Changes in HIF-1α protein, pyruvate dehydrogenase phosphorylation, and activity with exercise in acute and chronic hypoxia

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Submitted 7 February 2011; accepted in final form 14 July 2011

Le Moine CMR, Morash AJ, McClelland GB. Changes in HIF-1α protein, pyruvate dehydrogenase phosphorylation, and activity with exercise in acute and chronic hypoxia. Am J Physiol Regul Integr Comp Physiol 301: R1098–R1104, 2011. First published July 20, 2011; doi:10.1152/ajpregu.00070.2011.—Exercise under acute hypoxia elicits a large increase in blood lactate concentration ([La]b) compared with normoxic exercise. However, several studies in humans show that with the transition to chronic hypoxia, exercise [La]b returns to normoxic levels. Although extensively examined over the last decades, the muscle-specific mechanisms responsible for this phenomenon remain unknown. To assess the changes in skeletal muscle associated with a transition from acute to chronic hypoxia, CD-1 mice were exposed for 24 h (24H), 1 wk (1WH), or 4 wk (4WH) to hypobaric hypoxia (equivalent to 4,300 m), exercised under 12% O2, and compared with normoxic mice (N) at 21% O2. Since the muscle associated with a transition from acute to chronic hypoxia, a switch in metabolism remains unclear.

Central to mitochondrial carbohydrate metabolism, pyruvate dehydrogenase (PDH) is a multisubunit enzyme complex with a significant role in controlling the flux of pyruvate to acetyl-CoA. In acute hypoxia, where PDH is inactivated, pyruvate is readily converted to lactate by the near-equilibrium enzyme LDH due to high enzyme activity and a K_m that favors pyruvate to lactate conversion. In this way, PDH activity strongly influences the fate of pyruvate entering aerobic or anaerobic pathways. PDH enzymatic activity is under tight covalent control by the phosphorylation of three residues on the PDH1α subunit by the actions of the PDH kinase (PDK) family of proteins (19, 35). Typically, all four isoforms are capable of inactivating PDH through phosphorylation of sites 1 and 2 (Ser239, Ser359) but with differing affinities. However, the third phosphorylation site of PDH (Ser322) is specifically phosphorylated by the PDK1 isoform (22). Interestingly, PDK1 is induced by hypoxic stress via the hypoxia inducible factor-1α (HIF-1α; 32), a transcription factor involved in a suite of homeostatic responses in response to low oxygen availability (see Ref. 40). The oxygen-labile subunit, HIF-1α, is continuously targeted for degradation by the prolyl-hydroxylases (PHD) under normoxic conditions. When cellular oxygen tension drops, HIF-1α is stabilized and associates with the constitutively expressed HIF-1β subunit to induce the expression of several hypoxia-induced metabolic genes including PDK1 and the LDH-A subunit (32, 41). Therefore, HIF represents a putative regulator of metabolic function in the transition from acute to chronic hypoxia. Recent evidence from both muscle HIF-1α−/− mice and cancer cells suggests that regulation of PDH by a HIF-1α-dependent pathway affects cellular metabolism and lactate accumulation (4, 25). Furthermore, PDH−/− mice show that this oxygen sensor directly regulates HIF-1 levels, consequently affecting PDK1 regulation of PDH activity in muscle tissues (1). In addition, chronic normobaric hypoxia in mice induces PHD levels, increasing HIF-1α degradation and therefore reducing the HIF response despite continued hypoxia (14). Thus, we hypothesized that changes in this HIF-mediated cascade at different stages of hypoxia exposure modulate muscle metabolism. Specifically, we predicted that mice exposed to chronic hypobaric hypoxia will show a reduction in muscle HIF-1α compared with acute hypoxia levels. This decrease in HIF will result in a lower PDK1 levels that will relieve the inhibition of PDH activity, returning the muscle toward a normoxic phenotype and a reduction in pyruvate to lactate flux.
MATERIALS AND METHODS

Animals. All procedures were approved by the Animal Research and Ethics Board at McMaster University according to Canadian Council of Animal Care guidelines. Female CD1 mice were used to facilitate group housing in the hypobaric chambers (see below) and were obtained commercially (Charles River, Wilmington, MA) at ~10 wk of age. The mice were housed in groups (maximum of 5 per cage) and were provided standard chow and water ad libitum throughout the experiment. Mice maintained in hypobaric chambers were returned to normobaria once a wk for cage cleaning and to replenish water and food (~ 1 h).

Treatments. Mice were then divided into four experimental groups: normoxic controls (N) maintained and exercised under normobaric normoxic conditions and hypoxic mice maintained for 24 h (24H), 1 wk, or 4 wk (1WH and 4WH, respectively) in hypobaric chambers at ~60 kPa or the equivalent of 4,300 m altitude and run under hypoxic conditions (see below). All animals were familiarized with a small animal Plexiglas-enclosed treadmill (Columbus Instruments, Columbus, OH) for 5 min at a speed of 10 m/min before being subjected to the different experimental treatments. At the end of the treatments, in each group the body weights were (in g): 31.41 ± 1.09 for N, 32.31 ± 0.83 for 24H, 28.35 ± 0.81 for 1WH, and 29.11 ± 1.09 for 4WH.

Incremental exhaustion test. Mice were subjected to an incremental exercise test to exhaustion on a 10% inclined Plexiglas-enclosed treadmill (adapted from Ref. 3). Briefly, individual mice were rapidly transferred from their respective treatment conditions to the treadmill and kept at rest for 10 min. Runs were initiated at a speed of 10 m/min for 3 min, and speed was increased 2 m/min every minute until the mice could not maintain position on the treadmill belt. For normoxic mice, blood was sampled at different speeds during the run in the same mouse from one of the lateral veins of the tail using a 5-mm lancet (MEDIpoint, Mineo), while for the other treatment groups a single sample was obtained at one speed using the same procedure. For hypoxic exercise testing, 12% O2 (balance N2) was flowed through the treadmill chamber and, the O2 levels were verified by passing a subsample of the chamber air through an O2 analyzer (Sable Systems, Las Vegas, NV). For each blood sample, the mice were rapidly removed from the treadmill and blood (5–10 μl) was immediately collected from the puncture site using a capillary tube and the mouse returned to the treadmill (for N mice only). The blood was then transferred to a lactate test meter strip for concentration determination using a lactate analyzer (Lactate Pro; Arkray, Kyoto Japan), as described previously in mice (3).

Constant speed test. Since the incremental exercise experiment suggested that 1 wk of hypobaric hypoxia was sufficient to elicit marked changes in lactate accumulation (Fig. 1), we selected only three treatment groups (N, 24H, and 1WH) for further analysis. A separate group of mice, using the same treatments, were run at a constant speed. Again, mice were rapidly transferred to the treadmill chamber and allowed to settle for 10 min. The run was then initiated at 10 m/min for 3 min, and then over ~1 min, speed was raised progressively to 26 m/min for the remainder of the run (total time of 9 min). This speed was selected as it elicited a large increase in lactate production across treatment conditions. At the end of the run the mice were immediately removed from the treadmill, euthanized by cervical dislocation, and to minimize variation due to differences in muscle blood flow and therefore regional hypoxia, their entire lower hindlimb (including mixed gastrocnemius, tibia anterioralis, extensor digitorum longus) were immediately excised, skinned, and crushed in liquid N2-cooled aluminum tongs. The procedure took on average 70 ± 4 s for the left hindlimb and 107 ± 5 s for the right hindlimb from the end of exercise. Tissues were then kept at −80°C until further analysis. Whole lower hindlimb muscles were dissected free of bones and powdered under liquid N2. The left hindlimb was used for enzyme and Western blot analysis to preserve PDH phosphorylation state, while the right leg was used for metabolites.

Enzymes and metabolites assays. All assays were performed at 37°C in 96-well format on a Spectramax Plus 384 microplate reader (Molecular Devices, Sunnyvale, CA). Active PDH (PDHc) was assayed through acetylation of 4-aminooazobenzene-4′-sulfonic acid (AABS) by arylamine acetyltransferase (AAT) on homogenized muscles by adapting established protocols (7, 20, 29). Powdered tissues were homogenized on ice in extraction solution (in mM: 45 HEPES-KOH pH 7.5, 1.2 EDTA, 5 DTT, 20 dichloroacetate, and 0.1% Triton X-100) and immediately assayed for PDHc activity in assay buffer (in mM, 0.1 Tris pH 7.8, 0.5 EDTA, 1 MgCl2, 1 thymine pyrophosphate, 5 β-mercaptoethanol, 5 NAD+, 0.1 CoA, 0.1 AABS, and 20 μl purified AAT). The reaction was monitored by loss of absorbance of AABS at 460 nm after the addition of 0.8 mM pyruvate (final concentration) to initiate the reaction. The homogenates were then assayed for total protein using the Bradford protein assay (Bio-Rad, Mississauga, ON, Canada) and freeze thawed prior to citrate synthase (CS) and LDH assays as described previously (23, 28). Muscle glycogen and lactate levels were evaluated on lyophilized right lower hindlimb muscle tissues following standard protocols (13, 34).

Western blot analysis. Powdered hindlimb muscles were briefly homogenized in cold buffer (in mM: 50 HEPES pH 7.5, 150 NaCl, 10% glycerol, 1% Triton X-100, 1.5 MgCl2, 1 EGTA, 10 NaP2O7, 100 NaF) containing protease inhibitors (complete minitablets; Roche, Mississauga, ON). Equal amounts of soluble protein (40–60 μg) were loaded and separated on 7.5% polyacrylamide gels (Ready Gel Tris-Cl; Bio-Rad) and electrobotted to polyvinylidene difluoride membranes (Stratagene, LaJolla, CA). The antibodies were directed against PDK-1 (Novus Biological, Littleton, CO), PDK-2 (Santa Cruz Biotechnology, Santa Cruz, CA), PDHcE1α (PDHe; Santa Cruz Biotechnology), HIF-1α (Novus Biologicals), antibodies targeted to the phosphorylated sites of PDH [pSer201, pSer232, pSer300 (38)], and the respective anti-goat or anti-rabbit horseradish peroxidase-linked secondary antibodies (Perkin Elmer, Waltham, MA; Santa Cruz Biotechnology). Chemiluminescence (Perkin Elmer) was detected on autoradiographic films (Kodak XOR, Rochester, NY). Results are presented relative to PDHc content.
showed elevated \([\text{La}]_b\) after exhaustive exercise in hypoxia (14.86 ± 1.09 mM), significantly higher than any other treatment groups (\(P = 0.01\), Fig. 1). In contrast, both the 1WH and 4WH acclimated mice exhibited \([\text{La}]_b\) at exhaustion that were not significantly different from the N group (12.8 ± 0.7 mM and 12.4 ± 0.9 mM, respectively; \(P > 0.05\)). When comparing the maximal running speed, all the hypoxic groups reached exhaustion at a significantly slower speed (24H = 32.0 ± 1.8, 1WH = 28.7 ± 1.1, and 4WH = 31.3 ± 1.1 m/min) than the normoxic controls (36.3 ± 0.7 m/min, \(P < 0.05\)).

Metabolites were quantified in muscle after a run at constant speed (26 m/min), and hindlimb postexercise intramuscular lactate accumulation in the 24H group was approximately twice that found in normoxic mice (\(P < 0.01\), Fig. 2A), while lactate values in 1WH mice were significantly lower than the 24H groups (Fig. 2A). In contrast, postexercise muscular glycogen concentrations were remarkably constant between all groups (Fig. 2B). In these same muscle samples, the activity of the Krebs cycle enzyme CS did not change with treatments (in U/mg protein: 214.00 ± 28.79 in N, 161.83 ± 7.34 in 24H, and 175.80 ± 27.91 in 1WH; Fig. 3), but LDH activity was significantly decreased in 1WH mice (14.50 ± 0.98 U/mg protein) compared with the other two groups (in U/mg protein: 20.20 ± 1.53 in N and 19.21 ± 1.50 in 24H; Fig. 3, \(P = 0.02\)). At 24-h acute hypoxia exposure, activity levels of PDHa were lower compared with values for the N group (in mU/mg protein: 7.74 ± 0.77 in N and 5.25 ± 0.27 in 24H; \(P = 0.02\), Fig. 3), while in the 1WH group, PDHa activity increased to a value not significantly different from either N or 24H (6.87 ± 0.77 in 1WH, 5.25 ± 0.90 in N, and 6.87 ± 0.77 in 24H; Fig. 3). These changes in enzymatic activities modified the muscle stochiometries of the enzymes studied. Indeed, the ratio of LDH/CS activity was 30% higher in 24H mice compared with 1WH animals (\(P = 0.01\)); further muscle PDH/LDH was ~30% lower in the acute hypoxia group than in the normoxic group (\(P = 0.02\)) and increased by nearly 90% between 24 h and 1 wk of hypoxia (\(P = 0.002\)). To help explain changes in PDH activity, we examined relative levels of phosphorylation of the three main regulatory sites on PDH. The phosphorylation of sites \(\text{Ser}^{232}\) and \(\text{Ser}^{300}\) showed relatively similar levels across treatment groups. \(\text{Ser}^{232}\) phosphorylation did show a rise in signal in the 24H group compared with the other treatments but not to the level of statistical significance (Fig. 4). In contrast, the level of phosphorylation of \(\text{Ser}^{332}\) was approximately twofold higher in the 24H animals than in the other groups (\(P = 0.01\), Fig. 4A). Levels of PDK2

**Fig. 2.** Metabolites measured postexercise in lower hindlimb muscles of normoxic and hypoxic mice run at a constant speed of 26 m/min. Muscle lactate (A) and glycogen (B) were measured in normoxic mice in normoxia and mice exposed for 24 h and 1 wk to simulated altitude under 12% O\(_2\) exercised for 8 min. Metabolite content (\(\mu\)mol/g dry wt). Statistical significance between groups is indicated by different letters (\(n = 5–6\)) between values (means ± SE).

**Statistical analysis.** Data are presented as means ± SE, and all statistics (ANOVA, ANOVA on ranks, and post hoc tests) were performed using Sigma Stats 3.5 (Systat Software, Chicago, IL).

**RESULTS**

During incremental exercise to exhaustion in normoxic controls, \([\text{La}]_b\) increased with speed, with pronounced increase at ~20 m/min and a sharp rise at exhaustion (11.25 ± 0.76 mM, Fig. 1). After a 24-h exposure to hypobaric hypoxia, mice

![Fig. 3.](image-url) Relative enzyme activities in hindlimb muscle of normoxic and hypoxic mice exercised at a constant speed of 26 m/min. Apparent \(V_{\text{max}}\) (mean ± SE) of LDH, active form of pyruvate dehydrogenase complex (PDH\(_a\)), and citrate synthase (CS) were measured in the hindlimbs of normoxic mice postexercise in normoxia (white bars) and mice exposed for 24 h (light grey bars) and 1 wk (dark grey bars) to simulated altitude run under 12% O\(_2\). A value of 1 represents 20.2 U/mg protein of LDH, 214.0 mU/mg protein of CS, and 7.7 mU/mg protein of PDH\(_a\). Statistical significance between groups is indicated by different symbols (\(n = 5–6\)).

**AJP-Regul Integr Comp Physiol** • VOL 301 • OCTOBER 2011 • www.ajpregu.org
were slightly but not statistically elevated in the 24H mice ($P = 0.18$, Fig. 4B). In contrast, PDK1 levels were significantly higher in the 24H group compared with either normoxic or 1-wk hypoxia-acclimated mice ($P < 0.05$, Fig. 4B). As a major metabolic and transcriptional regulator under hypoxic conditions, we also investigated the protein levels of HIF-1α. 24H hypoxia stabilized and increased accumulation of HIF-1α (+70%, $P = 0.03$) but levels were reduced back to control values by 1 wk of hypoxia (Fig. 4B).

**DISCUSSION**

We demonstrate that CD-1 mice are an appropriate animal model to study regulation of lactate metabolism since they display lower peak [La]$_b$ after only 1 wk of acclimation to simulated altitude (~4,300 m, Fig. 1). Our findings support the hypothesis that the HIF-mediated cascade modulates muscle metabolism at different stages of hypoxia. We have shown that mice exposed to 1 wk of chronic hypoxia exhibit a reduction in HIF-1α that is correlated with lower PDK1, lower phosphorylation of the PDK1-specific Ser232 site on PDH, and an increase in the activity of PDHα from acute hypoxia values. We propose that a reduction in HIF-1α protein expression is responsible for restoring pyruvate oxidation potential back to normoxic levels in chronically hypoxic mice (Fig. 4B) and help explain reductions in blood and muscle lactate. These data provide a tantalizing muscle-level explanation for a long-standing observation (9).

**Exercise lactate accumulation in CD1 mice.** We confirmed with the CD-1 strain that mice show a progressive increase in [La]$_b$ with increasing exercise intensity in normoxia (2, 3). Furthermore, as in rats and humans (e.g., 2, 16, 24, 37), we show that peak exercise [La]$_b$ in CD-1 mice is reduced with the transition from acute to chronic hypoxia (Fig. 1). 24H mice exhibited [La]$_b$ values that were 32% higher (Fig. 1) than their normoxic counterparts, and 15% higher (Fig. 1) than hypoxia acclimated (both 1WH and 4WH) mice. It appears that the response of CD-1 mice to 1 wk of chronic hypoxia is comparable to humans or rats over longer hypoxic exposures (2, 16, 24, 37). To assess whether changes in [La]$_b$ after acclimation were independent of a reduction in peak performance as seen here (Fig. 1) and in other studies (e.g., 10, 18, 37, 43), we subjected the animals to a constant submaximal exercise challenge. When exercised at the same absolute submaximal intensity, 24H mice accumulated significantly more intramuscular lactate than controls, while 1WH animals had reduced intramuscular lactate with similar postexercise glycogen levels (Fig. 2, A and B) as previously observed for humans (2, 18). In contrast, rats run at the same relative exercise intensity as controls after long-term acclimation had equivalent [La]$_b$ lev-

**Enzymatic changes in acute and chronic hypoxic mice.** Lactate production results from a mismatch between glycolytic flux-generating pyruvate and the capacity to decarboxylate pyruvate for further oxidation. Therefore, changes in net lactate production by muscle could result from altered 1) muscle aerobic capacity, 2) capacity for pyruvate to lactate flux or...
lactate oxidation (27), or 3) capacity for the decarboxylation of pyruvate to acetyl-CoA by the PDH. Consistent with previous reports on rats, activity of the Krebs cycle enzyme CS was unchanged with varying hypoxia exposure times, suggesting that mitochondrial content and oxidative capacities were unaffected (Fig. 3 and Ref. 8). In contrast, activity of LDH significantly decreased in mice acclimated for 1 wk to hypobaric hypoxia (Fig. 3), thus lowering the capacity for pyruvate to lactate flux, also highlighted by an increase in PDH/LDH. Although previous reports in rats suggest that neither LDH protein nor activity change with extended hypoxia exposure (>8 wk) (27, 39), it does not preclude changes occurring with shorter exposures. However, it is difficult to elaborate on the cause for this change in LDH activity, since the different subunits of the allozyme appear to be differentially regulated under various conditions with unclear repercussions on enzymatic activity (11, 27, 42). Since muscles in humans appear to be net lactate consumers during exercise after chronic hypoxia (5), it has been suggested that increased capacity for lactate oxidation may contribute to reduced [La]b. However, long-term hypoxia in rats had a tissue-specific effect on the machinery for lactate oxidation, specifically changes in monocarboxylate transporters in muscle were inconsistent with putative increases in lactate disposal that would explain reduced [La]b (27).

**PDH phosphorylation, PDK, and HIF-1α proteins.** The activity of PDHα was depressed in the 24H mice compared with N mice (Fig. 3), lowering the capacity for pyruvate oxidation. To our knowledge this is the first report of a differential PDH activity during exercise after varying periods in hypoxia. Previous experiments in humans showed that a 1-min exercise bout upon acute exposure to hypoxia reduced PDHα compared with normoxia (33), but the effect of chronic exposure has been unexplored. PDH is strongly covalently regulated by phosphorylation/dephosphorylation of three critical residues on the PDHε1α subunit (19, 35). Overall, phosphorylated PDH (inactive) relative to total amount of PDH protein (PDHt) was significantly increased by 24 h of hypoxia and returned to normoxic levels after 1 wk of hypoxia (Fig. 4). Furthermore, while two of the three sites (Ser293, Ser300) showed similar phosphorylation status in all treatment groups (Fig. 4A), the regulatory site Ser232 was significantly more phosphorylated in 24H mice (Fig. 4A). Interestingly, this site is only phosphorylated by the hypoxia-responsive PDK1 isoform (22, 32) whose expression correlates with the phosphorylation state of its target amino acid (P < 0.05, Fig. 4B). Furthermore, HIF-1α levels were significantly higher in 24H mice compared with the other groups, as was its target PDK1 (6, 32; Fig. 4B). HIF-1α is strongly regulated at the posttranslational level by oxygen-sensitive PHD (for a review see Ref. 40). Moreover, there is a putative desensitization of the PHD-to-HIF-1 pathway after chronic exposure to hypoxia (14). Therefore, we propose that this mechanism accounts for changes in HIF levels and downstream targets with the transition from acute to chronic hypoxia. It is clear that HIF-1α plays a primordial role in the metabolic remodeling under hypoxic conditions, and recent evidence indicates that in skeletal muscle of mice, loss of HIF-1α results in a reduction in PDK1 levels and in exercise [La]b, providing evidence for a direct mechanistic link between the HIF-1 pathway and lactate production (25).

Taken together, these results show that HIF-1α protein levels are differentially regulated during different hypoxia exposures, leading to a reestablishment of normoxic levels of PDK1 in muscles and thus higher levels of PDHα under chronic vs. acute hypoxia. This restoration of PDH capacity, along with a decrease in LDH activity, are likely important contributors to a return of the intramuscular and circulating lactate levels to normoxic values upon chronic acclimation to hypobaric hypoxia in CD-1 mice. Certainly, additional intramuscular and extramuscular factors, beyond the scope of the present study, may be at play in this complex metabolic response. However, we propose that any changes in these factors would have a more fine-tuning role to the overall HIF signaling response. For example, both PDK and PDH activities are directly regulated by several modulators (e.g., pyruvate, acetyl-CoA, ATP, NADH, alkalosis), and these could have an important influence on our present findings (44). However, if these regulators were of prime importance in the regulation of PDK and PDH, one would expect global changes in both PDK isoforms and phosphorylation targets instead of the very specific PDK1 response. PDH phosphatases could reverse PDH phosphorylation by PDK (35), and contribute to the increased activity of PDHα after acclimation but likely would have pleiotropic effects on all phosphorylation sites of PDH rather than the specific changes in Ser232 phosphorylation status observed here. Extramuscular factors, such as adrenergic stimulation of muscle glycogenolysis, could also play a role in hypoxic acclimation. For example, arterial catecholamine and lactate production are both reduced in concert over altitude acclimation in humans (26, 37). However, acute hypoxia has been shown to reduce glycogen phosphorylase activity, a target of adrenergic stimulation, and change in flux through this step is reliant on posttranslational regulation via free P, or AMP (32). Nevertheless, the present study along with previous work on the HIF regulatory axis (e.g., 14, 25), highlight the modulation of a biochemical pathway at the level of the myocyte that provides an explanation for a decrease in lactate production in the early stages of hypoxic acclimation.

**Perspectives and Significance**

Hypoxia has long been known to induce many rescue pathways including augmenting glycolytic flux and reducing oxidative glucose oxidation, aided by changes coordinated by the transcription factor HIF-1α (40). Although reliance on anaerobic glycolysis is a suitable short-term strategy to surviving hypoxia, it is not sustainable beyond the point where glycogen reserves are depleted. It is therefore not surprising that we find HIF-1α protein returning to normoxia levels with chronic hypoxia, even when the muscle is most likely still hypoxic.

The present work provides some of the first evidence of a muscle-level mechanism underlying an observation made in humans almost eight decades ago (9). In addition, lactate is now recognized as an important intermediate in metabolism not only in response to natural environmental stress but also in pathological conditions of oxygen deprivation (e.g., cancer) (12, 15, 21, 36). Therefore, further understanding of the regulatory and metabolic cascade leading to changes in lactate production at different levels of organization can provide further insights into the overall homeostatic response of animals to oxygen deprivation in a variety of conditions.
ACKNOWLEDGMENTS

The authors thank G. Heigenhauser (McMaster University) for helpful comments regarding these experiments. Antibodies for individual PDH phosphorylation sites were a generous gift from M. Rardin (University of California, San Diego, San Diego, CA).

GRANTS

This work was funded through a Natural Science and Engineering Council of Canada Discovery Grant and an Ontario Ministry for Research and Innovation Early Researcher Award to G. B. McClelland. A. J. Morash was the recipient of a NSERC postgraduate scholarship.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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2. Bender PR, Groves BM, McCullough RE, McCullough ED. No conflicts of interest, financial or otherwise, are declared by the author(s).


