J Appl Physiol 117: 307–315, 2014.
First published June 12, 2014; doi:10.1152/japplphysiol.00973.2013.

Changing the paradigm for myoglobin: a novel link between lipids and myoglobin

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Submitted 26 August 2013; accepted in final form 10 June 2014

Schlater AE, De Miranda MA Jr, Frye MA, Trumble SJ, Kanatous SB. Changing the paradigm for myoglobin: a novel link between lipids and myoglobin. *J Appl Physiol* 117: 307–315, 2014. First published June 12, 2014; doi:10.1152/japplphysiol.00973.2013.—Myoglobin (Mb) is an oxygen-binding muscular hemeprotein regulated via Ca^{2+}-signaling pathways involving calcineurin (CN), with Mb increases attributed to hypoxia, exercise, and nitric oxide. Here, we show a link between lipid supplementation and increased Mb in skeletal muscle. C_2C_{12} cells were cultured in normoxia or hypoxia with glucose or 5% lipid. Mb assays revealed that lipid cohorts had higher Mb than control cohorts in both normoxia and hypoxia, whereas Mb Western blots showed lipid cohorts having higher Mb than control cohorts exclusively under hypoxia. Normoxic cells were compared with soleus tissue from normoxic rats fed high-fat diets; whereas tissue sample cohorts showed no difference in CO-binding Mb, fat-fed rats showed increases in total Mb protein (similar to hypoxic cells), suggesting increases in modified Mb. Moreover, Mb increases did not parallel CN increases but did, however, parallel oxidative stress marker augmentation. Addition of antioxidant prevented Mb increases in lipid-supplemented normoxic cells and mitigated Mb increases in lipid-supplemented hypoxic cells, suggesting a pathway for Mb regulation through redox signaling independent of CN.

calcineurin; cell culture; hypoxia; lipids; myoglobin

DURING ENDURANCE EXERCISE, terrestrial mammals rely primarily on erythrocytic oxygen stores bound to hemoglobin to fuel aerobic metabolism in working muscle. Physiological changes associated with endurance training elicit responses that increase muscular blood flow and subsequent oxygen delivery (e.g., increasing capillary density) (2, 13, 32). Muscle oxygen stores, alternatively, appear to bear little significance in sustaining aerobic metabolism during endurance exercise, evident by the inability to appreciably release intramuscular stored oxygen during exercise (29); however, terrestrial endurance athletes have more myoglobin (Mb) than their sedentary counterparts (10, 27). Accordingly, Millikan's coined alias "muscle hemoglobin" (28), in which the functional paradigm of Mb pertains to oxygen storage and transport, does not appear to be fully applicable to terrestrial mammals in vivo. Here, we provide data that offer an alternative paradigm for Mb increases associated with aerobic metabolism in the skeletal muscle of terrestrial mammals.

Mb is an oxygen-binding hemeprotein generally localized to oxidative muscle and functions as an oxygen store, nitric oxide (NO) scavenger, and reactive oxygen species (ROS) scavenger (11, 12, 14, 15, 17, 23, 26, 28, 35, 46). Interestingly, as deduced from the low p50 of its oxygen dissociation curve (p50 = 2.39 mmHg in equine Mb), Mb has a strong affinity for oxygen and only releases oxygen under a very low partial oxygen pressure (1, 14, 17, 30, 39). Thus, under standard conditions, muscle must necessarily be hypoxic to utilize Mb-bound oxygen for aerobic metabolism. This presents an interesting theoretical perspective of Mb as it assists cellular respiration via oxygen supply solely in a time of potential stress. Interestingly, oxymyoglobin measured in submaximally exercising adult humans desaturates only during the first 20–40 s of exercise; moreover, during its brief desaturation period, Mb never desaturates beyond 50% (29). Many terrestrial, athletic mammals, however, have relatively high levels of Mb within their skeletal muscle despite never being truly oxygen limited due to higher muscular capillary densities (10, 27).

Regarding a role in oxygen transport, muscular oxygen flux is attributed to competing contributions of Mb-facilitated oxygen diffusion and free oxygen. This relationship is described by the equipoise diffusion P_{O_2}, which is the P_{O_2} that allows Mb and oxygen to contribute equally to oxygen transport. Declines in the equipoise diffusion P_{O_2} indicate lower contributions of Mb to oxygen flux and can vary with changes in p50. Specifically, as the p50 of Mb increases, the equipoise diffusion P_{O_2} decreases. Temperature change has been characterized as a modifier of the p50 of Mb, such that temperature increases will increase the Mb p50. Taken together, this means that an increase in cellular temperature is concomitant with a decreased cellular dependence on Mb-facilitated oxygen diffusion. Therefore, during exercise, when cellular oxygen consumption rises and contractile activity increases temperature, Mb contribution to oxygen transport is actually decreased (7, 25, 39). Collectively, in light of its low p50, minimal oxygen desaturation, and decreased contribution to oxygen transport during exercise, high Mb levels in healthy terrestrial animals that do not experience routine hypoxic stress is nonsensical in the context of increasing oxygen storage and transport.

Physiological hypoxic stress can occur in high-altitude environments or in cardiovascular and/or pulmonary disease states. Because the aforementioned conditions cause low oxygen at the cellular level, they can ultimately manifest into pathological conditions at the tissue level over time; however, Mb is capable of providing cytoplasmic oxygen reserves that can be readily used in pathological states when erythrocytic oxygen becomes insufficient. As such, Mb presents a potential target for pharmacological intervention and treatment of human hypoxic disease or, more specifically, treatment of consequent tissue ischemia. Understanding regulation of Mb and

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potential stimuli thus bridges the gap between pharmacological potential and actual practice.

Although Mb regulation is not yet fully understood, understanding of Mb stimulation is escalating. Transcriptionally, the Mb gene is activated via CCAC box, A/T element, nuclear factor of activated T cells (NFAT), E box, and myocyte enhancing factor-2 motifs (16, 23, 47). Transcriptional regulation is sensitive to increases in intracellular calcium, which activate calcineurin (CN), a calcium-activated phosphatase. Activated CN, in turn, dephosphorylates NFAT, thus enabling NFAT translocation to the nucleus and subsequent stimulation of Mb expression (6, 23, 24, 31). This pathway, therefore, demonstrates a component of calcium dependency in Mb regulation. Beyond transcription, Mb increases are attributable to environmental factors including hypoxia (36), exercise (24), and NO (35). Recently, Mb has also been shown to increase in response to lipid supplementation in seal cells (8). Seals and other diving vertebrates have an extreme abundance of Mb in their skeletal muscles as an adaptation to chronic severe hypoxia (5, 18, 22, 41). Moreover, diving marine vertebrates have diets rich in lipids and protein, from the extremely lipid-rich milk consumed early in life (3) to consumption of fish and invertebrates as adults (4, 33). As such, it is unknown whether lipid-induced Mb increases observed in seal cells are relevant to Mb regulation across all vertebrates, both marine and terrestrial alike, or whether it is specific to hypoxia-adapted marine mammals.

Here, we aimed to determine the role of lipids in Mb regulation within the skeletal muscle of terrestrial model species. C_2C_{12} cells, immortalized mouse skeletal muscle cells, were differentiated in normoxic (21% O_2) or hypoxic (0.5% O_2) conditions with standard, glucose, or 5% lipid-supplemented differentiation media. Also, a subset of normoxic and hypoxic C_2C_{12} lipid-supplemented cells was differentiated with the addition of a ROS scavenger (i.e., antioxidant), phenyl-α-tert-butyl nitrone (PBN), starting on *day 3* of differentiation. Between treatment groups, Mb levels were determined via functional CO-binding Mb assays and immunoblots, CN levels were determined via immunoblots, citrate synthase (CS) levels were determined via CS assays, and oxidative stress was inferred via PCR transcript analysis. Also, normoxic cells were compared with normoxic whole tissue using soleus muscle from Sprague-Dawley rats fed high-fat diets [40% saturated fatty acids (SAT)].

MATERIALS AND METHODS

Cell culture. Normoxic C_2C_{12} cells were grown and differentiated in a 37°C humidified incubator with 5% CO_2. Hypoxic cells differentiated in a humidified hypoxic chamber (Coy Laboratories, Grass Lake, MI) at 37°C, 5% carbon dioxide, 0.5% oxygen, and 94.5% nitrogen. Standard growth media was used for myoblast proliferation [Dulbecco's modified Eagle's media high glucose (DMEM), 20% fetal bovine serum, 1% sodium pyruvate, 1% penicillin/streptomycin antibiotic]. At 90% confluency, myotube differentiation was induced with either glucose control media (high-glucose DMEM, 5% equine serum, 10 μg/ml insulin, 10 μg/ml transferrin) or 5% lipid-supplemented media [2 μg/ml arachadonic acid, 10 μg/ml each of linoleic, linolenic, myristic, oleic, palmitic, and stearic fatty acids (Sigma Aldrich, Milwaukee, WI)]. A subset of normoxic and hypoxic lipid-supplemented cells were differentiated with the addition of 1 mM PBN starting on differentiation *day 3* (19)(Sigma Aldrich). At differentiation *day 7*, all cells were harvested for protein or RNA with a standard homogenization buffer (20% glycerol, 1% Tween 20, 0.001 M dithiothreitol in PBS with a protease inhibitor table) or TriPure (Roche, Indianapolis, IN), respectively.

Table 1. *Macronutrient composition and caloric density of rat diets*

	CON	SAT
Protein, % kcal	20	20
Carbohydrate, % kcal	69	38
Fat, % kcal	11	42
Saturated fat, % total FA	43	92
Monounsaturated fat, % total FA	8	4
Polyunsaturated fat, % total FA	49	4
Kcal/g	3.6	4.5

CON, control; SAT, 40% saturated fat; FA, fatty acid.

High-fat rats. Soleus muscle came from rats fed control starch diets (CON) or high-fat diets (40% SAT) as previously described (20). Briefly, adult male Sprague-Dawley rats (CD IDS Rats; Charles River Laboratories, Wilmington, MA) were maintained in a temperature- and humidity-controlled environment at Colorado State University Laboratory Animal Resource Center under a normal 12-h:/12-h light/dark cycle. Rats were housed in pairs under regulations of the Animal Welfare Act, the Guide for Care and Use of Laboratory Animals, and the Guide for Care and Use of Agricultural Animals in Agricultural Research and Teaching, and all protocols were approved by the IACUC at Colorado State University. Before commencing dietary treatments, rats were acclimated to the facilities for 2 wk.

Diet. At 6 wk of age, rats were fed either control (CON) or 40% saturated fat diets (SAT) for a total of 32 wk. Diets were supplied by Harlan Teklad (Madison, WI) and are detailed in Tables 1 and 2. Body weight was measured weekly. Before terminal sample collection, rats were fasted overnight.

Tissue collection. Rats were placed in a commercial rodent anesthesia chamber for anesthetic induction using 4% isofluorane in a 95% O_2-5% CO_2 gas mixture, and anesthesia was maintained at identical gas concentrations administered via nosecone. Animals were euthanized by exsanguinations and removal of the heart. The soleus muscle was excised at 0°C and stored at −80°C until used.

Tissue homogenization. Tissue was mechanically homogenized at 0°C in lysis buffer (79% PBS, 20% glycerol, 1% Tween 20, 0.001 M dithiothreitol with a protease inhibitor tablet). Samples were centrifuged at 10,000 *g* at 4°C for 5 min, and the supernatant was frozen at −80°C until samples were used. Protein concentrations were determined using a Coomassie Plus Protein assay (Thermo Scientific, Rockford, IL).

Protein assays. Assays were performed using a BioTek Synergy HT Multi-Detection microplate reader. Protein concentrations were determined using Coomassie Plus (Thermo Scientific). CS assays were performed as previously described (22) to determine aerobic capacity of cells. CS assay buffers included: 50 mmol/l imidazole,

Table 2. *Fatty acid composition of diets (% of total diet)*

Fatty Acid	CON	SAT
8:0	0.14	1.6
10:0	0.09	1.1
12:0	0.72	8.6
14:0	0.24	2.9
16:0	0.28	2.0
18:0	0.24	2.9
18:1 n-9	0.33	0.8
18:2 (LA)	1.95	0.8
n-6	1.95	0.84

LA, linoleic acid.

0.25 mmol/l 5,5-dithiobis(2-nitrobenzoic acid), 0.4 mmol/l acetyl-CoA, and 0.5 mmol/l oxaloacetate, pH 7.5; ΔA_{412}, $\epsilon_{412} = 13.6$.

Mb assays were performed as adapted from Reynafarje (37) and Kanatous et al. (21). Briefly, protein homogenates were diluted with phosphate buffer (0.04/mol, pH 6.6) and subsequently centrifuged at 28,000 *g* at 4°C for 50 min. The resultant supernatant was then bubbled with 99.9% CO, which converts myoglobin to carboxymyoglobin. After 3 min of bubbling, samples were combined with 0.01 g sodium dithionite, a reducing agent, and then bubbled again for 2 min; this was done to account for Mb that may have been oxidized and thus not accounted for in the assay otherwise. The absorbance of the supernatant at 538 and 568 nm was measured using a Bio-Tek PowerWave ×340 microplate reader (Winooski, VT). A Mb standard (horse Mb, Sigma-Aldrich) was included with each set of samples. The Mb concentrations were calculated as described previously (37) and expressed in mg/mg protein. All assays were performed in triplicate.

Western blots. Changes in protein expression were determined using Western blots as previously described (22). Briefly, samples were mixed in a 1:1 ratio with SDS and 0.05% bromophenol blue, boiled for 5 min, and spun through glass wool spin columns. Then, 20 μg of protein were loaded into wells of precast, 4–20% polyacrylamide gels, and gel electrophoresis was run out in standard running buffer (1× tris-glycine SDS) at 150 V for ~40 min, until dye front reached the bottom of the gel. Gels were dry transferred onto nitrocellulose membranes using iBlot gel transfer stacks (Invitrogen, Grant Island, NY), and membranes were subsequently probed with primary antibodies. Polyclonal rabbit, anti-human myoglobin (1:3,000) (DakoCytomation, Carpinteria, CA), polyclonal rabbit anti-actin (1:5,000) (Thermo Scientific), and anti-mouse CN (1:250) (BD Transduction Laboratories, San Diego, CA) were the primary antibodies used; each primary antibody was detected with a horseradish peroxidase-conjugated secondary anti-serum. Resultant protein bands were visualized using the Supersignal West Dura Luminol chemiluminescent agent (Thermo Scientific). Band intensity was quantified using Bio-Rad Image Lab 3.0 software (Hercules, CA).

RNA transcript analysis. RNA was isolated using TriPure (Roche) and cleaned using RNeasy (Qiagen, Valencia, CA); yield was determined via optical density measurements on a DU580 spectrophotometer (Beckman Coulter, Indianapolis, IN). cDNA was synthesized from 500 ng RNA via first-strand synthesis kit (Qiagen) and thermocycler (MJ Research, St. Bruno, Quebec, Canada). PCR was performed with RT^2 profiler PCR array PAMM-065ZG-4 (Mouse Oxidative Stress and Antioxidant Defense superarray) with RT^2 Real-Time SYBR Green Mastermix on the Roche 480 Light Cycler for 10 min at 95°C, then 45 cycles of 95°C for 15 s and 60°C for 1 min. Gene expression changes were calculated using the Second Derivative Maximum analysis method, which uses cross-point analysis of the PCR reaction to obtain relative fold changes. Samples were run in replicates of three; difference in transcript expression was defined at less than twofold or greater than twofold change.

Statistical analysis. Student's *t*-test or one-way ANOVA with a Tukey's post hoc test were used for statistical analyses with SigmaStat version 2.0 (Ashburn, VA). Significance was considered at $P \leq 0.05$; all data are presented as means ± SE.

RESULTS

Mb. Cellular Mb assays showed Mb increased in normoxic 5% lipid C_2C_{12} cells compared with normoxic glucose C_2C_{12} cells (0.159 ± 0.00145 vs. 0.0560 ± 0.00169 mg/mg protein, respectively, $P < 0.001$, $n = 6$). Similarly, cells differentiated in hypoxia also showed Mb increases in 5% lipid C_2C_{12} cells compared with normoxic glucose C_2C_{12} cells (0.118 ± 0.00238 vs. 0.0278 ± 0.00111 mg/mg protein, respectively, $P < 0.001$, $n = 6$) (Fig. 1). Western blots for normoxic cells indicated an insignificant trend for an increase in Mb protein in 5% lipid cells [0.875 ± 0.0472 relative units (R.U.) in lipid cells compared with 0.636 ± 0.100 R.U. in control cells, $P = 0.1$] (Fig. 2*A*), and Western blots for hypoxic cells showed an increase in Mb protein (0.0809 ± 0.0029 R.U. in lipid cells compared with 0.0420 ± 0.0089 R.U. in control hypoxic cells, $P = 0.01$) (Fig. 2*C*).

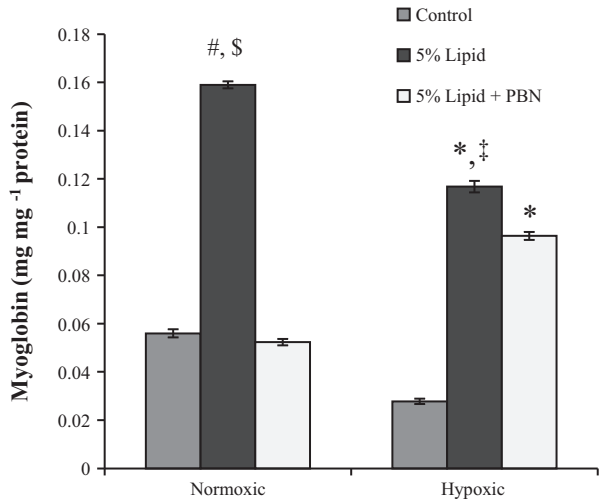

Fig. 1. Myoglobin (Mb) measured in C_2C_{12} cells. In normoxic cells, 5% lipid conditions increased Mb relative to control cells; interestingly, addition of a reactive oxygen species (ROS) scavenger, phenyl-α-tert-butyl nitrone (PBN), reversed this lipid-induced Mb increase in normoxic cells. In hypoxic cells, 5% lipid-supplemented cells showed an increase in Mb. Hypoxic, 5% lipid-supplemented cells supplemented with PBN showed a reduction in hypoxic lipid-induced Mb increase, but these cells still showed an Mb increase relative to hypoxic control cells (#significantly different from normoxic control, $P < 0.001$, $n = 6$) ($significantly different from normoxic 5% Lipid + PBN cells, $P < 0.001$, $n = 6$) (*significantly different from hypoxic control, $P < 0.001$, $n = 6$) (‡significantly different from hypoxic 5% Lipid + PBN cells, $P < 0.001$, $n = 6$).

Subsets of all lipid-supplemented cells were cultured with PBN, a ROS scavenger. Normoxic cells showed that addition of PBN in conjunction with 5% lipid caused a complete reversal of previously seen lipid-induced Mb increase. Specifically, 5% lipid + PBN cells showed no difference in Mb compared with glucose control cells (0.0523 ± 0.00132 vs. 0.0560 ± 0.00169, mg/mg protein, respectively) (Fig. 1). In regard to the hypoxic cohorts, 5% lipid + PBN cells showed a decrease in Mb relative to 5% lipid cells (0.0963 ± 0.00165 vs. 0.118 ± 0.00238 mg/mg protein, respectively, $P < 0.001$, $n = 6$); interestingly, the hypoxic 5% lipid + PBN cells still had more Mb than hypoxic control cells (Fig. 1).

At the tissue level, Mb assays showed no difference in functional CO-binding Mb between CON vs. 40% SAT diets in rat soleus muscle (data not included). Interestingly, Western blots for tissue indicated an increase in Mb protein in the SAT rat soleus compared with the CON (1.312 ± 0.109 R.U. in SAT rats compared with 0.596 ± 0.0906 in CON rats, $P = 0.01$) (Fig. 2*B*).

Calcium regulatory protein. Unlike previous studies that show increases in CN with increases in Mb (6, 24, 31), our study found an increase in Mb with either no change or a decrease in CN. Western blots comparing CN expression showed a decrease in CN protein in normoxic high-fat cells compared with normoxic control cells (0.529 ± 0.077 in lipid

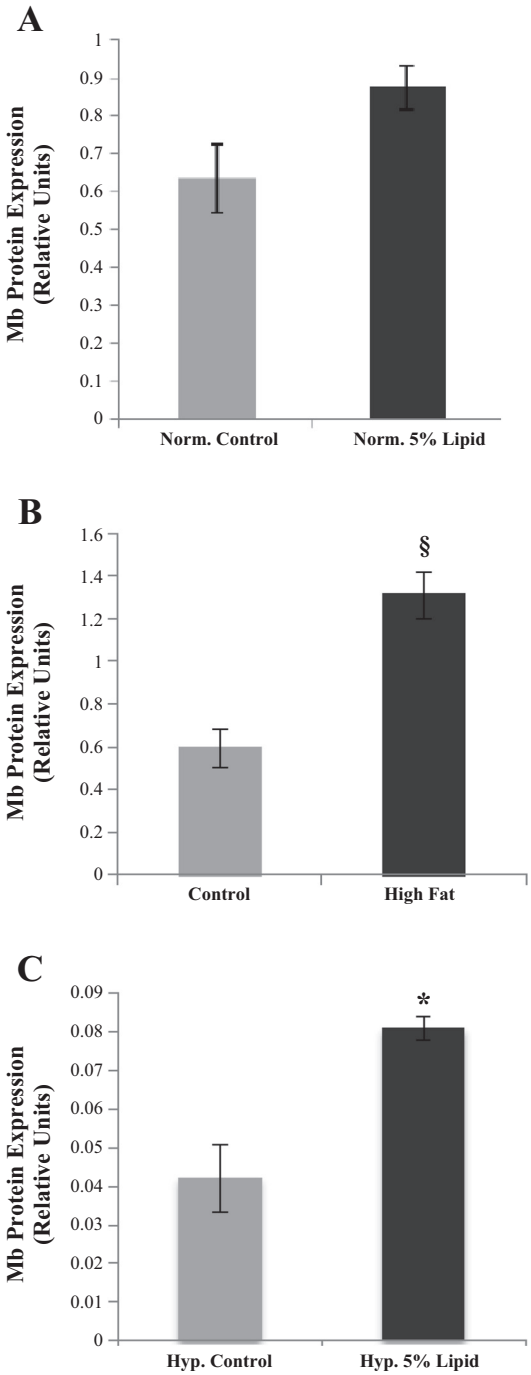

Fig. 2. Mb protein expression in C_2C_{12} cells and rat soleus. Total Mb protein expression, normalized to α-actin as determined by Western blot analysis showed that C_2C_{12} cells have a slight change toward increased Mb protein expression ($n = 6$, $P = 0.09$) (*A*), although this was not significantly different, soleus muscle from fat-fed rats has more total Mb protein expression ($n = 8$, $P = §0.013$) (*B*), and hypoxic C2C12 cells have a significant increase in total Mb protein when supplemented 5% lipid ($n = 3$, $*P = 0.014$) (*C*).

cells compared with 0.795 ± 0.0166 R.U. in control cells, $P = 0.03$), whereas hypoxic cells showed no difference in CN between glucose and high-fat cells (0.663 ± 0.0269 R.U. in lipid cells compared with 0.555 ± 0.0488 R.U. in control hypoxic cells) (Fig. 3*A*).

At the tissue level, there was an insignificant trend toward decreased CN protein in soleus muscle of rats fed high-fat diets relative to control rats fed high-starch diets (2.53 ± 0.442 R.U. in SAT rats compared with 3.46 ± 0.213 R.U. in CON rats, $P = 0.2$) (Fig. 3*B*).

Aerobic capacity. CS, the rate-limiting enzyme of the citric acid cycle, provides a measure of aerobic capacity in cells; CS activity measured in normoxic cells showed a decrease in CS activity in 5% lipid compared with glucose cells (2.56 ± 0.006 vs. 0.211 ± 0.006 U/mg protein, respectively, $P = 0.002$). CS activity measured in hypoxic cells also showed a decrease in CS activity in 5% lipid compared with glucose cells (0.130 ± 0.008 vs. 0.175 ± 0.006 U/mg protein, $P = 0.001$). Moreover, CS activity in normoxic glucose conditions was higher than both hypoxic conditions ($P < 0.001$) (Fig. 4*A*).

At the tissue level, CS activity showed no difference between SAT vs. CON rat soleus muscle tissue (2.54 ± 0.450 vs. 2.24 ± 0.214 U/mg protein, respectively, $P = 0.7$) (Fig. 4*B*).

RNA transcript expression. PCR array analysis in normoxic cells showed lipid cells as being more oxidatively stressed than control cells. Lipid cells had an increase (>2-fold difference) in nine transcripts of antioxidant genes, ten transcripts of genes involved in ROS metabolism, and five transcripts of oxygen transporter genes (Fig. 5). Mb transcript showed no difference between glucose and lipid normoxic cells. Glucose cells, alternatively, showed greater expression of only two antioxidant transcripts relative to lipid cells.

Interestingly, transcript analysis in hypoxic cells suggests that glucose hypoxic cells are more oxidatively stressed than

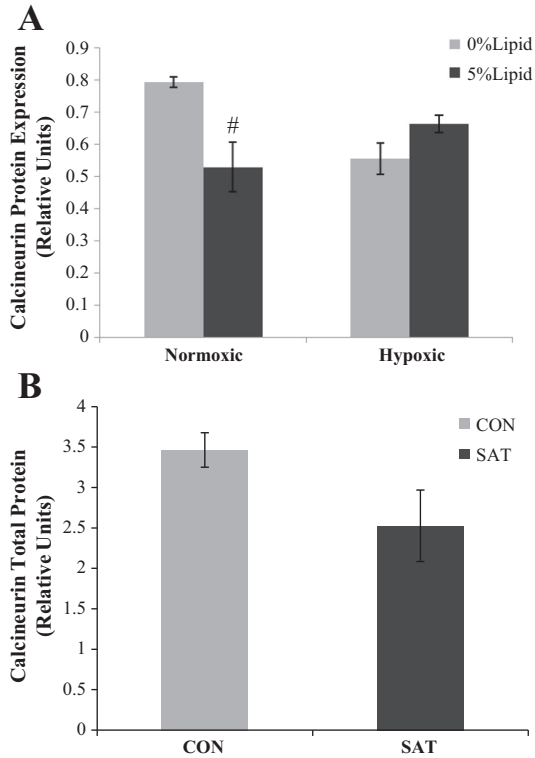

Fig. 3. Calcineurin (CN) total protein in hypoxic and normoxic C_2C_{12} cells. Total CN protein expression, normalized to α-actin, as determined by Western blot analysis showed a decrease in CN expression in normoxic high-fat cells compared with normoxic control cells ($n = 3$, $\#P = 0.028$) (*A*), with no difference in CN expression between control and high-fat cells ($n = 3$), and a trend toward decreased CN expression in 40% saturated fat diets (SAT) vs. control (CON) rats ($n = 3$, $P = 2.000$) (*B*).

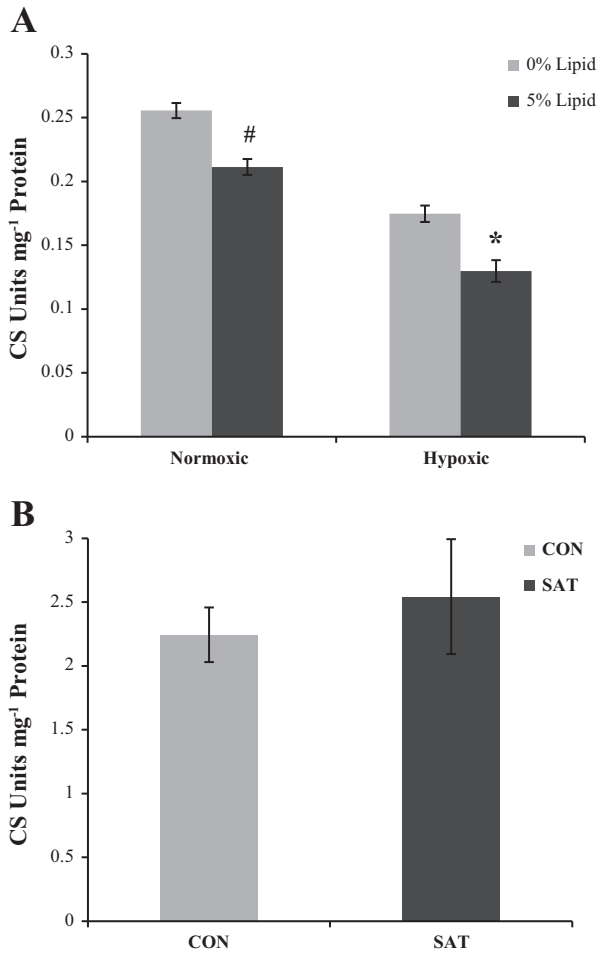

Fig. 4. Citrate synthase (CS) activity in C_2C_{12} Cells and rat soleus. CS activity determined by CS assay showed that in normoxic cells (*A*) CS decreased in 5% lipid conditions compared with control conditions ($n = 9$, $\#P = 0.002$). Similarly, CS assay showed in hypoxic cells that CS also decreased in 5% lipid conditions compared with control conditions ($n = 9$, $*P = 0.001$); whole rat soleus tissue (*B*) shows no difference in CS activity in SAT vs. CON rats ($n = 27$, $P = 0.71$).

lipid hypoxic cells. Transcripts of five genes involved in ROS metabolism, one antioxidant gene, and two oxygen transporter genes were upregulated in glucose hypoxic cells relative to lipid hypoxic cells. Lipid hypoxic cells, conversely, only showed three transcripts of genes involved in superoxide metabolism as being upregulated (Fig. 5).

Analysis of both lipid-supplemented cell groups suggests that lipid normoxic cells are more oxidatively stressed than lipid hypoxic cells. Transcripts of two antioxidant genes, thirteen genes involved in ROS metabolism, and four oxygen transporter genes were upregulated in normoxic lipid cells, whereas transcripts of one antioxidant gene and two genes involved in ROS metabolism were upregulated in hypoxic lipid cells (Fig. 5).

Lastly, results from rat soleus PCR array analysis suggests that SAT rats were more oxidatively stressed than CON rats. Specifically, transcripts of two antioxidant genes and three genes involved in ROS metabolism were upregulated in soleus tissue from SAT rats relative to CON rats (Supplemental Table S4; supplemental material for this article is available online at the *Journal of Applied Physiology* website).

DISCUSSION

As previously seen in diving mammals (8), here, for the first time in terrestrial models, we show a scenario whereby lipid increases Mb in the skeletal muscle without elevated CN, suggesting a pathway for Mb gene regulation independent of calcium signaling. On the cellular level, supplementation of a 5% lipid mixture into differentiation media increased functional CO-binding Mb in both normoxic and hypoxic C_2C_{12} cells. Addition of PBN, a ROS scavenger, to lipid-supplemented cohorts caused a complete reversal of lipid-induced Mb increases in normoxic cells, suggesting a connection between oxidative stress and Mb stimulation. Hypoxic cells also showed an inhibitory effect on lipid-induced Mb increases upon the addition of PBN to lipid-supplemented cohorts; however, hypoxic lipid + PBN cells still had more Mb than hypoxic controls, suggesting that Mb stimulation in hypoxic conditions may be in response to a secondary stimulus beyond oxidative stress and in relation to lower oxygen availability. Moreover, immunoblot analysis showed that total Mb protein increased in hypoxic lipid cells, with an insignificant trend of increasing in normoxic lipid-supplemented cells. On the tissue level, normoxic SAT rats showed no difference in functional CO-binding Mb compared with CON rats; however, immunoblot analysis showed an increase in total tissue Mb protein in SAT rats. This latter tissue Mb dataset parallels, in part, normoxic cellular Mb data, whereby increasing exogenous fat accompanies an increase in Mb protein. Taken together, these tissue data suggest that increasing lipid availability in normoxic environments increases a modified, non-CO-binding Mb in whole muscle. Moderate lipid signaling, thus, may initially be adaptive and beneficial in the skeletal muscle, whereas excessive, long-term lipid signaling may become maladaptive and lead to pathologies.

Another interesting perspective from these data is that high-fat conditions that produced an increase in Mb did not show the predicted increase in CN protein, which is reflective of CN activity (9). Normoxic, lipid-supplemented cells increasing Mb showed a decrease in CN, with normoxic tissue showing increased Mb protein with a decrease in CN, whereas hypoxic, lipid-supplemented cells showing an increase in Mb showed no change in CN. CN, a calcium-calmodulin activated phosphatase, has previously been established as a transcriptional regulator of Mb, whereby calcium released from the sarcoplasmic reticulum during contraction activates CN, which then dephosphorylates NFAT, allowing NFAT to translocate into the nucleus and subsequently bind the Mb promoter (6, 23, 24). Here, for the first time, we show Mb being stimulated in mammalian skeletal muscle in the absence of altered CN protein expression. Although these protein data suggest that lipid-induced Mb stimulation may be operating independent of CN, further studies into the role of calcium signaling with lipid stimuli are warranted.

If an increase in Mb observed in lipid-supplemented skeletal muscle is occurring independent of calcium signaling, then how is Mb being stimulated? Lipid metabolism is known to accompany increased ROS production (43). Skeletal muscle ROS are most commonly generated from superoxide radicals and subsequent H_2O_2 production. These are spawned in several sites throughout sarcomeres, including the mitochondria, sites of NADPH oxidases, and sites of xanthine oxidase (34).

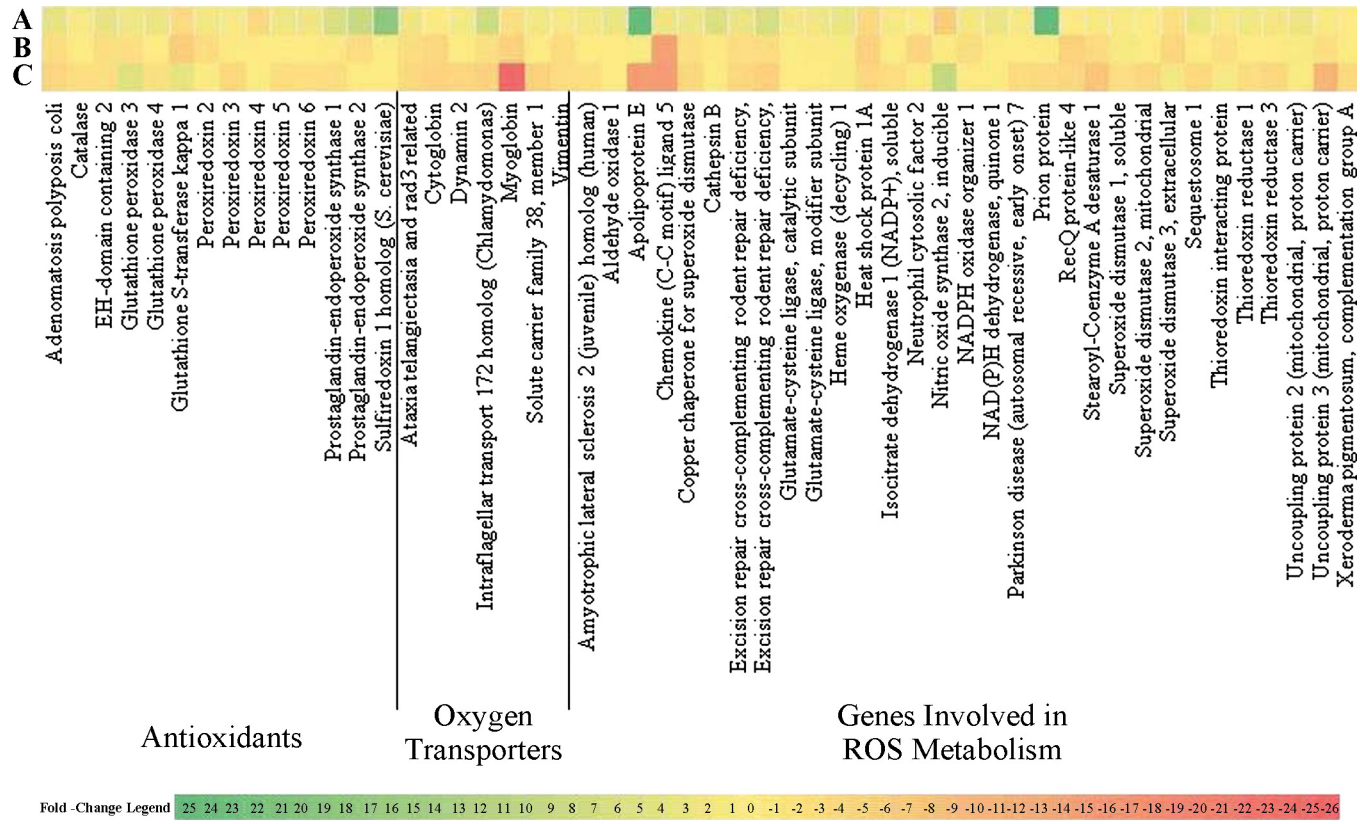

Fig. 5. Heat map of oxidative stress gene transcripts within cell culture treatments. Transcripts of genes related to oxidative stress with at least a 2-fold change difference between treatment groups ($P \leq 0.05$, $n = 3$) show that, in lipid normoxic vs. glucose normoxic cells (*A*), lipid cells appear to be more oxidatively stressed, whereas, in lipid hypoxic vs. glucose hypoxic cells (*B*), the lipid hypoxic cells are less oxidatively stressed than glucose hypoxic, and, in lipid hypoxic vs. lipid normoxic cells (*C*), lipid hypoxic cells are less oxidatively stressed than lipid normoxic cells.

Regarding the former, mitochondria generate ROS as a by-product of aerobic metabolism (specifically, the electron transport system, ETS). Because β-oxidation increases ROS production through the ETS, perhaps increasing lipid availability in skeletal muscle is stimulating Mb expression via ROS signaling. Suggested Mb functions include oxygen reservoir, NO scavenger, and ROS scavenger (11, 12, 14, 15, 26). Previous research has proposed roles of the former two functions in Mb regulation. Regarding oxygen storage, Mb increases in response to hypoxic exercise, which increases oxygen demand in an oxygen-limited environment (22). Alternatively, regarding the role of Mb as an NO scavenger, NO stimulates smooth muscle Mb, whereby addition of NO to vascular smooth muscle cells positively correlates with Mb gene and protein expression (35). The interpretation of this response is that the smooth muscle responds to increases in potentially dangerous reactive nitrogen species (RNS) by enhancing scavenging and subsequent protection from RNS. Building on this same rationale, if increases in oxygen demand in an oxygen-limited environment (i.e., hypoxic exercise) and increases in NO can stimulate Mb, then perhaps an alternative stimulus could pertain to third proposed role of Mb as a ROS scavenger. Although current knowledge of this function is limited to in vitro studies (11), increases in cellular ROS production could be capable of stimulating Mb in oxidative muscle.

In support of this theory, our data show an inverse relationship between Mb and CS; increased lipid availability in cells produced Mb increases coupled with CS activity decreases. In tissue, alternatively, there was no change in CS activity. Given its role as an oxygen reservoir (17, 28), Mb has a direct connection to aerobic metabolism. Thus, if Mb were increasing solely as a means of increasing cellular oxygen reservoirs, then we would not have expected a biomarker of aerobic metabolic activity to decrease. This result, therefore, suggests that measured increases in functional CO-binding Mb and Mb protein expression may actually reflect an increased cellular demand pertaining to one of the other functional roles Mb, specifically as a ROS scavenger; thus, increased Mb without a simultaneous increase in CS activity may be the response of the cell to increased ROS production from β-oxidation of lipids (Fig. 6).

β-Oxidation of fatty acids has been shown to increase mitochondrial ROS and subsequent H_2O_2 generation (43); thus, increasing aerobic metabolism through lipid oxidation in muscle will increase ROS generation, which may, in turn, stimulate Mb. Muscle antioxidants have been found to correlate positively with lipid supplementation; feeding mice high-fat diets increases activity and protein expression of catalase, an H_2O_2-metabolizing enzyme (38). Accordingly, this trend of lipid-stimulated increases in muscular oxidative defense holds true in regard to Mb as well. Moreover, this theory may better explain observed elevation in Mb in healthy, athletic, terrestrial mammals that are not oxygen limited. Terrestrial endurance athletes preferentially burn polyunsaturated fatty acids (PUFAs) in their skeletal muscle, which conserves oxygen, thus lowering oxygen consumption (and demand) in their working muscles.

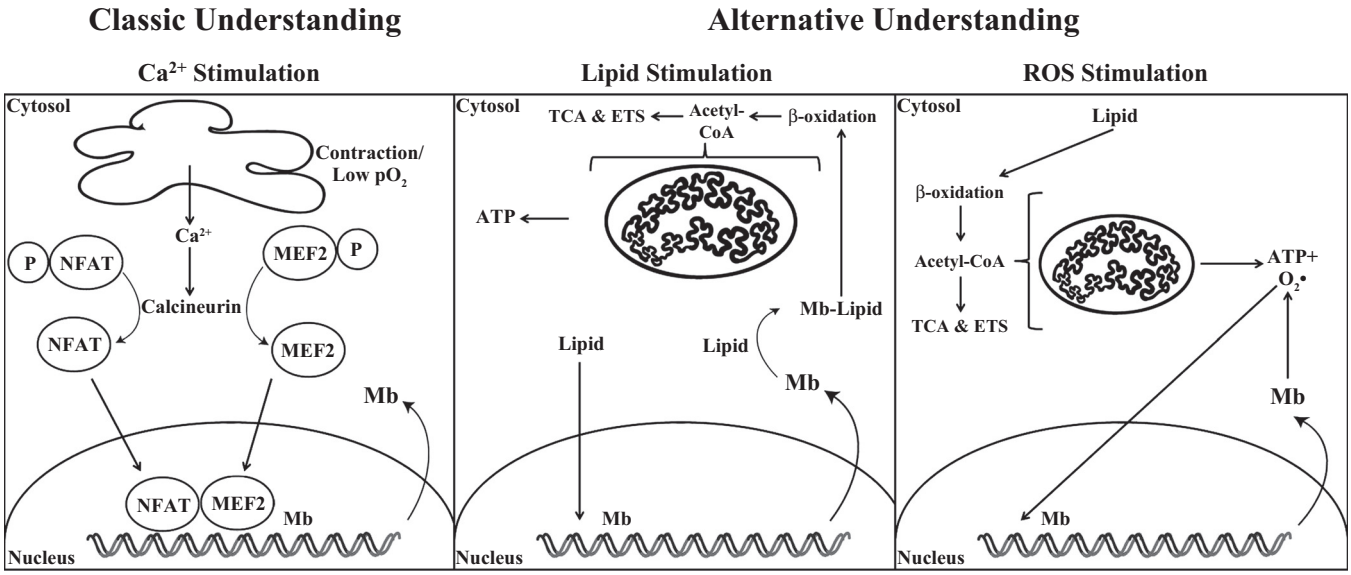

Fig. 6. Mb stimulation: classic vs. alternative understanding. Classically, Mb stimulation relates to exercise-associated muscle contraction releasing calcium from the sarcoplasmic reticulum, activating CN, and dephosphorylating and translocating nuclear factor of activated T cells (NFAT) and myocyte enhancer factor 2 (MEF2) to the nucleus (*left*). Alternatively, in the resting, nonexercising cell, lipids may stimulate the Mb promoter independent of a CN signaling pathway; this type of Mb increase may facilitate better fatty acid transport via Mb from the cytosol to the mitochondria for β-oxidation, or redox signaling may involve increased ROS associated with β-oxidation affecting the Mb gene, independent of calcium signaling, to increase transcription, providing more Mb to prevent accumulation of potentially harmful metabolic byproducts (*right*). TCA, tricarboxylic acid; ETS, electron transport system.

Increases in internal oxygen stores, therefore, do not appear physiologically necessary, given this lower oxygen demand, whereas oxidative scavenging appears to better match physiologically. PUFAs generate more ROS than saturated fatty acids; consequently, increased ROS production attributable to β-oxidation of PUFAs may account for Mb increases in terrestrial endurance athletes (44). Moreover, this information may better explain the somewhat disparate results between lipid normoxic cells, which were given a heterogeneous mixture of fatty acids, vs. fat-fed rats, which were fed exclusively saturated fatty acids.

Support of ROS-induced Mb stimulation is further evident through addition of an antioxidant to lipid-supplemented cell culture cohorts. Mb assay data from normoxic cells cultured with 5% lipid + 1 mM PBN, a scavenger of ROS, showed a complete reversal of previously seen lipid-induced Mb increases (Fig. 1). In other words, removing the predicted influx of ROS associated with augmented lipid metabolism prevented increases in Mb stimulation. These data imply that lipid-induced Mb increases occur through elevated ROS production consequent of amplified β-oxidation. Mb assay data from hypoxic cells cultured with 5% lipid + 1 mM PBN also showed a decrease in Mb compared with hypoxic lipid cells but not to the same degree as observed in normoxia (Fig. 1). This observation may be consequent of cellular Mb increasing via lipid and a secondary stimulus, hypoxia. Although hypoxia alone actually decreases cellular Mb, it has previously been established as an important secondary stimulus for increasing expression of Mb, particularly in the context of exercise (24). Accordingly, the presence of this secondary stimulus may account for the damped decrease of Mb in hypoxic lipid + PBN cells compared with the normoxic PBN response.

RNA transcript analysis of oxidative stress markers supports differential stress levels between experimental conditions. Of the normoxic cells, lipid-supplemented cells showed increases in 39 transcripts of oxidative stress markers (antioxidants, genes involved in ROS metabolism, and oxygen transporters). These data suggest that lipid-supplemented cells experience more oxidative stress than control cells. Moreover, substantial increases (24.6-fold) of the lipid transporter (48) apolipoprotein E in lipid-supplemented cells suggest that lipids are being metabolized, thus supporting lipid metabolism byproducts as Mb stimulators. According to soleus data, SAT rats were more oxidatively stressed than the CON rats. Whereas five transcripts indicative of oxidative stress were upregulated in SAT rats, no transcripts were upregulated from CON rats. Thus these tissue data support our cellular data, showing an increase in oxidative stress with lipid supplementation in normoxic environments. Oxidative stress in normoxic conditions despite elevated Mb may be explained by a change in oxidation state of the heme iron, which may, in turn, alter Mb ROS scavenging abilities.

Of the hypoxic cells, glucose hypoxic cells were more oxidatively stressed than lipid-supplemented hypoxic cells. Eight transcripts of genes involved in ROS metabolism and oxygen transportation were upregulated in glucose hypoxic cells (ranging from 2.9-fold increase to a 13.1-fold increase), whereas only three transcripts of genes involved in ROS metabolism were upregulated (ranging from 2.4–2.7-fold increases) in lipid-supplemented cells. These somewhat surprising data may be a result of Mb differences and, specifically, differences in Mb ROS scavenging, as Mb was significantly higher in lipid hypoxic cells relative to glucose hypoxic cells. Interestingly, Mb transcript was down 3.79-fold in lipid hypoxic cells relative to glucose. Although contradictory to protein and functional assay data, these data are likely due to early Mb increases during myotube differentiation, such that, by the time cells were harvested, cellular demand for Mb was

already being met, reflected as decreased transcript. Protein abundance in some human cell lines, for example, only partially correlates with relative mRNA abundances, thus illustrating the importance of protein abundance regulation and the effect on protein-to-mRNA ratios (45). Future research will explore temporal differences in lipid-induced Mb stimulation in hypoxic vs. normoxic myotube differentiation. Given the possibility of these temporal differences, future analyses of markers in the present study across several time cohorts (i.e., different days during the myotubes differentiation period) may offer insightful information regarding the timing of signal induction.

Of the lipid-supplemented cells, lipid normoxic cells appear to be more oxidatively stressed than their hypoxic counterparts. Nineteen gene transcripts were upregulated in lipid normoxic relative to lipid hypoxic cells (ranging from 2.4-fold increase to 24.4-fold increase), whereas lipid hypoxic cells show transcripts of only three genes being upregulated relative to normoxic lipid cells. These data initially seem paradoxical because hypoxic lipid cells have two confounding factors contributing to oxidative stress (increased lipid and insufficient oxygen); however, lipids associated with the hypoxic cells are clearly providing a beneficial physiological change that allows the cells to better adapt to ameliorating oxidative stress. The ability of hypoxic lipid cells to adaptively increase Mb earlier in differentiation (implicated by a 24.4-fold decrease in Mb transcript compared with normoxic lipid cells, despite both having elevated Mb protein) is likely mitigating physiological stress. This, in turn, may be compensating for the necessity to increase oxidative stress-related transcripts that would otherwise be prominent. This disparity between Mb protein and transcript expression, where transcript is down in experimental conditions where protein is up, mirrors Mb protein and transcript disparities in hypoxic cells. Again, these seemingly contradictory data are likely due to early Mb transcript and subsequent protein increases in the lipid hypoxic cells, whereby cellular Mb demands are being sufficiently met upon cellular harvesting.

An alternative theory to ROS stimulating Mb is that cells may be responding to increased insoluble lipid by increasing means of lipid transportation. Proton nuclear magnetic resonance data indicate that Mb can bind fatty acids. This trait is speculated to relate to a role for Mb in fatty acid transport, that is, the transport of an insoluble macromolecule in the aqueous cellular environment (40, 42). In this light, Mb increases here may be a response to increased exogenous lipids working in concert with fatty acid-binding proteins, thus making lipids accessible to the mitochondria for β-oxidation (Fig. 6). Despite the known ability of Mb to bind fatty acids, lipid has never been shown to stimulate Mb in a terrestrial species.

In summary, we show that lipid supplementation is associated with increased Mb expression in both C_2C_{12} mouse muscle cells and Sprague-Dawley rat soleus muscle independent of CN. This overarching pattern shows similar responses between normoxia and hypoxia, whereby lipids increase total Mb protein in addition to functional CO-binding Mb. Interestingly, addition of PBN, a ROS scavenger, inhibited lipid-induced Mb increases although this response differed between normoxic and hypoxic cohorts. In normoxia, addition of PBN to lipid-supplemented cells completely reversed lipid-induced Mb increases, whereas, in hypoxia, addition of PBN to lipid-supplemented cells decreased Mb relative to hypoxic lipid cells but did not decrease Mb down to hypoxic control cell levels, suggesting that, in hypoxia, Mb increases are in response to lipids and a secondary stimulus (i.e., lower oxygen availability). Moreover, because all cell culture experimental conditions in which Mb increases concomitantly show unchanged CS activity, and because all normoxic lipid-supplemented experimental conditions show increases in RNA transcripts associated with oxidative stress, we propose that lipid-stimulated Mb increases are consequent of redox signaling associated with increased ROS production via β-oxidation. Thus, in light of these novel data and in conjunction with the inability of terrestrial mammals to appreciably utilize Mb oxygen stores during exercise, we propose an alternative paradigm for Mb, whereby the role of Mb as an antioxidant defense during terrestrial exercise, which increases aerobic metabolism and ROS production, is more relevant and applicable than the role relevant to storage and transport of oxygen in healthy animals.

ACKNOWLEDGMENTS

The authors thank A. Masino for assistance with RNA experiments and A. Corley, T. Green, and A. Larson for assistance with preliminary cell culturing and enzyme assays.

GRANTS

This research was supported by the National Science Foundation (OPP no. 044-0713), Colorado State University, Baylor University, and Sigma Xi (G20120315159261).

DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Author contributions: A.E.S., M.A.F., and S.B.K. conception and design of research; A.E.S. and M.A.D.M. performed experiments; A.E.S. analyzed data; A.E.S. and S.B.K. interpreted results of experiments; A.E.S. prepared figures; A.E.S. drafted manuscript; A.E.S., M.A.D.M., M.A.F., S.J.T., and S.B.K. edited and revised manuscript; A.E.S. and S.B.K. approved final version of manuscript.

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