altered calcium release under environmental hypoxia prevented skeletal muscle myoglobin expression in mouse and mouse cell culture models (Kanatous et al., 2009). Hypoxia as the lone stimulus was shown to cause calcium release from the endoplasmic reticulum, which inhibits the translocation of NFAT into the nucleus, essentially preventing the expression of the myoglobin protein. The study concluded that in the absence of skeletal muscle contraction, myoglobin expression in mouse skeletal muscle decreases or remains the same under hypoxia compared with normoxic oxygen environments. When skeletal muscle is stimulated to contract, selective calcium release from the sarcoplasmic reticulum is able to activate the calcineurin/NFAT pathway, resulting in myoglobin gene expression (Kanatous et al., 2009). When skeletal muscles are exercised under normoxic conditions, myoglobin concentration does not significantly increase beyond pre-exercise values. However, artificially and naturally stimulated skeletal muscles do show a significant increase in myoglobin when coupled with hypoxia (Hoppeler and Vogt, 2001; Kanatous et al., 2009; Mammen et al., 2003; Vogt et al., 2001). These studies, using terrestrial models, have demonstrated that muscle contraction and hypoxia in combination are essential in invoking significant increases in myoglobin expression. Although the terrestrial mouse models featured in the study by Kanatous et al. (Kanatous et al., 2009) showed significant increases in myoglobin under hypoxia, diving mammal models may hold unique mechanisms to further our knowledge regarding myoglobin regulation in response to hypoxia (Kanatous et al., 2009).

Weddell seals, *Leptonychotes weddellii* (Lesson 1826), have developed a suite of unique skeletal muscle adaptations to diving that allow them to maintain muscle function despite increasing ischemia and subsequent increasing tissue hypoxia during long duration breath-hold dives (Kanatous et al., 2002; Kanatous et al., 2008). An important adaptation that Weddell seals possess in order to maintain aerobic metabolism under the hypoxic conditions associated with breath-hold diving is increased concentrations of myoglobin in their skeletal muscles, compared with the skeletal muscles of terrestrial non-diving mammals. Previous studies have measured myoglobin concentrations to be 10-fold greater in the skeletal muscles of diving mammals when compared with those of athletic terrestrial mammals (Hochachka and Foreman, 1993; Kanatous et al., 2002; Reed et al., 1994). High concentrations of myoglobin allow divers to store oxygen within skeletal muscle to fuel aerobic metabolism throughout the duration of the breath-hold dive. Specifically for an adult Weddell seal, myoglobin concentrations of the primary swimming muscle have been calculated to be between 45.9±3.3 and 55.9±2.9 mg myoglobin g^{-1} wet muscle mass (Kanatous et al., 2002; Kanatous et al., 2008; Noren et al., 2005). Weddell seal pups have been found to have less myoglobin than mature adults, yet still possess myoglobin at concentrations of about 35 mg myoglobin g^{-1} wet muscle, which is greater than the concentrations found in the swimming muscle of adult Stellar sea lions (*Eumetopias jubatus*) and Northern fur seals (*Callorhinus ursinus*) (Kanatous et al., 1999; Kanatous et al., 2008). Weddell seal pups are considered to be non-diving during the time they are nursing and relying on their mother for dietary intake. Thus, Weddell seal pups seem to have enhanced myoglobin concentrations without proper skeletal muscle stimulation and exposure to diving conditions, i.e. without the cues normally associated with classical myoglobin regulation pathways. This is also true for other seal species, as studies have found that myoglobin concentrations show a trend of increasing from birth to when the animal is weaned (Burns et al., 2010). While myoglobin expression in terrestrial mouse models appears to be regulated by a combination of several stimuli (hypoxia and skeletal muscle contraction), developing seals already express high levels of myoglobin before experiencing the same physiological cues. Diving mammals appear to possess an inherent ability to augment myoglobin concentrations to great levels before mature diving patterns develop. Understanding the inherent *de novo* metabolic properties and myoglobin concentrations of developing Weddell seal skeletal muscle cells can advance our understanding of the mechanisms by which these elite divers enhance myoglobin expression to such high levels.

The purpose of this study was to investigate myoglobin concentrations and the metabolic profile of cultured Weddell seal skeletal muscle cells to determine whether Weddell seal skeletal muscle cells are inherently adapted to possess enhanced myoglobin concentrations. To accomplish this we examined non-stimulated Weddell seal skeletal muscle cells under normoxic (21% O_2) and hypoxic (0.5% O_2) culture conditions. We measured myoglobin concentrations and the activity of metabolic enzymes in the cells after 7 days of differentiation into myotubes. The enzymes assayed included: citrate synthase (CS), the enzyme in the first step of the citric acid cycle and an indicator of aerobic capacity, lactate dehydrogenase (LDH), the enzyme responsible for the conversion of pyruvate to lactate and an indicator of anaerobic capacity, and β-hydroxyacyl CoA dehydrogenase (HAD), an indicator of β-oxidation of fatty acids. We also examined the role lipids may play in the seal cells to aid in myoglobin expression under each oxygen condition by varying the amounts of lipid (2.5% and 5%) supplemented to the culture medium. The C_2C_{12} mouse muscle cell line served as a control throughout the study and was subjected to the same oxygen conditions as the seal cells. We hypothesized that the Weddell seal skeletal muscle cells would have enhanced concentrations of myoglobin *de novo* that would be significantly higher than those from a C_2C_{12} control mouse cell line and reflect the enhanced concentrations of myoglobin observed in tissue. In addition we hypothesized that the seal cells would respond to environmental hypoxia similarly to the terrestrial mammalian cell line (C_2C_{12} cells) in that CS enzyme activity and myoglobin would remain the same or decrease under hypoxia and LDH activity would increase under hypoxia, as reported in previous studies (Bigard et al., 1991; Kanatous et al., 2009; Lundby et al., 2009; McClelland and Brooks, 2002). We further hypothesized that HAD activity would increase in response to the increasing amounts of lipid supplemented to the growth medium. The results of this study suggest the regulation of myoglobin expression is fundamentally different in Weddell seal skeletal muscle cells when compared with a terrestrial mammalian cell line in that hypoxia and lipids may initially prime the skeletal muscle for enhanced myoglobin concentrations; however, the cells are still in need of a secondary stimulus to further increase myoglobin to levels seen in the whole animal.

MATERIALS AND METHODS

Weddell seal primary skeletal muscle cell isolation

The animal care and use committee at Colorado State University, Fort Collins, CO, USA approved all protocols for this study. MMPA no. 10751788-00. Animal handling and biopsy techniques were performed according to previously published protocols (Kanatous et al., 2008; Trumble et al., 2010). Primary Weddell seal skeletal muscle cells were isolated from the primary swimming muscle (M. longissimus dorsi) of an adult male (8 years old, mass 430 kg) using a muscle biopsy taken on 25 October 2006 near McMurdo Sound, Antarctica. Isolation of the myoblasts was performed in the Albert P. Crary Science and Engineering Center at McMurdo Station. The muscle biopsy tissue was briefly dipped into 70% ethanol and placed into Ham's F-10 growth medium (Hyclone Laboratories Inc., Logan, UT, USA) supplemented with 20% fetal bovine serum (FBS) (Atlanta Biologicals, Norcross, GA, USA) and 1% penicillin/streptomycin antibiotic (Sigma-Aldrich, St Louis, MO, USA), and taken *via* snowmobile from the field site on the ice to the Crary Laboratory. The biopsy was placed in a 60 mm cell culture dish and minced with sterile razor blades into 1 mm pieces. The pieces of muscle tissue were placed into a new 60 mm cell culture dish and 3 ml of Ham's F-10 growth medium was added to the plate. The plate was placed into an incubator set to 37°C, 21% O_2 and 5% CO_2. The plate was monitored for myoblast migration for 6 days. Fresh Ham's F-10 growth medium was added as needed. The cells were then passaged in order to enrich the myoblast cell type. The cells were seeded on a new 60 mm culture dish; after 30 min, the supernatant containing myoblasts was removed and placed onto a fresh 60 mm culture dish, leaving the fibroblasts behind. After four more pre-plating passages the myoblasts were frozen in liquid nitrogen, and at the end of the field season (November 2006) taken to Colorado State University, USA. The Weddell seal primary cell line is currently stored in a liquid nitrogen dewar for future use.

Weddell seal skeletal muscle cell culture

The primary skeletal myoblasts isolated from the Weddell seal were grown in standard medium used for skeletal muscle cells containing high glucose Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich), 20% FBS, 1% sodium pyruvate and 1% penicillin/streptomycin antibiotic. However, previous studies

in our lab found Weddell seals to be highly reliant on fatty acid metabolism (Kanatous et al., 2008). Therefore, the growth and differentiation media were supplemented with Lipid mixture 1 (product number L0288, Sigma-Aldrich). The lipid content of this chemically defined mixture is composed of the following fatty acids: 2 μg ml^{-1} arachidonic acid and 10 μg ml^{-1} of each of linoleic, linolenic, myristic, oleic, palmitic and stearic acid. The lipid mixture also contained 0.22 mg ml^{-1} cholesterol from New Zealand sheep's wool, 2.2 mg ml^{-1} Tween-80, 70 μg ml^{-1} tocopherol acetate and 100 mg ml^{-1} Pluronic F-68 solubilized in cell culture water. Lipid supplemented to the media at concentrations of 2.5% and 5% produced the best overall cell growth. A lipid concentration of 1% was used initially after myoblast isolation, and lipid concentrations showed an incremental increase until optimum medium was achieved. Concentrations that exceeded 5% actually became toxic to the cells and overall myoblast growth rates slowed and eventually ceased. Therefore, for this investigation concentrations of 2.5% and 5% were used throughout myoblast growth and differentiation, as this range was greater than the initial concentration yet lower than the lethal limit of the cells. To induce formation of differentiated myotubes, the medium was switched to high glucose DMEM, 5% equine serum, 10 μg ml^{-1} insulin and 10 μg ml^{-1} transferrin. The differentiation medium contained the same concentrations of lipid supplementation as the growth medium (2.5% and 5%).

Mouse skeletal muscle cell culture

An immortalized C_2C_{12} mouse skeletal muscle cell line was used as a control and was grown and differentiated in parallel with the Weddell seal primary skeletal muscle cells throughout the experiment. The C_2C_{12} myoblasts were grown in high glucose DMEM, 20% FBS, 1% sodium pyruvate and 1% penicillin/streptomycin antibiotic. At 90% confluency, to induce formation of differentiated myotubes, the medium was switched to high glucose DMEM, 5% equine serum, 10 μg ml^{-1} insulin and 10 μg ml^{-1} transferrin (Kanatous et al., 2009).

Cell culture oxygen conditions

The cultured skeletal muscle cells were differentiated under normoxic (21% O_2) and hypoxic conditions (0.5% O_2) for 7 days. Cells in the normoxic oxygen condition were kept in a humidified incubator set at 37°C with 5% carbon dioxide. Medium was changed and cells were harvested in a laminar flow cell culture unit (Labconco Corporation Purifier Class II Biosafety Cabinet, Kansas City, MO, USA). Cells in the hypoxic oxygen condition were kept in a humidified hypoxic environmental chamber (Coy Laboratory Products Inc., Grass Lake, MI, USA) set to 37°C with 5% carbon dioxide, 0.5% oxygen and 94.5% nitrogen. Medium was changed and cells were harvested within the hypoxic chamber, where oxygen concentration varied by ≤0.5% oxygen for periods of 1–2 min during the procedure.

Protein harvesting

Muscle cells were harvested using a rubber policeman in lysis buffer containing 79% phosphate-buffered saline, 20% glycerol, 1% Tween-20, 1 mmol l^{-1} dithiothreitol and a protease inhibitor cocktail that included serine and cysteine proteases (Roche Applied Science, Indianapolis, IN, USA). The resulting homogenates were spun at 10,000 ***g*** at 4°C for 10 min. The supernatant was divided into equal portions and stored at –80°C until assayed. Protein concentrations were determined using a Pierce Coomassie Plus Protein Assay Reagent (Pierce Chemicals, Rockford, IL, USA).

Enzymatic assays

All enzyme activities were assayed using a BioTek Synergy HT Multi-Detection microplate reader (Winooski, VT, USA). All assay reagents were purchased from Sigma-Aldrich.

The assay conditions were adapted from previous studies (Kanatous et al., 2008; Reed et al., 1994). CS assay buffers included: 50 mmol l^{-1} imidazole, 0.25 mmol l^{-1} 5,5-dithiobis(2-nitrobenzoic acid) (DTNB), 0.4 mmol l^{-1} acetyl-CoA and 0.5 mmol l^{-1} oxaloacetate, pH 7.5 at 37°C; ΔA_{412}, ε_{412}=13.6. LDH assay buffers included: 50 mmol l^{-1} imidazole, 0.15 mmol l^{-1} NADH, pH 7.0 at 37°C and 1 mmol l^{-1} pyruvate; ΔA_{340}, ε_{340}=6.22. HAD assay buffers included: 50 mmol l^{-1} imidazole, 1 mmol l^{-1} EDTA, 0.1 mmol l^{-1} acetoacetyl-CoA and 0.15 mmol l^{-1} NADH, pH 7.0 at 37°C; ΔA_{340}, ε_{340}=6.22. CS was assayed to determine the aerobic capacity of the seal and mouse cells. LDH was used to determine the anaerobic capacity of the seal and mouse cells. In addition, LDH activity determined whether the seal and mouse cells were experiencing a hypoxic condition (Lundby et al., 2009). HAD activity was assayed to determine the capacity of the seal and mouse cells to oxidize lipids for energy production. Enzyme activity was calculated using the rate of change derived from the maximal linear slope. Activity was normalized to protein concentration and is presented as U mg^{-1} protein.

Myoglobin concentrations

Myoglobin was assayed using a BioTek Synergy HT Multi-Detection microplate reader following previous methods (Kanatous et al., 1999; Kanatous et al., 2002) (modified from Reynafarje, 1963). In brief, aliquots of total protein was diluted in 0.04 mmol l^{-1} potassium phosphate buffer (pH 6.6) and centrifuged for 50 min at 28,000 ***g*** at 4°C. The supernatant was removed and bubbled with 99.9% carbon monoxide for 3 min. The absorbance of the supernatant was measured at 568 and 538 nm. Myoglobin concentrations were calculated as described previously (Kanatous et al., 1999) and normalized to total protein concentration. Myoglobin concentrations are presented as mg myoglobin mg^{-1} protein. Horse myoglobin standards were purchased from Sigma-Aldrich and measured with each sample.

Statistical analysis

Statistical analysis was performed using a one-way analysis of variance (ANOVA) with a Tukey *post hoc* test. Significance or alpha was considered at $P \leq 0.05$. All samples were run in triplicate and each enzymatic and myoglobin assay was repeated three times for an overall N=9 for all samples. All statistical tests were performed using SigmaStat version 2.0 (Ashburn, VA, USA). All data are presented as means ± s.e.m.

RESULTS

Cultured Weddell seal cell morphology

Although larger than the C_2C_{12} mouse myoblasts, the cultured Weddell seal myoblasts (Fig. 1B) were mononucleated and triangular, and so appeared morphologically similar to control C_2C_{12} mouse myoblasts (Fig. 1A). Upon differentiation, the cultured Weddell seal myotubes (Fig. 1D) were tube shaped, which was also similar to the control C_2C_{12} cells (Fig. 1C). The transformation of myoblasts into myotubes observed with the Weddell seal cells indicates proper skeletal muscle culture conditions and, more importantly, that the Weddell seal primary cell line retained its ability to differentiate into myotubes, because of the presence of the muscle-specific protein myoglobin.

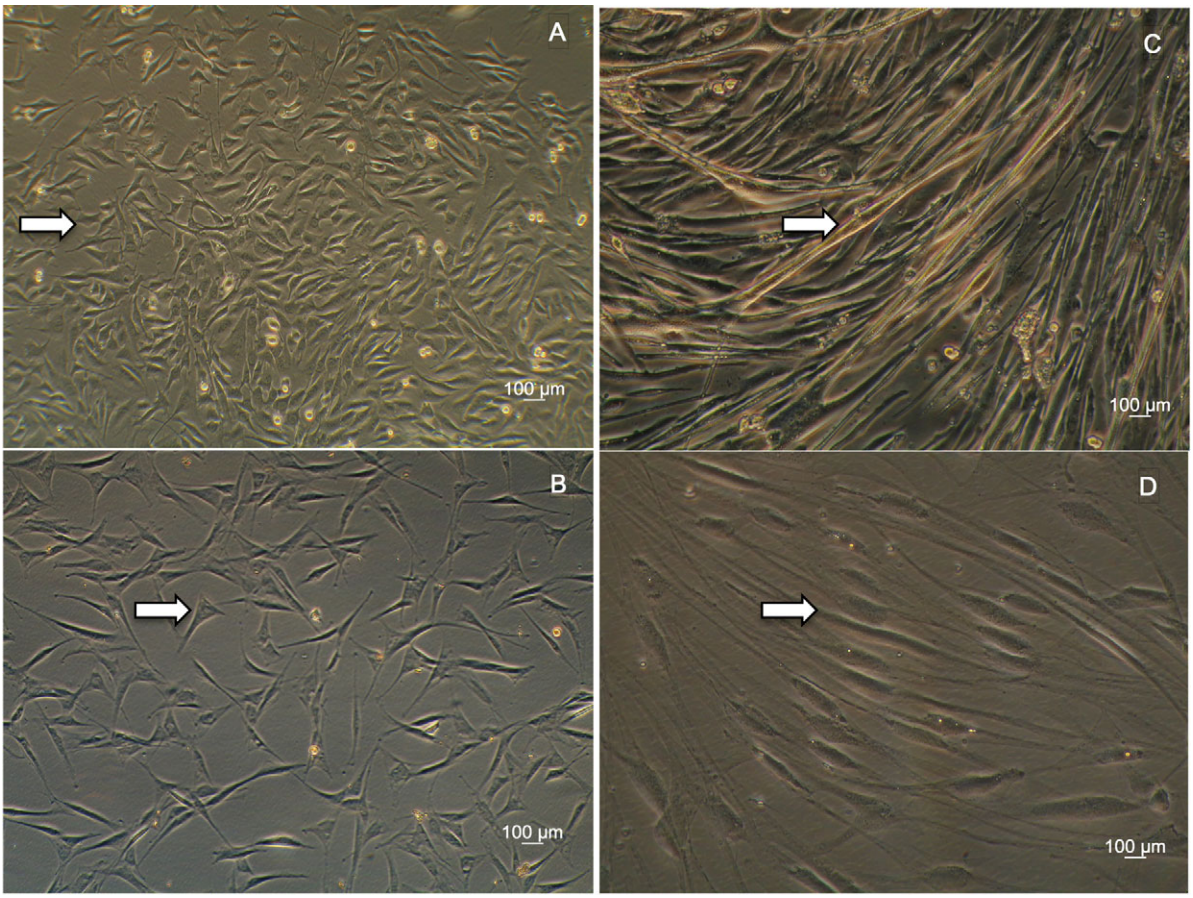

Fig. 1. (A) Control mouse skeletal muscle cell line (C_2C_{12}) myoblasts. (B) Primary Weddell seal skeletal muscle cell myoblasts. (C) Control mouse skeletal muscle cell line (C_2C_{12}) fully differentiated into myotubes. (D) Primary Weddell seal skeletal muscle cells fully differentiated into myotubes. Magnification for A–D, 20×. Arrows indicate individual myoblasts and myotubes. Scale bars, 100 µm.

Enzymatic activity

There was no significant difference in CS activity under the normoxic and hypoxic conditions in the Weddell seal cells supplemented with 2.5% lipid (0.066±0.021 and 0.084±0.020 U mg^{-1} protein, respectively; Fig. 2A, P=0.554). Similarly, there was no significant difference in CS activity between the Weddell seal cells supplemented with 5% lipid under the normoxic or hypoxic conditions (0.100±0.019 and 0.091±0.020 U mg^{-1} protein, respectively; Fig. 2A, P=0.758). In addition, the CS activity of the Weddell seal cells supplemented with 2.5% lipid under the normoxic and hypoxic oxygen condition was not different from that of the Weddell seal cells supplemented with 5% lipid under both oxygen conditions (Fig. 2A). CS activity in the C_2C_{12} control cells was significantly greater under the normoxic and hypoxic conditions when compared with that of the Weddell seal cells at both lipid concentrations (0.182±0.0027 and 0.302±0.0053 U mg^{-1} protein for normoxia and hypoxia, respectively; Table 1, P<0.001).

LDH enzyme activity increased significantly between the normoxic and hypoxic oxygen conditions in the Weddell seal cells supplemented with 2.5% lipid (0.63±0.026 *vs* 1.66±0.021 U mg^{-1} protein, respectively; Fig. 2B, P<0.001) and 5% lipid (0.62±0.012 *vs* 1.44±0.011 U mg^{-1} protein, respectively; Fig. 2B, P<0.001). There was no significant difference in LDH activity between the Weddell seal cells supplemented with 2.5% lipid and 5% lipid under the normoxic condition (0.63±0.026 *vs* 0.62±0.012 U mg^{-1} protein, respectively; Fig. 2B, P=0.669). Under the hypoxic condition, LDH significantly decreased between the Weddell seal cells supplemented with 2.5% lipid and 5% lipid (1.66±0.021 *vs* 1.44±0.011 U mg^{-1} protein, respectively; Fig. 2B, P<0.001). LDH activity in the C_2C_{12} control cells was also significantly greater under the normoxic and hypoxic conditions when compared with the Weddell seal cells in both lipid concentrations (2.769±0.0264 and 3.258±0.058 U mg^{-1} protein for normoxia and hypoxia, respectively; Table 1, P<0.001). LDH activity in the control cells also significantly increased under hypoxia when compared with the normoxic condition (Table 1, P<0.001).

Significant differences were found in HAD activity between the normoxic and hypoxic conditions in the Weddell seal cells supplemented with 2.5% and 5% lipid. In the Weddell seal cells supplemented with 2.5% lipid, HAD significantly increased in the hypoxic condition when compared with the normoxic condition (0.023±0.0002 *vs* 0.013±0.002 U mg^{-1} protein, respectively; Fig. 2C, P<0.001). In contrast, in the Weddell seal cells supplemented with 5% lipid, HAD activity significantly decreased in the hypoxic condition when compared with the normoxic condition (0.036±0.0018 *vs* 0.041±0.0012 U mg^{-1} protein, respectively; Fig. 2C, P=0.037). The Weddell seal cells supplemented with 5% lipid showed significantly increased HAD activity when compared with the Weddell seal cells supplemented with 2.5% lipid under both oxygen conditions (Fig. 2C, P<0.001). HAD activity in the control C_2C_{12} cell line was significantly increased under normoxia when compared with that in the Weddell seal cells supplemented

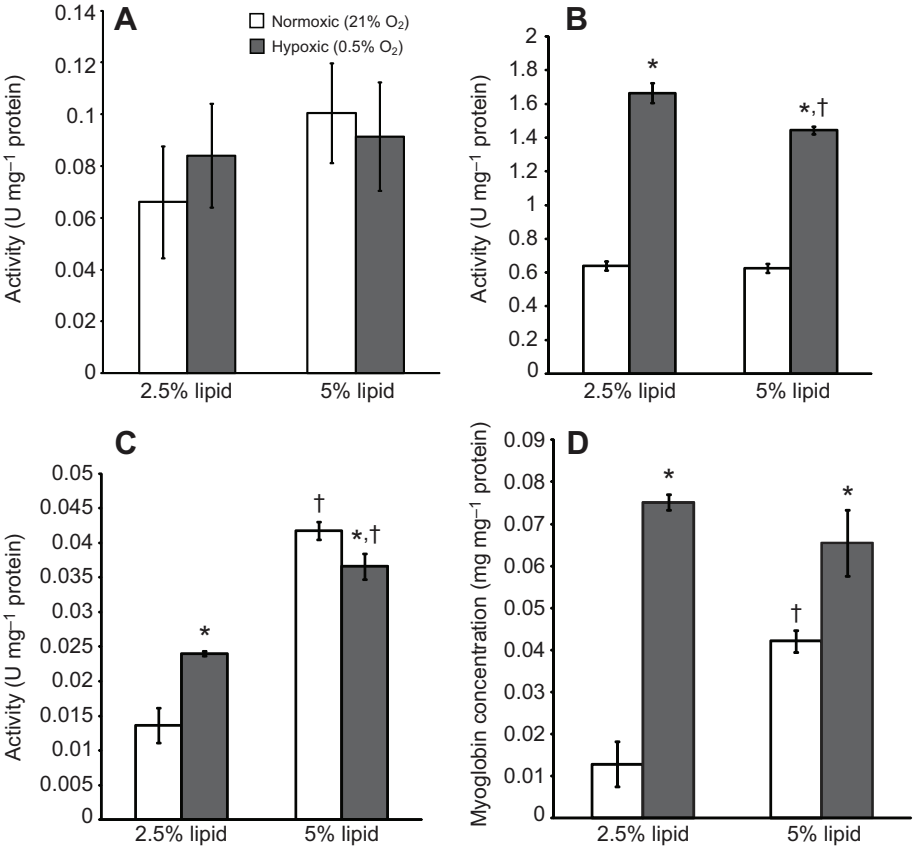

Fig. 2. Enzymatic activity and myoglobin concentrations in the cultured Weddell seal skeletal muscle cells under normoxic (21% O_2) and hypoxic (0.5% O_2) conditions and 2.5% and 5% lipid supplementation. Citrate synthase (A), lactate dehydrogenase (B) and β-hydroxyacyl CoA dehydrogenase (C) activity, and myoglobin concentration (D) are presented as means ± s.e.m. *Significant difference between the normoxic and hypoxic conditions. †Significant difference between the Weddell seal cells grown in 2.5% lipid and 5% lipid. *N*=9 for all enzymatic and myoglobin assays.

with 2.5% lipid (Table 1, $P<0.001$), but was not significantly different from that in the seal cells supplemented with 5% lipid (Table 1, $P=0.540$).

Myoglobin concentrations

Unexpected results were observed when myoglobin was assayed. In the Weddell seal cells supplemented with 2.5% lipid, we observed significantly greater concentrations of myoglobin in the cells under the hypoxic condition as compared with the normoxic condition (0.075±0.0078 *vs* 0.012±0.0025 mg myoglobin mg^{-1} protein, respectively; Fig. 2D, $P<0.001$). When myoglobin concentrations were measured in the Weddell seal cells supplemented with 5% lipid, we observed significantly greater concentrations in the cells under the hypoxic condition as compared with the cells under the normoxic condition (0.065±0.0068 *vs* 0.042±0.0067 mg myoglobin mg^{-1} protein, respectively; Fig. 2D, $P=0.021$). There was also a significant difference in myoglobin concentration under normoxia between the Weddell seal cells supplemented with 2.5% and 5% lipid (0.012±0.0025 and 0.042±0.0067 mg myoglobin mg^{-1} protein, respectively; Fig. 2D, $P<0.001$). This difference was not observed under hypoxia between the different lipid supplementation levels (Fig. 2D, $P=0.357$). Myoglobin concentrations in the C_2C_{12} control cells were not significantly different between the normoxic and hypoxic condition (0.031±0.0053 and 0.038±0.001 mg myoglobin mg^{-1} protein, respectively; Table 1, $P=0.366$).

DISCUSSION

The results of this study provide researchers with the first metabolic profile and myoglobin concentrations of cultured primary skeletal muscle cells isolated from a Weddell seal. The main findings from this study are: (1) myoglobin increases under environmental hypoxia in the non-stimulated Weddell seal cells to levels beyond those of the C_2C_{12} control cells, which remained the same under hypoxia; (2) when lipid supplementation was increased from 2.5% to 5% under hypoxia, myoglobin expression also increased in the seal cells; and (3) increasing lipid supplementation from 2.5% to 5% increased myoglobin expression in the seal cells under the normoxic oxygen condition. Contrary to our hypothesis, the cultured myotubes did not reflect the results of other studies using whole-muscle tissue in that we did not observe the levels of myoglobin previously reported (Kanatous et al., 2002; Kanatous et al., 2008; Ponganis et al., 1993). Our results indicate Weddell seal skeletal muscle cells are not inherently adapted to have such high myoglobin concentrations *de novo*; rather, there must be a combination of hypoxia, skeletal muscle activity and lipids acting in concert to create the unique myoglobin phenotype observed in Weddell seal skeletal muscle.

An important result from this study is that un-stimulated Weddell seal cells actually upregulated myoglobin under environmental hypoxia. Under hypoxia, myoglobin concentrations of the Weddell seal cells were found to be up to 1.7 times greater than those of the C_2C_{12} control cells (Table 1). The observed myoglobin concentrations were significantly greater in the Weddell seal cells (5% lipid) than the C_2C_{12} control cells under both normoxic and hypoxic conditions. This indicates that Weddell seal skeletal muscle cells have a unique response to hypoxia in terms of myoglobin regulation when compared with a terrestrial mammalian cell line. Although it is unknown whether the same results would have been observed in other diving marine species' skeletal muscle cells as a result of the uniqueness of the primary cell line used in this study, the result shows a clear capacity of Weddell seal muscle cells to respond to hypoxia in an adaptive fashion by upregulating myoglobin. The principal finding of this study, that Weddell seal

Table 1. Summary of enzymatic activities and myoglobin concentrations of Weddell seal skeletal muscle cells and control mouse skeletal muscle cells (C_2C_{12})

		Weddell seal		
		2.5% lipid	5% lipid	C_2C_{12}
Normoxic (21% O_2)	CS	0.066±0.021	0.100±0.019†	0.182±0.0027‡
	LDH	0.63±0.026	0.62±0.012	2.769±0.0264‡
	HAD	0.013±0.002	0.041±0.0012†	0.030±0.0017‡
	Myoglobin	0.012±0.0025	0.042±0.0067†	0.031±0.0053‡
Hypoxic (0.5% O_2)	CS	0.084±0.022	0.091±0.020	0.302±0.0084*,‡
	LDH	1.66±0.021*	1.44±0.011*,†	3.258±0.058*,‡
	HAD	0.023±0.0002*	0.036±0.0018*,†	0.050±0.0050*,‡
	Myoglobin	0.075±0.0078*	0.065±0.0068*	0.038±0.001‡

Enzyme activity is presented as U mg^{-1} protein. Myoglobin concentrations are presented as mg myoglobin mg^{-1} protein. All values are presented as means ± s.e.m. (N=9 for all assays).

*Significant difference from the normoxic (21% O_2) oxygen condition.

†Significant difference between the Weddell seal cells grown in 2.5% lipid and 5% lipid.

‡Significant difference between C_2C_{12} control cell line and Weddell seal cells.

cells upregulate myoglobin under hypoxia, is in direct contrast to recent studies undertaken to understand myoglobin expression in mouse models and mouse cell culture (Kanatous et al., 2009).

It has been shown, using C_2C_{12} cells and whole-animal mouse models, that chronic environmental hypoxia as the lone stimulus is not sufficient to increase myoglobin expression (Kanatous et al., 2009). It was only when the skeletal muscle was stimulated to contract under hypoxia that an increase in myoglobin was measured, indicating the need for contraction to activate myoglobin gene expression. The key factor in the expression of myoglobin under hypoxia is the need for simultaneous stimulation of specific calcium handling pathways associated with skeletal muscle activity (Kanatous et al., 2009). This is consistent with other studies that measured an increase in myoglobin mRNA content after providing skeletal muscle stimulation under simulated hypoxia (Hoppeler and Vogt, 2001; Vogt et al., 2001). Under normoxia, however, skeletal muscle activity (exercise) was not sufficient to increase myoglobin concentrations in exercising human subjects and trained laboratory rats. These studies show that myoglobin expression was unaffected by the demands of the exercising subject under normoxia, which may indicate the need for an additional stimulus to enhance myoglobin concentrations (Harms and Hickson, 1983; Masuda et al., 1999). It is important to note that changes in myoglobin have been shown to occur with and without activity-induced changes in skeletal muscle fiber type. In some cases myoglobin has been shown to increase in response to changes in fiber type during simulated exercise under normoxic conditions (Chin et al., 1998). In contrast, Kanatous and colleagues showed decreases in myoglobin under hypoxia without simultaneous changes in fiber type (Kanatous et al., 2009); however, it is unknown whether any changes in fiber type occurred in the Weddell seal skeletal muscle cells with the changes in myoglobin.

The relationship between skeletal muscle activity and hypoxia and its role in myoglobin expression has been explored in mammalian and non-mammalian breath-hold divers. Studies following the ontogeny of diving in mammalian species have shown that the highly active juvenile Weddell seals have significantly greater myoglobin concentrations than adults, which is presumed to be due to the high skeletal muscle activity during multiple breath-hold dives (Burns, 1999; Kanatous et al., 2008; Kanatous et al., 2009). In non-mammalian models using emperor penguins (*Aptenodytes forsteri*) it was shown that captive penguins, which have not experienced long breath-hold dives, had lower myoglobin concentrations in their primary swimming muscle than penguins from the wild (Ponganis et al., 2010). In addition, myoglobin mRNA content was found to be lower in pre-fledging non-diving chicks when compared with diving adults (Ponganis et al., 2010). These results from mammalian and non-mammalian divers indicate the need for a hypoxic dive bout, coupled with the skeletal muscle activity associated with swimming to enhance myoglobin expression.

Another result of significant interest from this study is that the addition of lipid to the growth and differentiation media of the Weddell seal cells enhanced myoglobin concentrations under normoxic oxygen conditions. Under normoxia, a 1.3 times increase in myoglobin expression in the Weddell seal cells compared with the C_2C_{12} control cells was measured when the medium was supplemented with lipid at a concentration of 5%. Our results show that lipids may prime skeletal muscle initially to allow Weddell seals to enhance myoglobin concentrations to levels well beyond those of terrestrial mammals. This finding may explain why non-diving weaned Weddell seal pups have myoglobin concentrations up to 35 mg g^{-1} wet tissue in their primary swimming muscle (Kanatous et al., 2008). Weaned harp seal (*Pagophilus groenlandicus*) and hooded seal (*Cystophora cristata*) pups have ~30% of the myoglobin content of adults in their skeletal muscle while weaned Weddell seal pups specifically have 35.5±3 mg myoglobin g^{-1} wet tissue or ~63% of that of adults (Burns et al., 2010; Kanatous et al., 2008). During development, seal pups rely on milk with a high fat content (>50%) and a relatively low carbohydrate content as an energy source before commencement of their first breath-hold dive (Burns et al., 2010; Oftedal, 1993). During this pre-dive development, seal pups show increasing myoglobin expression before becoming expert adult divers, indicating dietary lipids may be the key initial stimulus to possessing enhanced myoglobin stores (Burns et al., 2010; Kanatous et al., 2008; Lestyk et al., 2009; Noren et al., 2005). This idea is illustrated in developing harp seal pups when myoglobin was analyzed during fetal to late weaned time points. Harp seals are born relatively altricial when compared with other pack-ice seals and spend about 9 days on land nursing before the commencement of diving (Ronald and Dougan, 1982). During this time, when high fat milk was the only source of dietary intake, myoglobin was found to significantly increase from the fetal stage to a late weaned stage (Burns et al., 2010). The results from the present study taken with the results from harp seals (Burns et al., 2010) demonstrate the fundamental role lipids may play during the development of enhanced myoglobin stores in skeletal muscle of marine mammals.

A fundamental role for lipids in the development of myoglobin may also translate to non-mammalian breath-hold divers. Emperor penguin chicks (3–6 months old) have been shown to possess a relatively high myoglobin content before breath-hold diving. Because of the lack of skeletal muscle activity and hypoxia associated with diving, lipids from the diet of penguin chicks may be the key stimulus to augment myoglobin content up to 2.7±0.4 g $100\,g^{-1}$ muscle in 6 month old chicks (Ponganis et al., 2010). In pre-fledging emperor penguins, the occasional wing flapping to aid in maintaining body temperature coupled with dietary lipid may be responsible for the increases in myoglobin as the penguin develops into the diving adult stages of its life (Ponganis et al., 2010). Results from Ponganis and colleagues (Ponganis et al., 2010) are similar to those of this study because we found myoglobin significantly increased in the seal cells under the normoxic oxygen condition when we increased lipid concentrations from 2.5% to 5% (Fig. 2D). Our results suggest that a complex interaction between myoglobin and lipids is present in divers, and lipids may enhance myoglobin expression initially during the development of breath-hold divers. However, we hypothesize that there is still the need for additional external cues (hypoxia and skeletal muscle activity) to further stimulate the increase in myoglobin expression to levels seen in mature animals.

The enzyme activities showed classical responses to environmental hypoxia, except when we measured CS activity in the cells supplemented with 2.5% lipid. We measured a trend of increase in CS activity under hypoxia but this was not statistically significant. The increase actually correlated with an increase in HAD activity, suggesting an increase in flux of acetyl-CoA from lipid oxidation through the metabolic system. When we measured LDH, we observed a significant upregulation under hypoxia in the Weddell seal cells with 2.5% and 5% lipid, suggesting an increase in anaerobic capacity. We measured the same significant increase in LDH activity under hypoxia in the C_2C_{12} control cell line (Table 1). Previous studies measuring LDH activity in skeletal muscle of hypoxia-adapted mammals have also shown an increase in activity of the enzyme (Bigard et al., 1991; Lundby et al., 2009; McClelland and Brooks, 2002). This result is significant in that we observed evidence that the cells are actually experiencing a hypoxic situation, in which the response was to increase anaerobic capacity. More importantly, the LDH results show the Weddell seal cells and C_2C_{12} mouse cells are similar in their anaerobic response to environmental hypoxia, yet dissimilar in terms of the myoglobin expression response to hypoxia. When we compared CS and LDH activities of our Weddell seal cells (2.5% and 5% lipid supplementation) with our control C_2C_{12} cell line, we observed that the activity of both enzymes was significantly lower under the normoxic and hypoxic conditions (Table 1). The overall superior metabolic profile of the C_2C_{12} cell line was attributed to the properties of an immortalized cell line with optimized growth and differentiation media, which give the cells the best chance to grow into well-developed myotubes (Blau et al., 1983). Although the C_2C_{12} cells appeared to be in a better position to express more myoglobin by having a greater capacity to generate usable energy through aerobic metabolism, they actually had significantly less myoglobin than the Weddell seal cells.

In this study, environmental hypoxia and the addition of lipids to cell culture media was sufficient to increase myoglobin in non-stimulated cultured Weddell seal skeletal muscle cells. The results suggest fundamental differences at the cellular level in myoglobin regulation in Weddell seal skeletal muscle cells when compared with skeletal muscle cells of a terrestrial mammal. Although the cells enhanced myoglobin expression to levels greater than those in a control C_2C_{12} mouse cell line (Table 1), we did not measure the amounts seen in previous studies of Weddell seals. The results of the present study also showed that Weddell seal skeletal muscle cells increase myoglobin protein expression in response to increasing amounts of lipid added to the growth and differentiation media. These results suggest myoglobin regulation in cultured Weddell seal cells may initially be independent of skeletal muscle activity and rely on lipids to prime the skeletal muscle. However, to enhance myoglobin protein expression to levels seen in the whole animal, a secondary stimulus of hypoxia coupled with skeletal muscle activity is still needed. This study, which utilized a cell culture technique on a marine mammal, has revealed novel data that advance our understanding of enhanced myoglobin expression of elite diving mammals.

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