Regulation of cellular metabolism: programming and maintaining metabolic homeostasis

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Submitted 6 August 2013; accepted in final form 3 October 2013


This is accomplished through an intrinsic program that determines the metabolic [ATP]/[ADP]/[Pi], where [Pi] is the concentration of inorganic phosphate (energy state) and maintains it through a bidirectional sensory/signaling control network that reaches every aspect of cellular metabolism. The program sets the energy state with high precision (to better than one part in 10⁹) and can respond to transient changes in energy demand (ATP use) to more than 100 times the resting rate. Epigenetic and environmental factors are able to “fine tune” the programmed set point over a narrow range to meet the special needs associated with cell differentiation and chronic changes in metabolic requirements. The result is robust, across platform control of metabolism, essential to cellular differentiation and the evolution of complex organisms.

In living organisms, DNA and epigenetic systems contain the information required to construct the parts and guide the assembly. Life, however, requires a metabolic CPU programmed to maintain metabolic homeostasis through an integrated control network. The equilibrium value for the ATP/ADP inorganic phosphate concentration ([ATP]/[ADP][Pi] where [Pi] is the concentration of inorganic phosphate (energy state)) ratio is near 2 × 10⁻⁶ M⁻¹, whereas in living cells, this needs to be held between 10⁴ and 10⁵ M⁻¹. In mammals, excursions of more than a factor of five from the set point are pathological and can be tolerated for only short periods of time. This means that the CPU that controls metabolic homeostasis is programmed not only to drive the synthesis of ATP to a factor of 10¹⁰ from equilibrium but also to regulate metabolism precisely enough to hold that set point with an accuracy of better than one part in 10⁹, an extraordinary level of precision.

The CPU for cellular metabolism is a metabolic component(s) that receives sensory input from all of the different metabolic pathways of the cell. It integrates this information, compares it with the programmed set point, and generates outgoing control signals that increase or decrease the activity of the individual components of metabolism to maintain homeostasis. There are three important requirements for the metabolic CPU: 1) it has an internal program that determines the homeostatic set point; 2) it receives sensory input sufficient to provide a comprehensive picture of the current status of the critical components of the system; and 3) it outputs signals to key metabolic control points of the system that increase or decrease the activity as appropriate to maintain metabolism operating near the set point and to return to that set point following transient changes in metabolic requirements. To accomplish its role, the CPU needs to be the center of a network of sensory input/control output signaling pathways. These sensory/control pathways have the CPU as the “hub.”
and expand outward like the branches of a tree. The result is a control network centered on the CPU that interacts with all of the cellular metabolism, dynamically melding the individual functions into a coherent whole. It should be emphasized that the branch point or nodes in the network (such as pyruvate dehydrogenase and phosphofructokinase) have a key role in regulation. They are responsible not only for providing appropriate output to the downstream connections but also for helping to maintain the levels of metabolites on the upstream side. These nodes typically have several levels of regulation, often including hormonally induced covalent modification of the enzyme and/or altered gene expression.

In this paper, I will summarize evidence that mitochondrial oxidative phosphorylation is the CPU that sets and regulates metabolic homeostasis in higher organisms. Living systems require continuous input of energy for their existence, and in higher organisms, oxidative phosphorylation provides most of that energy (ATP). This is done by oxidizing substrates, such as glucose and fatty acids, all the way to carbon dioxide and water (burning them) and using much of the available energy from that combustion to synthesize ATP from ADP and Pi. Oxidative phosphorylation has a higher flux than any other metabolic pathway and must be continuously active, with any interruption causing progressive loss of viability. As a result, a priori considerations favor energy metabolism, oxidative phosphorylation, in particular, having a central role in setting and maintaining metabolic homeostasis. To show that oxidative phosphorylation is the metabolic CPU, however, I will focus on two critical characteristics: 1) oxidative phosphorylation is coupled to all aspects of cellular metabolism, largely through the energy state, and this coupling has the bidirectional sensory/control signaling (input/output) characteristics required for the CPU; and 2) oxidative phosphorylation has a built-in program that determines the homeostatic set point, and the signaling system acts to counter displacements from that set point. The programmed set point is very precise, but it can be “fine tuned” over a narrow range by epigenetic and environmental factors to meet the special needs associated with cell differentiation and longer-term changes in metabolic requirements. A schematic of the operation of oxidative phosphorylation as the metabolic CPU is presented in Fig. 1. Only a skeleton of the control pathways connecting oxidative phosphorylation to the rest of the metabolism will be discussed. Each reader will be able to think of many additional connections and control mechanisms that should be added to flesh out the skeleton. When completed, for mammalian cells, the regulatory “tree” encompasses all of metabolism.

**METABOLIC SIGNALING AND OXIDATIVE PHOSPHORYLATION**

**Catabolic and Anabolic Metabolism**

Oxidative phosphorylation depends on the citric acid cycle (CAC) to provide most of the NADH required to maintain the cellular energy-state ratio (9, 48, 49). Flux through the CAC is tightly coupled to the rate of oxygen consumption through the intramitochondrial NAD couple concentration ([NADH]/[NADH]) and energy state. In turn, flux through the CAC and oxidative phosphorylation is dependent on the cellular energy (ATP) demand and substrate availability. Regulation of the CAC and the many metabolic pathways that feed into the CAC is covered extensively in textbooks and reviews and need not be discussed further here. Glycolysis illustrates an important aspect of how oxidative phosphorylation maintains homeostasis. Glycolysis feeds pyruvate into the CAC through pyruvate dehydrogenase, but it is a special case, because it can also operate as an independent source of ATP by producing lactate or ethanol. The rate of oxygen consumption by oxidative phosphorylation is coupled to the cellular need for ATP (rate of ATP use) through the energy state, which involves ATP, ADP, and Pi equally, but [ATP] and [Pi] are much higher, by 50-fold, than that of ADP. As a result, changes in the energy state of most cells are reflected largely in [ADP] [for discussion, see Wilson and colleagues (9, 47)]. Adenylyl kinase, the enzyme that catalyzes the reaction

$$2 ADP \leftrightarrow AMP + ATP \quad \text{Keq} = \frac{[AMP][ATP]}{[ADP]^2} \quad (1)$$

where Keq is the equilibrium constant, is near equilibrium in most cells, and because the [ATP] remains nearly constant, [AMP] increases or decreases as the square of the change in [ADP] \([AMP] = (\text{Keq}[\text{ATP}]) \times [\text{ADP}]^2\). AMP is a powerful activator for phosphofructokinase, the rate-limiting step of glycolysis. When suspensions of cells with functional mitochondria, but without a supply of oxygen, have adequate levels of glucose, they consume glucose very rapidly to make ATP, with lactate or ethanol as the end-product. Providing oxygen activates oxidative phosphorylation, and ATP synthesized by oxidative phosphorylation generates a higher energy state than glycolysis alone, decreasing the level of AMP and thereby decreasing glycolytic flux and production of lactate or ethanol (Pasteur effect). The low levels of AMP are maintained for as long as adequate oxygen is available. When the oxygen is exhausted, the AMP level increases again, activating phosphofructokinase and increasing glycolytic flux. Almost all cells with mitochondria and the enzymes of glycolysis can exhibit the Pasteur effect. In mammals, the Pasteur effect is observed most easily in skeletal muscle due to the presence of stores of glucose as glycogen. Light exercise in aerobic skeletal muscle does not result in a significant increase in lactate concentration. If blood flow is interrupted, however, the oxygen levels are depleted rapidly, and then a very rapid increase in lactate begins, due to activation of glycolysis and inhibition of oxida-
tive phosphorylation. Restoration of blood flow (tissue oxygenation) inhibits glycolysis and production of lactate. Glycolytic activity in muscle is not regulated only by AMP, of course, and calcium ($Ca^{2+}$), in particular, has a major role. Many other small molecules, such as $Ca^{2+}$, augment the effects of AMP in meeting the specific requirements of each cell type.

Oxidative phosphorylation also has an important role in most biosynthetic (anabolic) pathways, including those for synthesis of fatty acids, glucose, glycogen, amino acids, proteins, and nucleic acids. Real-time control operates, in part, through the action of small molecules, including, but not limited to, NAD+, NADH, ATP, ADP, AMP, and Pi. These small molecules can also act on a longer time scale through their effects on gene expression (see below).

**Oxygen**

**Oxygen consumption.** In mammals, oxidative phosphorylation is responsible for >95% of the oxygen consumption by the body. Under conditions where the oxygen supply is maintained, oxidative phosphorylation has a very high capacity for ATP production. The rates of oxygen consumption (ATP production) can increase >100-fold in human skeletal muscle (25, 31, 35) and in flight muscle (2, 8, 11) as they go from rest to near-maximal work. The increase in oxygen consumption by the flight muscle was not measured in flight muscle per se, but whole-body oxygen consumption of the hummingbird increased 100-fold ongoing from rest to normal hover flight. Hover flight is a high, but not maximal, work rate, and the flight muscle is unlikely to contribute more than one-third of the oxygen consumption of the whole body at rest, suggesting that in the flight muscle, the maximal rates may be substantially >300 times the resting rate. Suspensions of cultured cells allow simultaneous measurements of the concentration of mitochondria and oxygen consumption, and these approximate the conditions in tissue. In suspensions of hepatocytes, as well as cultured cells derived from neurons and kidney, the calculated rate of mitochondrial respiration corresponds to a turnover number for cytochrome a of the respiratory chain of 3-6 s$^{-1}$ (9). The maximal turnover number for cytochrome a, measured in mitochondria, isolated from liver or muscle, is near 1,000 s$^{-1}$ (42, 43, 45). Thus measurements in vivo and in vitro show that mitochondrial oxidative phosphorylation in resting tissues operates at <1% of maximal capacity. Oxidative phosphorylation involves several reactions that act in sequence, and the maximal rate through each intermediate reaction must be at least high enough to account for the maximal rate of oxygen consumption and ATP synthesis. The net rate of ATP synthesis (oxygen consumption) is the difference between the forward and reverse rates (forward $\rightarrow$ reverse $= \text{net}$), leading to the conclusion that every step of oxidative phosphorylation has reverse rates that are very nearly equal to (>99%) of the forward rates. Reactions with nearly equal rates in the forward and reverse directions are considered near equilibrium, with equilibrium defined as the condition when the forward and reverse rates are equal. The very large capacity makes possible the large increases in ATP use/synthesis that occurs in flight and heavy exercise. Near equilibrium under resting (unstressed) conditions is also important to the program that determines the homeostatic set point (see below).

**Oxygen delivery.** Large alterations in the rates of oxygen use in vivo, such as occur in exercising muscle, must be matched by a rapid and nearly equally large increase in delivery of oxygen. That requires a hierarchy of control mechanisms that coordinates, in real time, oxygen delivery with oxygen demand. The pressure for half-maximal function of mitochondrial oxidative phosphorylation for oxygen is ~12 Torr at physiological pH (41, 43), about one-third of the mean oxygen pressure in most tissues (near 35 Torr), and overlaps the low end of the oxygen distribution in normoxic tissues (3, 14, 16, 24, 44). This makes energy metabolism very sensitive to the pericellular oxygen pressure in vivo. The rate of oxygen consumption and of ATP synthesis is tightly coupled to the rate of ATP use. In most cells, [ATP] is between 3 and 8 mM, and the rate of ATP use, even in resting cells, is high enough to consume this in ~1 min. This small “buffer capacity” means that the energy state responds rapidly (seconds) to changes in either ATP production or consumption (47), even under resting conditions.

ATP is consumed for active ion transport, synthesis of proteins, muscle contraction, etc.—reactions that are typically not directly dependent on oxygen pressure. A decrease in the oxygen pressure to below normoxic levels lowers the capacity for oxidative phosphorylation but does not significantly affect the rate of ATP use. At below-normal oxygen pressures, oxidative phosphorylation can still synthesize ATP fast enough to match the rate of use but only at a decreased energy state. With a progressive decrease in oxygen pressure, there is a progressive decrease in energy state, propagating the metabolic displacement (metabolic stress) throughout cellular metabolism. Numerous control pathways are activated that are designed to counter the decrease in oxygen levels and energy state. If the stress is too severe or sustained for too long of a time, it can lead to activation of apoptotic or necrotic cell-death mechanisms. When the oxygen pressure decreases to near zero (anoxia), the rate of ATP synthesis can no longer match the rate of use, even at a decreased energy state, and there is a catastrophic metabolic failure. From a survival viewpoint, it is the range of oxygen pressures below normal but above anoxia that is important. In the literature, this range of oxygen pressures is referred to as “hypoxia” and often not differentiated further, leading to much confusion. Experimental results for cells having a pericellular oxygen pressure of 20 Torr, for example, are different from those with a pericellular oxygen pressure of 2 Torr, but the results are typically lumped together as hypoxia.

In the broad range of oxygen pressures between normoxia and anoxia, there is a graded response of metabolism to oxygen pressure. This graded response allows cells and tissue to respond to the deficit in oxygen with corrective measures designed to increase delivery of oxygen. One level of control is through local control of vascular resistance, as exemplified by regulation of coronary flow in the heart (30). Energy metabolism plays an important role in regulation of flow in the coronary vessels, where flow is precisely matched to work, and has a role in blood flow in other organs. Blood flow has many roles, of which oxygen delivery is only one, albeit an important one. As a result, there are a large number of other parameters that affect flow, many of which are tissue specific. The requirement for precise matching of oxygen delivery with oxygen...
consumption and possible mechanism for regulation of local blood flow have been addressed in a recent paper (13).

A second level of control of oxygen delivery by energy metabolism is through regulation of the oxygen levels in the arterial blood, as exemplified by the carotid body (CB). The CB is a small organ lying on the side of the carotid artery. The blood supply is derived directly from the carotid artery, but otherwise, it has a quite normal vascular system. The carotid sinus nerve (CSN) is afferent from the CB, and the CSN activity is dependent on the oxygen pressure in the internal vessels of the CB, increasing with decreasing oxygen pressure. The CSN connects to the glossopharyngeal nerve, which leads directly to cardiorespiratory centers in the medulla oblongata, as well as the vagus and phrenic nerves, which are involved in control of breathing and blood pressure. The function of the communication between the CB and the brain is not well understood but is expected to be important in control of the cardiopulmonary system.

Oxygen sensing by the CB occurs through oxidative phosphorylation, as has been shown using the isolated perfused/superfused CB (no hemoglobin) to study the oxygen dependence of the CSN activity. The afferent CSN activity responds to changes in oxygen pressure very similarly to the organ in vivo. The use of perfusates, equilibrated with mixtures of carbon monoxide (CO) and oxygen gas, results in a higher afferent neural activity than does an equal level of oxygen alone—the difference consistent with CO being competitive with oxygen in the sensory activity. The inhibition by CO can be reversed very rapidly by light. Absorption of photons results in dissociation of the CO but does not affect the oxygen reaction, so light returns the afferent neural activity to that without CO. These changes in afferent CSN activity occur very rapidly, beginning <1 s after the light is turned on or off. The wavelength dependence of the efficiency of light in reversing the inhibition is a direct measure of the absorption spectrum of the inhibitory CO complex (photochemical action spectrum). The spectrum that results is that of the CO complex with mitochondrial cytochrome oxidase (39, 46). Thus oxygen sensing by the CB, which occurs in the glomus cells, is directly through the oxygen dependence of mitochondrial oxidative phosphorylation. It is reasonable to assume that other neurons can sense oxygen in a manner similar to those in the CB, including those in aortic bodies and the brain. This gives mitochondrial oxidative phosphorylation a central role in neuronal regulation of oxygen delivery to tissue, including breathing, heart activity, and vascular resistance.

Regulation of gene expression. The metabolic CPU must also be able to regulate metabolic capacity in response to chronic alterations in the metabolic requirements in the cells, such as those imposed by endurance training (18, 19, 32–34). As noted above, oxidative phosphorylation maintains the energy state of the cell and through this, determines the levels of many essential metabolites and signaling molecules. These include ADP and through the activity of the adenylyl kinase, AMP. In addition to the role already mentioned in regulation of glycolytic flux and modulation of the activity of a number of other pathways, AMP and ADP are important activators for the AMP-dependent protein kinase. AMP kinase regulates gene expression for many of the enzymes associated with energy metabolism (7, 10, 17, 21, 27, 29, 50). This exemplifies how cellular metabolism is adjusted to compensate for chronic changes in energy requirements by increasing or decreasing the levels of the enzymes responsible for ATP production. Exercise, for example, lowers the energy state, increasing the ADP and AMP. If these levels remain elevated for sufficiently long periods of time—either through endurance training or repetitive shorter periods of higher intensity (1)—then the increase in AMP kinase activity leads to increased levels of the enzymes responsible for ATP production. As the capacity for ATP production increases, the levels of AMP (and AMP kinase activity) fall until metabolic homeostasis is re-established but at an increased average turnover for ATP (15). It is interesting to note that in muscles of patients with vascular occlusive disease, there is an increase in the oxidative enzymes similar to that observed for exercise training (6, 20, 28). Reconstructive surgery increased oxygen levels in the muscles, and the oxidative enzyme content decreased. This is consistent with mild hypoxia in the ischemic muscles, resulting in decreased energy state and increased levels of AMP. It might be expected that high altitude and global hypoxia would induce similar changes in muscle. All of the other cell types in the body are affected, however, and many different compensatory pathways affected mask the local effects on muscle.

Other regulatory pathways coupled to oxidative phosphorylation include the stress-inducible factors. Control of these factors is not fully understood, but there is evidence that they are regulated, at least partially, through the energy state, a critical component of most conditions that initiate a stress response. In addition, the mitochondria carry out a wide range of other functions (38)—too many to be addressed here.

The Program for the Homeostatic Set Point

The basic program. The power source for oxidative phosphorylation is transfer of reducing equivalents from the pyridine nucleotide pool in the mitochondrial matrix to molecular oxygen. Transfer of reducing equivalents through the redox components of the respiratory chain is reversible and near equilibrium (nearly equal forward and reverse rates of transfer) under most physiological conditions. Since the forward and reverse rates are dependent on the concentrations of the reduced and oxidized forms, respectively, the optimal capacity for reversible transfer occurs at the one-half reduction potential, whereas the concentrations of the acceptor and donor forms are equal. For components that transfer two reducing equivalents, the ratio of the concentrations (oxidized/reduced) increases 10-fold for each 30 mV that the potential becomes more positive and decreases for each 30 mV that it becomes more negative. The capacity for forward transfer (concentration of the reduced form), for example, falls to 10% at +30 mV, 1% at +60 mV, and 0.1% at +90 mV from the midpoint potential. There is a “sweet spot” in reversible oxidation-reduction systems when both the low potential and high potential redox couples have equal oxidized and reduced forms. [NADH][NAD+] is the principal donor of reducing equivalents for oxidative phosphorylation, has a one-half reduction potential near −320 mV when free in solution but in the mitochondria, these cofactors are mostly bound to dehydrogenase enzymes. The potential of [NADH][NAD+] in liver can be measured by equilibrium with the β-hydroxybutyrate/acetocetate couple and is near −260 mV (9, 40). The reducing equivalents from NADH are transferred through the respiratory chain to cytochrome c oxidase, where the two-electron accep-
tor has been tentatively identified as peroxide bound between two metal centers (45). The one-half reduction potential for this intermediate at pH 7.0 is near 570 mV. As noted earlier, the reactions of oxidative phosphorylation are freely reversible (near equilibrium), and when both the NAD and bound peroxide couples are near 50% reduction, a total of 830 mV is coupled to synthesis of ATP.

The rate constants for the irreversible part of oxygen reduction by cytochrome c oxidase are designed to bias the control to give an acceptor potential of 60 mV more positive than the midpoint potential (~630 mV). At first glance, this bias might appear to compromise the set point, but cells spend most of their time with metabolic rates near the set point and experience only transient excursions to much higher rates—a behavior most obvious for muscle cells and neurons. The positive bias limits the capacity of the cells to decrease their metabolic rates, where little change is required, but increases the ability to respond to escalation in ATP use, which can be very large. With the positive bias, there is ~890 mV electrical potential coupled to ATP synthesis. Assuming three ATP are synthesized/NADH oxidized, this is 297 mV/ATP. The K\textsubscript{eq} and free energy for hydrolysis of ATP have been measured [(26); see also Golding and Golding (12) and Veech et al. (37)] and reported to be ~7.79 kcal/mol under physiological conditions (pH 7.2, 1 mM Mg\textsuperscript{2+}, 0.25 ionic strength). It can then be calculated that in liver, oxidative phosphorylation is programmed to operate with a set-point energy state near 1.4 \times 10^{-4} M^{-1}. Deviations from this set point automatically feed back through oxidative phosphorylation, altering the rate of oxygen consumption and other regulatory responses as appropriate for returning metabolism to the set point. The reproducibility in the measured values for the energy state in specific tissues is currently limited by the analytical methods used, and we can only conclude that the homeostatic set point is maintained to very high precision.

"Fine tuning" the set point through environmental or epigenetic demand. The redox potential of the intramitochondrial pyridine nucleotide couple can be modulated by regulating the activity of the dehydrogenases of the CAC, such as through intramitochondrial [Ca\textsuperscript{2+}], activation of fatty-acid oxidation, etc. The range through which the set point can be changed by such regulatory mechanisms is small, typically less than a factor of five in the energy state. Such changes include hormonally controlled alterations in response to environmental demands or substrate availability. Another mechanism for changing the set point is epigenetic. For example, in mammals and other complex organisms, there are many different types of cells, each with different metabolic requirements. To be fully responsive to these different conditions, epigenetics can "fine tune" the set point to the specific needs of the cell. As noted above, the calculated set point for liver is 1.4 \times 10^{-4} M^{-1}, based on the potential of ~260 mV for [NADH]/[NAD\textsuperscript{+}] (40). In skeletal and heart muscle, the energy state is significantly higher than in the liver. This higher energy state is correlated with a more negative potential for the intramitochondrial pyridine nucleotide pool by ~60 mV [for review, see Erecinska and Wilson (9)]. This more-negative potential increases the calculated set point (energy state) by approximately fivefold, to 7 \times 10^{-4} M^{-1}. To make the calculations appropriate to specific tissues, such as muscle, it is necessary to put in the appropriate tissue-specific variables. The [NADH]/[NAD\textsuperscript{+}] ratio allows calculation of the energy state and [P\textsubscript{i}], the [creatine phosphate]/[creatine] ratio. To calculate free [ADP] ([ADP\textsuperscript{f}]), it is necessary to know (put in) [ATP]. In skeletal muscle tissue, [P\textsubscript{i}] is near 4.0 mM and that for ATP, ~8.0 mM. With the use of these values and the set point of 7 \times 10^{-4} M^{-1}, the calculated [creatine phosphate]/[creatine] ratio would be 1.2, and the calculated [ADP\textsuperscript{f}] would be 28 \mu M. The calculated values are consistent with the measurements reported in the literature (4, 9, 22, 23, 37). The set points calculated for the program built into oxidative phosphorylation and the energy states measured in vivo in liver and muscle are very similar. The set point calculated for muscle is applicable to brain tissue, which has intramitochondrial NADH/NAD\textsuperscript{+} ratios more similar to muscle than liver. As a result, the calculated set point is also consistent with the energy state measured for the brain, as well as for neurons and glial cells derived from the brain (37, 42). It should be noted that the one-half reduction potential for the bound peroxide remains to be fully established, and the above calculations may need to be revised slightly as better values are obtained.

CONCLUDING COMMENTS

Control of metabolism through oxidative phosphorylation is an example of extraordinarily elegant and robust engineering. Oxidative phosphorylation is precise (to better than one part in 10\textsuperscript{9}), the response time is <1 s, rapid and large transients in ATP demand are easily accommodated, and it can withstand brief periods of severe metabolic displacement, such as those caused by hypoxia. Oxidative phosphorylation is programmed to set the metabolic energy state and is central to a "tree-like" regulatory network that is designed to maintain that energy state. This provides a logical and coherent framework for understanding how cellular and tissue metabolism is regulated. Within this framework, it is relatively straightforward to understand how metabolic homeostasis is set and maintained, with large numbers of seemingly uncoordinated metabolic pathways working together in harmony and toward a common goal. From an evolutionary viewpoint, development of this system provided for stable, across-platform control of metabolism. Undifferentiated cells can afford wider swings in energy state in individual cells, because each acts as an individual with little effect on its neighbors. With differentiation, cells become interdependent and much less tolerant of metabolic deviations, because malfunction of any one cell type pathologically affects survival of the organism as a whole. It can be argued that a robust metabolic control system had to develop before extensive biological differentiation. It is interesting to speculate that this could have been a factor in initiating the period of rapid increase in the number of phyla in the Cambrian period (5, 36).

DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Author contributions: D.F.W. conception and design of research; D.F.W. analyzed data; D.F.W. interpreted results of experiments; D.F.W. prepared figures; D.F.W. drafted manuscript; D.F.W. edited and revised manuscript; D.F.W. approved final version of manuscript.

J Appl Physiol • doi:10.1152/japplphysiol.00894.2013 • www.jappl.org
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