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MUSCLE FATIGUE AND MUSCLE WEAKNESS: WHAT WE KNOW AND WHAT WE WISH WE DID

Topic Editors
P. Bryant Chase and Christina Karatzaferi

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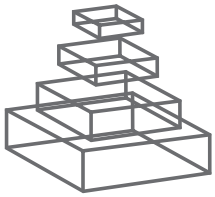

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MUSCLE FATIGUE AND MUSCLE WEAKNESS: WHAT WE KNOW AND WHAT WE WISH WE DID

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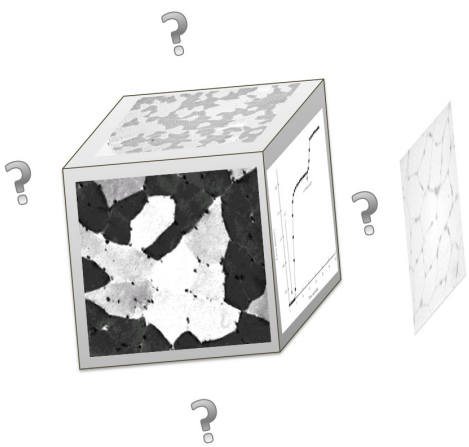

Image by P. Bryant Chase and Christina Karatzaferi.

The purpose of this Research Topic is to discuss evidence coming from macroscopic observations on the whole human/animal to investigations at the molecular level in an effort to identify critical factors in muscle fatigue and muscle weakness, in health and disease.

Why is this important?

Skeletal muscles confer movement to the human body using vast amounts of energy provided through complex metabolic pathways. Thus, whole body mobility and energy balance are dictated by muscle contraction. On the other side, muscle function reflects overall health status, as chronic conditions and/or ageing affect either or both of muscle quality (protein and fat content) and quantity (mass). In health, muscle fatigue is temporary and recovery occurs rapidly. In ageing or chronic disease however, muscle fatigue may occur prematurely and be persistent, endangering thus a person's safety (i.e. weakness leads to falls), and leading the sufferer in a self-perpetuating vicious cycle of inactivity -> disuse muscle atrophy/metabolic disturbance and so on, that compounds morbidity (i.e. causing metabolic syndrome, fatness, hypertension, muscle cachexia) and that eventually leads to premature death.

Research in skeletal muscle physiology and biophysics is at an exciting phase. The advent of new methodologies and technological advancements have allowed researchers to advance our knowledge on the "how's" and "why's" of muscle contraction – however there are still many unanswered questions especially when disease states are implicated.

Such questions need addressing if as a scientific community we aspire to secure better designed interventions to improve muscle function, and thus improve quality of life and life prognosis for the ageing population and chronic disease patients.

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EDITORIAL
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Muscle fatigue and muscle weakness: what we know and what we wish we did

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This Research Topic on muscle fatigue and muscle weakness presents the latest ideas, arguments, and evidence from investigations at the molecular level to macroscopic observations on whole animals including humans, in an effort to identify critical factors underlying fatigue and weakness in health and disease. Skeletal muscles confer movement to the human body using vast amounts of energy provided through complex metabolic pathways such that whole body mobility and energy balance are largely dictated by muscle activity. Conversely, muscle function reflects overall health status as exercise history and chronic conditions affect either or both muscle quality, including protein and fat content, and muscle mass. In health, muscle fatigue is temporary and recovery occurs rapidly, and recreational or competitive athletes are always pursuing the next best fatigue "fix." However, after inactivity—whether due to lifestyle choices, injury or chronic disease—muscle fatigue may occur prematurely and persist, endangering a person's safety because weakness can lead to falls that may result in loss of independence. Individuals are then trapped in a self-perpetuating, vicious cycle of inactivity, disuse muscle atrophy/weakness, and metabolic disturbance that compounds morbidity (i.e., causing metabolic syndrome, obesity, hypertension, cachexia) and eventually premature death. Such issues transcend many scientific disciplines and it becomes evident that not only recognizing fundamental factors in muscle fatigue and muscle weakness is necessary, but also evaluating their interaction with factors outside of the muscle is essential if we aspire to design better interventions that improve muscle function and thus improve quality of life and life prognosis for the ageing population and chronic disease patients.

Fatigue and weakness may stem from changes within myocytes that affect cross-bridge function or Ca^{2+} activation, to changes within the circulation or function of the nervous system. Within myocytes, metabolic products of ATP hydrolysis in the cytoplasm such as inorganic phosphate (Pi), protons (H^+ or pH), and ADP have often been considered as agents that could disrupt force generation at the sarcomere level (Fabiato and Fabiato, 1978; Cooke and Pate, 1985; Metzger and Moss, 1987; Nosek et al., 1987, 1990; Chase and Kushmerick, 1988, 1995; Cooke et al., 1988; Godt and Nosek, 1989; Pate and Cooke, 1989; Metzger and Moss, 1990a,b; Pate et al., 1995, 1998; Wiseman et al., 1996; Karatzaferi et al., 2003, 2008). These effects may be due to direct binding to proteins, or due to a more global alteration of cellular energetics (ΔG_{ATP}) in the myocyte (Karatzaferi et al., 2004).

In this Research Topic, Debold (2012) consolidates the most recent information, including single molecule assays and molecular biological approaches, about the mechanisms by which Pi, H^+, and ADP inhibit actomyosin cross-bridge cycling and thin filament Ca^{2+}-activation. Allen and Trajanovska (2012) provide a synthesis on the multiple roles of Pi in fatigue, including novel results from their group, showing that Pi is even more detrimental when its effects on Ca^{2+} release are combined with inhibition of actomyosin force generation and Ca^{2+} activation. In addition to activity-driven changes in metabolites and cellular energetics, mutations in sarcomeric proteins have been associated with prolonged muscle weakness in myopathies. Moving away from actomyosin events, Ottenheijm et al. (2012) consider the role of nebulin in sarcomere function, and how transgenic mouse models can inform us about mutations in the giant filamentous protein nebulin, and mutations in other thin filament and closely related proteins that are associated with nemaline myopathy.

To fully test our understanding of muscle fatigue, appropriately detailed models of muscle function will be necessary. Röhrle et al. (2012) make major advances in that arena by presenting a multi-scale, finite element model of the human tibialis anterior. Their model has the advantage of allowing simulation of fatigue at the cellular and motor unit levels, and can incorporate altered recruitment patterns of motor units due to central components of fatigue. Thus their model can serve an invaluable role as we bridge our understanding between the cellular and tissue levels.

Muscle's plasticity is most readily evident in its adaptation to repeated exercise, and conversely to inactivity that may be associated with various injuries and disease states. Bogdanis (2012) reviews the long-term changes in muscle at the molecular, cellular, and tissue levels, as well as the corresponding functional changes that are associated with these adaptations to activity level history. Fatigability is a key functional characteristic of different muscle fiber types, and can vary greatly with activity, or inactivity, and Bogdanis evaluates the utility of high-intensity bouts of exercise for modulating fatigability by training,

or as a component in therapy. Bogdanis' section on effects of reactive oxygen species (ROS) sets the stage for the succinct review on antioxidants by Hernández et al. (2012). Despite the popularity of antioxidants as nutritional supplements, Hernández et al. report that their utility for either minimizing or speeding recovery from fatigue appears to be limited to specific muscle types. Moreover, Bogdanis' (2012) section on neural factors opens the discussion on the role of non-muscle factors in fatigue and serves as a bridge to the articles by Kobilo and van Praag (2012), Sakkas and Karatzaferi (2012), and Noakes (2012).

What is the extent to which muscle activity and fatigue influence the function of other physiological systems of the body, particularly the nervous system upon which skeletal muscle depends for activation, and how much of fatigability is determined centrally? In the commentary by Kobilo and van Praag (2012), pharmacological activation of AMP-activated protein kinase (AMPK)—a metabolic regulator that is activated during exercise—is shown to alter performance in a test of spatial memory and hippocampal neurogenesis in mice in a time-dependent manner. How can diseases and treatments modify the experience and presentation of fatigue? In their opinion article, Sakkas and Karatzaferi (2012) consider available evidence on the complex symptomatology of fatigue in renal patients on hemodialysis treatment. By drawing analogies to Chronic Fatigue Syndrome, Sakkas and Karatzaferi (2012) present the view that fatigue, as experienced by patients undergoing routine hemodialysis, might be better addressed by caregivers as a syndrome and not with isolated measures since its apparent complexity requires a cross-disciplinary therapeutic approach. While hemodialysis and some other patients may be afflicted with specific syndromes, the rest of us have all heard the expression "mind over matter." Does it apply to muscle? Noakes (2012) concludes the series with a challenging review, partly historical in nature, arguing that the key component in fatigue is central. The author discusses the accepted models on the limits of human exercise performance, and presents his central governor model of exercise regulation, arguing that fatigue is brain-derived, being an important homeostatic mechanism that protects an organism from catastrophic overexertion.

It is our sincere hope that this Research Topic will not only provide readers with new insights and viewpoints on the issue of muscle fatigue and weakness, but will also stimulate novel ideas, experiments, and further advances in this research field.

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Recent insights into muscle fatigue at the cross-bridge level

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The depression in force and/or velocity associated with muscular fatigue can be the result of a failure at any level, from the initial events in the motor cortex of the brain to the formation of an actomyosin cross-bridge in the muscle cell. Since all the force and motion generated by muscle ultimately derives from the cyclical interaction of actin and myosin, researchers have focused heavily on the impact of the accumulation of intracellular metabolites [e.g., P_i, H^+ and adenosine diphoshphate (ADP)] on the function these contractile proteins. At saturating Ca^{++} levels, elevated P_i appears to be the primary cause for the loss in maximal isometric force, while increased [H^+] and possibly ADP act to slow unloaded shortening velocity in single muscle fibers, suggesting a causative role in muscular fatigue. However the precise mechanisms through which these metabolites might affect the individual function of the contractile proteins remain unclear because intact muscle is a highly complex structure. To simplify problem isolated actin and myosin have been studied in the *in vitro* motility assay and more recently the single molecule laser trap assay with the findings showing that both P_i and H^+ alter single actomyosin function in unique ways. In addition to these new insights, we are also gaining important information about the roles played by the muscle regulatory proteins troponin (Tn) and tropomyosin (Tm) in the fatigue process. *In vitro* studies, suggest that both the acidosis and elevated levels of P_i can inhibit velocity and force at sub-saturating levels of Ca^{++} in the presence of Tn and Tm and that this inhibition can be greater than that observed in the absence of regulation. To understand the molecular basis of the role of regulatory proteins in the fatigue process researchers are taking advantage of modern molecular biological techniques to manipulate the structure and function of Tn/Tm. These efforts are beginning to reveal the relevant structures and how their functions might be altered during fatigue. Thus, it is a very exciting time to study muscle fatigue because the technological advances occurring in the fields of biophysics and molecular biology are providing researchers with the ability to directly test long held hypotheses and consequently reshaping our understanding of this age-old question.

Keywords: muscle, fatigue, myosin, actin, phosphate, acidosis, troponin, tropomyosin

INTRODUCTION

The quest to identify the cause(s) of muscle fatigue has been a quintessential question in the field of physiology for more than 100 years. In that time our understanding of the etiology of fatigue has evolved greatly and we have a greater appreciation for the complexity of the phenomenon, recognizing that there are many potential factors that may contribute to fatigue. For example, the rate and extent of fatigue is highly dependent of the mode and intensity of contractility activity (Fitts, 1994). Based on these observations it is now clear that, in general, the factors that cause a muscle to fatigue from low intensity stimulation are distinctly different from the factors that elicit fatigue from high intensity stimulation (Fitts, 1994). This review focuses on the recent revelations with regard to fatigue resulting from short and intense bouts of contractile activity (i.e., high intensity fatigue) with a particular focus on the role of the cross-bridge cycle. This type of muscle fatigue was classically defined by a decrease in force in response to repeated intense contractile activity, however this definition has been broadened to a decrease in the expected or required power output (Fitts, 1994). This revision has two important implications; firstly it indicates that fatigue can occur at submaximal, as well as maximal, contractile intensities; and secondly that fatigue can result from a drop in force and/or the velocity of contraction. This more widely accepted definition of fatigue now defines for researchers in the field the parameters on which to focus on or identifying the underlying causes of this kind of transient loss of muscle function.

Work in the late 1970s and early 1980s used NMR spectroscopy on *in vivo* muscle to establish that the accumulation of metabolites, principally hydrogen ions (H^+, i.e., acidosis), inorganic phosphate (P_i), and adenosine diphosphate (ADP), were correlated with the development of fatigue in response to intense bouts of contractile activity (Dawson et al., 1978). Parallel efforts using chemically skinned single muscle fibers demonstrated that elevated levels of these ions directly inhibit muscle's ability to generate maximal isometric force and unloaded shortening velocity (Cooke et al.,

1988), providing strong evidence for a causative role in fatigue. While it is clear from the skinned single muscle fiber studies that elevated levels of metabolites directly affect the force and motion generating capacity of muscle, it is still not clear how this occurs at a molecular level. More sophisticated experiments in single fibers led to hypotheses about how these ions might inhibit force and velocity at the level of a single cross-bridge including how P_i might rebind to myosin and reverse the weak to strong-binding transition (Hibberd et al., 1985; Dantzig et al., 1992). Our current understanding of the role of the cross-bridge cycle in fatigue based on muscle fiber experiments has recently been reviewed (Fitts, 2008). In the present review we examine the research at the molecular level largely incorporating *in vitro* findings that provide more detailed insight into the underlying mechanisms of putative agents of fatigue on actomyosin function. The big advantage of using these methods is that the behavior of a single cross-bridge can be directly observed rather than inferred from the properties of a whole muscle or even single muscle fiber where the parameters measured represent the collective action of more than a billion individual cross-bridges. Furthermore, intact muscle contains a host of proteins in addition to actin and myosin that act to modulate and regulate contractile function, making it difficult to isolate which proteins are mediating the effects of fatigue. For these reasons researchers have resorted to *in vitro* approaches to understand both which proteins are involved and how the function of a single cross-bridge is affected.

Great technological advances in the fields of biophysics and molecular biology are now enabling researchers to gain unprecedented insight into some of the most fundamental mechanisms underlying the loss of the force and motion generating capacities of muscle during fatigue. It is these recent efforts that will be the focus of this review. It is important to note that at the molecular level the efforts to understand muscle fatigue are often confluent with the efforts to understand the basic molecular mechanism of contraction and thus this review incorporates some literature focused on the basic mechanism of contraction as it pertains to understand fatigue.

THE CROSS-BRIDGE CYCLE

The force and motion generated by muscle are ultimately the result of the cyclical interaction of myosin and actin in a process coupled to the hydrolysis of ATP. This process, referred to as the cross-bridge cycle, links myosin's ATPase cycle with the mechanical events that drive force and motion. Although many of the specific details remain controversial, extensive study over many years has provided a basic model for how a myosin molecule converts the energy from ATP hydrolysis into force and motion (Holmes and Geeves, 2000). This simple working model of the cross-bridge cycle that incorporates the salient features of the cycle can be used to explore the molecular basis of fatigue (**Figure 1**). In this basic model, P_i-release is closely associated with myosin's lever arm rotation, the key molecular event responsible for force and motion in muscle. This event is followed by two kinetic transitions that occur while myosin is strongly bound to actin. In the first step myosin, now strongly bound to actin, releases ADP from the active site putting actomyosin in a rigor state; subsequently, in the second step, the cross-bridge waits in a rigor state until a new ATP molecule rebinds to myosin's active site and facilitates the dissociation from actin. While myosin is detached (or more precisely weakly bound) from actin, ATP is hydrolyzed, a biochemical event coupled to resetting of the lever arm, ensuring that the next binding event causes another productive displacement. It is important to point out that more complex models have been proposed based on recent *in vitro* findings including additional AM.ADP states that could be coupled to a structural change in the position of the lever arm (Capitanio et al., 2006) and strongly strongly bound pre-powerstroke states (Takagi et al., 2004) but this simple model is consistent with much of the data from *in vitro* experiments (Palmiter et al., 1999; Baker et al., 2002; Debold et al., 2008, 2010) and provides the best starting point for understanding the effects of elevated levels of P_i, H^+, and ADP on actomyosin. In fact, years of investigations using skinned muscle fibers have led to several hypotheses regarding how elevated levels of P_i, H^+ and ADP during fatigue could directly inhibit specific steps in the cross-bridge cycle (Cooke, 2007). More recent *in vitro* approaches using isolated proteins have attempted to address these hypotheses

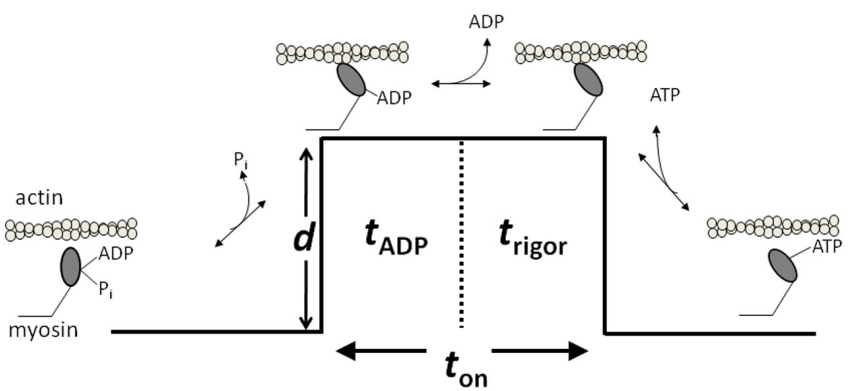

FIGURE 1 | A model of the cross-bridge cycle depicting the biochemical mechanical events. *d* indicates the powerstroke that causes the displacement. t_{on} refers to the duration of strong actomyosin strong-binding. t_{on} is made up of t_{ADP} corresponding to the ADP-lifetime and the rigor lifetime t_{rigor}. Figure is modified from Debold et al. (2011), reprinted with permission from the American Physiological Society.

more directly by taking advantage of the advances in the field of biophysics (Debold et al., 2008, 2011).

EFFECTS OF ACIDOSIS

As far back as 1880 acidosis was known to increase in contracting muscle and was postulated to be directly involved in the loss of force during periods of stress (Gaskell, 1880). The idea that the build-up of acidosis was causative in fatigue gained support from observations that the decrease in muscle performance could be temporally correlated with a decrease in the intracellular pH (Dawson et al., 1978). Consistent with this hypothesis early work on isolated muscle suggested that fatiguing levels of acidosis inhibit force production (Donaldson et al., 1978; Fabiato and Fabiato, 1978; Edman and Lou, 1990; Kentish, 1991; Ricciardi et al., 1994). However, more recent work in isolated single fibers demonstrates that the effect of acidosis on force is highly temperature-dependent (Pate et al., 1995). In fact, at physiological temperatures most studies now indicate that there is little, if any, effect of acidosis on maximal isometric force (Pate et al., 1995; Knuth et al., 2006). Some authors have even suggested that acidosis may actually prevent, rather than contribute to, the loss in force during fatigue (Pedersen et al., 2004), but this remains controversial (Kristensen et al., 2005). Thus at the very least it appears that acidosis contributes little if at all to the decrease in force during fatigue, at least at saturating levels of intracellular Ca^{++} (see Effects of Acidosis on Muscle Activation).

In contrast to its minimal effects on force, there is good evidence from muscle fibers that it may play a role in slowing the velocity of contraction during fatigue (Knuth et al., 2006; Karatzaferi et al., 2008). Single fiber studies demonstrate that decreasing pH from a resting value (∼7.0) to a value reached during fatigue (∼6.2) can slow unloaded shortening velocity by over 30% even near physiological temperatures (30˚C). These findings suggest acidosis may have other effects on the actomyosin interaction, specifically that it might slow the step in the cross-bridge cycle that limits shortening velocity. However, since these studies were done in muscle fibers it is not clear exactly which step in the cross-bridge cycle might be affected or which of the contractile proteins are helping to mediate the effect.

Confirmation that acidosis can directly affect the actomyosin interaction was demonstrated using an *in vitro* motility assay (a measure analogous to unload shortening velocity), where the velocity at which isolated muscle myosin translocates fluorescently labeled actin filaments can be quantified (Debold et al., 2008). Increasing acidosis from a resting level (7.4) to fatiguing levels (6.4), near physiological temperatures (30˚C), in this assay decreases actin filament velocity (V_{actin}) by over 65% (Debold et al., 2008), a finding qualitatively consistent with observations in single muscle fibers (Knuth et al., 2006). Interestingly, the acidosis-induced decrease in V_{actin} is greater than that observed in fibers, suggesting that the structure of the sacromere and/or the presence of additional contractile proteins present in intact muscle may attenuate some of the loss in unloaded shortening velocity. For example, the highly ordered arraignment of the thick filaments in muscle fibers absent in *in vitro* assays where myosin is randomly coated on the surface. And there is evidence that the orientation of myosin to actin affects the single molecule mechanics (Tanaka et al., 1998). Alternatively, the difference in magnitude may be related to the absence of the regulatory proteins in the *in vitro* assays discussed above. Here there is strong evidence for the involvement of the troponin and tropomyosin in the depressive effects of acidosis (as detailed below) and these proteins may act to attenuate the magnitude of the acidosis (Fujita and Ishiwata, 1999). It will be important and informative to reconcile this difference as we attempt to understand the potential role of acidosis in slowing velocity during fatigue.

In the simplest model (Huxley, 1990), V_{actin} is proportional to myosin's unitary step size (d) and the duration of the actomyosin strong-binding (t_{on}; i.e., $V_{actin} = d/t_{on}$). Using this paradigm the acidosis-induced decrease in V_{actin} could result from either a decrease in d and/or an increase in t_{on}. The advent of the three-bead laser trap assay (Finer et al., 1994) now provides an unprecedented means of directly determining which single molecule parameter is affected by acidosis (**Figure 2**). Using this assay Debold et al. (2008) found that while d was largely unaffected by acidosis at 30˚C, but that t_{on} was increased by almost threefold at low pH, which could quantitatively account for the 65% decrease in V_{actin} that was observed in the *in vitro* motility assay (Debold et al., 2008).

As illustrated in **Figure 1**, t_{on} is composed of two biochemical states of myosin; the ADP bound state (AM.ADP) and the rigor state (AM). By manipulating the ATP concentration in the single molecule laser trap assay one can further delineate whether the prolongation of t_{on} results from a slowing of the rate of ADP release or the rate of ATP-induced dissociation from rigor. The results of this series of experiments suggested that acidosis has little effect on the rate of ATP-induced dissociation of actin and myosin, but increases the duration of the ADP-bound state by over threefold (Debold et al., 2008). The latter effect can fully explain the acidosis-induced increase in t_{on} and therefore the slowing of V_{actin} at a fatiguing level acidosis. Thus these findings provided some of the first direct evidence of the effects of a fatiguing level of acidosis on a single cross-bridge, suggesting that the effect can be attributed to a single biochemical transition in myosin's cross-bridge cycle.

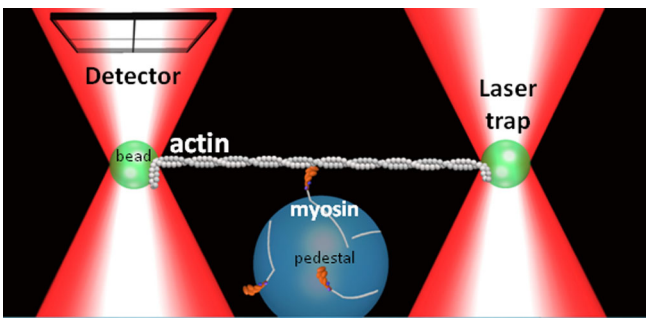

FIGURE 2 | Three-bead laser trap assay. A schematic representation of the three-bead laser trap assay showing a single myosin molecule interacting with a single actin filament. A quadrant photodiode detector tracks the motion a trapped bead connected to the actin filament.

Now that this information is known what we wish we knew is the specific structures and mechanisms responsible for mediating this effect. Given the knowledge gained from revelation of both the full amino acid sequence and myosin's atomic structure (Rayment et al., 1993) this task may now be a less daunting one. An interesting starting point to identify the important structures would be to take advantage of the differential sensitivity to acidosis of fast type II and slow type I muscle fibers (Metzger and Moss, 1990). Fibers expressing the fast type II, IIx, or IIb myosin heavy chain (MHC) seem to be more sensitive to a decrease in acidosis than fibers with slow type I MHC. A Comparison of all the different residues of each protein might be inefficient, however since velocity is strongly governed by the rate of ADP-release (Siemankowski et al., 1985) the structures affecting this rate present an attractive area to target in myosin's heavy chain. The sequences for type I and each type II isoform (IIa, IIx, and IIb) are highly homologous except for the surface binding loops (Chikuni et al., 2004). And there is evidence that these binding loops can influence the overall ATPase rate, shortening velocity, and the ADP-release rate (Kurzawa-Goertz et al., 1998) therefore this may be a key area to probe the differential sensitivity to acidosis.

EFFECTS OF ACIDOSIS ON MUSCLE ACTIVATION

In addition to direct effects on the actomyosin cross-bridge cycle, acidosis is also thought to indirectly affect the actomyosin interaction by altering the Ca^{++} sensitivity of muscle. A decreased Ca^{++}-sensitivity means that less force will be produced at the same level of activation, thus unlike the reduced effect of acidosis observed at saturating Ca^{++} levels (Pate et al., 1995) if acidosis disrupts muscle activation force will be compromised at sub-saturating Ca^{++} levels. This may play a particularly important role during the latter stages of fatigue, when the intracellular $[Ca^{++}]$ is thought to compromised, due to decreased release from the sarcoplasmic reticulum (Lee et al., 1991).

Muscle activation starts when a complex series of molecular motions following the binding of Ca^{++} to TnC ultimately lead to the movement of tropomyosin away from a position where it blocks the myosin-binding sites on actin. Improvements in biophysical techniques combined with new high resolution structures of the contractile proteins have revealed new details of this process (Galinska-Rakoczy et al., 2008). These findings are leading to more sophisticated hypotheses about how contraction is regulated at the molecular level and will be crucial to understanding how this process might be disrupted during fatigue. Much of these new data support a model that posits that Tm oscillates between three distinct positions on actin (McKillop and Geeves, 1993); the first being a "Blocked-state" where the myosin binding sites on actin are completely unavailable; a second, "Closed-state" where Tm only reveals the weak binding sites on actin; and a third, "Open-state" in which the sites for strong myosin-binding are available and myosin can therefore generate force and motion (Gordon et al., 2000). In this model the binding of Ca^{++} to TnC increases occupancy of the Closed-state, but only after myosin strongly binds to actin in the Open-state is the filament fully activated. Thus full activation of the thin filament requires both Ca^{++} dependent process and myosin being strongly bound to actin. We also know that this process is highly cooperative, meaning that the binding of one myosin increases the probability that neighboring myosin molecules will bind to actin. And there is evidence that the putative agents of fatigue might affect this cooperative behavior (Debold et al., 2006), which would likely show the greatest alterations under conditions where Ca^{++} release is compromised.

Support for the above model of activation and its cooperative properties have been provided from structural evidence showing that Tm can exist in three distinct positions on actin (Galinska-Rakoczy et al., 2008). In addition, recent single molecule laser trap experiments using actin filaments reconstituted with TnTm demonstrate that in the absence of Ca^{++} TnTm decrease the probability of myosin strong-binding by 100-fold, but that the binding of one myosin increases the probability of a neighboring myosin binding by more than 10-fold (Kad et al., 2005). The later result is consistent both with this "three-state model" of thin filament activation and nicely demonstrates the cooperative aspect at the single molecule level. This later technique could provide interesting insight into how acidosis might exerts its effects on during fatigue.

In addition to these studies which highlight the recent work characterizing Tm dynamics, the revelation of the crystal structure of the core domain of Tn has provided exciting details of the intra-molecular dynamics of Tn resulting from Ca^{++}-binding to TnC (Takeda et al., 2003), and the movements that might ultimately couple to the positions of Tm on actin. The most significant finding from these structural investigations suggests that in the absence of Ca^{++} actin and myosin are prevented from interacting because the C-terminal portion of the inhibitory subunit of Tn (TnI) is tightly bound to actin. This constrains Tm in a position that blocks the myosin binding sites on actin. Binding of Ca^{++} to TnC opens up a hydrophobic patch the N-terminal lobe TnC that has an affinity for a specific helix in the C-terminal portion of TnI causing it to dissociate from actin and bind to N-terminal lobe of TnC. This dissociation of TnI from actin frees Tm to move out of way of the myosin binding sites on actin, allowing actin, and myosin to bind. These new molecular insights are providing unprecedented detail of the molecular basis of muscle activation and will therefore help us to pinpoint where activation process acidosis might exert its depressive effects.

Early work, using skinned single muscle fibers, established that acidosis decreases Ca^{++} sensitivity (Fabiato and Fabiato, 1978) and more recent evidence indicates this effect can be recapitulated in the *in vitro* motility assay where acidosis can slow the V_{actin} of actin filaments reconstituted with TnTm (Sata et al., 1995; VanBuren et al., 2002). To better understand the molecular basis of this effect and isolate the specific structures involved researchers have used advances in molecular biology and biophysical techniques to both manipulate the structural of the regulatory proteins and then directly assess the impact on function *in vitro*.

The interest in identifying the structural elements responsible for the acidosis-induced decrease in Ca^{++}-sensitivity originally stemmed from an interest in understanding the effects of acute ischemia on cardiac function (Blanchard and Solaro, 1984; Solaro et al., 1988), a condition that shares with fatigue the rapid accumulation of metabolites. It was readily apparent that cardiac muscle was much more strongly affected by acidosis than skeletal muscle, particularly at sub-maximal levels of activation. In fact after

cardiac muscle, fast skeletal muscle is the next most sensitive followed by slow skeletal, which shows the smallest decrease in Ca^{++}-sensitivity under acidic conditions (Morimoto et al., 1999). The structural differences between skeletal isoforms may provide avenues to pinpoint the key structures and molecular motions which gives rise to these effects. Fortuitously, researchers have focused heavily on understanding the effects acidosis, often using pH levels experienced during fatigue (Fabiato and Fabiato, 1978; Solaro et al., 1988; Ball et al., 1994). Thus the findings provide important insight into the role of acidosis in the fatigue process as well.

Some of the initial work suggested that the differential sensitivity between cardiac and skeletal muscle might be attributable to the subtle structural differences in the isoforms of troponin (Blanchard and Solaro, 1984). A subsequent comparison of neonatal cardiac muscle, which expresses a slow skeletal isoform of TnI (ssTnI) with adult cardiac muscle, which expresses cTnI, revealed that the neonatal isoform of TnI was much less sensitive to acidosis (Solaro et al., 1988). This suggested that the differential response might be attributed to the specific regions in TnI that differ between ssTnI and cTnI. However, it is important to point out that in this experiment the isoforms of TnC and the tropomyosin binding subunit of TnT were also slightly different, preventing the authors from attributing the effect exclusively to TnI. To attempt to resolve this issue Ball et al. (1994) extracted Tn from fast skeletal muscle fibers (fsTn) and replaced the fsTnI with the cardiac isoform of TnI isoform and then incorporated the full Tn complex back into the skeletal muscle fibers. These manipulated skeletal fibers displayed the mild acidosis sensitivity characteristic of skeletal muscle fibers despite containing cardiac TnI, suggesting that TnI alone does not govern the differential response to acidosis. In a subsequent set of experiments both the fsTnI and fsTnC were replaced with cTnI and cTnC and under these conditions the fibers demonstrated this increased sensitivity to acidosis characteristic of cardiac muscle. This led the authors to conclude that the interaction between TnC and TnI is crucial for mediating the acidosis-induced depression in Ca^{++}-sensitivity. This finding may not be surprising in light of the recent structural evidence that the interaction of these two subunits is crucial for activation (Takeda et al., 2003) but clearly more precise manipulation of the structures was required to understand this more effect more fully.

Based on the above evidence researchers have focused on indentifying the crucial structural regions within the TnI subunit that might be responsible for the differential response of cardiac and skeletal muscle. Initial experiments in this process used chimeras of ssTnI and cTnI and measured the effect on the force-pCa relationship (Day et al., 2007). By systematically reducing the number of amino acids that differed between the cardiac and skeletal isoforms researchers were able to assign a good portion of the differential sensitivity to acidosis to the carboxy-terminus region of TnI. In fact the same research group used point mutations to suggest that the differential response to acidosis was largely attributable to a difference of one amino acid at residue 164 (Day et al., 2006). In an elegant follow-up study they directly tested this hypothesis by substituting the alanine at residue 164 in cTnI for the histadine, present in ssTnI, made the cardiac muscle respond nearly identical to slow skeletal muscle, essentially protecting it against the depressive effects of acidosis (Day et al., 2006). The authors postulated that the since pKa of histadine is in the physiological range, its presence makes ssTnI better able to buffer the free protons and therefore maintain its Ca^{++}-sensitivity under acidic conditions (Westfall and Metzger, 2007). This of course has important potential applications for the treatment of myocardial ischemia but also highlights nicely how functional biophysical assays paired with molecular biology provide exceptional insight into the molecular basis of the depressive effects of acidosis during fatigue.

Based on these experiments it is clear that the depressive effects of acidosis involve TnI, particularly in cardiac muscle, however there is also evidence that the other subunits of Tn may be involved in this decreased Ca^{++}-sensitivity, particularly in skeletal muscle. In an effort to delineate a possible role for TnC in the acidosis-induced depression of Ca^{++}-sensitivity, Metzger et al. (1993) replaced the TnC in skeletal muscle fibers (sTnC) with the cardiac isoform (cTnC) and found that this increased the sensitivity of the skeletal fibers to acidosis suggesting it may be more important to fatigue than TnI (Metzger et al., 1993). Based on this evidence and findings that indicate that acidosis directly reduces the affinity of Ca^{++} binding sites on TnC (Parsons et al., 1997) authors have suggested that high levels of H^+ may temporally alter the structure of the Ca^{++} binding sites on TnC. In addition, the C-terminal region of TnC makes important contacts with TnI during activation (Takeda et al., 2003) and this could help explain why both TnI and TnC are implicated in mediating this depressive effect of acidosis (Ball et al., 1994). Since the effects of acidosis on TnC's Ca^{++} binding affinity are significant in both cardiac and fast skeletal isoforms, but the effects mediated through TnI are more prominent in cardiac muscle, this suggests that the effects mediated through TnC may be more important for skeletal muscle fatigue. The greater potential involvement of TnC in the fatigue process could stem from the fact that the fsTnC has two low-affinity Ca^{++}-binding sites while cTnC has just one (Gordon et al., 2000), although this is an idea that needs further exploration.

There is also evidence that Tm-binding subunit of Tn (TnT) is involved in the acidosis-induced decrease in Ca^{++} sensitivity in skeletal muscle (Ogut et al., 1999). In support of this notion Nosek et al. (2004) demonstrated in mice, genetically engineered to express the fast skeletal isoform of TnT in the myocardium, that there was an increased sensitivity to acidosis compared to those with the normal those with the normal cTnT. This is particularly interesting for skeletal muscle fatigue because it suggests that while fsTnI may attenuate the Ca^{++}-sensitivity, fsTnT may actually accentuate the decrease in Ca^{++}-sensitivity in skeletal muscle. This would imply a greater role for TnT than TnI in skeletal muscle fatigue. Since the structural differences between the cardiac and skeletal isoforms of TnT are now well known the authors concluded that the subtle charge differences in the NH-terminal domain between cardiac and fast skeletal may account for the differential response to acidosis (Nosek et al., 2004). Here a systemic approach, similar to the one for taken for TnI, could be employed to identify the structures and mechanisms responsible TnT's role in the acidosis-sensitivity of skeletal muscle.

These investigations into the structure and function of Tn have been very important for understanding how the loss of contractility during ischemic heart disease and provide an important starting point for understanding the role in of acidosis in the decreased Ca^{++}-sensitivity during fatigue. Interestingly, while much of the effect in cardiac muscle can be localized to a specific region in TnI the evidence from skeletal muscle suggests a greater involvement of TnC and TnT. Future work in which systematic series of structural perturbations of each Tn subunit and Tm are paired with functional assessments in an *in vitro* setting will more clearly delineate the role that the regulatory proteins play in the acidosis-induced decrease in Ca^{++}-sensitivity.

EFFECTS OF P_i

One of the strongest correlates with the loss of force during fatigue is the accumulation of P_i in muscle, as demonstrated in NMR studies using *in vivo* muscle (Dawson et al., 1978). The associated decrease in force is believed to be due to a direct effect in the contractile proteins since in non-fatigued muscle fibers it is routinely observed that high levels of P_i reduce maximal isometric force (Cooke and Pate, 1985; Hibberd et al., 1985; Nosek et al., 1987; Cooke et al., 1988; Pate and Cooke, 1989; Debold et al., 2004, 2006).

Muscular force, at the molecular level, is dependent on the product of the unitary force of a single cross-bridge (F_{uni}) and the duty cycle (the percentage of the ATPase cycle spent strongly bound to actin; VanBuren et al., 1995). Therefore P_i could affect either one or both of these parameters to cause a reduction in force. The prevailing model suggests that the duty cycle is reduced because the rebinding of P_i rapidly reverses the lever arm rotation and leads to detachment of myosin from actin reducing the number of strongly bound cross-bridges (Takagi et al., 2004). Strong support for this model comes from the observation that elevated levels of P_i reduce maximal isometric force in skinned muscle fibers in a concentration-dependent manner (Pate and Cooke, 1989). Furthermore, elevating P_i in a single molecule assay suggests that P_i release and myosin's lever arm rotation are closely coupled (Baker et al., 2002). Therefore the presence of high levels of P_i would act to decrease myosin's duty cycle with little or no effect on F_{uni}. However, this mechanism of force production still remains controversial because isolated muscle preparations that incorporate spin-labeled probes to sensitive to myosin's lever arm rotation do not detect a reverse shift the distribution of myosin heads from the post-powerstroke to pre-powerstroke state following exposure to high levels of P_i. This finding suggests that rather than reversing lever arm rotation and inducing detachment elevated P_i may decrease the force per cross-bridge (Baker et al., 1999). Findings such as this have led to the proposal of thermodynamic models of muscular contraction (Baker et al., 1999; Karatzaferi et al., 2004) as opposed to the more conventional molecular models (Huxley, 1957). In support of these types of models maximal isometric force is linearly decreases with the logarithm of the [Pi] (Pate and Cooke, 1989). This suggests that elevated levels of P_i during fatigue decrease force by decreasing the available energy from the hydrolysis of ATP.

In addition, to the thermodynamic information we are also gaining important atomic level insight into the internal motions of myosin that involve P_i. In fact, the effects of P_i on the cross-bridge cycle are important to understanding the molecular basis of contraction because the release of P_i from myosin is believed to be the step most closely associated to the rotation of myosin's lever arm, the key molecular event underlying the generation of force and motion (Holmes and Geeves, 2000). In a conventional molecular model of the cross-bridge cycle P_i is thought to reduce force by rebinding to myosin in the AM.ADP state and rapidly, causing both reversal of myosin's lever arm rotation and the dissociation from actin (Takagi et al., 2004). In isometrically contracting muscle this molecular process would lead to a decrease in the number of strongly bound cross-bridges and therefore force. In the 1980s this may have provided a complete mechanistic explanation for the effect of P_i on force, however advances in structural biology leading to the revelation of the 3-D atomic structure of myosin (Rayment et al., 1993; **Figure 3**) means that we can now gather much more detail about how this process might occur. For example, what internal molecular motions within myosin's active site lead to the P_i-induced reversal of the lever arm rotation that ultimately lead to the reversal of the lever arm rotation and detachment from actin? Atomic resolution structures of myosin in different nucleotide states have provided important clues into these motions, at least from the perspective of P_i-release and lever are rotation(Holmes and Geeves, 2000). In fact, with all this new structural information, the suggestion that P_i reduces force during fatigue by simply reversing force generating step is analogous to suggesting that a clock tells time by the moving its hands. In the case of the clock you would be ignoring the intricate motions of gears and springs behind the face of the clock that precisely govern the rotation of the hands. Similarly, we now know that the reversal of force-generating step by myosin likely involves the coordinated movement of several regions within the myosin molecule.

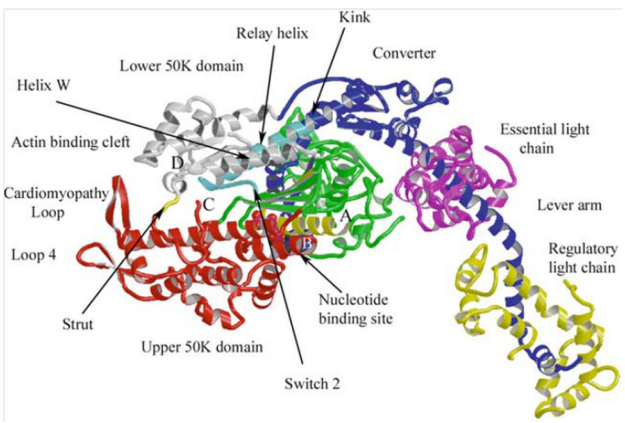

FIGURE 3 | Atomic structure of a myosin molecule A ribbon representation of the head portion myosin (S1). The heavy chain is colored in blue, gray, green, and red, denoting different regions of the protein. The light chains are colored in yellow (Essential) and pink (Regulatory). The upper portion of the motor domain is separated by a large cleft (actin-binding cleft), the opening and closing of which modulates actin affinity. Subtle changes in the nucleotide-binding site are amplified in the converter region resulting in a large rotation of the lever arm. Figure from Geeves and Holmes (2005), reprinted with permission from the publisher.

In recent years systematic and careful experimental designs have providing important insights into the basic intra-molecular motions deep within myosin that link subtle changes in the ATP binding site to large conformational changes in the converter region and lever arm that are associated with force generation (Holmes and Geeves, 2000). Since myosin is quite a large protein, there must be pathways of communication that allow distant regions of the protein coordinate the biochemical and structural to ensure energy is not wasted. Most importantly, strong-binding of myosin to actin must be precisely timed and coordinated with rotation of the lever arm to effectively displace an actin filament and/or generate force. Both of these events are also coupled to the release of P_i from myosin's active site, this mechanochemical coupling is thought to ensure efficient use of the energy from the hydrolysis of ATP (Holmes and Geeves, 2000). Coordination of these events is believed to be initiated through the opening and closing of a large cleft in the motor domain, involving a strut that spans the cleft (**Figure 3**). This large cleft, which reaches from the actin-binding domain deep into active site, closes upon strong-binding to actin, a movement coupled to subtle rearrangements within the nucleotide binding pocket and to the release of P_i. Through a series of linkages and switches these subtle motions in the active site are communicated and amplified by the converter domain to cause the lever arm to rotate and generate the ∼10 nm displacement of an actin filament (Holmes, 2005). The events of rebinding are not yet known in the same intricate detail, but it is generally assumed that the reaction is reversible and the rebinding of P_i during fatigue reverses this sequence of events to reduce force. Thus these basic findings have the potential to provide unprecedented molecular detail of the fatigue within muscle's molecular motor and it will be interesting to follow the developments as the number of structures and the resolution increases.

It is clear these and similar structural data are providing important insights into the molecular basis of muscle contraction and indirectly insight into the process of fatigue, however it is important to point out that structures are only "snapshots in time." Therefore they do not provide a full characterization of all the motions within the protein through time. What we wish we knew, and could see directly, is how P_i rebinds to the active site of myosin and how this event is linked to conformational changes in the actin-binding site, converter, and lever-arm in real-time. Given the time scales of these events this will be difficult but recent developments in molecular dynamics simulations may soon provide important insights into these events in the near future (Cecchini et al., 2010).

Despite extensive work on the effects of P_i on force and the wealth of knowledge of structural states there are still effects of P_i on contractile function that are not easily explained with existing models. For example, the effects of P_i on shortening velocity are much more equivocal, and seemingly more complex, than the effects on force. In skinned single muscle fibers high levels of P_i most often have little effect on unloaded shortening velocity (Cooke et al., 1988), however altering the conditions slightly can cause P_i to elicit a decrease (Pate and Cooke, 1989) or even a minor increase in velocity (Pate and Cooke, 1989). Similar observations have been made in *in vitro* motility experiments where P_i can either increase or decrease V_{actin} depending on the experimental conditions (Debold et al., 2011). In both preparations the effects of P_i are highly dependent on the ATP concentration, at high ATP P_i can cause a slight increases in V_{actin} (Homsher et al., 1992) while at μM ATP levels it slows V_{actin} (Warshaw et al., 1991). This issue has recently been re-examined and the data suggest that the opposing effects result from the ability of P_i to rebind to the either AM.ADP or rigor states (see **Figure 1**) of myosin (Debold et al., 2011). In this variation of the simple detachment-limited model (**Figure 1**), P_i rebinding to the AM.ADP state facilitates dissociation from a post-powerstroke state and therefore limits duration of strong-actin binding and increases velocity by ∼5%. However the lifetime of the AM.ADP state is very short in skeletal muscle myosin and the affinity rather low so this sequence is not readily observed under normal conditions. By contrast the when P_i rebinds to the rigor state it prevents ATP from binding, prolonging the strongly bound state (i.e., t_{on}) and therefore slows velocity. Simulations governed by this mechanism were able to accurately capture both the P_i-induced increase in velocity at ATP and the depression in velocity at low ATP (Debold et al., 2011). These findings were then extended to more fully understand the effect during fatigue-like conditions by manipulating the pH as well as the P_i levels (Debold et al., 2011). In this experiment it was demonstrated that the P_i-induced increase in V_{actin} was significantly enhanced at low pH. For example, at pH 6.5, the addition of 30 mM P_i caused V_{actin} to almost double (**Figure 4**). The effect was still significant but smaller in magnitude in a less acidic pH of 6.8 and recently

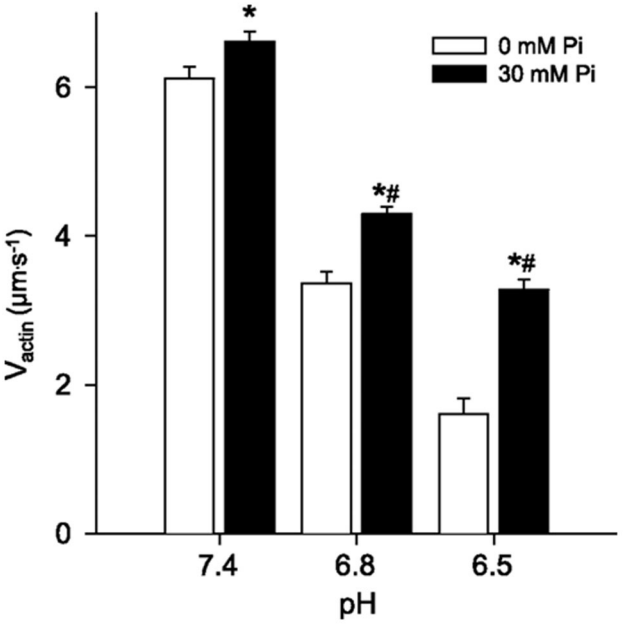

FIGURE 4 | The effect of P_i at low pH on V_{actin}. *In vitro* motility actin filament velocities from chicken skeletal myosin and actin under various levels of pH and P_i. Values represent mean ± SEM for V_{actin} at each pH and P_i level. Data were analyzed using a two-way ANOVA, * indicates significantly ($p < 0.05$) different from 0 mM P_i and # indicates a significant interaction, meaning that the increase in V_{actin} in response to P_i was greater at both pH 6.8 and 6.5 than at 7.4. Figure from Debold et al. (2011), reprinted with permission from the American Physiological Society.

a similar effect was reported under even more severe fatigue-like conditions (Greenberg et al., 2010). Taken together these findings strongly suggest the effects of Pi on velocity are dependent on the pH. Debold et al. (2011) interpreted the findings using the same modified simple model described above. The simulations using the model suggested that the changes in t_{on} were due to either acidosis prolonging the AM.ADP state or by P_i shortening the lifetime of the AM.ADP state. Since our earlier work demonstrated that pH prolongs the AM.ADP state this suggests that there is more time for P_i to rebind to actomyosin, making the increase in V_{actin} more pronounced at low pH, providing a kinetic basis for the pH dependence of the effect (Debold et al., 2011).

These findings have relevance to fatigue because they suggest that the depressive effects of acidosis on V_{actin} may be offset by the effects of P_i. However, since pH also slows myosin's overall ATPase rate (Stone and Prevost, 1973) these ions would likely act additively to reduce force in muscle and thus power. This hypothesis is supported by the observation that in a loaded *in vitro* motility assay a fatigue-like milieu (pH 6.2, 30 mM P_i, 0.3 mM ADP) reduced myosin's force generating capacity at a given velocity and translated into profound reduction in the peak power generating capacity of myosin (**Figure 5**). Therefore a P_i-induced increase in shortening velocity may come at the expense of force generation and ultimately power at the molecular level. Here we wish we knew how these ionic changes might affect force and power *in vitro* and with recent advances in the laser trap assay (Debold et al., 2005) this is something that could be answered in the near future.

EFFECTS OF P_i ON MUSCLE ACTIVATION

The effects of fatiguing levels of P_i, like acidosis, typically decrease the Ca^{++}-sensitivity of force in single muscle fibers (Millar and Homsher, 1990, 1992; Walker et al., 1992; Palmer and Kentish, 1994; Metzger, 1996). Furthermore, recent work suggests that this effect this is more pronounced near physiological temperatures vs lower temperatures (Debold et al., 2006; **Figure 6**). This effect may be particularly relevant for fatigue because it means that during the later stages of fatigue, when intracellular Ca^{++} is reduced (Westerblad et al., 1991), increases in P_i would have a more dramatic depressive effect on isometric force. Despite being a well-established phenomenon, the molecular mechanisms underlying this P_i-induced depression of Ca^{++}-sensitivity effect remain poorly understood.

In contrast to the putative effects of acidosis on muscle activation, most evidence indicates that P_i does not disrupt the function of Tn, including Ca^{++} binding to TnC (Palmer and Kentish, 1994). This suggests that the effect is mediated through a distinctly different mechanism than the acidosis-induced depression in Ca^{++}-sensitivity (Fabiato and Fabiato, 1978). Rather most evidence suggests that P_i's strong depressive effect on the force–pCa relationship is likely mediated through P_i's effect on a strongly bound actomyosin cross-bridge (Millar and Homsher, 1990, 1992; Walker et al., 1992; Palmer and Kentish, 1994; Metzger, 1996; Debold et al., 2006). Strong-binding of myosin to actin is required for full activation of the thin filament (McKillop and Geeves, 1993) therefore at high concentrations, P_i rebinding to myosin is thought to cause the reversal of the force generating step (Takagi et al., 2004). This is believed to have several effects on the level of thin filament activation; first the stabilization of Tm in the Open state (i.e., full activation) requires myosin strong binding (Gordon et al., 2000) thus this effect would be reversed by elevated P_i. Secondly, strong-binding also contributes to the cooperative binding of myosin through propagation of the motions of Tm on actin (Gordon et al., 2000) from one regulatory unit (7Actin monomers, 1Tn, 1Tm) to its nearest neighbors, here a P_i-induced decrease in strong-binding would be expected to reduce this effect and thus activation. Another source of cooperative activation that is also dependent on strong-binding is the force-induced increase in TnC's affinity for Ca^{++} (Gordon et al., 2000), an increase in P_i would reduce this effect, leading to a muscle that is less sensitive to Ca^{++} all of these contributing to a reduced sensitivity to Ca^{++} in a force–pCa relationship.

While the above findings provide plausible mechanisms for the rightward shift in the force–pCa relationship there are some findings that suggest our understanding is incomplete. For example, elevated levels of P_i can increase the steepness of the force–pCa curve suggesting an increase rather than a decrease in

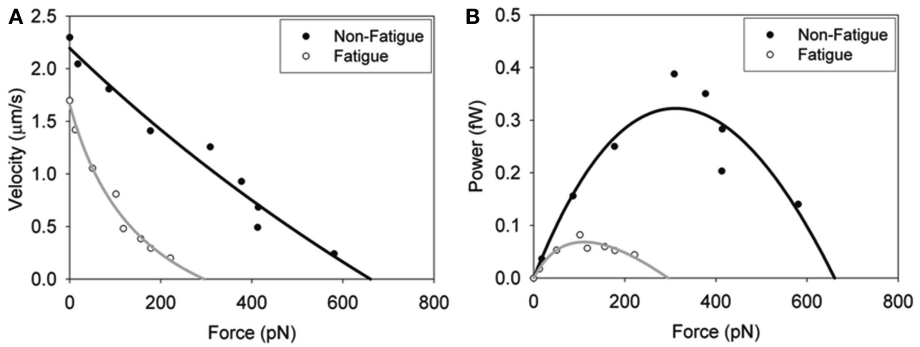

FIGURE 5 | ***In vitro*** **force–velocity (A) and force–power (B) relationships.** Fatigue was simulated by exposing the myosin pH 6.2, 30 mM P_i and 0.3 mM ADP and the effects on skeletal muscle myosin's force–velocity relationship determined. Load was applied by adding increasing amounts of an actin-binding protein (alpha-actinin). Data were fit with the Hill force–velocity equation (Hill, 1938) with the black line normal conditions and the gray line fatigue-like conditions. The force–power relationships **(B)** were derived by calculating the product of force and velocity and fit to the Hill force–power equation (Hill, 1938). Figure reprinted from Greenberg and Moore (2010) with permission from John Wiley and Sons.

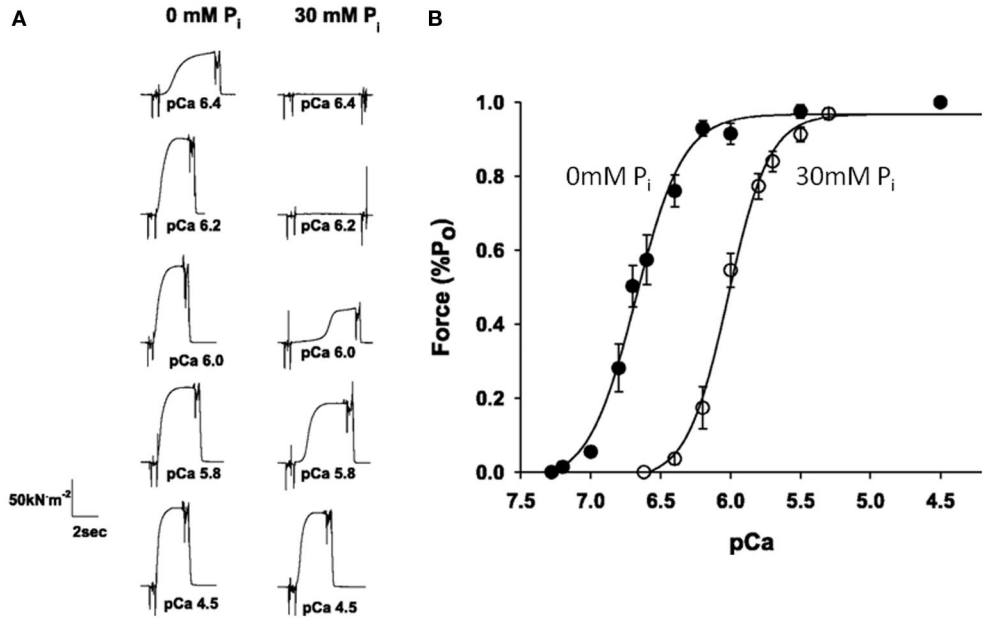

FIGURE 6 | Effect of P_i on the force–pCa relationship. Skinned single fiber muscle forces measured at increasing concentrations of free Ca^{++} (displayed in pCa, −log units) in the presence and absence of 30 mM P_i **(A)**. The forces are normalized to the maximum reached at saturating pCa (4.5) and fit with the Hill plot **(B)**. The leftward shift in the curve indicates that elevated P_i makes the fibers less sensitive to Ca^{++}. Figure is modified from Debold et al. (2006), reprinted with permission from the American Physiological Society.

cooperativity, even in the face of decreasing Ca^{++}-sensitivity as evidenced by a decrease in the pCa50 (Millar and Homsher, 1990). The increased cooperativity seems inconsistent with the mechanism outlined above and suggests that a cross-bridge to which P_i rebinds behaves differently than a myosin molecule in a state prior to P_i-release. In addition, the effects of the velocity–pCa relationship have been equivocal with the earliest work demonstrating that P_i decreases Ca^{++}sensitivity of V_{actin} (Sata et al., 1995), yet more recent work suggests that elevated P_i seems to have little if any impact on the velocity–pCa relationship (Gorga et al., 2003). This resolution of these issues will be crucial to understanding how this important ion mediates its potent effects on fatigue. Here it might be informative to directly observe the effects on P_i on the behavior of a single actomyosin cross-bridge by using a regulated thin filament in the laser trap assay (Kad et al., 2005) both under unloaded and loaded conditions.

EFFECTS OF ADP

At rest, ADP is typically maintained quite low (∼10 μM) inside the cell due to the creatine kinase reaction strongly favoring the formation of ATP. However, with intense contractile activity the concentration of creatine phosphate rapidly decreases and creatine reciprocally increases. These changes shift the equilibrium of the reaction so that when creatine phosphate is completely depleted intracellular levels of ADP can be as high as 1 mM (Nagesser et al., 1993). However others have argued that this might significantly overestimate the values reached during fatigue in *in vivo* muscle (Cooke, 2007). This controversy aside it is clear that even 0.5 mM ADP can significantly affect both the force and velocity generating capacity of myosin *in vitro* (Greenberg et al., 2010).

Observations from skinned single muscle fibers consistently reveal that mM levels of ADP slow unloaded shortening velocity (Cooke and Pate, 1985; Metzger, 1996). ADP has a similar effect on V_{actin} measured in a motility assay, suggesting that ADP slows velocity by directly inhibiting myosin function (Greenberg et al., 2010). In contrast high levels of ADP have the opposite effect on fiber force, consistently causing small but significant increases maximal isometric force (Cooke and Pate, 1985; Hoar et al., 1987; Metzger, 1996; Fukuda et al., 2000).

The opposing effects on force and velocity can be explained at the molecular level by assuming that ADP competes with ATP for binding to myosin's active site. In a simple detachment model (**Figure 1**), when levels are high ADP can readily rebind to myosin's empty nucleotide-binding site in the rigor state resulting in the reformation of the AM.ADP state. This state is a strongly bound post-powerstroke state, therefore high ADP acts to prolong t_{on}, while having no effect on d, and therefore slows velocity (according to $V = d/t_{on}$). This molecular concept is supported by observations from the single molecule laser trap assay where mM ADP levels have been shown to prolong the strongly bound lifetime of a single actomyosin bond without affecting d (Baker et al., 2002). Prolongation of t_{on}, while slowing velocity, would also increase the number of strongly bound cross-bridges at any given moment. Therefore, since force is directly dependent on the number of attached cross-bridges this would act to increase maximal isometric force in muscle. These data and accompanying interpretations provide a simple, molecular level explanation for the slowing of velocity but increased force observed with increasing ADP levels.

While these data provide important information, maximal isometric force and unloaded shortening velocity only represent two

endpoints on the force–velocity relationship continuum. Therefore to fully understand if ADP contributes to the loss of muscular power during fatigue it may be more informative to characterize its effects on myosin's load-dependent velocity. This was recently accomplished using a modified motility assay where myosin worked against the load of an actin-binding protein (Greenberg et al., 2010). The results revealed that elevated levels of ADP attenuated load-induced decrease in V_{actin} that characterizes muscle and myosin. This implies that elevated ADP levels may have little effect on myosin's power-generating capacity and thus might play only a small or even protective role in *in vivo* fatigue. Here, examining the effect of ADP the load dependence of a single actomyosin interaction, a technique recently used to characterize the load dependence of smooth muscle muscle (Veigel et al., 2003). These are related techniques may provide even more crucial insight into both the mechanism of fatigue and the role of ADP-release in contraction.

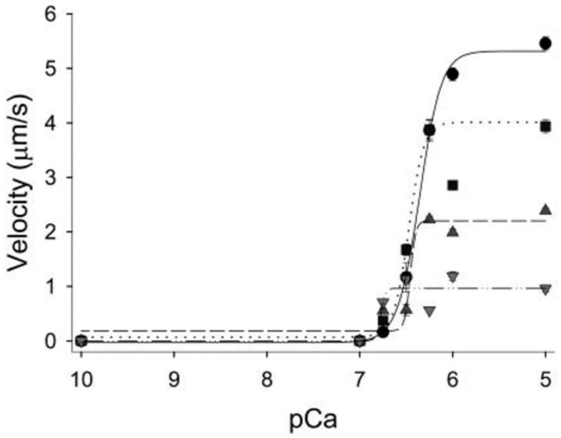

FIGURE 7 | Effect of ADP on the velocity–pCa relationship *in vitro*. Regulated actin filament velocities plotted as a function of increasing ADP concentrations. The ADP levels were increased from 0.5 mM ADP (squares, dotted line), to 2 mM (triangles up, dashed regression), and 5 mM (triangles down, dash-dot regression) and compared to control experiments with no added ADP (circles, solid regression). Elevated ADP significantly decreased maximal velocity but increased the pCa_{50} of the thin filament in a concentration-dependent manner. Figure modified from Gorga et al., 2003, reprinted with permission from Cell Press.

EFFECTS OF ADP ON Ca^{++}-SENSITIVITY

As detailed in the previous section the potential role of ADP in fatigue has been strongly questioned due to the debate over the concentrations achieved during high intensity exercise (Cooke, 2007). However the highest estimates suggest the ADP can reach 1 mM and this level of ADP can have a significant impact on the Ca^{++}-sensitivity both in muscle fibers and *in vitro* (Greenberg et al., 2010). In skinned single muscle fibers elevated levels of ADP slow unloaded shortening velocity but enhance maximal isometric force and it also increases the sensitivity to Ca^{++} evidenced by a leftward-shift in the force–pCa relationship (Hoar et al., 1987). This might seem beneficial during fatigue since it could potentially offset a loss in force caused by other metabolites, however ADP has the opposite effect on contraction velocity, slowing unloaded shortening velocity at both maximal and submaximal Ca^{++} concentrations (Metzger, 1996). Thus the ADP-induced slowing of velocity might offset an increase in force and as a result power may ultimately be compromised.

The ADP-induced slowing of V_{actin} is thought to be mediated by a direct effect on myosin since the observations from the *in vitro* motility assay using reconstituted thin filaments (actin + Tn + Tm) also show ADP slows actin filament velocity (Gorga et al., 2003). In these *in vitro* investigations, ADP levels of only 0.5 mM can significantly decreases V_{actin} at both maximal and submaximal Ca^{++} levels, and this effect is concentration dependent with 5 mM ADP causing V_{actin} to decrease by ∼80% at maximal Ca^{++} (Gorga et al., 2003; **Figure 7**).

In the model of the cross-bridge cycle, described above (**Figure 1**), elevated ADP is thought to rebind to myosin in the rigor state, pushing the cross-bridge back into the AM.ADP state. This scenario presumably prolongs the duration of the strongly bound state causing velocity to slow, but it would increase activation of the thin filament via strong-binding activation. This model nicely accounts for the increase in force and Ca^{++}-sensitivity via an increased number of strongly bound cross-bridges and also the decrease in velocity due to the same increase in t_{on}. Therefore while the increase in force due to elevated ADP might attenuate the effects of other metabolites (Hoar et al., 1987), any benefit to muscle performance would be offset by the much more pronounced decrease in velocity (Gorga et al., 2003). Thus this evidence implies a small but potentially significant role for elevated levels of ADP in fatigue.

EFFECTS OF REACTIVE OXYGEN SPECIES AND OTHER STRUCTURAL CHANGES

Reactive oxygen species (ROS) and nitric oxide (NO) are somewhat newer putative agents of fatigue, but an accumulating body of literature is beginning to indicate that they may play a significant role in the loss of muscular force and velocity during fatigue (Smith and Reid, 2006). ROS are oxygen containing molecules, such as superoxide ($O2^-$), that have unpaired electrons making them highly reactive and thus capable of damaging membranes and structurally modifying various contractile proteins. The potential structural modifications include the formation of disulfide bridges between neighboring cysteine residues as well as modifications of methionine residues. Many species of ROS and NO have now been demonstrated to accumulate inside contracting muscle cells during intense activity. In addition, there is also evidence that they may be playing a causative role in the fatigue process (Smith and Reid, 2006). However, the effects are concentration dependent in a complex manner. When levels of ROS are low, as might occur at rest or even in the early stages of fatigue, they appear to enhance force production in isolated muscle (Reid et al., 1993). In contrast, at higher concentrations (as might occur in the later stages of fatigue) certain species, such as H_2O_2, begin to inhibit contractile function (Andrade et al., 1998). Thus while small amounts of ROS may actually improve contractile function, the negative impact of higher concentrations suggests they can still play a causative role in the fatigue process.

Several groups are now beginning to explore the molecular basis of the effects of ROS and NO species on contractile function

in vitro. Snook et al. (2008), recently set out to the probe the molecular basis of the effects of the ROS peroxynitrite ($ONOO^-$) using an *in vitro* motility assay, exposing both myosin and actin to increasing concentrations of $ONOO^-$ and measuring its effects myosin's ability to move actin and generate force. At concentrations greater than 10 μM, $ONOO^-$ caused significant reductions in V_{actin}, suggesting that this species and concentration negatively can negatively affect actomyosin function. They complemented these functional assessments with biochemical experiments to determine the nature of these structural changes, and found that $ONOO^-$ caused an increase in tyrosine nitration of both actin and myosin, with myosin showing the strongest relationship between tyrosine nitration and the decrease in V_{actin} (Snook et al., 2008). Interestingly, exposing myosin to the same level of $ONOO^-$ actually increased its force-generating capacity, an observation that agrees with findings from intact muscle and skinned fibers (Allen et al., 2008). This suggests that this species might attenuate the loss of force during fatigue. It is interesting that the opposite effects on velocity and force are similar to the effects of ADP on myosin, suggesting that $ONOO^-$ may slow the same step in the cross-bridge cycle, ADP-release. In terms of fatigue, however, this makes interpretation of the role of $ONOO^-$ in the fatigue process more difficult because it has opposite effects on force and velocity. The implications for its role in the fatigue process may depend on the relative magnitude of the effect force vs velocity and ultimately on power generation. This will have to be delineated by assessing the effect of $ONOO^-$ on the force–velocity and force–power relationships of myosin *in vitro*. It should also be noted that the concentrations used in this and similar *in vitro* studies were well above the levels believed to be reached *in vivo* during fatigue, further complicating the interpretation of such studies (Allen et al., 2008).

Some of the strongest evidence for the role of ROS in fatigue comes from the observations that the progression of fatigue can be greatly slowed (Moopanar and Allen, 2005). In addition, in muscle that has been severely fatigued exposure to a ROS scavenger can readily reverse much of the loss in force generating capacity (Moopanar and Allen, 2006). The reversibility of these changes suggests they are only transient structural modifications but the specific nature of these modifications, such as the proteins modified and the nature of the structural changes are not yet clear.

Moopanar and Allen studied the effects of fatiguing levels of ROS on single fiber contractile properties and observed that this level caused a significant depression in force. Measures of intracellular Ca^{++} enabled them to attribute much of the depressive effect of ROS to an alteration in the Ca^{++}-sensitivity, suggesting the involvement of TnTm. In support of this notion, Snook et al. (2008) recently presented evidence that ROS can directly affect Tn/Tm function slowing velocity in an *in vitro* motility assay (Snook et al., 2008). In this experiment when both the myosin and the regulated filaments (Actin + Tn + Tm) were pretreated with peroxynitrite $ONOO^-$ the effects on V_{actin} were more pronounced than that observed when myosin alone was treated, suggesting in addition to myosin Tn/Tm are modified and functional altered by $ONOO^-$. Interestingly, the depressive effects using reconstituted thin filaments (Snook et al., 2008) were less pronounced than in muscle fibers (Moopanar and Allen, 2005) suggesting that other as yet unidentified factors may be contributing in *in vivo* muscle.

Thus the role of ROS in fatigue is beginning to be explored but future efforts will have to determine whether the ultimate effects on muscular performance in an *in vivo* setting are beneficial or detrimental. Other key issues to resolve related to ROS and fatigue include the determination of the actual concentration of ROS reached during fatigue and whether the structural alterations induced by ROS are readily reversed in a live cell where antioxidants and other natural reducing agents exist to reverse the changes to structure and function.

ADDITIONAL STRUCTURAL MODIFICATIONS

In addition, to the transient changes induced by ROS and NO species there may also be longer lasting structural modifications to the contractile proteins that occur during fatigue that in some cases may be secondary to ROS damage. For example, when diaphragm strips are repeatedly contracted under hypoxic conditions there is a loss of both TnI and TnC from the muscle (de Paula et al., 2001). The authors speculated that the loss might be due to cleavage and degradation of TnI and TnC during the fatigue and that these structural changes contributed to the loss in force. Furthermore, much of the loss in force was reversed when full-length exogenous TnI and TnC was added back into the fatigued fibers (de Paula et al., 2001), adding further support that the cleavage of these proteins is responsible for the reduction in force. While they could not identify what caused the cleavage the authors speculated that it may stem from increased ROS production directly damaging the proteins. This hypothesis could be tested by repeating the experiments in the presence of a reducing agent to determine if it prevents the degradation of TnTm and the associated loss in force. This kind of cleavage and degradation of the regulatory proteins is similar to the response observed in cardiac muscle following a transient ischemia/reperfusion injury (a.k.a. myocardial stunning; Gao et al., 1996). This very well-characterized phenomenon results in a progressive cleavage of TnI which is dependent on the severity of the injury, and is linked to a loss in Ca^{++}-sensitivity (McDonough et al., 1999). Thus a similar phenomenon may be occurring during hypoxic fatigue, although more investigation will be needed to confirm that this effect occurs during fatigue *in vivo*.

Phosphorylation of contractile proteins can also be classed as a transient structural modification, as it is a covalent modification of a protein. One particular phosphorylation event related to fatigue that has been the focus of considerable effort is the contraction-induced phosphorylation of myosin's regulatory light chain (RLC). Phosphorylation of myosin's RLC increases with repeated contractions and thus is high during fatigue from intense contractile activity. These observations led Karatzaferi et al. (2008) to examine the effects of P_i and H^+ on single fiber contractile properties at high and low levels of RLC phosphorylation. Interestingly, they found that at physiological temperatures, elevated levels of P_i and H^+ had their greatest depressive effect on single fiber force and velocity when the RLC was highly phosphorylated as they suggested would be the case during fatigue (Karatzaferi et al., 2008). However knocking out the light-chain phosphorylating kinase (MLCK) in a mouse model does not seem to prevent or even attenuate the decrease in tetanic force with fatigue, suggesting little or no role for this phosphorylation event in fatigue. Although the temperatures of the two studies were different (30 vs

25˚C) could help explain a portion of the discrepancy, the power of the later *in vivo* work strongly suggests a limited or non-existent role for MLC phosphorylation in fatigue. A similar conclusion was reached by Greenberg et al. (2010) based findings from an *in vitro* motility assay where elevated levels of H^+, P_i and ADP had a similar depressive effect on the phosphorylated and dephosphorylated myosin. Therefore while it is clear phosphorylation of MLC influences tetanic force it is not clear that it has a significant role in fatigue.

In addition to the RLC of myosin there are a many other potential phosphorylation sites on other contractile proteins, thus this may not be the only phosphorylation of contractile proteins associated with fatigue. One particularly interesting phosphorylation linked to oxidative stress in skeletal muscle, and thus possibly fatigue, is the phosphorylation of Tm (Houle et al., 2003). In response to elevated levels of H_2O_2 Houle et al. (2003) were able to detect an increase in the phosphorylation of Tm. In subsequent experiments it was demonstrated that *in vitro* phosphorylation of Tm can greatly increase the force and velocity of actin reconstituted with Tm in a laser trap assay (Rao et al., 2009). Interestingly, the results suggested that the effect was due to an increase in the size of the cooperative unit of the thin filament, implying that phosphorylation modifies the behavior of Tm on actin to promote strong actomyosin binding and ultimately activation of the thin filament. While such a change in Tm phosphorylation might occur during fatigue the increase force and velocity observed *in vitro* suggests this effect might help to prevent the loss of muscle function during fatigue rather that exacerbate it. However, more work will need to be done to definitely determine the role of this and other contraction dependent changes in phosphorylation in fatigue *in vivo*. In related work in the future it will be crucial to be able to link the structural modifications to the function changes. This will likely take a systemic approach to both locate each modification and assess its impact on the function of various contractile proteins in an *in vitro* setting.

CONCLUSIONS AND FUTURE DIRECTIONS

The cause of fatigue has been a fundamental question of science for many years and the cause of muscle fatigue specifically has been studied for more than 100 years. While understanding the limits of human performance drove much of the early efforts in this field it is now clear that identifying the molecular causes will have important clinical relevance to conditions such as chronic heart failure and ischemic heart disease where the effects of fatigue can be severe enough to limit the daily tasks required for independent living (Poole-Wilson and Buller, 1988). Many of the originally proposed mechanisms, established decades ago, are now able to be directly tested and challenged with the recent technological developments in molecular and structural biology as well as single molecule biophysics. These recent findings have confirmed some long-held theories but others have been sharply challenged by the first direct evidence. And maybe more importantly, the new findings are presenting the field with a new set of questions about the nature of the mechanism of fatigue, forcing us to think at an even more basic level about the process of fatigue. Thus it is a particularly exciting time to be studying this question as the rapid pace of technological advances will now allow the field to push our knowledge even further in the very near future.

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The multiple roles of phosphate in muscle fatigue

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Muscle fatigue is the decline in performance of muscles observed during periods of intense activity. ATP consumption exceeds production during intense activity and there are multiple changes in intracellular metabolites which may contribute to the changes in crossbridge activity. It is also well-established that a reduction in activation, either through action potential changes or reduction in Ca^{2+} release from the sarcoplasmic reticulum (SR), makes an additional contribution to fatigue. In this review we focus on the role of intracellular inorganic phosphate (P_i) whose concentration can increase rapidly from around 5–30 mM during intense fatigue. Studies from skinned muscle fibers show that these changes substantially impair myofibrillar performance although the effects are strongly temperature dependent. Increased P_i can also cause reduced Ca^{2+} release from the SR and may therefore contribute to the reduced activation. In a recent study, we have measured both P_i and Ca^{2+} release in a blood-perfused mammalian preparation and the results from this preparation allows us to test the extent to which the combined effects of P_i and Ca^{2+} changes may contribute to fatigue.

Keywords: skeletal muscle, fatigue, intracellular calcium, inorganic phosphate, myofibrillar performance

INTRODUCTION

Muscle fatigue can be defined as the decline of muscle performance associated with intense muscle activity. Many mechanisms contribute to this decline, from psychological factors within the cortex down to the state of individual proteins inside the muscle. To study mechanisms in detail it is usually necessary to use a simplified preparation and a well-defined pattern of stimulation. One approach has been to use isolated small preparations of muscle and stimulate with intermittent, maximal, isometric tetani; obviously central (cortical) mechanisms do not occur in this preparation and the effects of changes in blood flow are also absent. Another approach is to use human subjects and induce fatigue by maximal or near-maximal contractions repeated regularly. In suitable muscles both force and metabolite changes can be measured simultaneously and repeatedly. Two limitations to this approach are that both the force and the metabolites are usually averaged across a muscle which consists of various fiber types with different properties. In addition the degree of central vs. peripheral fatigue is often uncertain in human subjects. Given the interpretative difficulties of these two approaches, a third approach is of great importance; application of known metabolite changes to skinned muscle preparations. In this preparation, the metabolites are defined and can be changed at will and the fiber type can be determined. Extensive data is available documenting how the performance of the contractile proteins is affected by individual metabolite changes.

In this short review we will (1) discuss the evidence that inorganic phosphate (P_i) contributes to the changes in crossbridge behavior, (2) consider the evidence that phosphate contributes to the reduced Ca^{2+} release, (3) examine the correlation between force and P_i in fatiguing muscles, and (4) describe a new model of fatigue in which both P_i and intracellular tetanic Ca^{2+} concentration ($[Ca^{2+}]_i$) can be measured and analyse how these two factors in combination contribute to the force decline. This type of analysis involves many assumptions and can only give a general indication of the magnitude of the contribution that might arise from these sources. It is accepted that many other factors, with both positive and negative effects, contributed to the overall effect.

Many metabolites change in fatigue and a number of them have been shown in skinned fibers to affect the performance of the contractile proteins or Ca^{2+} release. Historically pH change has been widely considered a major cause of fatigue acting through a reduction in Ca^{2+} sensitivity (Fabiato and Fabiato, 1978). However, over the last two decades its contribution has been reassessed mainly because the effects of pH on isometric force are much reduced at mammalian body temperatures (Pate et al., 1995). In contrast it seems that acidosis reduces shortening velocity and muscle power at body temperature (Fitts, 2008) so that acidosis may still be of importance in many kinds of fatigue. This reassessment has been documented extensively elsewhere (for review see Westerblad et al., 2002; Cairns, 2006; Cooke, 2007). Other metabolites with probable roles in fatigue include ATP, ADP, PCr, Mg, and reactive oxygen and nitrogen species (Allen et al., 2008b). In addition many proteins display post-translational changes which affect their function, for instance phosphorylation, nitrosylation, and oxidation and the importance of these are starting to emerge (Bellinger et al., 2008; Mollica et al., 2012).

Muscles use ATP as the immediate source of energy but the ADP and P_i produced are regenerated by a series of metabolic pathways which utilize stores of PCr, glycogen, and lipid within the cell. Glycogen within the cell can be utilized by anaerobic glycolysis whose net products are lactate and protons. The metabolic pathways that regenerate ATP are turned on rapidly and tightly regulated so that the net effect is that ATP concentration is only

marginally reduced by moderate exercise but there are major increases in P_i, ADP, creatine, and protons while PCr and glycogen decrease. The changes in concentration of ATP, PCr, P_i, and ADP occur in a relatively stereotyped fashion which has been documented in numerous studies (Dawson et al., 1978; Cady et al., 1989; Lanza et al., 2006). Conversely the degree of acidosis observed is more variable because the rate and extent to which anaerobic glycolysis is turned on is dependent on the fiber type and the nature of the activity. Furthermore, skeletal muscle fibers contain variable amounts of the lactate transporter which reduce any intracellular accumulation of lactate and protons.

PHOSPHATE CONTRIBUTES TO THE DECLINE IN CROSSBRIDGE PERFORMANCE

Ruegg et al. (1971), working with skinned insect flight muscle, were the first to note that increased P_i inhibited contractile force. This observation was later repeated and extended by many groups working on skinned skeletal muscle (Cooke and Pate, 1985; Millar and Homsher, 1990). These groups demonstrated that, in skinned skeletal muscle at low temperatures (10–20°C), an increase in P_i produced a substantial reduction in both maximum Ca^{2+}-activated force and in Ca^{2+} sensitivity e.g., **Figure 1A** from Millar and Homsher (1990). It is generally accepted that this reduction occurs because P_i is released from crossbridges at a stage closely associated with force production so that elevation of phosphate accelerates the backward rate of this step and thus reduces force (Takagi et al., 2004; Cooke, 2007; Fitts, 2008). Cooke and Pate and Millar and Homsher showed that both the reduction of force and the shift of Ca^{2+} sensitivity were logarithmic functions of P_i. Thus, a 10-fold increase in P_i produced a reduction in maximum Ca^{2+}-activated force to 63% and a reduction of Ca^{2+} sensitivity of 0.2 pCa units (Millar and Homsher, 1990). In order to compare data from skinned fibers

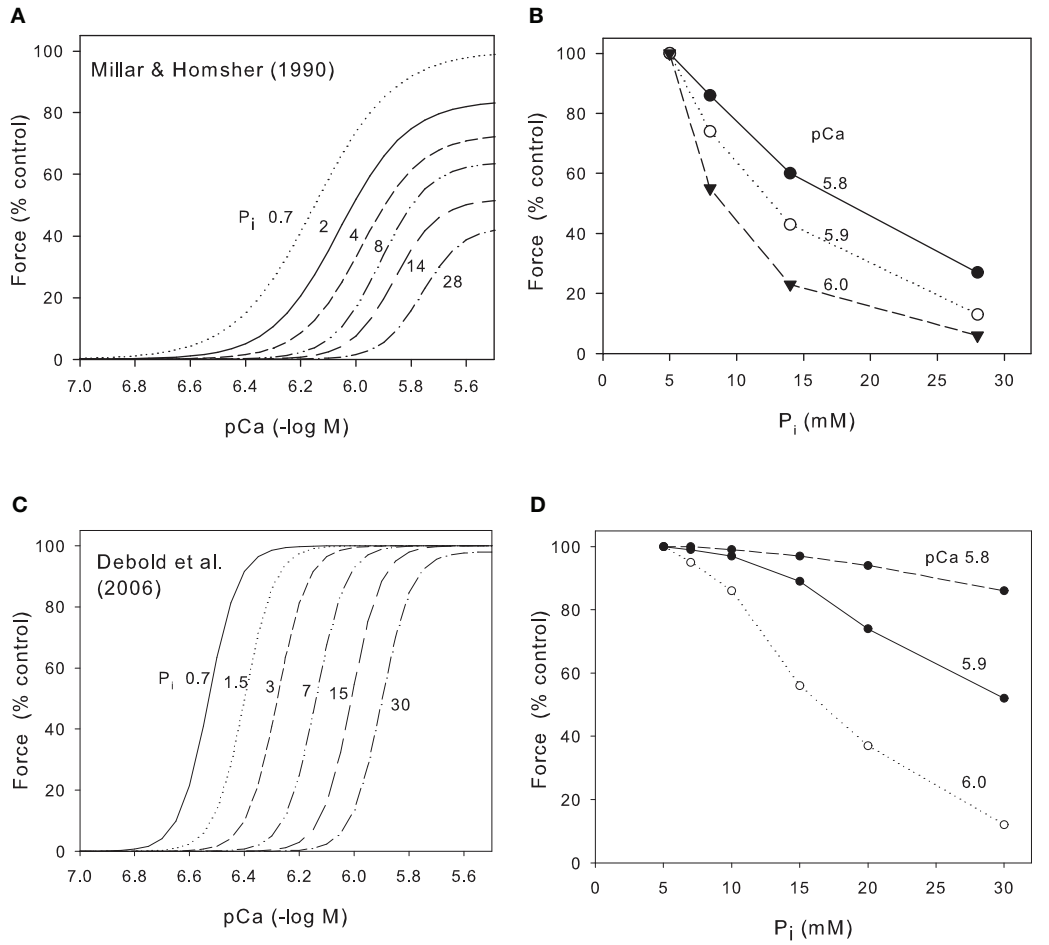

FIGURE 1 | Effect of P_i on relation between pCa and force for skinned skeletal muscle. (A) Data from rabbit psoas muscle at 10°C (Millar and Homsher, 1990). Ca^{2+} concentrations have been adjusted for the excessively high Ca^{2+} EGTA binding constant used in this study. P_i concentrations (mM) as indicated on Panel. Note large fall in maximum Ca^{2+}-activated force under these conditions. **(B)** Data from Millar and Homsher replotted to show how force falls as a function of P_i at various pCa levels. **(C)** Data from fast gastrocnemius muscle at 30°C (Debold et al., 2006). Published data were at 0.7 mM (no added P_i) and 30 mM P_i; we have interpolated curves on the basis that pCa50 is a logarithmic function of P_i. Note the absence of significant effect of P_i on maximum Ca^{2+}-activated force under these conditions and that the Debold et al. data is much steeper; Hill coefficients ~8 whereas for Millar and Homsher (1990) Hill coefficients were 2–6. **(D)** Data from Debold et al. (2006) redraw to show force as a function of P_i at various pCa levels.

with data from intact muscles, we have converted the data in **Figure 1A** to the relationship between force and P_i (**Figure 1B**). In mammalian muscles the resting myoplasmic $[P_i]$ is around 5 mM (Kemp et al., 2007; Fitts, 2008) so we have normalized the force to 100% at this $[P_i]$ and compared the relationship at three different pCa (5.8, 5.9, and 6.0) to represent the levels that may occur in near-maximally activated tetani. Even at high Ca^{2+}, the relation between force and P_i declines because of the effect of P_i on maximum Ca^{2+}-activated force and this effect is largest at low P_i and decreases as P_i increases. At lower Ca^{2+} levels the relationship falls more steeply as the effect of changes in pCa50 (pCa required to give half maximal force) add to the reduced maximum Ca^{2+}-activated force.

The early work on skinned muscle cited above was performed at 10–20°C and a major recent development was the discovery that the effects of both pH and P_i on skinned fibers are quantitatively and qualitatively different when repeated at near physiological temperatures (Pate et al., 1995; Coupland et al., 2001; Debold et al., 2006). Data from Debold et al. (2006) are shown in **Figure 1C** who studied fast and slow fibers at both 15°C and 30°C. Over the range of 0.7–30 mM P_i, maximum Ca^{2+}-activated force was reduced by 46% at 15°C but by only 2% at 30°C. In contrast, over the same range of P_i, the pCa50 was reduced by 0.28 log units at 15°C but was reduced by 0.63 log units at 30°C. Assuming the relation between P_i and Ca^{2+} sensitivity is logarithmic (Millar and Homsher, 1990), we have interpolated the data of Debold et al. (2006) at 30°C into a series of force/pCa curves at various P_i (**Figure 1C**). We have then converted this data into the relationship between force and P_i for various pCa levels (**Figure 1D**). Note that in contrast to the data at low temperatures (**Figures 1A,B**), there is little effect of P_i on maximum Ca^{2+}-activated force and consequently, at the highest Ca^{2+} (pCa 5.8) P_i has relatively little effect on force (**Figure 1D**). Only when the Ca^{2+} is lower (pCa 5.9 and 6.0) does the force fall substantially and the effect is least at low P_i and greater as P_i increases.

At low temperatures, increases in both P_i and H^+ depress muscle force and when applied simultaneously the effect is larger and generally assumed to be multiplicative. Nosek et al. (1987) factored out the effect of pH and noted that, after this procedure, the effect on force of a fixed concentration of P_i was very variable. However, when the total P_i was converted to the $H_2PO_4^-$ and HPO_4^{2-} forms, there was a good correlation with the diprotonated form and a poor correlation with the monoprotonated form. On this basis they suggested that it was the diprotonated form of P_i which inhibited force and that pH exerted two effects on force; a direct effect of protons and an indirect effect by converting more of the total P_i to the diprotonated form. However, some other studies have been unable to reproduce this effect (e.g., Chase and Kushmerick, 1988) and it is generally agreed that this effect is only seen in fast skeletal muscle but not in slow or cardiac muscle. It is also worth noting that the experiments on this topic were at 22°C and it is not known whether the effect persists at mammalian temperatures. Thus, the issue remains unresolved and some experimenters chose to display force data against total P_i while others compare force with $H_2PO_4^-$.

THE EFFECT OF P_i ON Ca^{2+} RELEASE

Fatigue is known to involve both reduced crossbridge performance and reduced activation. There are many possible mechanisms by which activation might decline during fatigue (for review see Allen et al., 2008a) but in the present article we focus on the possibility that P_i may have some direct effect on activation. This idea was first raised by Fryer et al. (1995) in a study on skinned fibers with intact sarcoplasmic reticulum (SR). Ca^{2+} release was triggered by caffeine and was found to decrease substantially after the preparation had been bathed in a high P_i solution. They suggested that during fatigue P_i entered the SR reaching concentrations at which it was capable of precipitating with the high levels of Ca^{2+} found in the SR. This $Ca^{2+}P_i$ precipitate would thus reduce the free Ca^{2+} available in the SR for release (for review see Allen and Westerblad, 2001). This theory was supported by a study in which P_i was microinjected into single muscle fibers (Westerblad and Allen, 1996). There was a small reduction in Ca^{2+} sensitivity but a substantial reduction in tetanic Ca^{2+} present after 4 min. These data suggest that much of the P_i entered the SR where it precipitated and subsequently reduced Ca^{2+} release. Further support was provided by Laver et al. (2001) who discovered a phosphate permeable channel in the SR which could provide the route of entry for P_i. This mechanism was strengthened by the observation that the decline in tetanic $[Ca^{2+}]_i$ which normally occurs during fatigue is reduced or delayed in creatine kinase knockout mice, in which the normal fatigue-induced rise in P_i is reduced (Dahlstedt and Westerblad, 2001).

More recently Dutka et al. (2005) have extended earlier work on skinned fibers by showing that the P_i-induced reduction in Ca release initially observed with caffeine triggered release is also present when Ca release is triggered by T-tubular action potentials. They also showed that the total SR Ca^{2+} was unaffected by P_i, supporting the idea that Ca^{2+} had precipitated as opposed to leaking out of the SR. In their experiments a 2 min exposure to 30 mM P_i was sufficient to reduce Ca^{2+} release to a steady level suggesting that this mechanism would have time to operate during a fatigue protocol of this or greater length. This mechanism appears to be substantially smaller in slow fibers compared to fast, which may contribute to the fatigue-resistance of slow fibers (Posterino and Dunn, 2008).

In summary, a substantial body of evidence points to the possibility that elevated P_i contributes to reduced Ca^{2+} release during fatigue. Definitive evidence probably would require measurements of $Ca^{2+}P_i$ precipitates in the SR and the timecourse of their change during fatigue and recovery. However, the small size of the SR makes this a challenging procedure.

THE CORRELATION BETWEEN FATIGUE AND $[P_i]$

Many studies have correlated the changes in myoplasmic $[P_i]$ with the changes in force during fatiguing activities. While the measurements of metabolites from isolated muscles and from biopsies of human muscles have been possible for many years, these methods are not easily scaled up to obtaining multiple samples. Thus, a rapid expansion in knowledge occurred with the development of nuclear magnetic resonance which allows non-invasive sampling of metabolites to be repeated frequently.

Dawson et al. (1978) pioneered this technique in muscle and were able to plot PCr, Cr, P_i, ADP, ATP, and H^+ as functions of the declining force in tetanically stimulated ischaemic frog muscle. H^+ showed a strong linear correlation with declining force and was therefore a good candidate. P_i also correlated well with force decline though at high P_i there was some deviation from linearity. This work was performed on frog muscles at 4°C; given the importance of temperature we focus in this section on subsequent studies in humans or mammals at or near body temperature.

Lanza et al. (2006) examined force and metabolite change in the human tibialis anterior in response to six maximal 12 s contractions separated by 12 s rest periods. In this protocol force declined to 74% control and over this period $H_2PO_4^-$ increased from 3.5 to 15 mM and showed a near linear relation with force (**Figure 2A**). We have calculated total P_i from their data and this also correlates well with force (**Figure 2A**). These authors repeated the protocol under ischaemic conditions with similar results. Jones et al. (2009) also studied the human tibialis anterior under ischaemic conditions but using electrical stimulation of the muscle and their data show a comparable relationship between P_i and force decline (**Figure 2B**). ATP consumption was calculated and, after correction for the decline of force, the economy of

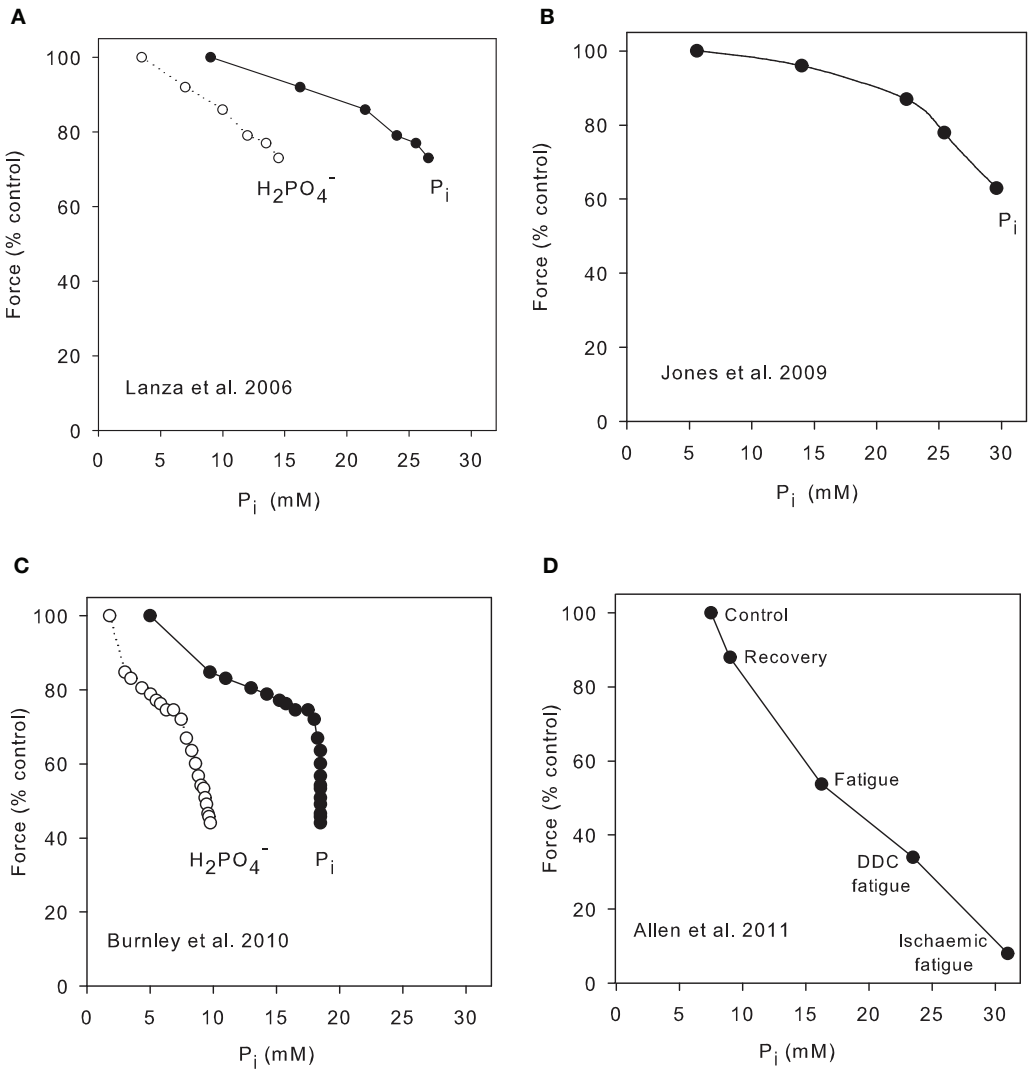

FIGURE 2 | Correlation between P_i and force in intact, fatiguing muscle. **(A)** Human subjects performed six 12 s maximal contractions involving dorsiflexion of the ankle and NMR spectra were collected from the tibialis anterior muscle in 12 s periods between contractions (Lanza et al., 2006). In the original publication only $H_2PO_4^-$ was plotted; we have calculated P_i on the basis $P_i = H_2PO_4^- (1 + 10^{pH-6.75})$. **(B)** Stimulated maximal tetani of the human tibialis anterior muscle under ischaemic conditions. 1.6 s tetani with 1.6 s intervals between tetani continued for 32 s (Jones et al., 2009). **(C)** Human subjects performed 60 maximal voluntary contractions of the quadriceps group, each contraction 3 s in duration with 2 s rest between (Burnley et al., 2010). P_i and $H_2PO_4^-$ were recorded between each contraction. Resting P_i assumed to be 5 mM. **(D)** Exteriorized mouse tibialis anterior stimulated with maximal tetani of 0.4 s duration repeated every 4 s. Muscles were sampled under control conditions and when fatigue had reached a steady state (12 min). A second protocol involved 0.4 s tetani repeated every 2 s (double duty cycle or DDC fatigue). Some fatigue protocols were recorded after death when circulation had ceased (ischaemic fatigue). Data from Allen et al. (2011).

contraction appeared unchanged during the period of fatigue. A different approach was used by Burnley et al. (2010) who studied the quadriceps group during repeated maximal voluntary contractions, 3 s on 2 s off, continued until the force was stable. P_i and $H_2PO_4^-$ were measured at 5 s intervals throughout, so that the timecourse of P_i and force could be followed throughout the procedure. The results show that P_i initially changed more quickly than force (**Figure 2C**) so that the plot has a region where force and P_i change reasonably linearly but, because force continued to fall while P_i was constant, this gives a near vertical line on the plot. Because pH continued to decline slowly throughout fatigue, the plot of $H_2PO_4^-$ is more nearly linear over the whole range. Thus, this data supports the idea that pH is making some contribution to fatigue, either directly or through the changing proportions of $H_2PO_4^-$/total P_i; alternatively the authors suggest that a central component to fatigue may become more prominent during the later part of the protocol. Another possibility is that Ca^{2+} release is reduced with a delay in this model as observed in many isolated muscle fatigue protocols (Westerblad and Allen, 1991) and in the exteriorized mouse muscle (**Figure 3B**). The final example is from Allen et al. (2011) who studied the exteriorized tibialis anterior of mouse (**Figure 2D**). Blood flow was intact and the muscle temperature was 30°C. The protocol involved direct muscle stimulation with 400 ms tetani repeated every 4 s (duty cycle = 0.1). Force reached a plateau at ~50% after 6–10 min and P_i was determined in whole muscle extracts at the beginning and end of the protocol and after recovery (a typical fatigue protocol for this preparation is shown in **Figure 3C**). The three upper most points are control, recovery and the standard fatigue protocol. The two lowest points are a more intense stimulation protocol (duty cycle 0.2) and a protocol in which the muscle was ischaemic. There is a reasonable correlation over the whole range between force decline and P_i in this preparation.

All the results cited demonstrate a negative correlation between P_i and force during fatiguing stimulation. Typically the effect of P_i appears smaller early in fatigue and the effect appears to be enhanced in the steady state. A simple explanation of this would be if P_i also decreases Ca^{2+} release but after a delay.

MEASUREMENTS OF P_i AND Ca^{2+} IN MOUSE MUSCLE DURING FATIGUE

The data in **Figure 1** make it clear that the expected reduction in force as a function of P_i is very dependent on the tetanic $[Ca^{2+}]_i$. Thus, to analyse the consequences of P_i during fatigue it is necessary to have measurements of force, tetanic $[Ca^{2+}]_i$ and P_i. We have recently developed a new model of fatigue in which, potentially, all these data can be determined (Allen et al., 2011). The approach uses the tibialis anterior muscle of the anaesthetized mouse. The distal tendon of the muscle is detached and tied to a force transducer while the proximal end is undisturbed so that normal blood supply occurs. Ca^{2+} is measured using genetically-encoded cameleons (Miyawaki et al., 1997) transfected into the muscle 5–10 days before the experiment. The indicator is expressed in a subset of fibers and those within one or two fibers diameters of the outer surface of the muscle can be examined. **Figure 3A** shows a fluorescent XY image of the muscle surface obtained with a 2-photon microscope. In this image the scanning occurred from left to right so that at the left of the image a group of five fibers showing variable degrees of expression are visible. Approximately half way through the image acquisition (total time 730 ms) the muscle was stimulated tetanically,

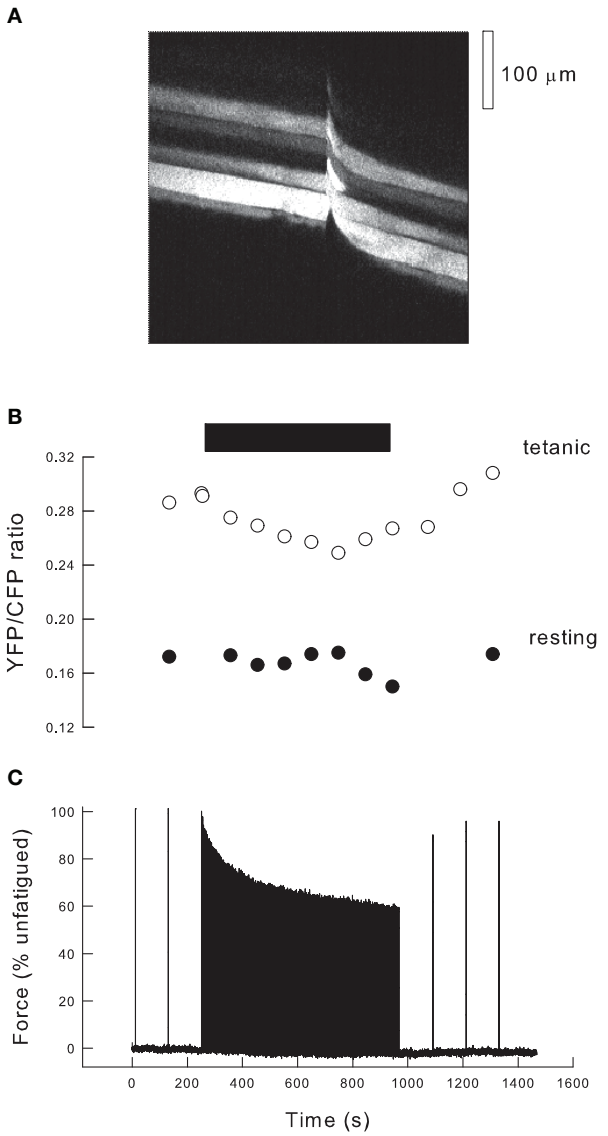

FIGURE 3 | Fatigue in the exteriorized mouse tibialis anterior muscle. **(A)** Fluorescence image of tibialis anterior muscle 10 days after transfection with cameleon (YC2). Image was scanned from L to R over 750 ms, and the muscle stimulated after 400 ms (indicated by movement artifact). Thus, in the L part of the image the fibers are resting whereas in the R part of the image the fibers are tetanized. **(B)** Records of the ratio of fluorescence of YFP/CFP obtained from images such as **(A)** during a fatigue protocol. Black bar indicates period of high duty cycle stimulation. Empty circles indicate ratios determined during tetani (R hand images in Panel **A**), filled circles show ratios from the resting muscle just prior to tetani (L hand images in Panel **A**). **(C)** Record of force during the stimulation protocol. Initially the muscle is stimulated every 2 min and force is stable. When duty cycle is increased to one tetanus every 4 s, force declines exponentially and approaches a steady state. Stimuli are then given every 2 min and force recovers to ~90% of the initial level.

indicated by the movement artifact, and the right hand part of the image shows the same group of fibers during a tetanus. Cameleons have two fluorophores (YFP and CFP) attached to the ends of a calmodulin molecule plus a linker segment. When Ca^{2+} binds to calmodulin, it undergoes a shape change and the CFP and YFP become sufficiently close that fluorescence resonance energy transfer (FRET) occurs between them. The molecule is illuminated with a wavelength that excites CFP which then emits at its characteristic wavelength. In the presence of Ca^{2+}, FRET occurs between CFP and YFP so that some fraction of the emitted light is now at the YFP emission wavelength. Thus, the ratio of YFP/CFP emitted fluorescence is Ca^{2+} sensitive.

Figure 3C illustrates our standard fatigue protocol. In the control period the muscle is stimulated with maximal tetani at 2 min intervals leading to stable tetanic force. During the fatiguing protocol the muscle is stimulated every 4 s (duty cycle = 0.1) for 12 min and force approaches a steady state by the end of this period. Subsequently the tetanic frequency is reduced so that at 2 min intervals tetani recover to around 90% of the initial force. **Figure 3B** shows the YFP/CFP ratios at rest and during selected tetani during the protocol. Resting $[Ca^{2+}]_i$ typically shows no significant change during this fatigue protocol. Tetanic $[Ca^{2+}]_i$ falls during fatigue and recovers afterwards. If the difference between tetanic and resting $[Ca^{2+}]_i$ is defined as 100%, then the average tetanic ratio during fatigue declined to 67%. The ratio then recovers with a timecourse similar to that of force. We have not calibrated our YFP/CFP ratios but data from Miyawaki et al. (1999) suggest that the relationship between YFP/CFP ratio and Ca^{2+} should be linear for our indicator (YC2) over the lower part of its range.

Metabolic measurements were made by rapidly freezing the muscle in liquid N_2 and extraction with perchloric acid. P_i was measured in the supernatant using 31P-NMR spectroscopy. The relation between force and P_i in control (upper leftmost point) and various types of fatigue is depicted in **Figure 2D** and shows a robust linear relationship between P_i and force over the whole range of force outputs. In order to decide to what extent changes in P_i can explain the reduction in force one needs to compare data in **Figure 2D** with the data in **Figure 1D**. There are several problems involved in such a comparison. (1) One needs to know the appropriate level of Ca^{2+} in order to make the comparison. (2) Both the force and the metabolic measurements are averaged from the whole muscle which will consist of a mixture of fiber types. Percival et al. (2010) have determined the fiber type composition of mouse tibialis anterior and the main fiber types are Type IIB (54%) and Type IIA (34%) with smaller amounts of various intermediate fiber types. The Type IIB fibers predominate on the superficial half of the muscle while the Type IIA fibers are mainly located in the deeper portion. As a consequence, our Ca^{2+} measurements, which are made from superficial fibers, are likely to be from Type IIB fibers. Larsson et al. (1991) have determined the fatiguability of these fiber types in rat tibialis anterior using the single motor unit stimulation protocol. Using a duty cycle of 0.4, compared to our 0.1, Type IIB fibers fatigued to 2% control in 4 min whereas Type IIA fibers fatigued to 93% over 4 min. Assuming rat and mouse fibers are similar in fatiguability, then it would seem that Type IIA fibers should show very little fatigue at our duty cycle and, conversely, most of the observed fatigue in our preparation is likely to be in the Type IIB fibers.

To understand how P_i and Ca^{2+} interact during fatigue, in **Figure 4** we plot skinned fiber data at 30°C (Debold et al., 2006) showing pCa/force curves at the resting P_i (7 mM) and the fatigued P_i (15 mM). Our Ca^{2+} measurements are not calibrated but, since we stimulate at a frequency that gives maximal force, we can assume that Ca^{2+} is at least supramaximal on the appropriate P_i curve (for recent review of calcium measurements in skeletal muscle see Baylor and Hollingworth, 2011). Thus, for the unfatigued muscle ($P_i = 7$ mM) reading from **Figure 4**, we can see that the tetanic $[Ca^{2+}]_i$ should be ~pCa = 5.9 giving a tetanic $[Ca^{2+}]_i = 1.26\,\mu M$. This point is marked on **Figure 4** as a black circle. From our Ca^{2+} measurements we found that tetanic $[Ca^{2+}]_i$ reduced to 67% in the fatigued muscle giving a tetanic $[Ca^{2+}]_i$ of 0.83 μM (pCa 6.08). Simultaneously during fatigue P_i rises to 15 mM so the expected force for these conditions is given by the empty circle (22% control force). Thus, under these assumptions, the combination of Pi and $[Ca^{2+}]_i$ predict a force of 22% in our fatigue protocol whereas the measured fatigue force was 49%. This analysis assumes that both the fast and slow fibers had similar changes in Ca^{2+} and P_i and that the properties of the slow and fast fibers are similar with respect to P_i. On the first point, Karatzaferi et al. (2001) measured metabolic changes in both Type IIA fibers and the human equivalent of Type IIB (Type IIXa). Although they did not measure P_i, the decline in PCr was substantially greater in the IIXa fibers (103 mmol/kg dry weight) compared to the Type IIA fibers (71 mmol/kg dry weight).

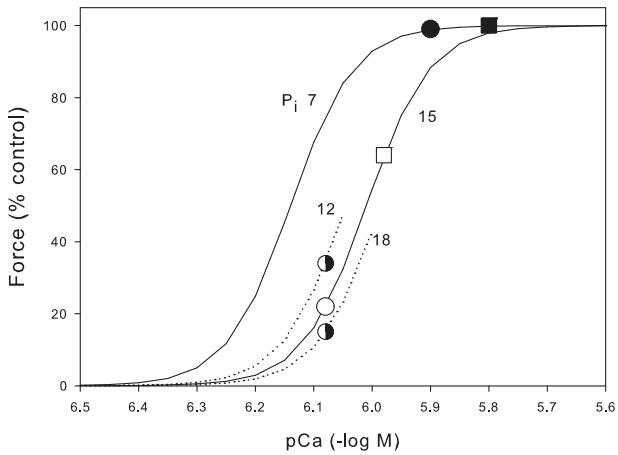

FIGURE 4 | Prediction of the force during fatigue based on the P_i and Ca^{2+} data from the mouse tibialis anterior. Skinned fiber data (continuous lines) is from Debold et al. (2006) and shows pCa force data at 7 mM, the resting P_i in mouse tibialis anterior, and 15 mM, the P_i reached after the standard fatigue protocol shown in **Figure 3**. Black circle indicates the resting unfatigued muscle with P_i 7 mM and Ca^{2+} assumed just sufficient to fully saturate the contractile machinery (pCa 5.9). Open circle shows the predicted force when Ca^{2+} falls by 33%, as measured during fatigue, and P_i rises to that observed in fatigue. The skinned fiber data predict a force of 22% under these conditions. The closed and open square show another estimated with the Ca^{2+} higher by 25%. Also shown are parts of the pCa force curves for P_i 12 and 18 mM (see text for details).

This suggests that P_i would be higher than the average in Type IIB fibers and correspondingly lower in the Type IIA fibers. On the second point Debold et al. (2006) state that Type IIB and IIA fibers had similar properties with respect to Ca^{2+} and P_i sensitivity. To further investigate this point we have recalculated the expected force assuming the muscle consists of 60% Type IIB and 40% Type IIA. We further assume that P_i is 20% greater in the Type IIB fibers (18 mM) and 20% less in the Type IIA fibers (12 mM). The half shaded circles (**Figure 4**) show that on these assumptions, the Type IIB fibers would generate 15% force while the Type IIA fibers would generate 34%. The whole muscle would then generate 23% ($0.4 \times 0.34 + 0.6 \times 0.15$). These new assumptions only change our estimated force from 22 to 23%. Thus, these two areas of uncertainty probably would not make a very substantial change in the estimated force under the fatigue conditions. On the other hand the estimated tetanic $[Ca^{2+}]_i$ makes a large contribution. For instance if we assume the tetanic $[Ca^{2+}]_i$ is 25% higher than required to saturate (black square in **Figure 4**), then the estimated force during fatigue after the appropriate reduction in tetanic $[Ca^{2+}]_i$ (open square) becomes 64%. Thus, a 25% change in our estimated Ca^{2+} leads to large (64 vs. 21%) estimate in the fatigued force. Furthermore, we have no information on the tetanic Ca^{2+} in Type IIA fibers or how it might change during fatigue. Our conclusion as present is that the combination of declining Ca^{2+} and rising P_i during fatigue are probably capable of explaining much of the observed force reduction. To make this estimate more precise we would need calibrated Ca^{2+} measurements in both fiber types and more details on the metabolite changes in different fiber types.

An unresolved difficulty with these calculations is to explain the observation of Larsson et al. (1991) showing that Type IIB fibers would be expected to fatigue to near 0 whereas Type IIA fibers show very little fatigue. In contrast our modeling suggests Type IIB fatigue to 15% while Type IIA fatigue to 34%. There are many possible causes of this discrepancy; one is that factors other than P_i and Ca^{2+} are important in fatigue. A second possibility is that the difference in P_i is greater between the two fiber types. A further possibility is that the Ca^{2+} changes occurring during fatigue are smaller in the Type IIA fibers and larger in the Type IIB fibers which would be expected if the Ca^{2+} changes are a function of the P_i changes as discussed earlier.

CONCLUSIONS

Current data on the changes in P_i in muscle during fatigue suggest that the combination of the increase in P_i and the decrease in tetanic Ca^{2+} are capable of explaining much of the observed decline in force. This analysis is highly dependent on skinned fiber data obtained at 30°C and more data at 37°C would be desirable. Another area of uncertainty is the level of tetanic Ca^{2+} in the various fibers types and the extent to which this changes during fatigue protocols. While the mechanism of change in tetanic Ca^{2+} remains uncertain, a strong possibility is that the change in P_i is instrumental in the reduction of tetanic Ca^{2+} suggesting that evolution has utilized one metabolite to reduce both the myofibrillar performance and the Ca^{2+} release. The many other factors that modulate fatigue would appear to do so by fine-tuning the substantial reduction in force caused by the interactions between P_i and myofibrillar proteins and the Ca^{2+} release mechanism.

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The sarcomeric protein nebulin: another multifunctional giant in charge of muscle strength optimization

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The sliding filament model of the sarcomere was developed more than half a century ago. This model, consisting only of thin and thick filaments, has been successful in explaining many, but not all, features of skeletal muscle. Work during the 1980s revealed the existence of two additional filaments: the giant filamentous proteins titin and nebulin. Whereas the role of titin rapidly progressed, nebulin's role in muscle structure and function remained long nebulous. An important feature of muscle structure and function that has remained relatively obscure concerns the mechanisms that are involved in regulating thin filament length. Filament length is an important aspect of muscle function as force production is proportional to the amount of overlap between thick and thin filaments. Recent advances, due in part to the generation of nebulin KO models, reveal that nebulin plays an important role in the regulation of thin filament length, most likely by stabilizing F-actin assemblies. Another structural feature of skeletal muscle that has been incompletely understood concerns the mechanisms involved in maintaining Z-disk structure and the regular lateral alignment of adjacent sarcomeres during contraction. Recent studies indicate that nebulin is part of a protein complex that mechanically links adjacent myofibrils. In addition to these structural roles in support of myofibrillar force generation, nebulin has been also shown to regulate directly muscle contraction at the level of individual crossbridges: cycling kinetics and the calcium sensitivity of force producing crossbridges is enhanced in the presence of nebulin. Thus, these recent data all point to nebulin being important for muscle force optimization. Consequently, muscle weakness as the lead symptom develops in the case of patients with nemaline myopathy that have mutations in the nebulin gene. Here, we discuss these important novel insights into the role of nebulin in skeletal muscle function.

Keywords: nebulin, skeletal muscle, thin filament

NEBULIN IS INVOLVED IN SPECIFYING THIN FILAMENT LENGTH

Skeletal muscle produces efficient contractile force because they contain thin and thick filaments of well-defined lengths that are organized into regular, symmetric arrays that interdigitate. Filament length is an important aspect of muscle function because a muscle generates force in proportion to thin and thick filament overlap. Whereas thick filament length is considered a constant 1.6 μm, thin filament lengths are fine-tuned at ∼1.0–1.3 μm, depending on species and muscle type (Littlefield and Fowler, 2008) to overlap with thick filaments and to meet the muscle's physiological demands (Granzier et al., 1991; Burkholder et al., 1994; Littlefield and Fowler, 2008).

Changes in thin filament length affect thin–thick filament overlap and impact a muscle's force generating capacity at a given sarcomere length: thus, thin filament length is a key aspect of muscle function. Since length is not an intrinsic property of actin filaments (actin monomers assemble *in vitro* to highly variable polymer lengths (Pollard and Borisy, 2003), thin filament length is likely to be specified *in vivo* by an actin-binding protein; for this, nebulin has been considered for a long time a prime candidate (Wang and Wright, 1988; Labeit et al., 1991; Labeit and Kolmerer, 1995), but critical evidence has been lacking up until recently.

Nebulin is a giant protein (Mw 700–800 kDa) expressed in skeletal muscle, and makes up 2–3% of the myofibrillar protein mass. Immuno-electron microscopy revealed that a single nebulin molecule spans the thin filament with its C-terminus anchored at the Z-disk and its N-terminal region directed toward the thin filament pointed-end (for a schematic representation, see **Figure 1**; Wang and Wright, 1988). The first evidence for nebulin's proposed role in specifying thin filament length came from the analysis of nebulin's cDNA sequence. This revealed that the bulk of the molecule is comprised of modules with the centrally located modules, M9 to M162, each thought to represent individual actin-binding motifs, and organized into seven-module super-repeats that match the repeat of the actin filament (**Figure 1**). This precise arrangement is thought to allow each nebulin module to interact with a single monomer of the actin filament (Labeit et al., 1991; Labeit and Kolmerer, 1995), and each nebulin super-repeat to associate with a single tropomyosin (Tm)/troponin (Tn) complex (Jin and

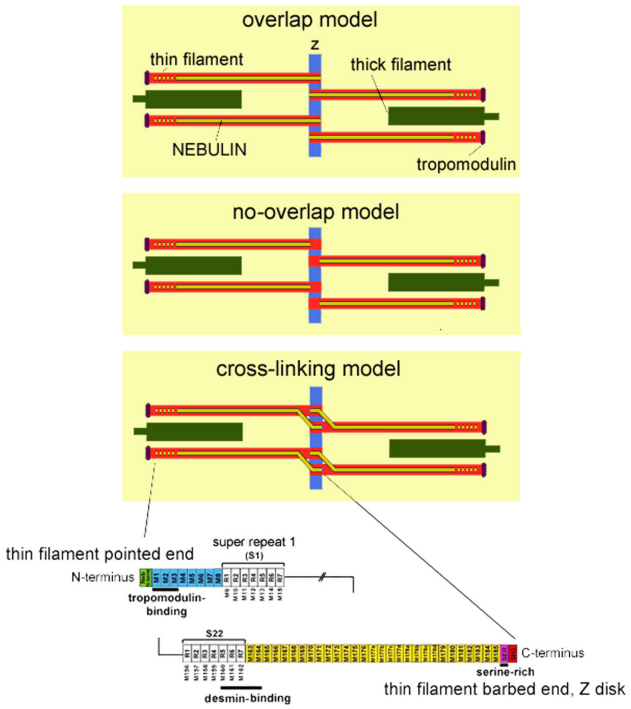

FIGURE 1 | Top: Three schematics of the skeletal muscle sarcomere depicting the three proposed models of nebulin in the Z-disk: the *overlap model* in which nebulin penetrates the Z-disk creating a ∼75 nm wide nebulin overlap zone in the center of the Z-disk; the *no-overlap model* in which nebulin penetrates the Z-disk by only about 25 nm, and nebulin filaments from neighboring sarcomeres do not overlap; the cross-linking model in which nebulin fully penetrates the Z-disk and cross-links thin filaments from adjacent sarcomeres. Also note that a single nebulin molecule spans most of the length of the thin filament, but that according to recent studies the thin filament pointed-end is nebulin-free. The length of the nebulin-free thin filament extension is variable (as indicated in the drawing by the dotted nebulin filament) and depends on species and muscle type. Bottom: Schematic of the human nebulin sequence. Nebulin has a highly modular structure, with in the central region (M9–M162) seven modular repeats arranged into 22 super-repeats.

Wang, 1991; McElhinny et al., 2003; Ogut et al., 2003). Nebulin's extreme N-terminal modules M1–M3 (**Figure 1**) contain a high-affinity binding site for the thin filament pointed-end capping protein tropomodulin (McElhinny et al., 2001). Tropomodulin, in addition to binding nebulin's N-terminus, binds actin and tropomyosin with high-affinity and prevents actin filaments from elongating or shortening at the pointed-end (dos Remedios et al., 2003). Furthermore, earlier studies revealed that the electrophoretic mobility of nebulin from different muscle types correlates with thin filament length (Kruger et al., 1991; Labeit et al., 1991).

Although the findings discussed above were consistent with the hypothesis that nebulin is involved in specifying thin filament length, direct evidence was lacking. More conclusive evidence for a role for nebulin in specifying thin filament length required studies of muscle that lack nebulin. To test the role of nebulin in skeletal muscle *in vivo*, nebulin KO mouse models were generated (Bang et al., 2006; Witt et al., 2006). The first work on these models revealed that in nebulin-deficient skeletal muscle the thin filaments are on average shorter, thus supporting a role for nebulin in the *in vivo* regulation of thin filament length (Bang et al., 2006; Witt et al., 2006). Witt et al. (2006) performed an immunoelectron microscopy study and reported that thin filament lengths in wildtype tibialis cranialis muscle are a constant 1.2 μm, but in nebulin-deficient muscle are on average ∼0.8 μm, and range from ∼0.4 to 1.2 μm. That such reduction in thin filament length greatly affects force production was illustrated by Ottenheijm et al. (2009) by plotting force as a function of sarcomere length for both wildtype and nebulin-deficient muscle. In these experiments, skinned muscle fibers were activated by exogenous calcium at various sarcomere lengths and the force response was measured (note that in skinned fiber preparations factors outside of the myofilaments, e.g., calcium handling by the sarcoplasmic reticulum, do not contribute to force production). The force-sarcomere length relation of wildtype muscle is characterized by a force plateau reflecting optimal thick–thin filament overlap, followed by a descending limb at higher sarcomere lengths reflecting the decreased filament overlap. That the descending limb in wildtype muscle starts at a sarcomere length of ∼2.6 μm and ends at ∼4.0 μm suggests a thin filament length of ∼1.2 μm, which is in line with the previously mentioned electron microscopy data. In nebulin-deficient muscle, the shortened thin filaments reduce thin–thick filament overlap at a given sarcomere length, impairing force production and resulting in a leftward shift of the force-sarcomere length relation (see **Figure 2B**). Furthermore, when thin filaments are non-uniform in length no optimal thick–thin filament overlap exists, and therefore the force-sarcomere length relation of nebulin-deficient muscle lacks the characteristic plateau. Consistent with these findings on demembranated muscle, studies on *intact* nebulin-deficient muscle from another nebulin knockout model (Gokhin et al., 2009), in which muscles were activated at various lengths by electrical field stimulation, also revealed a leftward shift of the force-muscle length relation of nebulin-deficient muscle. Thus, the force-length relation of nebulin-deficient muscle is altered in a manner that is consistent with the presence of shorter thin filament lengths.

Work by Bang et al. (2006) on their nebulin KO model, using confocal microscopy on 1-day-old mice, indicated that in the absence of nebulin thin filament lengths are reduced from ∼1.15 to 1.3 μm (depending on muscle type) in wildtype muscle to a consistent ∼1.0 μm in all muscles types. These findings led to the proposal (Littlefield and Fowler, 2008) that a nebulin-independent mechanism specifies uniform thin filament lengths of ∼1.0 μm in all muscle types, whereas nebulin is responsible for specifying longer thin filament lengths in a muscle-specific manner. An opposite conclusion was drawn by Castillo et al. (2009) who used immuno-fluorescence microscopy on rabbit muscle and concluded that nebulin specifies the minimum thin filament length (∼1.0 μm) with a nebulin-independent mechanism regulating the final length according to the requirements of a particular muscle. Similar conclusions were drawn recently by Gokhin et al. (2012), showing that human thin filaments have nebulin-free pointed-end extensions that comprise up to 30% of total thin filament length,

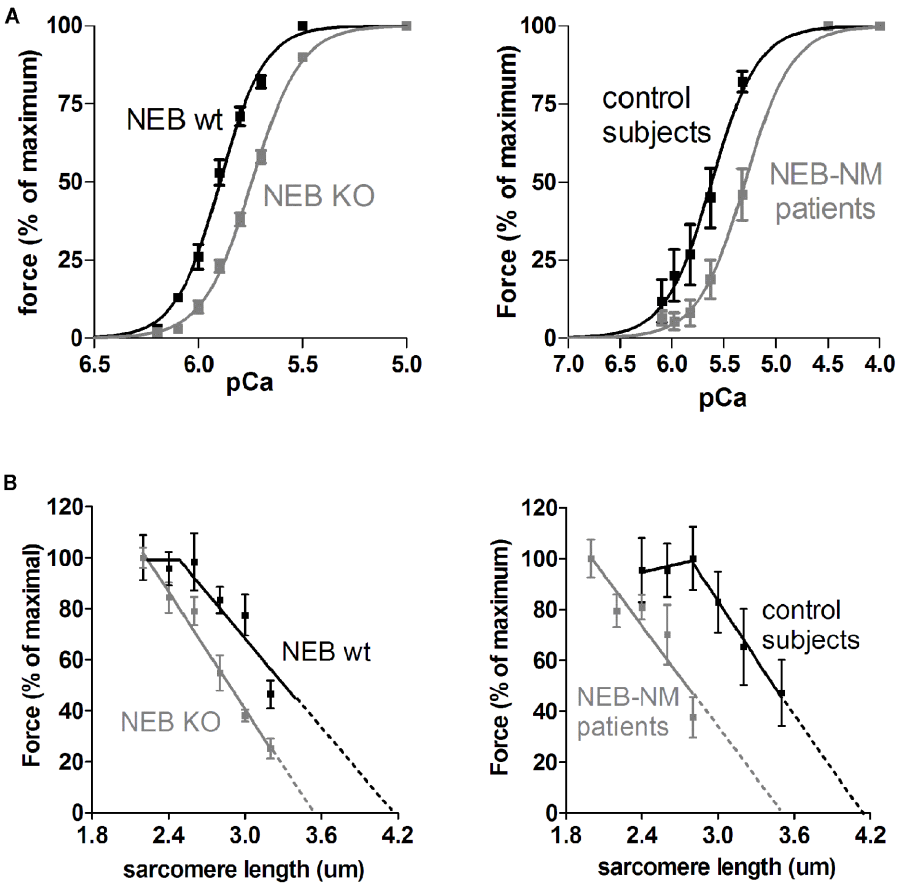

FIGURE 2 | (A) Force–Ca^{2+} characteristics of murine skinned nebulin-deficient muscle fibers from m. tibialis cranialis (NEB-KO, left panel), and of skinned muscle fibers from m. quadriceps of patients with nebulin-based nemaline myopathy (NEB-NM, right panel). Note that the force generated in response to incubation with incremental increase of $[Ca^{2+}]$ is significantly decreased in both NEB-KO and NEB-NM tissue, resulting in a rightward shift of the force–Ca^{2+} relationship (Figures adapted from Chandra et al., 2009; Ottenheijm et al., 2010). **(B)** Left: the force-sarcomere length relation of murine wt (NEB-wt) fibers has a characteristic force plateau followed by a descending limb. The force-sarcomere length relation of NEB-KO fibers is shifted leftward compared to control fibers, and the force plateau is absent. Right: the force-sarcomere length relation of muscle fibers from NM-NEB patients is shifted to the left and is very similar to that found in NEB-KO fibers (Figures adapted from Ottenheijm et al., 2008).

and by Pappas et al. (2010) who showed that when endogenous nebulin is replaced with a mini-nebulin in skeletal myocytes, thin filaments extend beyond the end of mini-nebulin. It is unclear as to what causes this apparent discrepancy between these studies. Detecting differences in thin filament length, especially length gradients, with high precision is challenging and might be hard to accomplish with confocal microscopy, as was done in the two aforementioned studies. In contrast, Witt et al. (2006) used electron microscopy and decorated thin filaments with gold beads (attached to actin monomers with the actin-binding peptide phalloidin). This made it possible to determine thin filament length gradients and showed that thin filaments varied in length and were on average shorter than in wildtype muscle. Thus, this study does not support the conclusion by Bang et al. (2006) that there is a nebulin-*independent* mechanism that sets a constant thin filament length of 1.0 μm, but is consistent with Castillo et al. that there is a nebulin-dependent mechanism that sets a minimum thin filament length. To resolve these discrepancies additional studies on a range of mouse muscle types are needed that measure by electron microscopy thin filament length and the location of nebulin's N-terminus. It is clear, however, from the above referenced studies that nebulin does play a critical role in regulating thin filament length: in its absence the average thin filament length is shorter and force is reduced.

NEBULIN AS REGULATOR OF Z-DISK STRUCTURE

To understand the layout of the C-terminal region of nebulin in the Z-disk, immunoelectron microscopy (IEM) has been used on human soleus muscle and nebulin's C-terminus has been labeled with the nebulin-specific anti-SH3 antibody and the more N-terminal M177–181 domains with anti-neb177–181 (Millevoi et al., 1998). Results showed that the nebulin SH3 domain is located about 25 nm inside the Z-disk, and the repeats neb176 to neb181 near to the edge of the Z-disk. These results are consistent with two distinct models of the layout of nebulin in the Z-disk (for details, see Millevoi et al., 1998; **Figure 1**). In the first model, nebulin penetrates the Z-disk by only about 25 nm, and nebulin filaments from neighboring sarcomeres do not overlap (*no-overlap model*). In the

second model, nebulin penetrates the Z-disk by about 100 nm and this creates a ∼75 nm wide nebulin overlap zone in the center of the Z-disk (*overlap model*). It has been argued that the *no-overlap model* is more likely to be correct (Millevoi et al., 1998), but definite experimental evidence for either model is lacking. In a more recently proposed model, nebulin fully penetrates the Z-disk and cross-links thin filaments from adjacent sarcomeres (Pappas et al., 2008). A drawback of this model is that it predicts that the SH3 epitope is further from the center of the Z-disk than the M177–181 epitope, which is opposite of what has been measured. It also remains to be explained why nebulin leaves one actin filament to which it is anchored and moves to a neighboring filament, and how the thin filament spanning region of nebulin accommodates the myofilament lattice spacing changes that occur during muscle contraction. Clearly further work is needed to map the layout of nebulin in the Z-disk.

Evidence suggests that nebulin's C-terminus regulates Z-disk width. Z-disks of different muscles can vary greatly in width, from less than 100 nm in fast skeletal muscle to more than 150 nm in slow skeletal muscle (Tonino et al., 2010). The importance of regulation of Z-disk width is illustrated by muscle from patients with nemaline myopathy, which displays greatly widened Z-disks, including the characteristic nemaline rods (Wallgren-Pettersson et al., 2004). Previously titin has been suggested to play a role in Z-disk assembly (Gautel et al., 1996). The Z-disk region of titin contains a family of differentially expressed repeats, the titin Z-repeats (Gautel et al., 1996). These Z-repeats are a family of α-actinin-binding motifs, which are differentially expressed in a tissue- and developmental-stage-specific fashion (Gautel et al., 1996). As previously pointed out, it is unlikely that the differential expression of the titin Z-repeats alone can determine the Z-disk width, because too few isoforms exist to account for the wide range of different Z-disk widths (Millevoi et al., 1998). cDNA sequencing of rabbit nebulin has demonstrated that the Z-disk region of nebulin is differentially expressed (Millevoi et al., 1998). Several different isoforms have been identified that result from the skipping of various combinations of seven Z-disk domains (Millevoi et al., 1998), leading to the suggestion that nebulin is one of several proteins that are important for Z-disk width regulation (Millevoi et al., 1998). Consistent with this notion, the ultrastructural characterization of the Z-disk in NEB-KO mice revealed that average Z-disk width is increased by 40–80 nm depending on muscle type (Bang et al., 2006; Witt et al., 2006; Tonino et al., 2010). In addition, inclusion-like bodies of mis-assembled Z-disks are detected that resemble the rod bodies that are a hallmark of nemaline myopathy (Bang et al., 2006; Witt et al., 2006). Further support was obtained by a recent study on nebulin in a range of muscle types during postnatal development of the mouse in which transcript studies were performed with a mouse nebulin exon microarray (Buck et al., 2010). During postnatal development of the soleus muscle major changes in splicing were detected in the Z-disk region of nebulin. Three differentially spliced Z-disk exons were upregulated during postnatal development of soleus muscle and this correlated with a significant increase in Z-disk width. The increase in Z-disk width of the soleus muscle might reflect the increasing stress exerted on this muscle due to the rapid increase in body weight during postnatal development.

Together, these findings support a model in which titin and nebulin together specify Z-disk width, with titin constructing the central region of the Z-disk, including the number and positions of α-actinin cross-links and nebulin determining the ending of the Z-disk structure and its transition to the I-band, i.e., nebulin functions as a Z-disk terminator. The mechanism by which nebulin terminates the Z-disk might involve interaction between nebulin and Z-disk-localized proteins, such as CapZ. CapZ is a barbed-end actin capping protein that binds near the C-terminus of nebulin (Pappas et al., 2008). In muscle fibers devoid of nebulin (Witt et al., 2006; Pappas et al., 2008), CapZ does not localize properly, allowing the barbed ends of thin filaments to continue to grow beyond the Z-disk resulting in widened Z-disks. Thus, nebulin is involved in regulating Z-disk width. When this feature of nebulin is not present, Z-disks widen, ultimately culminating in the formation of nemaline rods.

NEBULIN MAINTAINS INTERMYOFIBRILLAR CONNECTIVITY

In addition to regulating Z-disk width and structure, nebulin is suggested to play a role in laterally linking myofibrils at the Z-diks. Longitudinally, sarcomeres are connected by Z-disk lattices that anchor thin filaments and transmit force along the myofibril. In the transverse direction, linkage of myofibrils at the Z-disks allows for lateral force transmission and limits the degree to which adjacent myofibrils translocate relative to each other during active contraction or passive stretch, thereby preventing damage to intermyofibrillar membrane systems, such as T-tubules and the sarcoplasmic reticulum. An important protein involved in linking adjacent Z-disks is the intermediate filament protein desmin, which forms a network of filaments that surrounds myofibrils at the level of the Z-disk (Wang and Ramirez-Mitchell, 1983; Capetanaki et al., 2007). The subunit proteins of desmin filaments are elongated coiled-coils with extensive intermolecular ionic and hydrophobic interactions between individual subunits, giving rise to filaments with high tensile strength as well as plasticity (Costa et al., 2004). That desmin tethers adjacent Z-disks is supported by work on a desmin KO mouse in which Z-disk misalignment was shown to occur in stretched muscle (Shah et al., 2002). *In vitro* work, using a yeast two-hybrid approach, suggested that desmin binds to the C-terminal region of nebulin (Bang et al., 2002), which is anchored in the Z-disk, and recently it was shown that nebulin modules M160–170 are involved in this interaction (Conover et al., 2009; Conover and Gregorio, 2011). These findings lead Bang et al. to speculate that this desmin-nebulin interaction links myofibrillar Z-disks to the intermediate filament system, thereby forming a lateral linkage system which maintains adjacent Z-disks in register. This role for nebulin in intermyofibrillar connectivity was tested recently by studies using a nebulin KO mouse model (Tonino et al., 2010). In these studies it was found that upon stretch, myofibrils devoid of nebulin translocate to a much higher degree than WT muscle, resulting in much larger Z-disk displacement. Although desmin is present in muscle devoid of nebulin, it is reduced in the intermyofibrillar spaces that surround the Z-disks, suggesting that nebulin is required for proper localization of desmin at the Z-disk. Consistent with this, both knockdown of nebulin with siRNA and overexpression of M160–M170 did not interfere with the formation of normal striation patterns but did prevent

desmin localization at the mature Z-disk. Thus, nebulin is required to laterally link myofibrils at the Z-disk by desmin filaments; in the absence of nebulin myofibrillar connectivity is significantly reduced leading to Z-disk displacement.

NEBULIN AND MUSCLE HYPERTROPHY

Exciting recent work revealed that nebulin's C-terminal SH3 domain is involved in the induction of muscle hypertrophy. Takano et al. (2010) found that Insulin-like growth factor 1 induces skeletal muscle maturation and hypertrophy by forming a complex of nebulin and N-WASP at the Z-disks of myofibrils by interfering with glycogen synthase kinase-3β. Their results convincingly show that N-WASP interacts with nebulin's SH3 domain, and that N-WASP is rapidly recruited to the Z-disk after treatment with insulin-like growth factor 1, which stimulates myofibrillogenesis and suppresses sarcomere breakdown via the PI3K–Akt pathway. The notion that nebulin is important in maintaining muscle mass is consistent with the previous observation that nebulin-deficient mice display severe muscle fiber atrophy (Bang et al., 2006; Witt et al., 2006) – although thin filament instability caused by nebulin-deficiency is likely to be involved as well in the mechanisms underlying muscle atrophy.

NEBULIN REGULATES CONTRACTION

Recent work shows that nebulin's role is not merely structural, but that nebulin also regulates contraction. Muscle contraction is driven by the cyclic interaction between the myosin-based cross-bridges and actin and the level of force a muscle generates is proportional to the force generated per crossbridge and the number of crossbridges in the force generating state. It is generally accepted that this interaction between actin and myosin is regulated through a steric hindrance mechanism in which tropomyosin and troponin control the conversion between interaction permissive and non-permissive states (Gordon et al., 2000). This view needs to be extended as two independent studies (Bang et al., 2009; Chandra et al., 2009) recently identified that nebulin contributes to the regulation of crossbridge cycling kinetics, with one of the two (Chandra et al., 2009) also identifying a role for nebulin in the calcium sensitivity of force generation.

During the crossbridge cycle, unbound non-force generating crossbridges move to an actin-bound force generating state followed by ATP-driven crossbridge release back to the non-force generating state (Huxley and Simmons, 1971; Lymn and Taylor, 1971). Brenner et al. (Brenner, 1988) proposed an analytical framework in which this transition between force and non-force generating crossbridge states can be described by two apparent rate constants; one for crossbridge attachment (f_{app}) and one for crossbridge detachment (g_{app}). These two rate constants determine the fraction of force generating crossbridges during activation, and a change in one or both will affect this fraction and thus force production. g_{app} is directly proportional to the ATP consumption rate normalized to tension generation (i.e., tension cost), and can therefore be estimated from the simultaneous determination of ATP consumption rate and tension in activated muscle fibers. Such studies on nebulin-deficient muscle (Chandra et al., 2009) revealed significantly higher tension cost in nebulin-deficient muscle, thus indicating a faster g_{app} and crossbridge detachment rate when nebulin is absent. Likewise, studies on another nebulin KO mouse model (Bang et al., 2009) reported higher velocity of unloaded shortening in nebulin-deficient muscle, also suggesting that g_{app} is higher when nebulin is absent. It is also important to highlight that these findings are consistent with results of *in vitro* motility assays in which nebulin fragments were found to reduce the sliding velocity of F-actin over myosin (Root and Wang, 1994).

In Brenner's framework (Brenner, 1988), the rate constant of force redevelopment (Ktr) is proportional to $f_{app} + g_{app}$, and the fraction of force generating crossbridges to $f_{app}/(f_{app} + g_{app})$. The rate constant of force redevelopment can be estimated by imposing a rapid release-restretch protocol on an activated fiber, mechanically disengaging all bound crossbridges so that force drops to zero and then measuring force redevelopment. Such experiments revealed that force redevelopment is slower in nebulin-deficient muscle (Bang et al., 2009; Chandra et al., 2009). Thus, the decrease in Ktr of nebulin-deficient muscle, together with the notion that g_{app} is increased, indicates that f_{app} must be reduced and that the reduction must be larger than the increase in g_{app}. Combined, this leads to the conclusion that the fraction of force generating crossbridges $[f_{app}/(f_{app} + g_{app})]$ is reduced in nebulin-deficient muscle. Furthermore, stiffness measurements indicated that the force per crossbridge was not affected by the absence of nebulin (Bang et al., 2009; Chandra et al., 2009). In summary, recent studies suggest that nebulin increases the rate of crossbridge attachment and reduces the rate of crossbridge detachment, and that as a result the number of force generating crossbridges is increased. Although the mechanism by which nebulin affects crossbridge cycling needs further investigation, previous work (Root and Wang, 1994) has shown that nebulin associates with the actin N-terminus in subdomain 1, where also the myosin crossbridge binds. Thus, the presence of nebulin at or near the S1 binding site might enhance the binding of crossbridges and slow their detachment. Chandra et al. (2009) estimated that the effect of nebulin on crossbridge kinetics enhances a muscle's force generating capacity by ∼50%, and increases the economy of contraction by ∼35%. These estimations are in line with findings reported by Bang et al. (2009), and they can largely account for the more pronounced leftward shift of the *measured* force-sarcomere length relation of nebulin-deficient muscle when compared to the *predicted* relation based on only thin filament length measurements. Clearly, nebulin is a major factor in determining the level of force and the energetic cost of force production in skeletal muscle. In line with this role of nebulin in the regulation of crossbridge cycling kinetics, recent studies on muscle fibers from patients with nemaline myopathy with severely reduced nebulin protein levels revealed that in addition to altered thin filament length, changes in crossbridge cycling kinetics contribute to the muscle weakness observed in these patients (Ottenheijm et al., 2010).

The studies discussed above were carried out at a maximal activating calcium level. Chandra et al. (2009) also measured active force at a range of calcium levels and the obtained force-pCa relations were markedly shifted to the right in nebulin-deficient muscle fibers (see **Figure 2A**), with a 0.16 unit reduction in pCa_{50} (pCa that gives the half-maximal force level). To

rule out a possible difference in the troponin complex (isoform composition and posttranslational modification) between wt and KO fibers, these studies were carried out on wildtype and KO fibers that had been reconstituted by the same recombinant troponin complex. An analysis of expression levels of tropomyosin, myosin heavy chain, myosin light chain did not reveal a significant difference in these proteins between the fibers, suggesting that the absence of nebulin is the most likely explanation for the lower calcium sensitivity of the KO fibers. Interestingly, the studies by Witt et al. (2006) and Bang et al. (2009) found no difference in calcium sensitivity. It is possible that the discrepancy is due to the fact that those studies did not carry out a troponin exchange and that differences in the troponin complex between wt and KO fibers could have negated nebulin's effect on calcium sensitivity. An alternative explanation involves the difference in sarcomere length between the studies. The two studies that did not detect a difference in calcium sensitivity were carried out at long sarcomere lengths [$\sim$2.5 μm (Witt et al., 2006) and $\sim$2.6 μm (Bang et al., 2009)] whereas the study that did show a difference (Chandra et al., 2009) was performed at $\sim$2.0 μm. The implication is that nebulin plays a role in the length dependence of activation with a much larger ΔpCa_{50} in the nebulin KO fibers than in wt fibers. It is a well known phenomenon that as sarcomere length increases muscle becomes more calcium sensitive. This length dependence of activation is most prominent in cardiac muscle (and is thought to underlie the Frank–Starling law of the heart) but is much less pronounced in skeletal muscle (Konhilas et al., 2002). The presence of nebulin provides an explanation for why skeletal muscle has less length dependence: the presence of nebulin increases calcium sensitivity at short length. Thus nebulin is an important player in a wide range of skeletal muscle characteristics.

The structure and protein binding properties of nebulin support a role in thin filament activation. Nebulin contains $\sim$200 domains of $\sim$35 amino acids that are characterized by the actin-binding sequence SDXXYK; these domains make up seven domain super-repeats characterized by the Tm/Tn binding motif, WLKGIGW (McElhinny et al., 2003). Biochemical studies have shown that a single nebulin module interacts with a single actin monomer and that each nebulin super-repeat interacts with a Tm/Tn complex of the thin filament (Ogut et al., 2003), binding characteristics that support that nebulin follows the helical path of F-actin. It is interesting that similar to Tm, nebulin appears to have different binding sites on F-actin with one site in close proximity to both the strong binding site for myosin and the blocked state of tropomyosin (Lukoyanova et al., 2002). This leads to the intriguing possibility that nebulin acts in concert with Tm and that it promotes the transition of contractile regulatory units (Tm/Tn) from the non-permissive to the permissive states, thereby increasing myofilament calcium sensitivity.

It is striking that nebulin-deficient skeletal muscle shares the low calcium sensitivity and low maximal active force with cardiac muscle, where stoichiometric levels of nebulin are absent (Bang et al., 2006; Witt et al., 2006). Cardiac muscle contains the nebulin-homolog nebulette (Moncman and Wang, 1995). However, nebulette is much smaller than nebulin ($\sim$100 vs $\sim$800 kDa for nebulin) and its location is restricted to the Z-disk and the near Z-disk I-band region (Millevoi et al., 1998). Thus, the thin filament in cardiac muscle is largely nebulette-free. Although it can not be ruled out that nebulette induces long-range conformational effects that propagate along the thin filament to affect its function, such effects would have to be first demonstrated in a direct manner, and in absence of such findings we consider it not likely that nebulette is intimately involved in thin filament activation. Cardiac muscle has multiple mechanisms for enhancing thin filament activation, such as, enhanced length-dependent activation, and the presence of multiple cardiac-specific phosphorylation sites in various thin and thick filament based protein sites (Solaro and de Tombe, 2008), which allow cardiac muscle to grade its force response to different loading conditions. Since skeletal muscle lacks the aforementioned features unique to cardiac muscle, nebulin might be essential for tuning thin filament activation for optimal skeletal muscle function.

Changes in calcium homeostasis have also been noted in nebulin-deficient muscle. A striking upregulation of sarcolipin (SLN), an inhibitor of SERCA, occurs in NEB-KO mice (Bang et al., 2006; Gokhin et al., 2009; Ottenheijm et al., 2008). This upregulation might be viewed as an adaptation in NEB-KO mice as an attempt to increase cytosolic calcium levels and counteract reduced myofilament calcium sensitivity. The mechanism by which nebulin-deficiency upregulates SLN requires further future studies, as does the functional role of SLN upregulation, for example by crossing SLN-KO mice and nebulin KO mice. Taken together, the above discussed studies clearly indicate that nebulin is not merely involved in regulation sarcomere structure, but also acts as a regulator of muscle contraction.

NEBULIN AND NEMALINE MYOPATHY

Dysfunctions of the skeletal muscle thin filament have emerged as important causes of skeletal myopathies of which nemaline myopathy is the most common disease with a previously estimated prevalence of 0.002% (Sanoudou and Beggs, 2001). However, because of its heterogeneous nature and often milder phenotypes, the actual frequency of nemaline myopathy in older patients may be considerably higher. Genetically, NM is heterogeneous and so far seven genes have been identified as NM-causing, namely alpha-tropomyosin-3 and beta-tropomyosin (TPM3 and TPM2), NEB, actin alpha 1 (ACTA1), troponin T type 1 (TNNT1), cofilin-2, and kbtbd13 (Laing et al., 1992, 1995; Nowak et al., 1999; Pelin et al., 1999; Ryan et al., 2001; Wallgren-Pettersson et al., 2002, 2004; Agrawal et al., 2007; Sambuughin et al., 2010). An unbiased linkage analysis of 45 NM families derived from 10 different countries implicated nebulin mutations as disease causing in 41 of these families. The typical form of NM presents with early onset in infancy, has a non-progressive or slowly progressive course and is caused by mutations in the nebulin gene. These mutations are often missense mutations, but in addition a 2502-bp deletion causing a 33-residue in frame deletion of exon 55 was identified as a prominent recessive disease causing allele initially in the Ashkenazi Jewish population, and more recently also in non-Jewish patients (Anderson et al., 2004; Lehtokari et al., 2006, 2009).

Recent studies on muscle fibers from patients with nemaline myopathy due to the deletion of nebulin exon 55 showed that these patients have severely reduced nebulin protein levels and that they show remarkable phenotypic similarities to fibers from nebulin KO mice, i.e., shorter and non-uniform thin filament lengths (**Figure 2B**) and significantly impaired force generating capacity (Ottenheijm et al., 2009). Thus, loss of thin filament length regulation appears to be an important contributor to muscle weakness in patients with nemaline myopathy. In addition to altered thin filament length, changes in crossbridge cycling kinetics and reduced calcium sensitivity of force production contribute to the muscle weakness observed in these patients (see **Figure 2A**; Ottenheijm et al., 2010), in line with the role of nebulin in these processes (Chandra et al., 2009; Ottenheijm and Granzier, 2010). Thus, nebulin's role in thin filament length regulation and contraction deduced from work on NEB-KO mice provides a mechanism for the first time to explain severe muscle weakness in patients with nemaline myopathy. Interestingly, recent work form our lab demonstrates that patients with nemaline myopathy due to mutations in the tropomyosin gene (TPM3) display a contractile phenotype that is distinct from that of patients with *NEB*-based myopathy (Ottenheijm et al., 2011). Whereas both show severe myofilament-based muscle weakness, the contractile dysfunction in *TPM3*-based myopathy can be largely explained by changes in crossbridge cycling kinetics, whereas dysregulation of thin filament length and altered crossbridge cycling kinetics play a prominent role in *NEB*-based myopathy (Ottenheijm et al., 2009). Furthermore, the loss of force generating capacity in *TPM3*-based myopathy appears to be partly compensated by enhanced calcium sensitivity of force generation, whereas decreased calcium sensitivity of force generation further depresses the capacity for force production in *NEB*-based myopathy (Ottenheijm et al., 2010). Whereas these findings support the existence of distinct genotype-phenotype correlations in NM, it is yet unclear whether they extrapolate to other patients with mutations in *NEB* or *TPM3*, and how they compare to the phenotype of NM patients with mutations in *ACTA1*, *TPM2*, *TNNT1*, *CFL2*, and *KBTBD13*. Understanding the genotype-phenotype correlations is important, as it allows the development of genotype-targeted treatment strategies. For example, analogous to current clinical studies that address whether contractility during heart failure can be augmented by actomyosin activating small molecules (Malik et al., 2011), a rationale for similar approaches in *NEB*-based nemaline myopathy now exists.

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A physiologically based, multi-scale model of skeletal muscle structure and function

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Models of skeletal muscle can be classified as phenomenological or biophysical. Phenomenological models predict the muscle's response to a specified input based on experimental measurements. Prominent phenomenological models are the Hill-type muscle models, which have been incorporated into rigid-body modeling frameworks, and three-dimensional continuum-mechanical models. Biophysically based models attempt to predict the muscle's response as emerging from the underlying physiology of the system. In this contribution, the conventional biophysically based modeling methodology is extended to include several structural and functional characteristics of skeletal muscle. The result is a physiologically based, multi-scale skeletal muscle finite element model that is capable of representing detailed, geometrical descriptions of skeletal muscle fibers and their grouping. Together with a well-established model of motor-unit recruitment, the electro-physiological behavior of single muscle fibers within motor units is computed and linked to a continuum-mechanical constitutive law. The bridging between the cellular level and the organ level has been achieved via a multi-scale constitutive law and homogenization. The effect of homogenization has been investigated by varying the number of embedded skeletal muscle fibers and/or motor units and computing the resulting exerted muscle forces while applying the same excitatory input. All simulations were conducted using an anatomically realistic finite element model of the tibialis anterior muscle. Given the fact that the underlying electro-physiological cellular muscle model is capable of modeling metabolic fatigue effects such as potassium accumulation in the T-tubular space and inorganic phosphate build-up, the proposed framework provides a novel simulation-based way to investigate muscle behavior ranging from motor-unit recruitment to force generation and fatigue.

Keywords: skeletal muscle mechanics, multi-scale, continuum mechanics, excitation-contraction coupling, motor-unit recruitment, tibialis anterior

1. INTRODUCTION

Research on investigating and analyzing functional or structural properties of skeletal muscles, e.g., fatigue, injury, aging, or muscle fiber composition, focuses almost entirely on *in vitro* or *in vivo* experiments. The restricted knowledge on the underlying complex mechanisms and their causal correlations often fosters a research environment focusing on mechanisms and components in isolation. Each knowledge gain is invaluable and provides a valuable step toward understanding skeletal muscle mechanics and the musculoskeletal system as a whole. However, if experimentalists worked hand-in-hand with theorists to exploit the dormant power of detailed biophysical computer models, it might be possible to approach this goal much quicker and more efficiently.

Using computer simulations in conjunction with experimental findings can provide an invaluable tool to test and evaluate complex hypothesis and conclusions. Comprehensive *in silico* analysis are able to identify important aspects or correlations needing further insights, and hence provide, *a priori*, valuable information for experimental research.

The limiting factor of combining *in vivo* or *in vitro* experiments with *in silico* ones is often the acceptance and/or the simplicity of existing (detailed) biophysical models. Moreover, in case of analyzing muscle fatigue, injury, or aging of skeletal muscles, the models need to extend beyond modeling "only" skeletal muscle tissue. They need to touch and embrace related research areas and fields. For example, comprehensive skeletal muscle models should also take into account neurophysiological aspects, such as motor-unit recruitment principles, functional aspects of motor unit, and muscle fiber type distributions, sub-cellular mechanism, as well as the mechanical behavior of adjacent tissue, and/or the dynamics of (parts of) the musculoskeletal system.

Current computational models of skeletal muscle models do typically either focus on sub-cellular processes of a (half) sarcomere or on simplified phenomenological relationships mimicking the overall (mechanical) behavior of a single skeletal muscle. Based on the focus of the respective skeletal muscle models, one can divide most of the existing skeletal muscle models into two very broad categories: (i) biophysically based models and (ii)

phenomenologically based models. Biophysically based skeletal muscle models calculate the output of skeletal muscle using an analysis of intrinsic physiological properties (e.g., Hodgkin and Huxley, 1952; Huxley, 1957). Phenomenological ones use mathematical representations to describe the relationships between input and output properties (e.g., Hill, 1938; Winters and Stark, 1987) – mainly to describe the mechanical properties of skeletal muscle.

Phenomenological models often use the findings of experimentalists to describe their input-output relationship and hence are less suitable for testing hypothesis and assumptions on the same scale, e.g., the scale of a single skeletal muscle. However, they can provide great insights into the applicability and validity of the input-output relationships by using these models on larger scales, e.g., the musculoskeletal system. Hill-type skeletal muscle models (e.g., Hill, 1938; Winters and Stark, 1987) appeal, for example, to a simple mechanical system, e.g., often to a three-element spring-dashpot system, to describe the force-generating properties of skeletal muscles. Clearly, they cannot be used to investigate intrinsic properties of skeletal muscle force generation, but they can be used to test the force-generating relationships of single skeletal muscles in the overall musculoskeletal system, e.g., to answer the question of whether experimentally derived force relationships are sufficient to act against physiological loads. Hill-models, like any other model, do exhibit modeling deficiencies; here for example on the organ system scale (the musculoskeletal system), where they cannot account for the three-dimensional structural complexity of an individual muscles and therefore cannot account for its interaction with surrounding tissue. Further, the model reduces a skeletal muscle to a force acting between an insertion point and an origin point, i.e., to an one-dimensional object.

Recently, full three-dimensional models of skeletal muscles have been created by a number of authors (Johansson et al., 2000; Oomens et al., 2003; Blemker et al., 2005; Lemos et al., 2005; Röhrle and Pullan, 2007; Böl and Reese, 2008). These models have led to a fuller understanding of muscle force distributions. The three-dimensional nature of the models resulted in the ability to analyze dynamic changes to the line of muscle action that cannot be determined from the more common one-dimensional models (Röhrle and Pullan, 2007), as well as elucidating possible causes of non-linear muscle strains (Blemker et al., 2005). These models are all based on the principles of continuum mechanics and result in macroscopic models that do not explicitly include any information from finer scales, e.g., the cellular level. This finer detail, however, is required to represent the changes in muscle properties as a result of disease or injury. The continuum representation also prohibits the use of functional information, which is important for rehabilitation techniques (e.g., functional electrical stimulation). Examples of such functional information include, motor-unit distributions, fiber firing rates, and different locations of fiber types. Furthermore, continuum-mechanical models cannot account for physiological changes, e.g., fatigue induced changes to mechanical output. Within these continuum-mechanical models, fatigue can essentially only be considered in a phenomenological way (e.g., Böl et al., 2009). This however, is of limited use, if one wants to jointly investigate functional and physiological aspects in conjunction with experimental studies.

More recently, researchers have focused on extending the continuum-mechanical models by taking into account the underlying electrophysiology. The aim is to drive the mechanics of full three-dimensional skeletal muscle models by electro-physiological principles, i.e., Fernandez et al. (2005) and Böl et al. (2011). These models, while providing more realistic responses, however do not fully represent the detailed electrophysiology (electrically isolated, independent motor units) of skeletal muscle.

The shortcoming of linking an electrical stimuli at the neuromuscular junction with mechanical output through a biophysically based cellular model, which is also capable of mimicking muscle fatigue, was first addressed in Röhrle et al. (2008) and then further extended to a specific muscle geometry in Röhrle (2010). Röhrle et al. (2008) also demonstrated on a cube that the proposed homogenization methodology, which links the output of a detailed biophysical model to a continuum-mechanical constitutive law, is feasible and delivers for different finite element (FE) discretizations FE convergence rates, which are comparable to a continuum-mechanical model. The key difference of Röhrle et al. (2008) and Röhrle (2010) to all other existing electromechanical skeletal muscle models (e.g., Fernandez et al., 2005; Böl et al., 2011) is the fact that in the proposed model the active contribution within the continuum-mechanical constitutive law is directly coupled to a detailed skeletal muscle model of the (sub-)cellular processes (Shorten et al., 2007) through a multi-scale constitutive law.

This paper aims to extend the framework proposed by Röhrle et al. (2008) and Röhrle (2010) to include a much larger array of anatomical and physiological properties; properties that are the key to modeling the underlying mechanisms behind many diseases and rehabilitation techniques. The main focus therefore is to provide the fundamental algorithms and modeling considerations for incorporating muscle fiber and motor-unit distributions within skeletal muscles and the ability to link a neurophysiological model of motor-unit recruitment to the electromechanical model. The methodology, here enhanced by structural and functional components, is applicable to any skeletal muscle. Herein, the feasibility of developing such a framework is demonstrated on the tibialis anterior (TA) muscle. The result is one of the most advanced and detailed skeletal muscle model currently available.

Such a detailed model becomes necessary, if one strives to obtain a deeper understanding of skeletal muscle function during muscle recruitment and to obtain a better understanding of how the interplay between muscle fiber recruitment mechanisms and mechanical force generation can be affected by alterations to the underlying muscle properties. This model will be able to provide for many different fields a framework capable of investigating injuries and disease processes that affect skeletal muscle encompassing the cellular composition, the functional recruitment processes, and the gross mechanical structure. Moreover, the proposed model will have potential to generate impact to the general field of computational neuroscience, in which all state-of-the-art recruitment models link their findings to simplistic muscle force models neglecting any kind of spatial characteristics. In general, the modular structure of the proposed framework shall allow easy model adaptations, such that the overall framework can be successfully applied to many different fields of musculoskeletal research.

2. MATERIALS AND METHODS

The skeletal muscle framework proposed in Röhrle et al. (2008) and Röhrle (2010) focused on developing the underlying methodology to embed electrically isolated skeletal muscle fibers within a three-dimensional skeletal muscle geometry and to link their cellular behavior to mechanical output. Based on the same tibialis anterior mesh as introduced in Röhrle (2010; cf. Section 2.1.1), the modeling framework presented herein is extended by the following anatomical and physiological enhancements:

1. Grouping of skeletal muscle fibers into functional units, i.e., the motor units (cf. Section 2.1.2),
2. fiber type segregation of motor units into fast- or slow-twitch type (Henneman and Olson, 1965; Wuerker et al., 1965; Andreassen and Arendt-Nielsen, 1987; Monti et al., 2001; Duchateau et al., 2006),
3. a physiological distribution of fibers within motor units (Enoka and Fuglevand, 2001),
4. an anatomically based spatial distribution of fibers into motor units (Bodine-Fowler et al., 1990; Roy et al., 1995; Monti et al., 2001),
5. motor-unit territory sizes proportional to the number of fibers per motor unit (Fuglevand et al., 1993; Roy et al., 1995; Yao et al., 2000; Monti et al., 2001),
6. recruitment of each skeletal muscle fiber of a particular motor unit through the neural input of an α-motor neuron (cf. Section 2.4), and
7. controlling the muscle force output by recruitment and rate coding of motor units (Fuglevand et al., 1993).

In summary, the proposed modeling framework encompasses (i) an anatomically based, three-dimensional mechanical model of a skeletal muscle, (ii) the electrophysiology of a single muscle fiber, (iii) the coupling of the electrophysiology (cellular) to the mechanical description through a cellular based multi-scale constitutive law, and (iv) the mechanical response of an entire muscle due to neural stimulation using a phenomenological model of motor-unit recruitment.

2.1. A THREE-DIMENSIONAL, ANATOMICALLY BASED MUSCLE GEOMETRY

2.1.1. The FE mesh and the embedding of the muscle fibers

The basis of this framework is a three-dimensional, anatomically based representation of the TA muscle. To generate the geometrical representation of the muscle, two-dimensional photographic slices of the Visible Human male data set (Spitzer and Whitlock, 1998) were manually digitized to create a three-dimensional data cloud. The data cloud was used to fit a three-dimensional quadratic Lagrange FE mesh using a least-squares minimization algorithm. For details about the fitting algorithm, the reader is referred to Bradley et al. (1997). The generated FE mesh distinguishes between the superficial and deep compartments of the TA, which are separated through an aponeurosis, and is depicted in **Figure 1**. To simplify the embedding of the muscle fibers, the FE mesh of the TA has been constructed in such a way that the muscle fibers within the TA will be aligned with a direction of the local FE coordinate system of each element. All fibers within the three-dimensional FE mesh are represented as one-dimensional lines consisting of evenly spaced grid points. Moreover, all fibers are assumed to have the same cross-sectional fiber area. The necessary fiber angle data for achieving the respective fiber angles within the FE mesh are taken from the fiber pennation angle data published in Lansdown et al. (2007). The pennation angle measurements in Lansdown et al. (2007) are based on *in vivo* diffusion-tensor magnetic resonance imaging (DT-MRI).

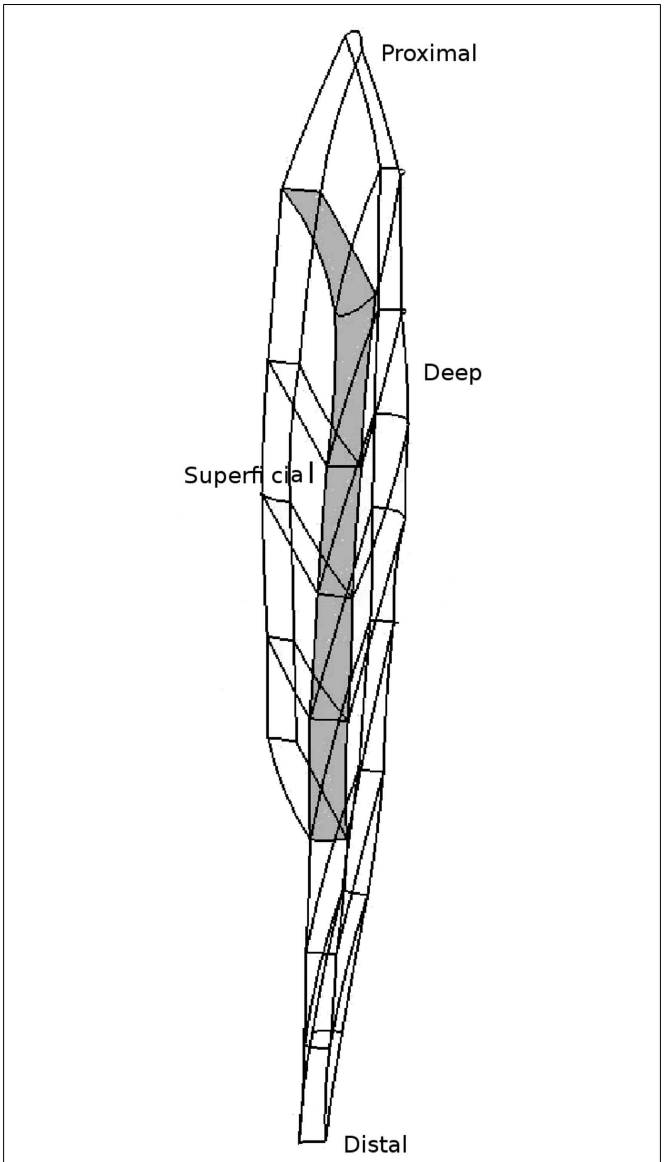

FIGURE 1 | The tri-quadratic FE mesh of the tibialis anterior (TA). The shaded section represents the element boundaries that are aligned with the aponeurosis separating the superficial and deep compartments of the TA.

2.1.2. Functional grouping of muscle fibers

Within this work, the distribution of muscle fibers per motor unit is based on the work of Enoka and Fuglevand (2001). They assume that the number of muscle fibers per motor unit exhibits an exponential distribution: many motor units exert small forces,

i.e., contain a small number of muscle fibers, and relatively few motor units exert large forces, i.e., contain a large number of muscle fibers. Such an exponential distribution of muscle fibers per motor unit can be determined for a fixed number of total muscle fibers, f^M, by computing for each motor unit i the number of associated fibers through

$$f_i = f_1 \cdot \exp\left(\frac{i-1}{m} \cdot \ln f_{\text{ratio}}^{\text{m/1}}\right), \tag{1}$$

while ensuring that $\sum_{i=1}^{m} f_i = f^M$. In equation (1), $f_{\text{ratio}}^{\text{m/1}}$ determines the ratio between the number of muscle fibers of the largest motor unit, f_m, and the smallest motor unit, f_1. As the number of fibers within this work is dictated by the number of embedded fibers, equation (1) has to be solved iteratively. As the solution of equation (1) typically results in non-integer values, each f_i is rounded to its nearest integer. To fulfill the constraint that the sum of all assigned muscle fibers has to equal f^M, the number of fibers of the last motor unit is adjusted by $f_m = f^M - \sum_{i=1}^{m-1} f_i$.

Each motor unit is assumed to be composed of either fast- or slow-twitch muscle fibers, with smaller motor units composed of slow type muscle fibers (Henneman et al., 1965b; Wuerker et al., 1965). The proportion of slow versus fast muscle fibers is specified by $p^{\text{s/f}}$ (about 70% of a human TA's muscle fibers are of slow type). Hence, by defining the first s motor units to be slow, and the last $m-s$ motor units to be fast, one can determine for a pre-defined $p^{\text{s/f}}$ the number of s slow motor units by the following relationship:

$$s = \underset{l}{\text{argmin}} \left| \frac{\sum_{i=1}^{l} f_i}{\sum_{i=l+1}^{m} f_i} - p^{\text{s/f}} \right|. \tag{2}$$

The assignment of embedded fibers to a specific motor unit is achieved by defining motor-unit territories within the mesh and then assigning random fibers within the territories to a motor unit. As fibers within a motor unit are defined to be of the same physiological type (i.e., fast or slow type) and as muscle fibers of a certain type are preferentially located in a certain region of the muscle (Polgar et al., 1973; Henriksson-Larsén et al., 1983), additional weighting centers $\mathcal{W}^t$ with $t \in \{s, f\}$ are introduced. The distance between a weighting center, $\mathcal{W}^t$, and a particular center of a motor-unit territory, $\mathcal{C}_i$, is assumed to be normal distributed, i.e., $\mathcal{N}(\mu_t, \sigma_t^2)$. The mean, μ_t, is given by the location of the weighting center, $\mathcal{W}^t$, while the variance is determined based on the location of the muscle fiber center points, f_k^c, and is therefore dependent on the anatomical arrangement of the muscle fibers and on the muscle geometry itself. The two variances for $t \in \{s, f\}$ are given by

$$\sigma_t = \frac{2\omega_t}{3}\left(\max_k \left\{d_{k,t}\right\} - \min_k \left\{d_{k,t}\right\}\right), \tag{3}$$

where $k = 1,\ldots, f^M$ is an index for a particular fiber, ω_t denotes a scaling factor, and

$$d_{k,t} = \| f_k^c - \mathcal{W}^t \|_2 \tag{4}$$

defines the Euclidean distance between the muscle fiber midpoint of the kth-fiber, f_k^c, and the respective weighting center $\mathcal{W}^t$. The scaling parameter, ω_t, is introduced to account for cases in which the motor-unit territory centers are defined to be in close proximity to the weighting center or for cases in which this condition shall be relaxed. In case that no weighting points can be specified, or if one prefers to distribute the motor-unit territory center points over the entire geometry of the muscle, the normal random distribution is substituted by an uniform random distribution. Within this work, the scaling parameter, ω_t, is chosen to be 1.

The motor-unit territory centers, $\mathcal{C}_i$ for $i = 1,\ldots, f^M$, are selected by choosing s random numbers $X_i^s \sim \mathcal{N}(\mu_s, \sigma_s)$ and $m-s$ random numbers $X_i^f \sim \mathcal{N}(\mu_f, \sigma_f)$, and by determining the closest muscle fiber midpoint

$$\mathcal{C}_i = \underset{f_k^c \in \mathcal{F}}{\text{argmin}} \left\| f_k^c - (\min_k\{d_{k,t}\} + X_i^t) \right\|_2 \tag{5}$$

with $i = 1,\ldots, m$ and $t \in \{s, f\}$ accordingly.

To determine the spatial distribution of muscle fibers for each motor unit i, single fibers from the pool of all available fibers, which is denoted by $\mathcal{M}^r$ and coincides with $\mathcal{F}$ prior of selecting the first fiber, are successively selected and removed from the set of all remaining fibers, $\mathcal{M}^r$. The selection algorithm for $\mathcal{F}_i$, which is the set of all muscle fibers numbers associated with motor unit i, is described in the following in more detail: First, all potential muscle fiber midpoints for motor unit i are determined by:

$$\mathcal{P}_i = \left\{k \mid f_k \in \mathcal{M}^r \text{ and } \left\| f_k - \mathcal{C}_i \right\|_2 \leq R_i \right\}, \tag{6}$$

where the radius,

$$R_i = \sqrt{\frac{f_i}{\rho_i \cdot \pi}}, \tag{7}$$

denotes a spatial constraint depending on the number of fibers, f_i, and the fiber density, ρ_i, of the respective motor-unit territory. The fiber density of a motor-unit territory, ρ_i, is based on physiological data (Roy et al., 1995; Monti et al., 1999). A total of f_i muscle fiber midpoints are randomly selected and removed from the sets $\mathcal{P}_i$ and $\mathcal{M}^r$, i.e., $\mathcal{P}_i := \mathcal{P}_i - \{k\}$, $\mathcal{M}^r := \mathcal{M}^r - \{k\}$, and $\mathcal{F}_i := \mathcal{F}_i \cup \{k\}$. Note, in some cases, the choice of R_i might be too restrictive resulting in an initial set $\mathcal{P}_i$ (before selecting any muscle fibers for motor unit i) that does not contain enough elements, i.e., card $(\mathcal{P}_i) = |\mathcal{P}_i| \leq f_i$. In this case, the radius, R_i is successively increased by 5% until the set of potential assignable muscle fibers, $\mathcal{P}_i$, is sufficiently large. The same procedure is repeated m-times, starting from the smallest motor unit and ending with the largest one. Following this procedure, all muscle fibers are uniquely assigned to a particular motor unit.

2.2. MODELING THE PHYSIOLOGY OF A SKELETAL MUSCLE FIBER

Although fibers within a skeletal muscle are mechanically coupled, from an electro-physiological point of view, they are independent. We assume that the activity of all fibers in a motor unit can be modeled as identical and that all fibers are innervated at their midpoint. Hence, it is sufficient to model the activation of a single muscle fiber per motor unit, and use its output for all associated

ones. The overall electro-physiological model of a single muscle fiber can be split up in two parts: (i) the first part focuses on modeling the (sub-)cellular behavior at a particular point along the muscle fiber and (ii) the second part focuses on the propagation of the AP along its muscle fiber. The first part can be described by a Hodgkin–Huxley-like cellular model and is elucidated in more detail in Subsection 2.2.1. The propagation of the AP along a single skeletal muscle fiber is an interplay between the cellular response of the muscle fiber at a particular location and of the propagation of the electrical signal (AP). The homogenization of the intra- and extracellular processes and the propagation of the AP along the muscle fiber can be modeled using the bidomain equations, which are described in detail in Subsection 2.2.2.

2.2.1. The cellular model

Many electro-physiological models describing various aspects of (sub-)cellular processes have been introduced. The specific skeletal muscle cell model used within this work is, like in Röhrle et al. (2008) and Röhrle (2010), the model developed by Shorten et al. (2007). This cell model describes by means of two separate sets of parameters the electro-physiological behavior of a (half-)sarcomere within slow- and fast-twitch type skeletal muscle fibers using a Hodgkin–Huxley-like description. The resulting system of coupled ordinary differential equations (ODEs) has been implemented using CellML, which is a Markup-Language specifically designed to describe cellular processes (cf. Lloyd et al., 2008). The model can be downloaded[1] from the CellML-website, www.cellml.org.

In brief, the Shorten et al. (2007) model can be described as an amalgamation of cell models, which each individually describe parts of the muscle cell physiology. The model contains descriptions of cellular processes such as the sarcolemmal membrane potential, excitation-contraction coupling, and the dynamics of the actin-myosin crossbridges. In modeling the membrane potentials, the sarcolemmal and T-tubular membranes are represented separately allowing the representation of fatigue effects through potassium accumulation in the T-tubular space. In addition, the effects of inorganic phosphate build-up are modeled to represent metabolic fatigue properties. The actin-myosin crossbridge model is an "eight-state model," in which the eight "states" can be associated with regulatory units on the thin filaments and, therefore, can be are related to different states of the troponin-tropomyosin complex. Specifically, the different states within this model are distinguished into six detached states and two attached states, i.e., the concentration of attached myosin crossbridges during the pre-powerstroke state, A_1, and the concentration of attached myosin crossbridges during the post-powerstroke state, A_2. The parameters A_1 and A_2 will be used later within the multi-scale constitutive law describing the overall electromechanical behavior of the skeletal muscle.

The Shorten et al. (2007) cell model is selected, because it represents the cellular properties of skeletal muscle fibers from action potential (AP) activation right through to the crossbridge dynamics. The main advantage of this model is that the entire chain of processes allows for a more physiologically realistic representation of complex cellular behavior such as membrane fatigue, metabolic fatigue, force summation, potentiation, and the catch-like effect (Shorten et al., 2007). Further, it allows the modeling of different muscle fiber types, e.g., fast and slow-twitch muscle, without the necessity of separate cell models.

[1] http://models.cellml.org/exposure/159ba2f081022ca651284404f39eeb40/view

2.2.2. The bidomain equations

The behavior of AP propagation in biological tissue is typically modeled using the bidomain equations (Pullan et al., 2005), which are a set of coupled reaction-diffusion equations. The reactive part stems from the cellular behavior, while the diffusive part describes the propagation of the AP. For examples of implementation of ODE models of cellular activity refer to Fernandez et al. (2005), Kim et al. (2007), and Röhrle and Pullan (2007).

Within this work, we assume a fiber diameter of $80\,\mu$m, which is within the range of a human fiber diameter of 80–$100\,\mu$m (e.g., Lexell et al., 1986; Sjöström et al., 1991; Miller et al., 1993) motivating the surface-to-volume ratio of $50\,\mathrm{mm}^{-1}$. The capacitance of the membrane was set at $0.01\,\mu\mathrm{Fmm}^{-2}$ for the fast-twitch fibers and $0.0058\,\mu\mathrm{Fmm}^{-2}$ for the slow-twitch fibers (cf. Shorten et al., 2007). The intracellular and extracellular conductivity tensors are, in the case of solving the bidomain equations for a one-dimensional fiber, scalars and are chosen to be $\sigma_i = 0.893\,\mathrm{mSmm}^{-1}$ (Bryant, 1969) and $\sigma_e = 0.67\,\mathrm{mSmm}^{-1}$ (Schwann and Kay, 1957; Rush et al., 1963), respectively.

The bidomain equations are discretized in space using linear Lagrange finite elements and the resulting system of ODEs is then solved using LSODA (Hindmarsh, 1982; Petzold, 1983). The key electro-physiological parameters that will be used later within a multi-scale constitutive law (cf. Section 2.3.1) are the concentration of actin-myosin crossbridges in the pre- and post-powerstroke, A_1 and A_2.

2.3. THE CONTINUUM-MECHANICAL SKELETAL MUSCLE MODEL

This work appeals to the same mechanical model as proposed and used in Röhrle et al. (2008) and Röhrle (2010). For completeness, however, a brief overview is given in the following.

The continuum-mechanical model proceeds form the local form of the balance of linear momentum,

$$\rho\,\ddot{\mathbf{x}} = \mathrm{div}\mathbf{T} + \rho\,\mathbf{b}\,, \tag{8}$$

where ρ denotes the mass density, $\mathbf{x}$ is a material point position in the current configuration and $\ddot{\mathbf{x}}$ its second time derivative, $\mathbf{b}$ are the body forces, and $\mathbf{T}$ denotes the Cauchy stress tensor. By assuming quasi-static conditions and small body forces (in comparison to the forces generated by the muscle), the local form of the balance of linear momentum reduces to $\mathrm{div}\,\mathbf{T} = 0$. For biological tissues, it is often advantageous to express the stress-strain relationship with respect to anatomical features, which are typically defined in the reference state. To do so, one can express the Cauchy stress tensor in terms of the second Piola–Kirchhoff stress tensor via the push-forward operation $\mathbf{T} = J^{-1}\mathbf{F}\mathbf{S}\mathbf{F}^{-1}$, where $J = \det\,\mathbf{F}$ and $\mathbf{F} = \partial\mathrm{x}/\partial\mathrm{X}$ is the deformation gradient tensor mapping points

between the reference configuration, **X**, and the current configuration, **x** (deformed state). The strain is typically measured by the Green strain tensor

$$\mathbf{E} = \frac{1}{2}(\mathbf{F}^T\mathbf{F} - \mathbf{I}) = \frac{1}{2}(\mathbf{C} - \mathbf{I}), \tag{9}$$

where **C** is the right Cauchy–Green deformation tensor and **I** is the identity tensor. The relationship between stress and strain can be specified for hyperelastic materials by a strain energy function ψ. A detailed introduction and overview to non-linear continuum mechanics and tensor analysis can be found in Holzapfel (2004).

A skeletal muscle tissue's overall mechanical behavior can be modeled by distinguishing between an passive and an active behavior. During contraction (the active part) the muscle generates a contractile force in the longitudinal direction, which is (locally) described by a vector a_0. The material behavior of the passive part is described through the mechanical behavior of the tissue's ground matrix, i.e., the extracellular matrix consisting of a network of collagen, fat, etc. Given the mechanical behavior's additive nature, the free energy of the entire muscle tissue, ψ^{muscle}, can be written as the sum of the free energy of the ground substance, ψ^{matrix}, and the free energy of the active part, ψ^{active}:

$$\begin{aligned}\psi^{\text{muscle}}(\mathbf{C}, \mathbf{a}_0) =& \psi^{\text{matrix}}(\mathbf{C}, f^{\text{passive}}(\lambda), \mathbf{a}_0) \\ &+ \psi^{\text{active}}(\mathbf{C}, \mathbf{a}_0, \alpha, f^{\text{active}}(\lambda)),\end{aligned} \tag{10}$$

where $\alpha \in [0, 1]$ is an internal variable that describes the level of activation, $\lambda = \sqrt{\mathbf{a}_0 \cdot \mathbf{C}\mathbf{a}_0}$ is the fiber stretch, and $f^{\text{passive}}(\lambda)$and $f^{\text{active}}(\lambda)$ are the normalized force-length relationships. For the active part, $f^{\text{active}}(\lambda)$ describes the overlap of actin and myosin and hence the ability to generate tension through crossbridge dynamics. The normalized force-length relationship is a commonly used tool to incorporate the physiological behavior of fiber stretch in purely mechanical models (e.g., Blemker et al., 2005; Röhrle and Pullan, 2007; Böl and Reese, 2008).

Differentiating the free energy with respect to **C** and assuming a simple and isotropic Mooney–Rivlin-type material behavior for the ground matrix, results in the following definition of the second Piola–Kirchhoff stress tensor

$$\begin{aligned}\mathbf{S}^{\text{muscle}} =& \underbrace{c_1\mathbf{I} + c_2(I_1\mathbf{I} - \mathbf{C}) - p\sqrt{I_3}\mathbf{C}^{-1}}_{=:\,\mathbf{S}^{\text{iso}}} \\ &+ \underbrace{\left[\frac{\sigma^{ff}_{\text{pass}}}{I_4} f^{\text{passive}}(I_4)\right](\mathbf{a}_0 \otimes \mathbf{a}_0)}_{=:\,\mathbf{S}^{\text{aniso}}} \\ &+ \underbrace{\alpha\left[\frac{\sigma^{ff}_{\text{ten}}}{I_4} f^{\text{active}}(I_4)\right](\mathbf{a}_0 \otimes \mathbf{a}_0)}_{=:\,\mathbf{S}^{\text{active}}},\end{aligned} \tag{11}$$

where

$$I_1 = tr(\mathbf{C}), \tag{12}$$

$$I_2 = \frac{1}{2}[(tr(\mathbf{C}))^2 - tr(\mathbf{C}^2)], \tag{13}$$

$$I_3 = det\mathbf{C}, \tag{14}$$

are the standard invariants and invariant

$$I_4 = \mathbf{a}_0 \cdot \mathbf{C}\mathbf{a}_0 \tag{15}$$

is associated with the fiber stretch in the current configuration, $\sigma^{ff}_{\text{pass}} = \sigma^{ff}_{\text{ten}} = 0.03$ MPa are the maximal passive and active stiffness in the along-the-fiber direction, p is the hydrostatic pressure and $\mathbf{S}^{\text{matrix}} = \mathbf{S}^{\text{iso}} + \mathbf{S}^{\text{aniso}}$. The dyadic product, **P**, between two three-dimensional vectors is defined by

$$\mathbf{P} = \mathbf{u} \otimes \mathbf{v} = \left(\sum_{i=1}^{3} u_i\mathbf{e}_i\right) \otimes \left(\sum_{j=1}^{3} v_j\mathbf{e}_j\right) = \sum_{i,j=1}^{3} u_i v_j \mathbf{e}_i \otimes \mathbf{e}_j, \tag{16}$$

where

$$\mathbf{e}_i \otimes \mathbf{e}_j = \mathbf{e}_i\mathbf{e}_j^T = \begin{bmatrix} e_{i,1} \\ e_{i,2} \\ e_{i,3} \end{bmatrix}[e_{j,1}, e_{j,2}, e_{j,3}] = \begin{bmatrix} e_{i,1}e_{j,1} & e_{i,1}e_{j,2} & e_{i,1}e_{j,3} \\ e_{i,2}e_{j,1} & e_{i,2}e_{j,2} & e_{i,2}e_{j,3} \\ e_{i,3}e_{j,1} & e_{i,3}e_{j,2} & e_{i,3}e_{j,3} \end{bmatrix}. \tag{17}$$

At each point, the local fiber direction, $\mathbf{a}_0$, can be expressed in terms of the global basis spanning the overall world coordinate system, i.e., $\mathbf{e}_i$, $i = 1,2,3$. Hence, the dyadic product of the local fiber orientation describes the contribution of the fiber orientations to the overall second Piola–Kirchhoff stress tensor in terms of the global coordinate system. In the special case that the local fiber direction is aligned with the first basis vector of the global coordinate system, i.e., $\mathbf{a}_0 = \mathbf{e}_1 = [1,0,0]$, the tensor resulting from the dyadic product results into a tensor $\mathbf{a}_0 \otimes \mathbf{a}_0$ with a 1 in (1,1)-component and zeros for all other components. Hence, the fiber will only have a contribution to the (1,1)-component of the second Piola–Kirchhoff stress tensor.

The mechanical model itself is based on solving the governing equations of finite elasticity theory using the FE method. The unknowns describing the displacements are discretized using tri-quadratic Lagrange FE basis functions whilst the unknowns for the hydrostatic pressure are discretized by linear FE basis functions. Solving for the mechanical deformation due to skeletal muscle activity or due to a change in the muscle attachment location (i.e., movement of the bone) requires the evaluation of the second Piola–Kirchhoff stress tensor.

2.3.1. A multi-scale constitutive law

The continuum-mechanical constitutive law does not yet contain any information from the smaller scales such as the cellular level. For a given time t, the activation level, α, in equation (11) is substituted by cellular variables, i.e., A_1 and A_2. Moreover, the mechanical description of the contractile response is split into two parts: the first one is based on the generation of tension in the post-powerstroke state and the second one is related to the change of passive stiffness due to the attached crossbridges in the pre- and post-powerstroke states. As both mechanisms act in the direction along a muscle fiber, the second Piola–Kirchhoff stress tensor

for the multi-scale constitutive law can be expressed in terms of cellular parameters by

$$\mathbf{S}^{\text{muscle}} = \mathbf{S}^{\text{iso}} + \mathbf{S}^{\text{aniso}} + \left[\frac{A_1 + A_2}{c_{\text{trop}}} \frac{\sigma^{ff}_{\text{pass}}}{I_4} f^{\text{active}}(\lambda) + \frac{A_2}{A_2^{\max}} \frac{\sigma^{ff}_{\text{ten}}}{I_4} f^{\text{active}}(\lambda) \right] (\mathbf{a}_0 \otimes \mathbf{a}_0),$$

where c_{trop} is the normalization factor and is the maximum possible amount of troponin within the cellular model (here: $c_{\text{trop}} = 140$) and $A_2^{\max}$ presents the maximal concentration of attachable crossbridges in the post-powerstroke. Hence, the term containing $A_2/A_2^{\max}$ is comparable to α in equation (11), while the term containing $(A_1 + A_2)/c_{\text{trop}}$ relates to an additional passive tension due to the activation.

2.3.2. Upscaling

From a computational point of view, the mechanical model of the skeletal muscle cannot be discretized with the same fine resolution as the embedded fibers due to accuracy constraints dictated by solving the system of ODEs describing the cellular processes. This mismatch in mesh resolutions requires a homogenization procedure to upscale the fine-grid solutions of the cellular variables to the nearest Gauss points of the mechanical FE mesh, at which the second Piola–Kirchhoff stress tensor is evaluated. This is done by computing in the vicinity of each Gauss point the average concentration of attached crossbridges. This homogenization procedure has been proposed and validated in Röhrle et al. (2008).

2.4. MOTOR-UNIT RECRUITMENT

Motor units are recruited in an order determined by the number of muscle fibers in each motor unit, the so-called size principle (Henneman and Olson, 1965; Henneman et al., 1965a,b). The number of active motor units and their firing frequency is modulated as a result of the excitatory drive coming from the motor cortex. This framework assumes that the drive to each motor unit in the pool is equal (De Luca et al., 1982; Yao et al., 2000), though this is still an unresolved issue (Heckman and Binder, 1988; Heckman, 1994). The activation of each motor unit is calculated using a method derived by Fuglevand et al. (1993). Briefly, the recruitment of a specific motor unit and its resulting firing frequency is dependent on a single pre-defined variable $E(t)$, where, $0 \leq E(t) \leq 1$, is a user defined activation parameter. If $E(t)$ is greater than the recruitment threshold of motor unit i, then motor unit i becomes active with a firing rate which is linearly dependent on $E(t)$. The recruitment thresholds of the motor units are defined to be exponentially varying over the pool of motor units

$$T_i^{\text{excite}} = \exp^{i \cdot (ln(RR)/m)}, \tag{18}$$

where T_i is the recruitment threshold of motor unit i, RR is the range of recruitment thresholds in the motor pool, and m is the number of motor units. Active motor units, i.e., $E(t) \geq T_i^{\text{excite}}$, are assumed to increase their firing rate linearly from the minimum firing rate to the maximum firing rate. Although it is possible to model variations in minimum and maximum firing rates over the motor pool (Fuglevand et al., 1993), all simulations within this work assume for each active motor unit an uniform minimum firing rate of 8 Hz and a maximum firing rate of 40 Hz. The minimum firing rate is based on the average reported rates in Grimby and Hannerz (1977), Bellemare et al. (1983), Broman et al. (1985), Kamen and Du (1999), McNulty and Cresswell (2004), and Do and Thomas (2005), while the maximum is based on the average value reported in Bellemare et al. (1983) and Enoka and Fuglevand (2001). Hence, the firing times for motor unit i can be computed by the following equation:

$$t_i^{\text{next}} = t_i^{\text{last}} + \frac{1}{(1 + \eta \cdot c_v)(g_e \cdot \left[E(t_i^{\text{next}}) - T_i^{\text{excite}}\right] + F_i^{\min})}, \tag{19}$$

if $E(t) \geq T_i^{\text{excite}}$. In equation (19), t_i^{last} is the point in time motor unit i has fired last, η a Gaussian-distributed random number mimicking the natural variability of motor-unit activation, c_v a coefficient of variation, g_e the gain of motor unit i, and $F_i^{\min}$ the minimum firing rate of motor unit i. The exact parameter values for equation (19) are given in **Table 3**.

The firing times of each motor unit are used in conjunction with the one-dimensional fiber models to produce the cellular output for each motor unit. It is important to note that each muscle fiber of a specific motor unit has approximately the same behavior as they are innervated by the same motor neuron and are of the same physiological type. Hence, the distribution of the pre- and post-powerstroke concentrations, i.e., the cellular variables A_1 and A_2, are computed for all fibers and serve, after homogenization, as input to the multi-scale constitutive law. Therefore, a change in the state of activation or a displacement boundary conditions at the end of the muscle causes a change in the muscles mechanical state and, hence, the exerted muscle force of the overall skeletal muscle.

3. RESULTS

3.1. MUSCLE FIBER DISTRIBUTION WITHIN THE TA

A particular choice of muscle fiber distribution for the TA, which will be used for most numerical investigations within this section, is depicted in **Figure 2**. The allocation of the muscle fibers to particular motor units has been carried out as described above. A (homogenized) muscle fiber diameter of 2000 μm has been assumed resulting in a total of 903 muscle fibers for the TA muscle geometry depicted in **Figure 1**. Further, within the muscle-fiber-to-motor-unit-allocation algorithm described above, a total of 30 motor units and a ratio between the number of fibers of the largest and the smallest motor unit of 10, i.e., $f_{\text{ratio}}^{\text{m/l}} = 10$ in equation (1), is considered. The discretization with a grid spacing of 0.0625 mm results in a total of about 50,000 grid points, at which cellular variables need to be computed/assigned. The grid spacing of 0.0625 mm is justified in a convergence study of the AP propagation speed, which is presented within Section 3.2. **Figure 2** depicts the anatomical location of the TA (in blue), the muscle fibers associated with motor units 1, 5, and 10, as well as the motor unit territory center $\mathcal{C}_1$ of the first motor unit. This muscle fiber allocation provides the basis for the remaining simulations, except for the simulations considering a total of 10 and 50 motor units in Section 3.4.

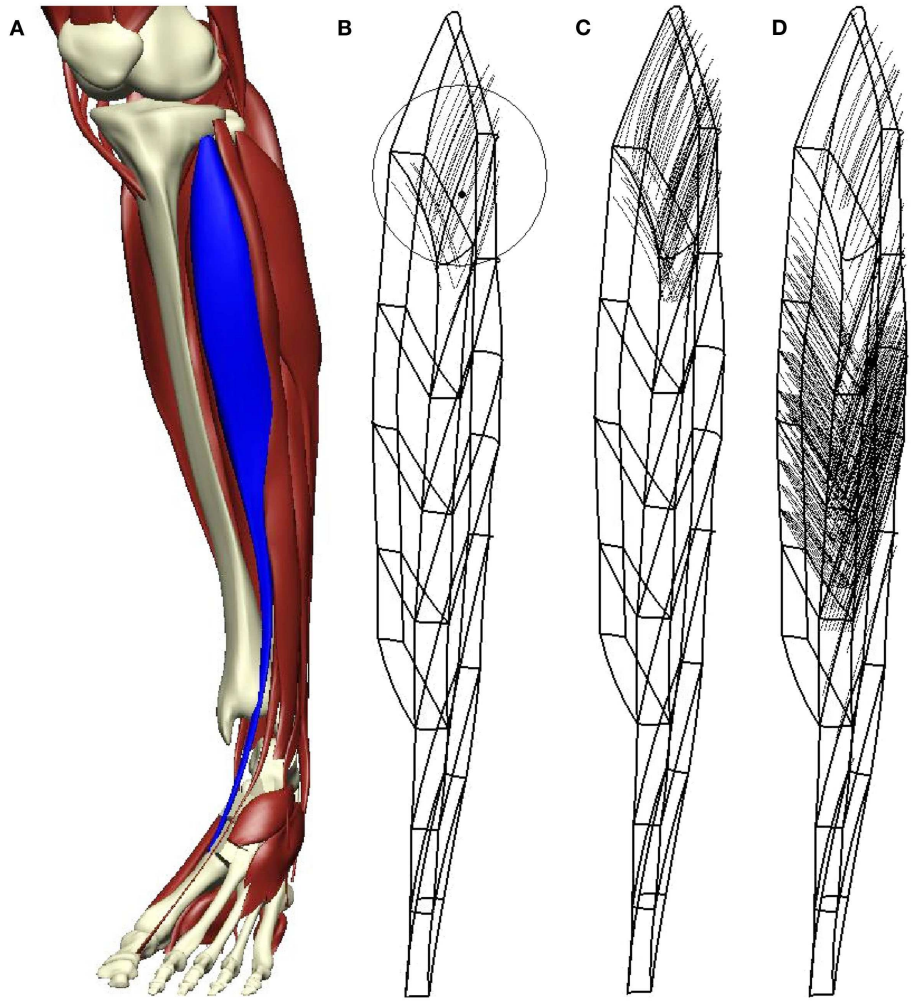

FIGURE 2 | Subfigure (A) shows the location of the TA within the lower limb; (B–D) show the muscle fibers associated with motor units 1, 5, and 10. Further **(B)** depicts the motor-unit territory center midpoint, $\boldsymbol{C}_1$, for motor unit 1 and (the sphere with) radius R_1 that was used to select the fibers for motor unit 1.

3.2. VERIFICATION OF COMPUTATIONAL PARAMETERS

The contractile properties of the multi-scale constitutive law are directly linked to the electro-physiological behavior of single skeletal muscles. Hence, it is of importance to properly model the physiological behavior of the AP propagation, i.e., the shape of the AP and its conduction velocity along the fiber. All single muscle fiber simulations carried out within this section aim to determine the appropriate temporal and spatial discretization parameters resulting in a correctly simulated AP propagation. The parameters for the bidomain equations are given in **Table 1**. The values for the Shorten et al. model are outlined in Shorten et al. (2007).

As simulation parameters for solving the Shorten et al. (2007) model using LSODA, a time increment of $\Delta t = 0.01$ and an absolute error $\epsilon_{\text{tol}} = 0.1^{-4}$ have been identified to provide a good balance between accuracy and computational speed. The spatial discretization of the muscle fibers is determined by a grid convergence analysis with respect to the conduction velocity. For this purpose, a 32 mm long muscle fiber has been discretized using different grid spacings and the AP propagation is calculated based on the bidomain equations. The AP conduction velocity is determined by the distance that the maximum positive gradient in membrane potential traveled for a given period of time. The results of the computed conduction velocities for different grid point spacings are presented in **Table 2**.

Based on the computed conduction velocities, a grid point spacing of 0.0625 mm (16 grid points per mm) is assumed to be adequate for solving the bidomain equations for fast- and slow-twitch muscle fibers.

3.3. PHYSIOLOGICAL RECRUITMENT OF A TA

Choosing an excitatory drive function $E(t)$, i.e., the temporal recruitment of single motor units and, hence, the motor-unit-associated fibers, the resulting electro-physiological changes within the fibers, and the multi-scale constitutive law, allows to compute the respective forces exerted by the TA. The TA is assumed to undergo only isometric contractions, i.e., the attachment areas

Table 1 | Values for the fiber model (bidomain equations).

Variable	Value	Description	Sources
A_m	$50\,\mathrm{mm}^{-1}$	Surface-to-volume ratio	Calculated based on a cell diameter of 80 μm: cf. Lexell et al. (1986) Miller et al. (1993) Sjöström et al. (1991)
C_m	$0.01\,\mathrm{mFmm}^{-2}$ $0.0058\,\mathrm{mFmm}^{-2}$	Slow-twitch Fast-twitch fiber membrane capacitance	Shorten et al. (2007)
σ_i	$0.893\,\mathrm{mSmm}^{-1}$	Internal fiber conductivity	Bryant (1969)
σ_e	$0.67\,\mathrm{mSmm}^{-1}$	External fiber conductivity	Rush et al. (1963) Schwann and Kay (1957)
I_s	$8000\,\mu\mathrm{A/mm}^2$	External stimulus current	Assumed

Table 2 | Computed conduction velocities based on different grid spacing for slow- and fast-twitch muscle fibers.

Discretization (grid points/mm)	Conduction velocities (m/s)	
	Slow-twitch muscle fiber	Fast-twitch muscle fiber
2	1.529	2.174
4	1.488	2.101
8	1.471	2.049
16	1.466	2.033
32	1.466	2.033
64	1.466	2.033
128	1.466	2.033

of the TA are fixed by assuming zero-displacement boundary conditions. Further, as mentioned earlier, the TA model consists of 903 fibers (cf. Section 3.1), which are distributed over 30 motor units. The total simulation duration is 500 ms. All the parameters of the recruitment model used within this section are listed in **Table 3**.

Herein, a step function is assumed as a particular choice of $E(t)$. Every 100 ms, $E(t)$ increases by 0.25 [the first 100 ms $E(t)$ is 0]. The time instances at which all the muscle fibers of a single motor units are activated through a nerve signal, are computed based on $E(t)$. The arrival of a nerve signal at the neuromuscular junction of a skeletal muscle fiber has been modeled by applying a stimulus at the midpoint of the fibers of the respective motor unit as boundary condition within the bidomain equations. The stimulus has been chosen such that the midpoint of the skeletal muscle fiber initiates a depolarization. As one computes first the cellular variables A_1 and A_2 before solving the governing equations of finite elasticity with the already updated and homogenized cellular variables, the coupling between the electrophysiological and the mechanical model can only be considered as weak. There exists

Table 3 | Values of the coefficients and variables of the recruitment and rate coding model used within this framework.

Variable	Value	Description	Sources
$F_i^{\min}$	8 Hz	Mean firing rate of motor unit i	Averaged from several sources (see Section 2.4)
$F_i^{\max}$	40 Hz	Peak firing rate of motor unit i	Averaged from several sources (see Section 2.4)
$\Delta F^{\max}$	0	Difference in peak firing rate between the smallest and largest MU	Assumed
c_v	0.2	Coefficient of variation	Fuglevand et al. (1993)
η	$\mathcal{N}(0,1)$	Gaussian-distributed random number	Fuglevand et al. (1993)
RR	30	Range of recruitment threshold values	Assumed
g_e	2	gain of the motor units	Assumed

no coupling between the mechanical deformation and the cellular model as of yet.

The electrophysiological and mechanical problem is solved in 1 ms increments. Based on the finite elasticity solution, one can compute the exerted muscle force at the attachment areas of the TA. **Figure 3** depicts the exerted muscle force as a result of the above described excitatory drive function, $E(t)$. For each (second) motor unit, a separate time line has been included in **Figure 3**. The small motor unit numbers correspond to the motor units that contain fewer muscle fibers and are sorted in an ascending order (the smallest at the bottom, the largest motor unit at the top). The y-axis on the right reflects the exerted muscle force (in kN).

3.4. IMPLICATIONS DUE TO DIFFERENT NUMBERS OF FIBERS AND MOTOR UNITS

The number of muscle fibers and motor units in a human TA varies greatly, e.g., between 96,000 and 162,000 fibers (Henriksson-Larsén et al., 1983) and 150 ± 43 motor units (McNeil et al., 2005). Modeling each fiber with a spatial resolution of 0.0625 mm would result in more than 50 Mio. computational nodes, at which cellular parameters need to be computed/stored. Moreover, the multi-scale constitutive law will not benefit from such a high resolution after homogenization. Hence, it is of interest to investigate the effects of the number of embedded fibers and the number of motor units on the force-generating capabilities of the TA.

3.4.1. Number of embedded muscle fibers

To investigate the effects of changing the number of embedded fibers within the TA geometry, different muscle fiber diameters have been considered, i.e., diameters of 2000, 1000, and 500 μm. Given actual muscle fiber diameters in humans of approximately 80–100 μm (Lexell et al., 1986; Sjöström et al., 1991; Miller et al., 1993), the simulation with the smallest muscle fiber diameter represents already a 1:25 fiber ratio reduction. All simulations in **Figure 4** assumed a constant number of motor units, i.e., 30 motor units, while the muscle fiber diameter has been varied. The same motor-unit recruitment protocol as described in Section 3.3 has

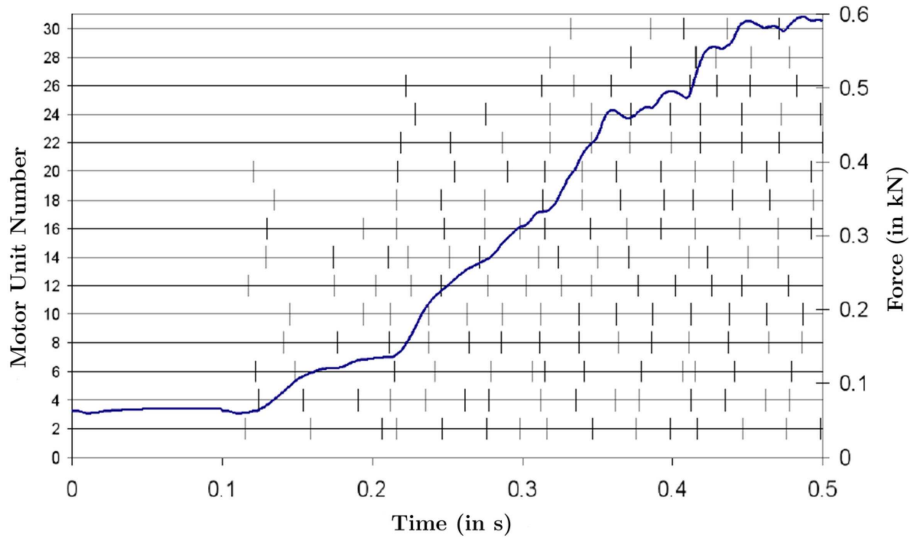

FIGURE 3 | The stimulation times of every second motor unit are shown with vertical strikes. It can be seen that the larger motor units become active later in the simulation and the average frequency of all motor units increases throughout the simulation. The sigmoidal shape of the force curve can be seen, with a slow average change in curvature at the beginning and end of the simulation and a relatively linear section in the middle.

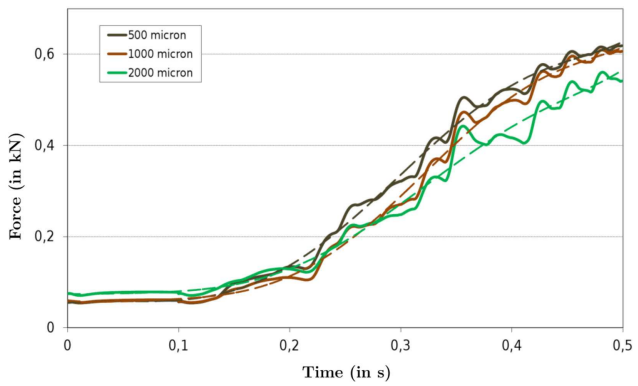

FIGURE 4 | The force output of TA muscle simulation with mechanical fiber spacings of 2000, 1000, and 500 μm.

been applied to compute the resulting muscle forces. The three computed force profiles for muscle fiber diameters of 2000 μm (in green), 1000 μm (in brown), and 500 μm (in black) muscle fiber diameter are plotted in **Figure 4**. Note, a variation in muscle fiber diameter also requires a recalculation of the muscle fibers association with a particular motor unit. Hence, due to the randomness in selecting muscle fibers to particular motor units, there is the possibility that the location of the muscle fibers belonging to a particular motor unit varies.

Figure 4 contains in addition to the force plots also "smoothed" force profiles (dashed lines of the respective fiber diameter colors). The smoothed force profiles have been computed through a least-squares fit to a 6th-order polynomial. The purpose of fitting the force profile to a smooth polynomial is to investigate the fluctuations of the muscle forces. Hence, the deviation of the force output from the best-fit curve is then given by

$$R^2 = 1 - \frac{\sum (y_k - \hat{y}_k)^2}{\sum y_k^2 + \frac{1}{n}(\sum y_k)^2}, \tag{20}$$

where y_k is the computed muscle force at time $k\Delta t$ with $k = 1,\ldots, n$ and $n \cdot \Delta t = 0.5$ s and $\hat{y}_k$ is the respective value obtained from evaluating the polynomial. For mechanical fiber spacings 2000, 1000, and 500 μm, the R^2 values are 0.9884, 0.9950, and 0.9958, respectively. In **Figure 4**, the increase in R^2 can be noticed by observing the fact that the force profiles exhibited less variations as the number of fibers increased. The increase in the force as the muscle fiber diameter increases is due to the proportionally larger increase in the number of fibers in the distal section of the muscle with a smaller pennation angle.

3.4.2. Number of motor units

Representing the TA with fewer than the actual number of motor units has similar benefits and drawbacks as reducing the number of embedded fibers. Considering fewer motor units reduces the computational cost for calculating the electro-physiological behavior of muscle fibers associated with a motor unit, but increases the possibility of less smooth force outputs. Like above, different simulations with 10, 30, and 50 motor units were carried out. The number of fibers were set to be constant at 903 fibers, i.e., a fiber diameter of 2000 μm (cf. Section 3.3). The excitatory input to the motor-unit pool is the same in each case. The respective force output based on simulations with 10 (in green), 30 (in brown), and 50 motor units (in black) are depicted in **Figure 5**.

To investigate the smoothness of the different force profiles, the least-square fits are repeated for each muscle force profile and the measure of smoothness, R^2 as defined in equation (20), is computed for each force profile. For the 10, 30, and 50 motor units

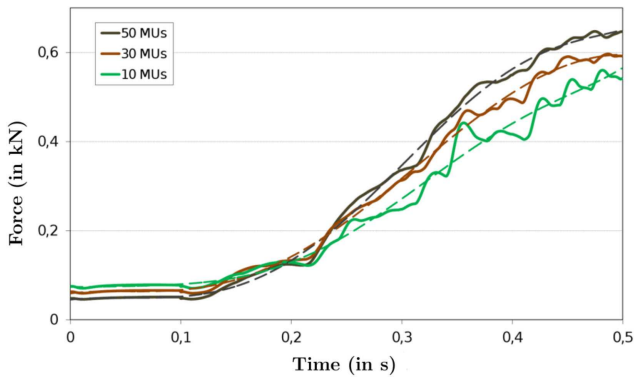

FIGURE 5 | The force output profiles of the TA with 10, 30, and 50 motor units. The sigmoidal shapes of the force profile can be clearly seen in the best-fit curves of the plots of 30 motor units and 50 motor units (dashed lines). The change in curvature of the 10 motor-unit simulation is more subtle. The variations in the base line force of the simulations are due to the changing location of the slow and fast fiber types. Increasing the number of motor units increases the maximum value of the force, which is also a result of fiber type location.

per muscle, the R^2 values are 0.9884, 0.9967, and 0.9972, respectively. The 30 and 50 motor-unit muscle exhibits an even higher smoothness coefficient than the TA with a 500 μm fiber diameter.

4. DISCUSSION

The proposed skeletal muscle modeling framework is capable of replicating many structural and functional aspects in skeletal muscles. The model's complexity and versatility to examine many different aspects of a skeletal muscle's function and structure through simulation is certainly the model's key strength. At the same time, this is also the model's weakness if muscle-specific simulations are desired. The complexity and versatility requires many input parameters about a skeletal muscle anatomy and functional distribution. The current body of knowledge for some of the input data is quite limited. This is in particular true for data concerning the motor-unit territories and a skeletal muscle's active and passive mechanical behavior, and, hence, the proper definition of the (multi-scale) constitutive within the theory of finite elasticity. Nevertheless, the forces predicted by the model (i.e., peak forces of 0.5–0.6 kN) agree well with experimental findings, e.g., Brand et al. (1986), Maganaris (2001), or Maganaris et al. (2001).

4.1. UNCERTAINTIES IN MOTOR-UNIT TERRITORY DATA

The limitations regarding the motor-unit territories stem from the fact that a rigorous description of all the parameters, which define the muscle fiber distribution across the motor units, is not available from the literature (Monti et al., 2001). Due to physical limitations in experiments, it is difficult to spatially analyze more than one motor unit at a time. Further, such analysis are generally based on 2D sections. These two factors combined lead, from an experimentally point of view, to limited insights into motor-unit distributions. Nevertheless, investigation such as those carried out by Bodine-Fowler et al. (1990), Roy et al. (1995), and Monti et al. (2001) can provide enough data for the generic assumptions made in this study.

Although three-dimensional descriptions of motor-unit fiber distributions within a muscle have been attempted, e.g., by analyzing multiple 10–20 μm thick cross-sectional segments of the muscle, the data is essentially only valid for the analyzed muscle. As far as the locations of a motor-unit's muscle fibers is concerned, there exists a substantial inter-subject variability of the location.

The definition of motor-unit territory weighting centers and the proposed random muscle-fiber-selection algorithm for populating motor units (cf. Section 2.1.2) provides in combination with the proposed continuum-mechanical framework (cf. Section 2.3) an alternative but biophysically based way to analyze the influence of different muscle fiber and motor-unit distributions on its mechanical behavior.

For example, from **Figure 5**, it becomes apparent that the force output becomes smoother as more motor units are added. In case of adding more motor units, however, there is a greater likelihood that distally located fibers are being activated first. Activating those fibers first would likely cause an increase in force as early activation allows the fibers to obtain a higher total force by the end of the simulation and their distal location means that their pennation angle is less and thus their effect on total force is greater. This is only one example that provides evidence that internal structures of the muscle can significantly contribute to the overall mechanical behavior of the muscle and that more information concerning the force transduction pathways within the muscle should be incorporated.

Further, one can carry out in analogy to the previous conclusion many other simulations aiming to investigate the effect of different muscle fiber and motor-unit distributions on the exerted muscle force. A systematic approach to setup such numerical experiments can lead, together with additional knowledge about the muscle's function and the purpose of other surrounding tissues, to a reverse-engineering approach of deducing certain (additional and unknown) information on motor-unit distributions.

4.2. LIMITATIONS IN CONSTITUTIVE MODELING

Despite the fact that the proposed multi-scale constitutive law does not incorporate many (micro-structural) detailed information on force transduction pathways, one obtains from the overall continuum-mechanical framework muscle forces that are similar to other numerical and physiological studies. Brand et al. (1986), for example, report 535 N as maximal exerted muscle force of the TA and Fukunaga et al. (1997) report for the force-length relationship of TA muscle fibers a maximum force of slightly more than 400 N.

Further, a mathematical validation of the multi-scale constitutive law has been carried out in Röhrle et al. (2008). In this work, Röhrle et al. (2008) showed that the multi-scale constitutive law exhibits for different activation principles, different finite element basis functions, and sequentially refined meshes similar finite element convergence rates as for the mechanical-only problem. This, however, only provides evidence that the homogenization of the cellular parameters for the multi-scale constitutive law have been defined in a mathematical consistent way. A full experimental validation of the coupling method is in fact currently almost impossible as the data required to generate a fully accurate constitutive law does not exist. A large amount of experimental and modeling work needs to be undertaken to fill the gap in

the literature regarding the three-dimensional mechanical properties of skeletal muscle tissue in both passive and active states. This work will almost definitely have to begin by looking at the micro-structural linkages between muscle fibers and the role that the epimysium, perimysium, and endomysium plays in modifying both the force output of muscle fibers, and the path of the generated force through the muscle. A more detailed mechanical description of the structure of skeletal muscle is desirable.

Another drawback of the current implementation of the coupling between the zero-dimensional cellular model (the Shorten et al., 2007 model), the one-dimensional fiber model (bidomain equations), and the three-dimensional continuum-mechanical model is the lack of force or length feedback from the continuum-mechanical model to the Shorten et al. (2007) transmembrane model and the recruitment model. The only data needed for each bidomain simulation was the activation timing (from rate coding). This is a considerable drawback, as much of the afferent input to the central nervous systems stems form feedback mechanisms within the muscle and tendon, e.g., muscle spindles and Golgi tendon organs.

4.3. THE CELLULAR MODEL AND MUSCLE FIBER RECRUITMENT

Feedback of mechanical data to the cellular level can currently not be directly included in the Shorten et al. (2007) model. Moreover, the Shorten et al. (2007) model is only valid for isometric contractions. Therefore the pre- and post-powerstroke concentrations (A_1 and A_2) have been multiplied by the normalized length relationship for active contractions, i.e., $f^{\text{fibre}}_{\text{active}}(\lambda)$, to account for the probability by how much the actin and myosin overlap. The Shorten et al. (2007) model can easily be extended to reflect non-isometric contractions as well. In such a case, the actin-myosin overlap might be directly considered within the cell model and hence would make an additional multiplication with a force-length relationship, which is typically derived from whole-muscle experiments, redundant. While such an extension is a necessary enhancement for a more general case, it would also provide a direct and strong coupling between the cellular and continuum-mechanical model. Another drawback of the Shorten et al. (2007) model within the proposed framework is the fact that the cell model has been precisely parameterized for mouse TA and not for a human TA. However, no parameters for a human skeletal muscle model are available. The bidomain parameters were taken from experimental data on a mouse's skeletal muscle. The parameters for LSODA were selected to maximize accuracy and minimize computational cost.

The major advantage of the proposed framework is that the motor-unit recruitment model can be replaced, in a straight forward fashion, by any other motor-unit recruitment model. In particular in the field of computational neurophysiology, there exist state-of-the-art motor neuron recruitment models that are no longer phenomenological, e.g., the motor-unit recruitment model used within this framework, but describe neural recruitment based on complex networks of cellular models (e.g., Cisi and Kohn, 2008). The drawback of all existing computational skeletal muscle mechanical models used in the field of computational neurophysiology is the fact that the calculations of the exerted muscle forces are extremely simplified neglecting any anatomical arrangements or mechanical behavior. This is an even more severe restriction, if researchers draw conclusions from their neurophysiological models based on computed muscle forces without considering an anatomically detailed skeletal muscle mechanics model. Linking both modeling approaches, i.e., the systems biological approaches for neural networks and the proposed skeletal muscle mechanics model, can provide a powerful computational framework to gain new insights in neurological disorders associated with muscle diseases. The proposed model is ideal for such research.

4.4. FORCE-VELOCITY RELATIONSHIP

The current framework considers only the special case of isometric contractions of the TA. Throughout this paper, the authors assumed that the force-velocity relationship provides only a very minor contribution within the considered experiments and, hence, can be neglected. Nevertheless, the force-length relationship can be easily implemented within the proposed framework in a number of different ways. Firstly, one can include the force-velocity relationship in a biophysical sense by alternating the Shorten et al. (2007) model. This can be achieved by incorporating biophysical effects altering the crossbridge kinetics as a result of the local contraction velocity. Secondly, the force-velocity relationship can be incorporated into the multi-scale constitutive law in a similar way as the force-length dependency, i.e., by multiplying the active stress tensor, $\mathbf{S}^{\text{active}}$ by a length dependent hyperbolic force-velocity relationship as it has been introduced, for the first time, by Hill (1938) or by Johansson et al. (2000) within a FE framework.

4.5. SUBJECT-SPECIFIC VARIABILITY

The modeling of the muscle fiber distributions, cellular processes, and its link to a continuum-mechanical skeletal muscle model requires a large set of input variables. To be able to find adequate model parameters, many parameters from different studies had to be combined. In some cases, the parameter did not even originate from humans as ethical measurements of such data in (living) persons would not be able to be conducted. Due to the great inter-subject variability of many of those parameters, it is essentially an impossible task to obtain a realistic set of model parameters for a subject-specific case.

Nevertheless, the proposed skeletal muscle model provides a detailed biophysical skeletal muscle mechanics model. Almost all of the parameters used within the framework are based on some experimental studies. In particular following the described methods for embedding fibers within a three-dimensional geometry and assigning the fibers to a specific motor unit results in anatomically detailed models that could exist in such a way. For instance, the origin and insertion of the skeletal muscle fibers are anatomically realistic and their orientation agrees with published data (Lansdown et al., 2007). The motor-unit distribution is consistent with published physiological data (Monti et al., 2001) and is also similar to the method used by other numerical studies (Yao et al., 2000; Enoka and Fuglevand, 2001). The geometry and the muscle fiber growth algorithm lead to a inherent description of the motor endplate band, which is defined by the center points of all the muscle fibers. The motor endplates within the proposed model form a

parabola as it is described in Aquilonius et al. (1984). Hence, the presented framework presents a great resource for exploring and investigating many open questions in skeletal muscle physiology and mechanics in a general and qualitative way.

4.6. VALIDATION AND HUMAN SPECIFIC PARAMETERS

Any physiologically based mathematical model relies on experimentally determined parameters. The comparative scarcity of human data generally means that models of human systems rely on the assumption that meaningful results can be gained when using parameters determined from different species. The benefit of building models of human tissue is that the assumed accuracy of parameters can be tested in a computational setting. Discrepancies between experimentally determined parameters in one species and the computationally predicted results of another species using these parameters can provide an insight into areas where more specific inquiry is needed.

Validation of the framework is therefore of key importance to any model. This work aims to provide a qualitative validation of the model. The trends predicted by the model, e.g., effects of motor-unit location and recruitment effects, fit within known experimental bounds. A more in-depth validation procedure would see a number of subject-specific models created using as many of the subject-specific material properties as possible. In contrast to the simulated approach, a rigorous experimental validation procedure can only be achieved for very simple systems, such as artificially activated *ex vivo* rodent muscle between force transducers. In addition to artificial activation of a muscle, the models predictive capabilities could be tested by accounting for the roles of synergist and antagonist muscle groups similar to the experimental approaches of Maganaris (2001) or Maganaris et al. (2001). Each of the above mentioned validation procedure would require a separate and thorough study. Other validation options include numerical validation procedures to investigate the effects of discretization errors, approximation errors, and errors introduced through the homogenization process of coupling the cellular level with the organ level. A *in silico* validation of the multi-scale constitutive law has been provided previously (Röhrle et al., 2008).

CONCLUSION

A novel approach to the three-dimensional modeling of skeletal muscle function is presented. This method has the potential to represent more of the known anatomical and physiological information than other modeling techniques currently available. This approach incorporates cellular physiology, anatomical structure, and functional grouping into a finite elasticity simulation of skeletal muscle. This multi-scale approach allows analysis of the effects of alterations to a large range of physiological and structural parameters, which is important when investigating physiological diseases, mechanical injury, or changes resulting from training and aging. While this framework still requires rigorous validation, it provides one of the most integrated electromechanical model of a skeletal muscle, which is currently available.

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Effects of physical activity and inactivity on muscle fatigue

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The aim of this review was to examine the mechanisms by which physical activity and inactivity modify muscle fatigue. It is well known that acute or chronic increases in physical activity result in structural, metabolic, hormonal, neural, and molecular adaptations that increase the level of force or power that can be sustained by a muscle. These adaptations depend on the type, intensity, and volume of the exercise stimulus, but recent studies have highlighted the role of high intensity, short-duration exercise as a time-efficient method to achieve both anaerobic and aerobic/endurance type adaptations. The factors that determine the fatigue profile of a muscle during intense exercise include muscle fiber composition, neuromuscular characteristics, high energy metabolite stores, buffering capacity, ionic regulation, capillarization, and mitochondrial density. Muscle fiber-type transformation during exercise training is usually toward the intermediate type IIA at the expense of both type I and IIx myosin heavy-chain isoforms. High-intensity training results in increases of both glycolytic and oxidative enzymes, muscle capillarization, improved phosphocreatine resynthesis and regulation of K^+, H^+, and lactate ions. Decreases of the habitual activity level due to injury or sedentary lifestyle result in partial or even compete reversal of the adaptations due to previous training, manifested by reductions in fiber cross-sectional area, decreased oxidative capacity, and capillarization. Complete immobilization due to injury results in markedly decreased force output and fatigue resistance. Muscle unloading reduces electromyographic activity and causes muscle atrophy and significant decreases in capillarization and oxidative enzymes activity. The last part of the review discusses the beneficial effects of intermittent high-intensity exercise training in patients with different health conditions to demonstrate the powerful effect of exercise on health and well being.

Keywords: high-intensity exercise, training, repeated sprints, aerobic training

INTRODUCTION

Muscle fatigue can be defined as the inability to maintain the required or expected force or power output (Edwards, 1981; Fitts, 1994). Due to the fact that a decrease in muscle performance may ensue even during a submaximal activity, a more appropriate definition of fatigue for any population may be: "any decline in muscle performance associated with muscle activity at the original intensity (Simonson and Weiser, 1976; Bigland-Ritchie et al., 1986). Muscle fatigue is a common symptom during sport and exercise activities, but is also increasingly observed as a secondary outcome in many diseases and health conditions during performance of everyday activities (Rimmer et al., 2012). In many of these health conditions, physical inactivity is a major contributing factor to the increased fatigability of the patient. Deconditioning as a result of restricted physical activity results in large decreases in muscle mass and strength, as well as increased fatigability due to changes in muscle metabolism (Bloomfield, 1997; Rimmer et al., 2012). On the other end of the physical activity spectrum, chronic exercise training increases muscle strength and function, and enhances the ability of the muscles to resist fatigue in healthy individuals and patients of all ages (Bishop et al., 2011; Hurley et al., 2011).

The aim of the present review is to investigate and explain the differences in muscle fatigue between individuals with different physical activity levels histories. The effects of different types of training will be evaluated and compared, while the factors that contribute to muscle fatigue in healthy individuals will be analyzed. Also, the outcomes of an acute or chronic decrease in physical activity due to injury, immobilization, or illness will be examined. Finally, the beneficial effects of exercise in patients with different health conditions will be presented in an attempt to demonstrate the powerful effect of exercise training not only on sport performance, but also on health and well being.

MUSCLE FATIGUE IN INDIVIDUALS WITH DIFFERENT TRAINING BACKGROUNDS

Training history has an impact on muscle fatigue profile during high-intensity exercise. It is well known that power trained athletes are stronger and faster than both endurance athletes and untrained individuals. Previous studies have shown that power trained athletes have 25–35% higher maximal voluntary contraction (MVC) force and maximal rate of force development (RFD), as well as peak and mean power compared to endurance athletes (Paasuke et al., 1999; Calbet et al., 2003). When comparing the fatigue profiles of those athletes, a lower peak power but a slower rate of muscle power decline is observed in endurance athletes than in power athletes. This is due to the ability of endurance trained athletes to better maintain their performance during the test as shown

by their lower fatigue index, calculated as the rate of drop from peak to end power output.

The differences in fatigue between power and endurance trained athletes are more evident when repeated bouts of maximal exercise are performed with short recovery intervals. A common method to examine fatigue in maximal repeated muscle performance is to calculate fatigue during a protocol of short-duration sprints, interspersed with brief recoveries (Bishop et al., 2011). In that case, fatigue index is expressed as the drop of peak or mean power from the first to the last sprint (Hamilton et al., 1991), or preferably as the average decrement of power in all sprints relative to the first sprint (Fitzsimons et al., 1993). According to the later calculation of fatigue, endurance runners had a 37% smaller power decrement over five 6 s maximal sprints interspersed with 24 s rest, compared with team sports players (Bishop and Spencer, 2004). This was accompanied by smaller disturbances in blood homeostasis as reflected by lower post-exercise blood lactate concentration (Bishop and Spencer, 2004). One important factor that may contribute to the slower rate of fatigue and the smaller metabolic disturbances of endurance trained individuals is their higher aerobic fitness. It has been shown that endurance athletes have higher oxygen uptake during a repeated sprint test, indicating a greater contribution of aerobic metabolism to energy supply (Hamilton et al., 1991). The comparison of fatigue profiles between athletes with different training background reveals some possible mechanisms that determine the ability of the muscle to maintain high performance. It is now accepted that the factors causing fatigue may range from central (e.g., inadequate generation of motor command in the motor cortex) to peripheral (e.g., accumulation of metabolites within the muscle fibers (Girard et al., 2011). High-intensity exercise, usually in the form of repeated bouts interspersed with a short interval, can be used as a model to examine muscle fatigue both in health and disease. The recent use of intense interval exercise as a time-efficient and highly effective strategy for training healthy individuals (Burgomaster et al., 2008) and patients with various health conditions (e.g., chronic obstructive pulmonary disease, COPD patients; Vogiatzis, 2011), necessitates understanding of the factors that cause muscle fatigue in this type of exercise.

FACTORS MODIFYING FATIGUE IN PHYSICALLY ACTIVE INDIVIDUALS

MUSCLE FIBER COMPOSITION

It is known for many decades that muscle fiber composition differs between sprint/power trained and endurance trained athletes and untrained individuals (Costill et al., 1976). The traditional distinction between slow and fast muscle fibers based on myosin ATPase, has been replaced by the characterization according to the expression of myosin heavy-chain (MHC) isoforms. The classification of fibers according to MHC can provide an informative picture about functional characteristics such as strength, power, and fatigue resistance (Bottinelli, 2001; Malisoux et al., 2007). Based on the major MHC isoforms, three pure fiber types can be identified: slow type I and fast type IIA and IIX (Sargeant, 2007). Although these fiber types have similar force per unit cross-sectional area (CSA), they differ considerably in maximum shortening velocity (type I about four to five times slower than IIX) and power generating capacity (Sargeant, 2007). Furthermore, type IIX fibers have an enzymatic profile that favors anaerobic metabolism, namely, high resting phosphocreatine (PCr) content (Casey et al., 1996) and high concentration and activity of key glycolytic enzymes such as glycogen phosphorylase and phosphofructokinase (Pette, 1985). This profile makes the fiber more vulnerable to fatigue due to energy depletion or accumulation of metabolites (Fitts, 2008). On the other hand, type I fibers have a higher content and activity of oxidative enzymes that favor aerobic metabolism and fatigue resistance (Pette, 1985). Thus, muscles with a greater proportion of type I fibers would be more fatigue resistant compared with muscles with a greater proportion of type IIA and type IIX fibers. In this context, endurance trained individuals have a higher percentage of type I slow/fatigue resistant fibers (about 65% type I fibers in the gastrocnemius muscle; Harber and Trappe, 2008), compared with sprinters (about 40% type I fibers in the quadriceps; Korhonen et al., 2006), and recreationally active individuals (about 50% type I fibers in the gastrocnemius muscle; Harber and Trappe, 2008).

The greater fatigability of individuals whose muscles have a high percentage of type II fibers was demonstrated in several studies. For example, Hamada et al. (2003) reported a more than twofold greater decrease in force during repeated maximal voluntary isometric contractions of the quadriceps, in individuals with a high percentage of type II fibers (72%) compared with individuals with much lower (39%) type II fibers. Similar findings were reported by Colliander et al. (1988) using repeated bouts of isokinetic exercise. An interesting finding in that investigation was that when blood flow to the leg was occluded using a pneumatic cuff, the decrease in peak force was fivefold greater in the group of subjects with higher percentage of type I muscle fibers. This indicates the reliance of these fibers on blood flow, oxygen availability, and aerobic metabolism (Colliander et al., 1988).

Single fiber studies show that there is a selective recruitment and selective fatigue of the fast fibers containing the IIX MHC isoform, as shown by a large (70%) decrease of single fiber ATP within 10 s of sprint exercise (Karatzaferi et al., 2001b). At the same time, type I fibers showed no change in ATP. This may suggest that the contribution of the faster and powerful fibers that contain the IIX isoform may be decreased after the first few seconds of high-intensity exercise (Sargeant, 2007). The greater metabolic disturbances in type II compared to type I fibers may be due to the faster rate of PCr degradation and anaerobic glycolysis and thus lactate and H^+ accumulation (Greenhaff et al., 1994).

IONIC REGULATION

During high-intensity exercise large changes in metabolites and ions are observed within the working muscles (Juel et al., 2004; Mohr et al., 2007; Cairns and Lindinger, 2008). Disturbances in the concentration of muscle lactate, hydrogen (H^+), potassium (K^+), and calcium (Ca^{2+}) ions are linked with fatigue (McKenna et al., 2008) and thus ionic regulation becomes critical for muscle membrane excitation, contraction, and energy metabolism (Allen et al., 2008).

The extensive activation of glycogenolysis and anaerobic glycolysis result in H^+ accumulation that decreases muscle pH by about 0.5 unit (McCully et al., 1994; Bogdanis et al., 1995). The ability of

the muscle to regulate H^+ and lactate homeostasis during high-intensity exercise may play an important role in the fatigue process (Juel, 2008). Several mechanisms contribute to muscle pH regulation, including release of H^+ to the blood via different transport systems and buffering of H^+ within the muscle (Juel, 2008). The most important membrane transport systems involved in pH regulation are the Na^+/H^+ exchange, which is considered as the most important, the Na^+/bicarbonate co-transport, and the lactate/H^+ co-transport (Juel, 2008).

Although removal of H^+ and lactate out of the muscle cell are considered important for the restoration of muscle performance following intense contractions, there is evidence to suggest that low pH and high lactate have far less inhibitory effects on the activation of the contractile apparatus than previously assumed (Allen et al., 2008). This evidence comes mainly from isolated animal muscle and single fiber experiments and suggests that a small part of fatigue is due to increased inorganic phosphate (Pi), that reduces force output, and K^+ accumulation inside the t-tubules, that affects action potential (Allen et al., 2008). According to this evidence, the greatest part of fatigue, is due to reduced sarcoplasmic reticulum calcium (Ca^{2+}) release and decreased Ca^{2+} sensitivity of the contractile proteins (Allen et al., 2008). An increasing body of data shows that oxidative stress may influence Ca^{2+} sensitivity and Ca^{2+} reuptake by the sarcoplasmic reticulum and therefore muscle function and fatigue (Westerblad and Allen, 2011).

EFFECTS OF REACTIVE OXYGEN SPECIES ON SKELETAL MUSCLE MASS AND FUNCTION

There is growing evidence that reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced in skeletal muscles under physiological (exercise) as well as under pathological conditions (Lamb and Westerblad, 2011; Pellegrino et al., 2011; Westerblad and Allen, 2011). The most important ROS are: (a) the superoxide anion that is mainly produced in the mitochondria as a by-product of oxidative phosphorylation and by NADPH and xanthine oxidases (b) hydrogen peroxide (H_2O_2) and (c) hydroxyl radicals (Allen et al., 2008). Although increased ROS are implicated in muscle fatigue, it is becoming increasingly clear that ROS are important components in normal cellular signaling and adaptation (Westerblad and Allen, 2011). ROS can cause muscle fatigue by decreasing maximum Ca^{2+} activated force, Ca^{2+} sensitivity, and Ca^{2+} release, and this was demonstrated by experiments where administration of ROS scavengers/antioxidants delayed fatigue development (Lamb and Westerblad, 2011). From all the administered antioxidant supplements (e.g., ubiquinone-10, vitamins C and E), the antioxidant *N*-acetylcysteine (NAC) has proven to be the most effective (Hernandez et al., 2012). NAC easily enters cells and contains a thiol group that can interact with ROS and their derivatives (Ferreira and Reid, 2008). Also, as a thiol donor, NAC also supports resynthesis of one of the major endogenous antioxidant systems, glutathione (Hernandez et al., 2012). The endogenous ROS scavenging pathways, such as glutathione peroxidase (GPX) and superoxide dismutase (SOD) activities are substantially up-regulated by exercise training (Allen et al., 2008).

Reactive oxygen species have also been implicated in damage of cell proteins, DNA, and lipids through oxidation and thus have been related with muscle damage and muscle wasting observed in heavy exercise, disuse, and various pathological conditions (Pellegrino et al., 2011). In models of disuse muscle atrophy using hindlimb unloading and limb immobilization, the potential role of oxidative stress in determining muscle wasting has been manifested as an increase in oxidative stress (free iron, xanthine oxidase activity, lipid peroxidation, and oxidized/reduced glutathione ratio), together with an impairment in antioxidant defense systems (decreased catalase and GPX activities) and other protective proteins such as heat shock proteins (Lawler et al., 2003, 2006; Pellegrino et al., 2011). However, the co-existence of oxidative stress and muscle atrophy does not necessarily imply a cause and effect relationship for the hindlimb unloading model. Similarly, data from the few human bed rest studies suggest a decrease in protein synthesis, suggesting anabolic resistance, and not mainly protein breakdown due to oxidative stress (for a review see Pellegrino et al., 2011). However, in respiratory, kidney, and cardiac disease and muscular dystrophy the pivotal role of oxidative stress and increased proteolysis has been suggested (Moylan and Reid, 2007).

CONTRIBUTION OF OXIDATIVE METABOLISM TO ENERGY SUPPLY

In recent years, data have been accumulated showing the significant contribution or oxidative metabolism to the energy supply during short bouts of all-out exercise, such as sprinting (Bogdanis et al., 1996, 1998; Spencer et al., 2005). An early study by Gaitanos et al. (1993) noted that although the decrease in mean power during 10 × 6 s sprints with 30 s rest was 27%, anaerobic energy supply was reduced by almost threefold (70%), due to diminished contribution of glycolysis to anaerobic ATP turnover. They were the first to suggest that power output during the last sprints was probably sustained by increased contribution of oxidative metabolism. The enhanced contribution of oxidative metabolism to repeated all-out exercise was quantified in a later study using a protocol of two 30 s sprints separated by 4 min of passive rest (Bogdanis et al., 1996). Aerobic energy contribution during the first 30 s sprint was about 29% and this increased to 43% during the second 30 s sprint performed 4 min later (**Figure 1**). Interestingly, aerobic contribution was further increased to 65% of the energy during the last 20 s of the second sprint, at which point 85 ± 3% of $V_{O_2\,max}$ was attained (Bogdanis et al., 1996 and unpublished calculations from biopsy and oxygen uptake data). An increased aerobic contribution to energy supply during repeated bouts of high-intensity exercise has been reported for endurance trained individuals (Hamilton et al., 1991; Tomlin and Wenger, 2002; Calbet et al., 2003). It is noteworthy that oxygen uptake increases very fast during repeated short-duration sprints (15 m × 40 m with 25 s rest), reaching 80–100% $V_{O_2\,max}$ during the last sprints (Dupont et al., 2005).

RATE OF PHOSPHOCREATINE RESYNTHESIS

Phosphocreatine degradation provides the most immediate and faster source of ATP resynthesis during high-intensity exercise (Sahlin et al., 1998). However, due to the relatively low intramuscular stores, PCr is exhausted early during a single bout of high-intensity exercise. However, PCr is rapidly resynthesized during the recovery following exercise and thus the rate of PCr

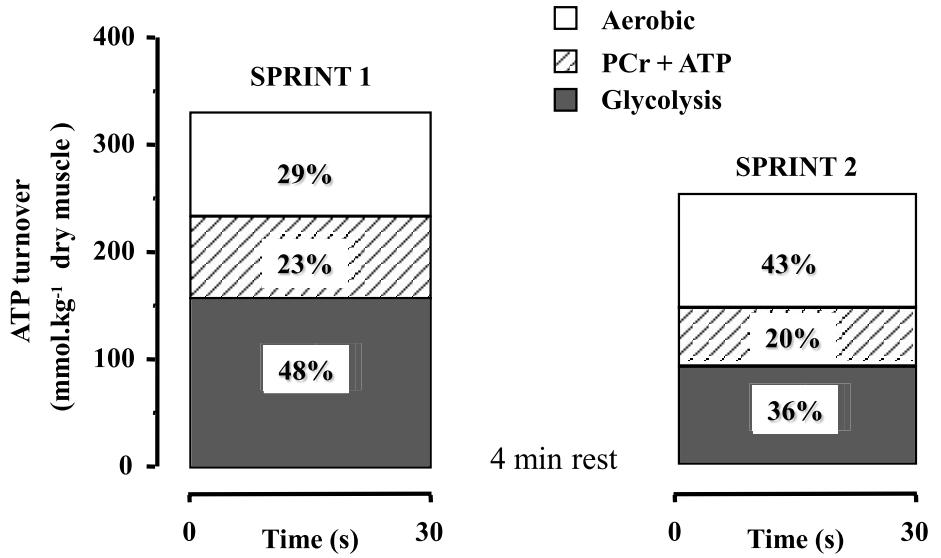

FIGURE 1 | Calculated ATP turnover (percent contribution) from ATP + PCr degradation, anaerobic glycolysis, and aerobic metabolism during two 30 s sprints separated by 4 min of recovery Redrawn using data from Bogdanis et al. (1996).

resynthesis determines its availability for the next exercise bout. Consequently, individuals with fast PCr resynthesis exhibit greater fatigue resistance during repeated bouts of high-intensity exercise (Bogdanis et al., 1996; Casey et al., 1996; Johansen and Quistorff, 2003). PCr resynthesis depends highly on oxygen availability (Haseler et al., 1999, 2007). The rate of PCr resynthesis measured by phosphorus nuclear magnetic resonance spectroscopy has been widely used as an index of muscle oxidative capacity (Haseler et al., 2004). Johansen and Quistorff (2003) have examined the differences in PCr resynthesis and performance recovery between endurance trained, sprint trained, and untrained individuals using phosphorus nuclear magnetic resonance spectroscopy. Participants performed four maximal isometric contractions of 30 s duration, interspersed by 60 s recovery intervals. Endurance trained athletes showed almost twice as fast PCr resynthesis rate compared to sprint trained and untrained participants (half time, $t_{1/2}$: 12.5 ± 1.5 vs. 22.5 ± 2.5 vs. 26.4 ± 2.8 s, respectively). This resulted in almost complete restoration of PCr stores prior to each contraction for the endurance athletes, whereas the untrained and the sprinters started the subsequent contractions with a PCr level of about 80% of baseline. There is evidence to suggest that the faster rate of PCr resynthesis in endurance athletes is probably not related to $V_{O_2\,max}$. The relationship between $V_{O_2\,max}$ and PCr resynthesis has been questioned, since it has been shown that individuals with high and low $V_{O_2\,max}$ (64.4 ± 1.4 vs. 46.6 ± 1.1 ml kg^{-1} min^{-1}, $P < 0.01$) have similar PCr resynthesis rates (Cooke et al., 1997). Furthermore, individuals with equal $V_{O_2\,max}$ levels may have remarkably different endurance capacity because of differences in "peripheral" or muscle characteristics such as muscle capillary density and onset of blood lactate accumulation (Coyle et al., 1988). Thus, it may be suggested that the faster rate of PCr resynthesis in endurance trained individuals is most probably related with adaptations that favor blood flow and oxygen delivery and utilization in the muscle, such as increased mitochondrial content, capillary density and oxidative enzyme content and activity (Andersen and Henriksson, 1977; Tesch and Wright, 1983; Tesch et al., 1985; Karatzaferi et al., 2001a). The role of peripheral adaptations on PCr resynthesis has also been indirectly demonstrated by the high positive correlation ($r = 0.89$, $P < 0.01$) between the percent PCr resynthesis 4 min after a 30-s sprint and endurance fitness as determined from the percentage of $V_{O_2\,max}$ corresponding to a blood lactate concentration of 4 mmol l^{-1} (Bogdanis et al., 1996).

NEURAL FACTORS

The level and the type of physical activity have an impact on the functional organization of the neuromuscular system. Power trained athletes have been shown to be more affected by fatiguing exercise than endurance athletes (Paasuke et al., 1999). Electromyographic (EMG) activity of the agonist and antagonist muscles and the level of voluntary activation of motor units have been traditionally used to examine the contribution of neural factors on muscle fatigue. A failure to fully activate the muscles that contribute to force or power output would imply the importance of neural factors in affecting the rate of muscle fatigue.

Changes in the normalized EMG amplitude (root mean square, RMS) of the vastus lateralis muscle during 10×6 s sprints interspersed by 30 s rest explained 97% of the total work done, suggesting that fatigue is accompanied by reductions in neural drive and muscle activation (Mendez-Villanueva et al., 2008). However, the parallel decrease of EMG activity and power output may imply that the decreased neural drive may be the consequence and not the cause of the decreased performance. Amann and Dempsey (2008) demonstrated that feedback from group III/IV muscle afferents exerts an inhibitory influence on central motor drive, so that to avoid excessive development of peripheral fatigue beyond a sensory tolerance limit associated with potential muscle tissue damage.

A common finding in many studies assessing neuromuscular activity is that fatigue in high-intensity exercise is characterized by a shift in the EMG power spectrum of the muscles involved, possibly indicating selective fatigue of fast-twitch fibers (Kupa et al., 1995; Billaut et al., 2006). This selective fatigue of the fast-twitch fibers may be related with increased fatigue in individuals with a high percentage of fast fibers.

Another neuromuscular characteristic that may be affected by physical activity level is voluntary activation. Voluntary activation of a muscle during an MVC can range from 80 to 100% (Behm et al., 2002). When a muscle is sub maximally activated during an MVC (e.g., 70% of its full capacity), fatigue is likely to develop at a slower rate than if it was fully activated. Sub-optimal muscle activation during maximal effort is commonly observed in children performing high-intensity exercise and is one of the reasons that young individuals fatigue less compared with adults (Ratel et al., 2003). However, sub-optimal muscle activation is not uncommon in adults. Nordlund et al. (2004) reported a wide range of voluntary activation (67.9–99.9%) for the plantar flexors of healthy habitually active males. A novel finding of that study was that a large percentage (58%) of the variance in fatigue during repeated short maximal isometric contractions was explained by the magnitude of MVC torque and the initial percent voluntary activation. This finding provides support to the suggestion that individuals who cannot fully activate their muscles fatigue less but are able to generate much less force and muscle power. This may be related with a failure to recruit all fast-twitch fibers results, which, in turn, results in less metabolic disturbances and less fatigue during high-intensity exercise. It must be noted that the level of voluntary activation is reduced with fatigue, as shown by a study by Racinais et al. (2007), who reported a decrease in voluntary activation from 95 to 91.5% ($P < 0.02$), along with a 10% decrease in peak power and an about 17% decrease in MVC following ten 6 s sprints.

Fatigue during high-intensity dynamic exercise may become greater due to the loss of synchronization between agonist and antagonist muscles and the increased level of co-contraction of the antagonists muscles. This would decrease the effective force or power generated by a joint, especially during faster movements where neuromuscular coordination is more important. Garrandes et al. (2007) reported that the co-activation level of the antagonist muscles during knee extension was increased by 31% after fatigue only in power trained and not in endurance athletes. Earlier findings by Osternig et al. (1986) showed a four times higher hamstrings co-activation during isokinetic knee extensions in sprinters compared with distance runners (57 vs. 14%), probably indicating a sport-specific adaptation. The higher antagonist co-activation in sprint/power trained individuals may partly explain their greater fatigue during dynamic exercise, since part of the agonist force/power is lost to overcome antagonist muscle activity. However, Hautier et al. (2000) reported that the lower activation of the antagonist knee flexor muscled due to fatigue appeared to be an efficient adaptation of the inter-muscular coordination to modulate the net force generated by the fatigued agonists and maintain the force applied on the pedals.

INFLUENCE OF INITIAL FORCE OR POWER ON MUSCLE FATIGUE

Fatigue is traditionally calculated as the drop of force or power from an initial value to the lowest or end value. A common observation when examining fatigue is that individuals who can generate high force or power per kg body or muscle mass, usually fatigue quicker (Girard et al., 2011). Previous studies have reported that initial sprint performance is strongly correlated with fatigue during a repeated sprint test (Hamilton et al., 1991; Bishop et al., 2003) and inversely related to maximal oxygen uptake (Bogdanis et al., 1996). In fact, when comparing endurance and sprint trained athletes, relative power output (per kg body mass) is only different at the initial part of the exercise bout and thereafter performance is similar or even greater in endurance athletes (Calbet et al., 2003). A high force or power output (per kg body mass) during the first part of a high intensity bout may imply high reliance on fast-twitch fibers and anaerobic metabolism and thus greater metabolic disturbances. Thus, the greater fatigue of more powerful athletes may be more related to differences in fiber-type contribution and energy metabolism than a greater initial force or power.

Tomlin and Wenger (2002) and later Bishop and Edge (2006) investigated the influence of the initial power output on fatigue during high-intensity exercise, by comparing two groups of female team sports athletes who had similar peak and mean power output in a 6-s cycle ergometer sprint, but different maximal oxygen uptake values (low $V_{O_2\,max}$: 34–36 vs. moderate $V_{O_2\,max}$: 47–50 ml kg^{-1} min^{-1}). These athletes were required to perform five 6 s sprints with 24 s recovery (Bishop and Edge, 2006) or ten 6 s sprints with 30 s recovery (Tomlin and Wenger, 2002). Even though the two groups were matched for initial sprint performance, the moderate $V_{O_2\,max}$ group had a smaller power decrement across the 10 (low vs. moderate: 18.0 ± 7.6 vs. $8.8 \pm 3.7\%$, $P = 0.02$) or the 5 sprints (low vs. moderate: 11.1 ± 2.5 vs. 7.6 ± 3.4, $P = 0.045$). These results point to an important role of aerobic fitness on the ability to resist fatigue.

Mendez-Villanueva et al. (2008) investigated this issue by calculating the anaerobic power reserve of each individual. This was quantified as the difference between the maximal anaerobic power measured during a 6-s sprint and the maximal aerobic power determined during a graded test to exhaustion. Individuals with a lower anaerobic power reserve, who had less reliance on anaerobic metabolism, showed a greater resistance to fatigue. This suggests that the relative contribution of the aerobic and anaerobic pathways to energy supply and not the initial power *per se*, provide a better explanation for fatigue during repeated bouts of high-intensity exercise (Mendez-Villanueva et al., 2008).

CHANGES IN FATIGABILITY FOLLOWING EXERCISE TRAINING

A systematic change in functional demands posed on skeletal muscle will result in adaptations that increase performance toward the characteristics of the exercise stimulus. Depending on the stimulus, skeletal muscle can increase its size (D'Antona et al., 2006), alter muscle fiber-type composition (Malisoux et al., 2007), increase enzyme activities (Green and Pette, 1997), and modify muscle activation (Bishop et al., 2011). The adaptations that may reduce muscle fatigue during high-intensity exercise depend on the characteristics of the training program, i.e., type, intensity, frequency, and duration. Muscle fatigue will be reduced by appropriate shifts in fiber type, enhanced enzyme activity, regulation of ionic balance, and changes in muscle activation.

SKELETAL MUSCLE FIBER-TYPE SHIFTING DUE TO TRAINING

Differences in muscle fiber-type distribution between athletes of various sports reflect a combination of two factors: (a) natural selection, i.e., individuals with a high percentage of fast-twitch fibers follow and excel in a sport that requires speed and power, and (b) training-induced fiber-type transformation, i.e., small changes in muscle fiber-type distribution due to long term sport-specific training. Training studies show that it is possible to attain some degree of MHC transformation even with shorter term training (Malisoux et al., 2007). Transitions between MHC isoforms are done in a sequential, reversible order from type I ↔ type IIA ↔ type IIX and vice versa (Pette and Staron, 1997; Stevens et al., 1999). This shifting is determined by the neural impulse patterns, the mechanical loading characteristics, and by alterations in the metabolic homeostasis (Pette, 1998). In addition to the pure fiber types there are hybrid fibers co-expressing I and IIA or IIA and IIX MHC isoforms. There is evidence to suggest that the relative proportion of hybrid fibers may increase with training, so that the functional characteristics of the muscle are improved. For example, endurance training may increase the percentage of type I fibers co-expressing fast and slow isoforms, making them faster without losing fatigue resistance (Fitts and Widrick, 1996; Fitts, 2003).

The typical response following high-intensity sprint or heavy resistance training is a shifting of the faster (type IIX) fibers toward the intermediate type IIA fibers, with the percentage of type I fibers either decreasing or remaining unchanged (Esbjörnsson et al., 1993; Ross and Leveritt, 2001; Andersen and Aagaard, 2010). Most data from sprint training studies show that the MHC IIX isoforms are down-regulated and there is usually a bidirectional change toward IIA at the expense of both I and IIX MHC isoforms (Esbjörnsson et al., 1993; Andersen et al., 1994; Ross and Leveritt, 2001). However, an increase in slow twitch at the expense of the fast-twitch fibers has been reported following 7 weeks of sprint training (Linossier et al., 1993). It should be noted that there are very few pure type IIX fibers in skeletal muscles of healthy individuals, while most of the MHC IIX protein is found together with MHC IIA protein in hybrid fibers (Andersen et al., 1994; Malisoux et al., 2007). As will be discussed later in this review, pure type IIX fibers appear most often in disused muscles.

The functional adaptations of muscle fibers following sprint and strength training depend mainly on increases in fiber CSA, with the force per unit of CSA remaining unchanged in most (Widrick et al., 2002; Malisoux et al., 2007), but not in all studies (D'Antona et al., 2006). Maximal shortening velocity of single fibers also seems to be unchanged after resistance (Widrick et al., 2002) or sprint training (Harridge et al., 1998) in healthy young individuals, but there is some evidence that plyometric training may increase maximal shortening velocity in single fibers (Malisoux et al., 2007).

Training the muscle with lower intensity and longer duration stimuli, as used in endurance training, brings about different adaptations. Studies preformed over the last four decades by Pette and colleagues demonstrated the remarkable degree of transformation of fast, fatigable muscles toward slower, fatigue resistant in terms of both fiber type and metabolism using chronic low-frequency stimulation (Pette and Vrbova, 1999). Although this situation is not realistic, it demonstrated that activity may have a large impact on the phenotype and fatigue profile of skeletal muscle. Similar, but to a much lesser degree, effects on muscle fiber composition are seen during endurance training. Trappe et al. (2006) trained recreational runners so that they could compete in a Marathon after 16 weeks (13 weeks training and 3 weeks tapering). They reported a decrease in slow (MHC I) and fast (MHC IIA) fiber CSA by about 20%, but an increase in the percentage of MHC I fibers (from 48 ± 6 to $56 \pm 6\%$, $P < 0.05$), while the percentage of MHC IIA fibers remained unchanged ($30 \pm 5\%$,). A significant finding of that study was that single fiber muscle power expressed per unit fiber volume as measured *in vitro*, was increased by >70% in both MHC I and IIA fibers. These increases of power demonstrate that high-volume endurance training (30–60 km running per week) can modify the functional profile of the fibers that are most involved.

It seems therefore that fiber-type profile can be affected to some extent by both high intensity (sprint, strength, power) and endurance training in healthy individuals. The bidirectional shifts of the fast (type IIX) and slow (type I) fiber types toward the intermediate IIA isoform do not guarantee that fatigability will be improved. Factors such as changes in the metabolic properties (e.g., oxidative capacity) of all fiber types with training (Fitts and Widrick, 1996) as well as neural activation patterns of the contracting muscle may play an important role in fatigue resistance and should also be considered together with fiber type shifts. There is growing evidence suggesting that the functional properties of muscle fibers can change in several physiological and pathological conditions with no significant shift in myosin isoforms. This does not negate the important role of muscle fiber composition on fatigue, but rather shows that a "fine tuning" of one or more characteristics of a given fiber may be done according to functional demands (Malisoux et al., 2007).

INCREASES IN ENZYME ACTIVITIES

The metabolic profile of each muscle fiber is sensitive to training, even when no fiber-type transformation occurs (Pette, 1998). The majority of investigations have reported increases in the activity of key enzymes of glycolysis, such as glycogen phosphorylase, phosphofructokinase (PFK), and lactate dehydrogenase (LDH) following sprint training (Linossier et al., 1993, 1997; Dawson et al., 1998). Linossier et al. (1993) trained young students with repeated short sprints (5 s sprint 55 s rest) for 7 weeks with four sessions per week. The number of sprints per session was increased every week from 16 to 26 sprints per session. This program resulted in increased energy production from anaerobic glycolysis, as indicated by the increased muscle lactate accumulation after compared to before training (Δ lactate 37.2 ± 17.9 vs. 52.8 ± 13.5 mmol kg^{-1} dry weight $P < 0.01$) and the 20% higher PFK and LDH activity. A similar training study by Dawson et al. (1998) involving short running sprints of comparable duration to the previous study (30–60 m) found a 40% increase in glycogen phosphorylase, but no increase in PFK. A common finding of these two studies involving short sprints was that the activities of key oxidative enzymes involved in carbohydrate metabolism, e.g., citrate synthase (CS) or lipid oxidation, e.g., 3-hydroxyacyl-CoA dehydrogenase (HAD), were either unchanged (Linossier et al., 1993,

1997) or decreased (Dawson et al., 1998) with this type of repeated short sprint exercise.

However, data from sprint training studies using longer sprint durations such as 30 s sprints, showed increases in oxidative enzymes. For example, MacDougall et al. (1998) trained their subjects three times per week for 7 weeks using repeated 30 s sprints with 3–4 min rest in each session. The number of sprints increased progressively from 4 to 10 per session. This training program resulted in a significant increase in the total work done during the last three of the 4 × 30 s sprint test protocol. This was accompanied by a 49% increase in PFK activity ($P < 0.05$) and 36 and 65% increases in CS and succinate dehydrogenase (SDH, $P < 0.05$). Also an increase in $V_{O_2\,max}$ from 51.0 ± 1.8 to 54.5 ± 1.5 ($P < 0.05$) was reported, suggesting that repeated long sprints (30 s) constitute a powerful aerobic stimulus. In a similar training study with repeated 30 s cycle ergometer sprints, there was a 7.1% increase in mean power over a 30-s sprint and an 8% increase in $V_{O_2\,max}$ (Barnett et al., 2004). Interestingly, these authors reported a 42% increase in CS activity but no increase in PFK or anaerobic energy provision from PCr or glycolysis. They suggested that the improvement in 30 s sprint performance was probably mediated by increased energy provision from oxidative metabolism.

HIGH-INTENSITY TRAINING: A POWERFUL AND TIME-EFFICIENT EXERCISE STIMULUS

The adaptations caused by high-intensity exercise training have first been examined by Dudley et al. (1982), who reported that fast-twitch fibers respond to training by increasing cytochrome *c*, only when intensity was high. A decade later, McKenna and his research group started investigating the effects of sprint training on ionic balance (McKenna et al., 1993). As discussed in a previous section of this review, Bogdanis et al. (1996) were the first to demonstrate a large increase in oxidative metabolism coupled by a decrease in anaerobic glycolysis when a 30-s sprint was repeated after 4 min or recovery. The increase in aerobic metabolism and the decrease in glycolysis were possibly mediated by changes in key enzyme activities, such as glycogen phosphorylase, PFK, and pyruvate dehydrogenase (PDH). Parolin et al. (1999) reported an inhibition of glycogen phosphorylase transformation to the more active form due to increased H^+ concentration at the last of three 30 s sprints performed with a 4-min rest. At the same time PDH activity was enhanced possibly due to the increased H^+ concentration, resulting in a better matching between pyruvate production and oxidation and minimal muscle lactate accumulation. Repeated high-intensity bouts lasting from 30 s (Stepto et al., 1999) to 4 min (Helgerud et al., 2001) are used since then to improve endurance performance in several sports. These early studies indicated that repeated bouts of intense exercise rely heavily on aerobic energy supply and formed the bases for the increasingly popular high-intensity exercise interval training (high-intensity training, HIT) concept.

A series of more recent studies by Burgomaster et al. (2005, 2006, 2008) have shown that training with repeated 30 s sprints results in large increases in oxidative enzymes such as CS (by 38%), cytochrome *c* oxidase (COX), and HAD. These adaptations were achieved with only six training sessions performed over 2 weeks with 1–2 days rest (four to seven sprints 30 s sprints per session with 4 min rest) and were accompanied by a remarkable 100% increase in endurance capacity as defined by time to exhaustion at 80% $V_{O_2\,max}$ (from 26 ± 5 to 51 ± 11 min, $P < 0.05$). The authors have proposed the repeated 30 s sprint method as a time-efficient training strategy to simultaneously improve aerobic and anaerobic fitness and reduce fatigue. The extremely low time commitment (2.5 min per session for 5 × 30 s sprints, or less than 20 min including the 4-min rest intervals) makes this method attractive and further research is warranted to examine its possible applications in health and disease. The basis for the usefulness of this exercise scheme in both sports and clinical settings is that the exercise stimulus induces rapid phenotypic changes that resemble traditional endurance training and promotes mitochondrial biogenesis (Gibala, 2009) which appear to stimulate other healthy metabolic adaptations in skeletal muscle, such as improved insulin action, improved lipoprotein lipase activity and greater clearance of plasma triglycerides (Coyle, 2005).

Following the pioneering study by Burgomaster et al. (2005), Gibala et al. (2006) compared the typical HIT protocol (i.e., 4–6 × 30 s sprints with 4 min rest) with traditional endurance exercise (90–120 min of continuous cycling at 65% $V_{O_2\,max}$) performed three times per week for 2 weeks. The two protocols resulted in similar increases in muscle oxidative capacity as reflected by the activity of COX and a similar improvement in an endurance time trial (by 10.1 and 7.5%). The key role the increase of the active form of pyruvate dehydrogenase (PDH) after this type of training was highlighted in the study of Burgomaster et al. (2006) who also reported a concomitant reduction in glycogenolysis (from 139 ± 11 to 100 ± 16 mmol kg^{-1} dry weight, $P = 0.03$) and lower lactate accumulation possibly due to greater mitochondrial pyruvate oxidation. The lower level of acidification due to decreased glycogenolysis may have contributed to reduced fatigability following this type of training.

It should also be stressed that this type of repeated sprint exercise also increases $V_{O_2\,max}$ and improves cardiovascular function. Astorino et al. (2012) reported a 6% increase in $V_{O_2\,max}$, oxygen pulse and power output, in only six sessions of HIT involving repeated 30 s sprints over 2–3 weeks. However, in athletic populations, the importance of lower intensity–high-volume training should not be overlooked. Laursen (2010) in a critical review of low and high volume and intensity training suggested that training for sports performance should have an appropriate blend of both HIT and high-volume training, otherwise performance ability can stagnate. A polarized approach for optimal intensity distribution for the training of elite athletes of intense events (rowing, swimming, track running, and cycling) was suggested by Laursen (2010), whereby 75% of total training volume should be performed at low intensities, and 10–15% should be performed at very high intensities.

Another form of high-intensity interval training is called "aerobic interval training" and usually consists of four exercise bouts of 4 min each, at an intensity corresponding to 90–95% of peak heart rate or 85–90% $V_{O_2\,max}$, with 2–3 min or rest in between (Wisloff et al., 2007). This type of training is commonly used in soccer in the form of running or small sided games and has been proved to be very effective in delaying soccer specific or game fatigue. A comparison between the effectiveness of this training protocol

with a repeated sprint protocol has been performed by Ferrari Bravo et al. (2008). They compared the effects of training with a 4 × 4 min running at 90–95% of maximal heart rate, with 3 min active recovery vs. a repeated sprint training protocol that included three sets of six 40 m all-out "shuttle" sprints with 20 s passive recovery between sprints and 4 min between sets. The repeated sprint group, compared with the aerobic interval training group, showed a greater improvement not only in repeated sprint performance, but also in the soccer specific "Yo–Yo" intermittent recovery test (28.1 vs. 12.5%, $P < 0.01$). A similar improvement in $V_{O_2\,max}$ (6%) was found for the two groups. As noted above, the adaptations and improvements following HIT of either form (aerobic interval and repeated sprints) are far superior and time-efficient compared with longer duration continuous training. As will be discussed later, the benefits of high-intensity interval exercise of both forms (30 s–4 min high-intensity bouts) extend to health promotion and are currently proposed for improving health and reducing fatigue in many diseases (COPD and cardiac patients).

MOLECULAR BASES FOR ADAPTATIONS TO HIT

Understanding the multiple benefits of HIT requires investigation of the molecular signals that cause adaptations at the level of the skeletal muscle fiber. According to Coffey and Hawley (2007), there are at least four primary signals, as well as a number of secondary messengers, that are related with mitochondrial adaptations and glucose transport capacity across the sarcolemma:

(1) Mechanical tension or stretch,
(2) Oxidative stress manifested by an increase in ROS.
(3) Increase in intracellular calcium with each contraction.
(4) Altered energy status, as reflected by a lower ATP concentration.

Some putative signaling cascades promoting skeletal muscle mitochondrial biogenesis in response to high-intensity interval training may be as follows (Gibala et al., 2012): during intense muscle contractions, the rise in intracellular calcium activates the mitochondrial biogenesis messenger calmodulin kinase. At the same time, the "energy crisis" that results in decreased ATP and increased adenosine mono phosphate (AMP) activates the AMP-activated protein kinase (Gibala, 2009; Laursen, 2010). Activation of p38 mitogen-activated protein kinase (MAPK), possibly via increase generation of ROS may also be involved (Gibala et al., 2012). These signals can increase a key transcriptional coactivator, namely the peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), which is a key regulator of oxidative enzyme expression in skeletal muscle. PGC-1α has been described as a "master switch" that coordinates mitochondrial biogenesis by interacting with various nuclear genes encoding for mitochondrial proteins (Gibala, 2009; Gibala et al., 2012). Previous work has shown that an increased expression of PGC-1α in the muscle results in the conversion of the muscle from glycolytic to oxidative with a dramatic up-regulation of typical oxidative genes/proteins like COX. This results in a shift of the functional capacity of the muscle toward a more fatigue resistance profile found in the endurance trained state. Calvo et al. (2008) demonstrated that up-regulation of PGC-1α in transgenic mice, results in far superior exercise performance and 20% higher peak oxygen uptake compared with wild-type control mice. It is noteworthy that in the study of Burgomaster et al. (2008) which compared typical endurance training with HIT, PGC-1α protein content of the quadriceps muscle was equally increased in both protocols, demonstrating the large potential of the repeated sprint protocol to produce rapid mitochondrial adaptations. As suggested by Coyle (2005), one of the advantages of the repeated sprint protocol over the traditional endurance exercise, lays on the high level of type II muscle fiber recruitment that is not achieved in the traditional low intensity endurance exercise. Thus, HIT results in mitochondrial adaptations also in type II fibers that are absent when lower intensity/high-volume endurance training is performed. These adaptations of type II fibers would also increase their fatigue resistance and this is beneficial for high-intensity performance.

CHANGES IN MUSCLE FIBER CAPILLARY SUPPLY AND REGULATION OF IONIC BALANCE

As noted in the previous sections, the improvement in fatigue resistance is partly due to an increase in the enzymes that favor oxidative metabolism. However, a proliferation of capillary supply to muscle fibers would cause an additional improvement in fatigue resistance by enhanced lactate and H^+ elimination and oxygen supply (Tesch and Wright, 1983). Additional to the role of the different lactate and H^+ transport mechanisms out of the exercising muscle, improved perfusion contributes to the increased release from muscle to the blood (Juel, 2008). HIT training with intense leg extension exercise three to five times per week for 7 weeks (1 min exercise, 3 min rest for 1 h at ∼150% of leg $V_{O_2\,max}$) resulted in an increase of capillary-to-fiber ratio from 1.74 ± 0.10 to 2.37 ± 0.12 capillaries per fiber, and a 17% increase in capillary density (Jensen et al., 2004). These adaptations would increase oxygen extraction and facilitate aerobic metabolism during exercise as well as the rate of PCr resynthesis during the recovery intervals (McCully et al., 1991).

In a recent review, Iaia and Bangsbo (2010) presented the benefits of "speed endurance" training, which is a form of repeated HIT. The characteristics of this type of training are as follows: The form or exercise is running and the intensity is between 70 and 100% of the maximum running speed, which corresponds to a cardiorespiratory load very close or well above $V_{O_2\,max}$. The number of repetitions is between 3 and 12 repetitions and the duration of each bout is 10–40 s (usually 30 s) with a recovery interval greater than five times the exercise duration (usually 2–4 min). In well trained athletes, this type of training causes adaptations that do not appear to depend on changes in $V_{O_2\,max}$, muscle substrate levels, glycolytic and oxidative enzymes activity. Instead they appear to be related to improved running economy, and a higher expression of muscle Na^+, K^+ pump α-subunits, which may delay fatigue during intense exercise by increasing Na^+–K^+ pump activity and a reduced contraction-induced net loss of K^+, thus preserving muscle excitability (Iaia and Bangsbo, 2010). These conclusions were based on previous studies that compared the effects of two different intense training regimens on changes in muscle ATPase subunits and fatigue.

Mohr et al. (2007) divided participants into a sprint training group (15 × 6 s sprints with 1 min rest) and a speed endurance

group (8×30 s runs at 130% $V_{O_2\,max}$, with 1.5 min rest). Training was performed three to five times per week and lasted for 8 weeks. The fatigue index during a 5 m × 30 m sprint running test with 25 s active recovery, was reduced by 54% only in the speed endurance group, and remained unchanged in the sprint group. The reduction in fatigue was accompanied by a 68% increase in Na^+–K^+ ATPase isoform α2 and a 31% increase in the amount of the Na^+/H^+ exchanger isoform, only in the speed endurance group. These adaptations are possibly related with the metabolic responses (and thus metabolic load) during each session of speed endurance training, where peak blood lactate (14.5–16.5 mmol l^{-1}) and plasma K^+ (about 6.4 mmol l^{-1}) were higher compared to the sprint training responses (blood lactate: ∼8.5 and K^+: ∼5.5 mmol l^{-1}).

The marked increases in extracellular K^+ that are commonly observed during high-intensity exercise contribute to muscle fatigue by causing depolarization of the sarcolemmal and t-tubular membranes (McKenna et al., 2008). A training-induced increase in Na–K^+ ATPase activity has been shown to contribute to the control of K^+ homeostasis and reduce fatigue (Mohr et al., 2007).

However, the importance of pH regulation, especially in less trained and non-athletic populations and patients with various diseases should not be overlooked. It is well established that the pH regulating systems in skeletal muscles are very responsive to HIT (Juel, 2008). During high-intensity exercise and the subsequent recovery period, muscle pH is regulated by three systems: (1) lactate/H^+ co-transport by two important monocarboxylate transporter proteins: MCT1 and MCT4, (2) Na^+/H^+ exchange by a specific exchanger protein, and (3) Na^+/bicarbonate transporters (Juel, 2008). The MCT1 and MCT4 transporters are considered as the most important during exercise and thus their changes following training have been extensively studied in animal and human muscle. Animal studies have shown that HIT in rats for 5 weeks results in 30 and 85% in the MCT1 and Na^+/bicarbonate transporter, respectively, while MCT4 remained unchanged (Thomas et al., 2007). In humans, changes in the Na^+/H^+ exchanger protein levels by 30% have been reported in the 4-week high-intensity sprint training study of Iaia et al. (2008). Moreover, significant increases in MCT1 and Na^+/H^+ exchanger protein densities have been found after HIT, especially when training bouts cause a significant accumulation of H^+ in the muscle (Mohr et al., 2007). Increased expression of lactate and H^+ transporters results in faster H^+ and lactate release. Juel et al. (2004) used the one-legged knee extensor exercise model to examine changes in muscle pH regulating systems following intense training. Following 7 weeks of training with 15×1 min bouts of single knee extensions at 150% $V_{O_2\,max}$ per day, time to exhaustion was improved by 29%. The rate of lactate release at exhaustion was almost double (19.4 ± 3.6 vs. 10.6 ± 2.0 mmol min^{-1}, $P < 0.05$) and the rate of H^+ release was ∼50% higher (36.9 ± 3.1 vs. 24.2 ± 1.5 mmol min^{-1}, $P < 0.05$) for the trained than for the untrained leg. The membrane contents of the MCT1 lactate/H^+ co-transporter and Na^+/H^+ exchanger proteins were increased by 15 and 16%, while blood flow was also increased by 16% in the trained compared to the untrained leg. This study demonstrated that when muscle is stressed with training stimuli that cause high intramuscular lactate and H^+ concentration, it adapts by increasing the rate of lactate and H^+ transport out of the muscle. These adaptations are done by both changes in specific membrane proteins and structural changes, such as increased capillary density (Jensen et al., 2004), that enhance blood flow and thus transport of lactate and H^+ away from the working muscle.

Within the muscle cell, the ability to buffer the build-up of free H^+ in the muscle during high-intensity exercise is an important determinant of fatigue resistance and may be improved by training. To test this hypothesis, Edge et al. (2006) trained recreationally active female team sport players for 3 days per week for 5 weeks, using two protocols with matched for total work but different intensity. The high-intensity group performed six to ten 2-min bouts of cycling with 1 min rest at an intensity that was 120–140% of that corresponding to the 4-mmol l^{-1} blood lactate threshold. The moderate-intensity group performed continuous exercise at 80–95% of that corresponding to the lactate threshold for 20–30 min, so that the total work was the same with the high-intensity group. Blood lactate at the end of a typical training session was 16.1 ± 4.0 mmol l^{-1} for the high-intensity group and only 5.1 ± 3.0 mmol l^{-1} for the moderate-intensity exercise group. $V_{O_2\,max}$ and the intensity corresponding to lactate threshold were equally improved (by 10–14%) in both groups, but only the high-intensity group showed a significant increase in buffering capacity by 25% (from 123 ± 5 to 153 ± 7 μmol H^+ g dry muscle^{-1} pH^{-1}, $P < 0.05$), coupled with a greater improvement in a repeated sprint exercise performance compared with the low intensity group (13.0 vs. 8.5%, $P < 0.05$, Edge et al., 2005). Taken collectively, the above results emphasize the importance of exercise intensity for achieving the most favorable adaptations that delay muscle acidification and increase fatigue resistance. A reduced rate of H^+ accumulation, by transporting more H^+ out of the muscle and/or by intracellular buffering, would allow a greater contribution of glycolysis to energy supply and thus higher muscle performance.

EFFECTS OF PHYSICAL INACTIVITY ON MUSCLE FATIGUE

DETRAINING

Detraining is a period of insufficient or reduced training stimulus that causes reversal of adaptations at rates depending on the magnitude of physical activity reduction and by the length of the deconditioning period. Muscular and neural adaptations may be reversed at different rates, while muscle fiber phenotype is altered toward an increased expression of the fast MHC IIX phenotype (Andersen and Aagaard, 2000).

A common practice in detraining studies is to train the participants for a short or longer period and then remove the training stimulus and measure the detraining adaptations. Andersen et al. (2005) trained sedentary young males using knee extension exercises three times per week for 3 months using moderate to heavy resistances (from 10 to 6 repetition maximum, RM). Testing was performed before the start of training, after 3 months of training and again 3 months after detraining. Following 3 months of training, the CSA of quadriceps and EMG activity both increased by 10%. Also, isokinetic muscle strength at 30 and 240° s^{-1}, was increased by 18% ($P < 0.01$) and 10% ($P < 0.05$), but power, velocity, and acceleration of unloaded knee extension was unchanged. The proportion of MHC IIX decreased from 5.6 ± 0.8 to 0.8 ± 0.3% ($P < 0.001$), with a corresponding increase of MHC

IIA proportion from 34.0 ± 2.5 to $39.4 \pm 2.0\%$ ($P < 0.001$). After 3 months of detraining isokinetic CSA, EMG and muscle strength and power at 30 and 240° s^{-1} returned to pre-training levels. However, unloaded knee extension angular velocity and power were increased remarkably by 14 and 44% in relation to pre and post training. This was accompanied by an increase in MHC IIX isoform from 0.8 ± 0.3 to $7.7 \pm 1.1\%$, which was significantly higher compared with both pre and post training levels ($P < 0.001$). This phenomenon, i.e., an increase of the fast MHC IIX isoform is a typical adaptation to detraining following systematic training and has been observed in even greater extent (from 2.0 ± 0.8 to $17.2 \pm 3.2\%$, $P < 0.01$), after a similar protocol of training and detraining (Andersen and Aagaard, 2000). However, this is accompanied by a reduction in type II fiber CSA, which would actually make the muscle weaker when higher loads than only the weight of the limb are to be moved (e.g., body weight).

From a metabolic point of view, detraining results in a marked decrease in muscle oxidative capacity, as indicated by a large decrease in mitochondrial enzyme activities. In the 10-week training study of Linossier et al. (1997) presented earlier in this review, the increased activities of the glycolytic enzymes were not reversed after 7 weeks of detraining. However, $V_{O_2\,max}$ and oxidative enzymes (CS and HAD) were decreased at or below the pre-training values. Simoneau et al. (1987) reported similar results of no significant change in glycolytic enzymes, but a significant reduction of oxidative enzymes after 7 weeks of detraining. In a more recent study (Burgomaster et al., 2007), cytochrome *c* oxidase subunit, a marker of oxidative capacity, remained elevated even after 6 weeks of detraining following 6 weeks of HIT. However, some studies have reported decreases in glycolytic enzymes in highly trained athletes who stop training for 4–8 weeks (Mujika and Padilla, 2001).

As discussed earlier in the review, exercise-induced angiogenesis (increased capillarization) is an important adaptation to HIT that is possibly mediated by the increased expression of PGC-1α (Tadaishi et al., 2011). Earlier studies reported that a short period of detraining does not seem to significantly decrease capillary density of the previously trained muscle, possibly due to the concomitant decrease in muscle fiber area (Klausen et al., 1981; Coyle et al., 1984). However, more recent data suggest that only a short period of detraining is adequate to reverse training-induced angiogenic remodeling, as seen by the regression of capillary contacts and individual capillary-to-fiber ratio in the plantaris and soleus muscles of rats (Malek et al., 2010). These authors suggested that this was modulated by vascular endothelial growth factor (VEGF).

The reductions in oxidative enzyme activities together with the shifting of muscle fiber type to the fast fatigable MHC IIX isoform would increase fatigue during high-intensity exercise following a period of detraining. However, short (∼2 week) "tapering" period of decreased training volume (by 40–60%) without changes in training intensity and frequency is commonly used by athletes to maximize performance gains (Bosquet et al., 2007). This short period of reduced training volume, would take advantage of the positive adaptations of detraining, while at the same time would avoid the negative long term effects of reduced activity.

IMMOBILIZATION AND DISUSE

Athletes and physically active individuals may be forced to short term immobilization of a limb or even to bed rest due to acute injury or illness. The consequences of gravitational unloading have been extensively investigated in recent years (Ohira et al., 2002; Urso, 2009). One of the most typical adaptations to immobilization is muscle atrophy, accompanied with decreases in functional capacity. Antigravity muscles (e.g., gastrocnemius and soleus) are most susceptible to atrophy following bed rest (Clark, 2009). The loss of muscle strength during a period of 4–6 weeks of unloading has been largely attributed to the loss of contractile proteins (Degens and Always, 2006; Urso, 2009), but it exceeds the loss of muscle mass due to neurological factors (Clark, 2009). Disuse-induced deficits in central activation may account for about 50% of the between – person variability in the loss of knee extensor strength after 3 weeks of bed rest (Kawakami et al., 2001). Deschenes et al. (2002) hypothesized that the loss of strength resulting from a 2-week unilateral lower limb unloading, was due to impaired neural activation of the affected muscle. In that study they immobilized the lower limb of healthy young college students in a light weight orthopedic knee brace at an angle of 70°, with the purpose to eliminate weight bearing activity. After 2 weeks of immobilization, peak isokinetic torque of the knee extensors across a range of velocities was reduced by an average of 17.2% with greater losses in slow than in fast contraction velocities. The reduction in torque was coupled by reduced EMG activity, but the ratio of total torque/EMG was unchanged. Muscle fiber composition remained unchanged in the 2-week unloading period.

Studies performed using animal models of hindlimb unloading showed that there is a shift of MHC isoforms from slow to fast, accompanied by significant muscle atrophy (Leterme and Falempin, 1994; Picquet and Falempin, 2003). It is noteworthy that chronic electrostimulation prevented the shift in fiber types, but failed to counteract the loss of muscle mass and force output (Leterme and Falempin, 1994). Similarly, tendon vibration applied daily on the unloaded hindlimb significantly attenuated, but did not prevent the loss of muscle mass and the changes in fiber type (Falempin and In-Albon, 1999).

A decrease in capillary supply and blood flow during rest and exercise is common in unloaded muscle. Degens and Always (2006) reported that the capillary loss and reduction in maximal blood flow are largely proportional to the loss of muscle mass, maintaining blood flow per unit muscle mass. However, a recent investigation looking at the effects of a 9-day hind limb unloading on both capillarization and expression of angio-adaptive molecules reported differences in capillary regression between fast and slow rat skeletal muscles (Roudier et al., 2010). In that experiment, both soleus and plantaris muscles were atrophied similarly, but capillary regression occurred only in the soleus, which is a slow twitch and oxidative postural muscle. Conversely, capillarization was preserved in the plantaris, a fast twitch, glycolytic muscle. The authors reported that the key pro- and anti-angiogenic signals (various types of VEGF) play a determinant role in regulating this process.

The muscle fatigue profile following muscle disuse atrophy involve both the loss of strength, transition from slow to fast myosin, a shift toward glycolysis and a decreased capacity for fat

oxidation (Stein and Wade, 2005). However, caution should be exercised when measuring fatigue on a disused muscle. In the immobilization study of Deschenes et al. (2002), fatigue resistance was assessed during a 30 repetition set of isokinetic knee extensions at 3.14 rad s^{-1}, as the difference in total work produced during the first 10 repetitions compared with the last 10 repetitions. By calculating this percent decrease of work, fatigue resistance was enhanced instead of decreased following immobilization (drop in total work 29.8 ± 2.5 vs. $20.6 \pm 6.5\%$, pre vs. post immobilization; $P < 0.05$). However, the total work generated over the 30 contractions was 15% less after immobilization (2735.3 ± 207.6 vs. 2339.0 ± 163.3 J, $P < 0.05$). This artifact, i.e., an improvement rather than a reduction of fatigue resistance should be interpreted with caution because this is simply due to the lower total work in the first 10 repetitions after immobilization.

The length of immobilization plays an important role in the negative adaptations resulting from muscle unloading. When immobilization is longer than 4 weeks, there is a large increase in fatigability linked with reductions in oxidative capacity due to decreases in CS and PDH. Indeed, Ward et al. (1986), showed that after 5 weeks of immobilization, the proportion of PDH in the active form was only 52%, compared with 98% after training 5 months training. This results in greater lactate and H^+ accumulation during exercise after the immobilization period.

USE OF HIGH-INTENSITY INTERMITTENT EXERCISE TRAINING IN PATIENT POPULATIONS

Many chronic diseases, such as coronary artery disease (CAD), COPD result in a progressively reduced exercise capacity due to both biochemical and morphological changes in skeletal muscles. Abnormal fiber-type proportions have been found in COPD patients, with markedly lower type I oxidative fibers (16 vs. 42%) compared to controls (Gosker et al., 2002). Also, oxidative capacity of type I, as well as of type IIA fibers was lower than normal, thus making those patients more susceptible to peripheral muscle fatigue. The reduced exercise capacity and increased muscle fatigue of those patients is not only due to intolerable sensations of breathlessness, but also due to peripheral muscle discomfort (Vogiatzis, 2011). The inability of those patients to be physically active reduces even more their exercise capacity and this vicious circle increases the risk of negative health outcomes due to the sedentary lifestyle (Rimmer et al., 2012). COPD patients have a reduced tolerance of continuous exercise and different rehabilitative strategies and training modalities have been proposed to optimize exercise tolerance. Several recent investigations have shown that the greater physiological benefits can be obtained through high-intensity intermittent, compared with moderate-intensity continuous training. Vogiatzis (2011) has shown that using interval exercise in the form of 30 s on and 30 s off, at an intensity of 100% of maximum work rate, COPD patients can almost triple the total exercise duration (30 vs. 12 min), with significantly lower and more stable metabolic and ventilator responses compared with continuous exercise. Patients with severe COPD can endure high-intensity interval training in a rehabilitation setting for long periods of time with lower symptoms of dyspnea and leg discomfort compared with the conventionally implemented continuous training (Kortianou et al., 2010). This is due to the beneficial effects of the recovery intervals that allow PCr resynthesis and lactate removal. The increased availability of PCr in each short exercise bout and its short-duration result in a decreased reliance on anaerobic glycolysis that results in less lactate accumulation and allows more intense exercise stimuli to the peripheral muscle with less cardiac and respiratory strain. A recent study showed that this type of training performed by severe COPD patients allowed them to exercise at a sufficiently high intensity to obtain true physiological training effects manifested by improvements in muscle fiber size, type, and capillarization (Vogiatzis et al., 2011).

High-intensity interval training in the form of four repeated 4 min bouts at 90–95% of peak heart rate, separated by 2–3 min or active recovery at ∼60–70% of peak heart rate, has been used successfully in cardiac patients (Wisloff et al., 2009). In those patients fatigue occurs not only because of reduced cardiac function but also due to skeletal muscle fatigue (Downing and Balady, 2011). Decreased muscle mass and capillarization, shifting of slow to fast-twitch fibers that rely more on glycolysis, as well as reduced mitochondrial size and oxidative enzymes are typically found in heart failure patients and cannot be explained by deconditioning alone (Downing and Balady, 2011). The role of inflammatory mediators, such as tumor necrosis factor and interleukin-6, in the pathogenesis of skeletal muscle wasting and fatigue in numerous clinical settings including heart failure, is an area of active investigation. Interestingly, inflammatory cytokines are reduced following exercise training, in parallel with the improved fatigue resistance (Downing and Balady, 2011). Supervised high-intensity intermittent training can be safely prescribed as a time-efficient strategy in those patients because it results in far superior adaptations compared with conventional low intensity exercise training (Moholdt et al., 2012). This type of exercise not only reduces muscle fatigue but also improves cardiorespiratory fitness, endothelial function, left ventricle morphology and function (e.g., ejection fraction) in all cardiac patients, with no adverse or other life-threatening events occurring secondary to exercise participation (Cornish et al., 2011).

This type of aerobic interval training has also been used for the treatment of metabolic syndrome (Tjonna et al., 2008). Patients exercised three times per week for 16 weeks and compared to the traditional low intensity training group, the high-intensity exercise group demonstrated a larger improvement in $V_{O_2\,max}$ (35 vs. 16%, $P < 0.01$), endothelial function (9 vs. 5%, $P < 0.001$), insulin signaling in fat and skeletal muscle, fasting blood glucose, and lipogenesis in adipose tissue. Furthermore, both the continuous and the intermittent exercise programs were equally effective in reducing mean arterial blood pressure and body weight and fat.

The use of high-intensity interval training in the form of short cycle ergometer sprints lasting 10–20 s has been recently used as a time-efficient alternative to traditional cardiorespiratory training with a target to improve metabolic health (Metcalfe et al., 2011). The subjects were healthy but sedentary men and women who trained three times per week for 6 weeks, with sessions lasting only 10 min, including only one or two 10–20 s sprints and a warm-up and cool-down. Insulin sensitivity in the male training group was increased by 28%, while V_{O_2} peak was increased by 15 and 12% in the males and females, respectively.

CONCLUSION AND FUTURE PERSPECTIVES

Muscle fatigue is not only important for sports settings but may be vital during everyday life because it may pose a barrier to normal physical activity. Deconditioning due to sedentary lifestyle and/or cardiovascular and pulmonary diseases may limit exercise capacity and increase fatigability, resulting in further deterioration of health and well being. However, the adverse effects of physical inactivity can be reversed by exercise training and the extended use of high-intensity interval training as a time-efficient strategy for improving both sports performance and health-related fitness requires further investigation. Since exercise intensity and duration are key variables for adaptations, more research is needed to reveal the best combination of those variables for each population group. Also, the safety of this type of training in the short and longer term and the possibility of overtraining should be examined in larger patient cohorts as well as in different age groups of healthy individuals.

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Antioxidants and skeletal muscle performance: "Common knowledge" vs. experimental evidence

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Antioxidants are assumed to provide numerous benefits, including better health, a reduced rate of aging, and improved exercise performance. Specifically, antioxidants are commonly "prescribed" by the media, supplement industry, and "fitness experts" for individuals prior to training and performance, with assumed benefits of improved fatigue resistance and recovery. This has provoked expansion of the supplement industry which responded by creation of a plethora of products aimed at facilitating the needs of the active individual. However, what does the experimental evidence say about the efficacy of antioxidants on skeletal muscle function? Are antioxidants actually as beneficial as the general populous believes? Or, could they in fact lead to deleterious effects on skeletal muscle function and performance? This Mini Review addresses these questions with an unbiased look at what we know about antioxidant effects on skeletal muscle, and what we still need to know before conclusions can be made.

Keywords: muscle, antioxidants, reactive oxygen species, reactive nitrogen species, performance, exercise, recovery, fatigue

INTRODUCTION

Commoner et al. (1954) reported that reactive oxygen species (ROS) intermediates were present in a wide range of animal tissues, including whole blood, brain, liver, and muscle. They proposed that ROS production was related to metabolic activity. It was later found that exercise results in elevated ROS, and that endurance was reduced by ∼40% in vitamin E deficient rats (Davies et al., 1982). The authors surmised that the peroxidative damage induced by ROS in the absence of vitamin E was responsible for the reduced exercise performance. Accordingly, a multitude of scientific investigations were launched to look at the effects of ROS, and also reactive nitrogen species (RNS), in relation to physical exercise and skeletal muscle fatigue (for in depth review, see Ferreira and Reid, 2008; Powers and Jackson, 2008; Westerblad and Allen, 2011).

It has become "common knowledge" that ROS generated during exercise are bad, and usage of antioxidant supplements to ameliorate their effects promotes health. The various makers of dietary supplements have taken full advantage of this phenomenon by including antioxidants in their supplements; or as concentrated products. However, experimental evidence shows that increased ROS production is not necessarily bad: ROS are important for a wide range of normal exercise-related physiological processes, including a role in contraction-mediated glucose uptake (e.g., Sandström et al., 2006) and promotion of the adaptive responses to training (Ristow et al., 2009). Accordingly, the use of antioxidants has been shown to blunt training responses (Ristow et al., 2009; Petersen et al., 2011; Strobel et al., 2011). But what does scientific evidence say about antioxidant usage prior to a single exercise bout? Do performance and recovery become enhanced and, if so, what are the underlying mechanisms? In this Mini Review we will survey potential effects on performance and recovery of antioxidants frequently used in association with physical exercise. We specifically focus on whether effects seen in the exercising human can be explained by effects observed in experiments on isolated muscle or muscle fibers. For more detailed descriptions of properties of different ROS and RNS that may increase during physical exercise and endogenous antioxidant systems we refer to more comprehensive reviews (Dröge, 2002; Powers and Jackson, 2008; Westerblad and Allen, 2011).

ANTIOXIDANT SUPPLEMENTATION AND MUSCLE FATIGUE

UBIQUINONE-10

Ubiquinone-10 is a lipid soluble antioxidant found in high concentrations in meat and fish (Powers et al., 2004). Concentrated ubiquinone-10 supplements are readily available. Early evidence indicated intramuscular ubiquinone-10 content had a positive relationship with exercise capacity (Karlsson et al., 1996). However, the greater exercise capacity was more likely a function of decreased fatigability based on the muscle properties (e.g., oxidative capacity) and not ubiquinone-10 content. Whereas supplementation with ubiquinone-10 may provide assistance to individuals with mitochondrial disease (Glover et al., 2010), most investigations on healthy individuals show no effect (Braun et al., 1991; Mizuno et al., 1997; Weston et al., 1997; Bonetti et al., 2000) or a deleterious effect (Laaksonen et al., 1995; Malm et al., 1997) on exercise performance. The lone exception is a recent study with a dose ∼3× that used in previous studies (Mizuno et al., 2008). Thus at present time, there is not enough evidence to support a role for ubiquinone-10 as an antioxidant having an ergogenic effect in healthy individuals.

VITAMINS C AND E

Vitamin C is hydrophilic and widely distributed in mammalian tissues. It can act as a radical scavenger and recycles vitamin E

(Powers et al., 2004; Powers and Jackson, 2008). Vitamin E is lipid soluble and the major chain-breaking antioxidant found in cell membranes (Powers et al., 2004; Powers and Jackson, 2008). These two vitamins are "expected" to improve exercise performance based on their antioxidant properties and are commonly used by athletes and active individuals. However, experimental evidence to support beneficial effects on physical performance does not exist. Neither vitamin C (Clarkson, 1995; Ashton et al., 1999) nor vitamin E supplementation (Shephard et al., 1974; Lawrence et al., 1975; Sumida et al., 1989; Rokitzki et al., 1994a,b; Bryant et al., 2003; Gaeini et al., 2006) improves exercise performance in humans. Further, no beneficial effects have been observed with the combination of vitamins C and E (Bryant et al., 2003), or vitamins C and E with ubiquinone-10 (Nielsen et al., 1999). Thus, claims as to the efficacy of vitamins C and E to improve exercise performance are without experimental support.

N-ACETYLCYSTEINE

The antioxidant *N*-acetylcysteine (NAC) got its start in the 1990s and has grown in popularity, now being readily available for daily use. NAC easily enters cells and contains a thiol group that can interact with ROS, RNS, and their derivatives (Aruoma et al., 1989; Dekhuijzen, 2004; Ferreira and Reid, 2008). As a thiol donor, NAC also supports resynthesis of one of the major endogenous antioxidant systems, glutathione (Dekhuijzen, 2004). The first report of beneficial effects of antioxidant supplementation on fatigue in humans came after NAC infusion (Reid et al., 1994). NAC was infused into the subjects for 1 h prior to low-(10 Hz) and high-frequency (40 Hz) stimulation of the tibialis anterior muscle. NAC infusion resulted in significantly less fatigue during 10-Hz stimulation; but not during 40-Hz stimulation. These data indicated two potential, important features of NAC supplementation: (1) fatigue can be reduced by NAC supplementation; and (2) the effect depends on the exercise protocol in that the effect is larger with submaximal contractions. Accordingly, a later study showed a beneficial effect of NAC during fatigue induced by repetitive submaximal handgrip exercise but not during maximal contractions (Matuszczak et al., 2005). The specific effect of NAC on submaximal contractile force has also been extended to cycling exercise (Medved et al., 2003, 2004a,b; McKenna et al., 2006; Corn and Barstow, 2011).

NAC has been shown to have beneficial effects on contractility and fatiguability of human ventilatory muscles (Travaline et al., 1997; Kelly et al., 2009). Using the murine diaphragm contracting *in situ*, Shindoh et al. (1990) measured a beneficial effect of NAC on fatigue resistance. They speculated that the mechanism of action could be through NAC effects on blood flow or directly on the muscle fibers themselves. Similar effects on fatigue resistance in the diaphragm have been reported by other groups (Diaz et al., 1994; Khawli and Reid, 1994; Supinski et al., 1997). Results from isolated diaphragm strips contracting *in vitro* indicate that the effects of NAC on fatigue resistance are at the muscle fiber level (Diaz et al., 1994; Khawli and Reid, 1994). Furthermore, using diaphragm bundles contracting *in vitro*, Mishima et al. (2005) reported less fatigue in fibers treated with NAC and this effect was independent of changes in sarcoplasmic reticulum (SR) Ca^{2+} release and uptake.

MECHANISMS BY WHICH ROS/RNS MAY AFFECT FATIGUE

Proposed mechanisms intrinsic to the muscle fibers by which ROS/RNS can accelerate fatigue development include: (1) reduced membrane excitability, (2) impaired SR Ca^{2+} release, (3) inhibition of SR Ca^{2+}-ATPase (SERCA), and (4) deleterious effects on myofibrillar function. Accordingly, antioxidants such as NAC may enhance fatigue resistance by hindrance of any of these proposed effects. NAC supplementation increased the time to fatigue in humans during submaximal cycling exercise and analyses of muscle biopsies suggest that the improved performance could be due to preserved function of Na^{+}-K^{+} ATPase (McKenna et al., 2006). This indicates that ROS may accelerate fatigue development by impairing membrane excitability. However, studies on isolated intact muscle fibers do not show any evidence of action potential failure induced by exposure to ROS either in the unfatigued state (Andrade et al., 1998a, 2001) or during fatiguing stimulation (Place et al., 2009).

Results from experiments with intact single fast- and slow-twitch fibers from limb muscles do not support a role for ROS in decreasing SR Ca^{2+} release during high-intensity fatiguing stimulation (Moopanar and Allen, 2005; Bruton et al., 2008). For example, SR Ca^{2+} release, and hence contractile force (**Figure 1**), can be well maintained even when fatigue is induced in the presence of a high concentration of the ROS hydrogen peroxide

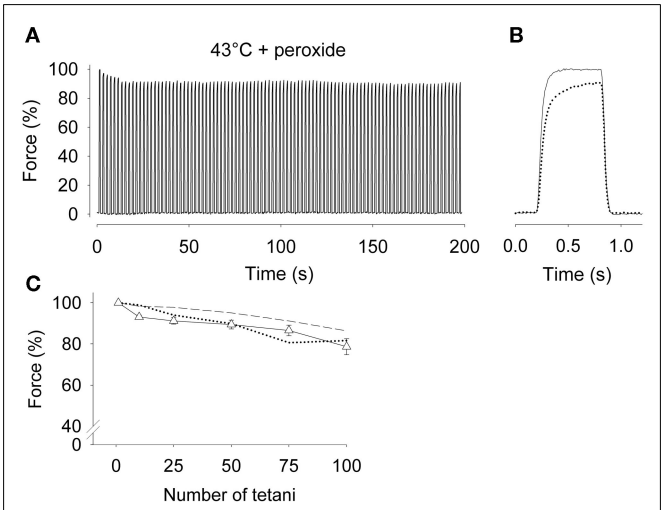

FIGURE 1 | Tetanic force was well maintained in intact soleus fibers during fatiguing stimulation at 43°C in the presence of peroxide. **(A)** Typical continuous force records from a soleus fiber fatigued by 100 Hz, 600-ms tetanic contractions repeated every 2 s at 43°C in the presence of 10 μM hydrogen peroxide. Force is expressed relative to the first tetanus, which was set to 100%. **(B)** Superimposed force records on an expanded time axis from the first (solid) and last (dotted line) tetani of the fatigue run. **(C)** Mean data (±SEM) of relative force measured during the 1st, 10th, 25th, 50th, 75th, and 100th fatiguing tetani at 43°C in the presence of 10 μM hydrogen peroxide (Δ, $n = 9$). For comparison, mean data from soleus fibers fatigued at 37°C (dashed line) and 43°C (dotted line) in the absence of peroxide are also shown. Fatigue in fast-twitch fibers was unaffected by elevated temperature. Contractile force in rested fibers was unaffected by 5 min of 10 μM hydrogen peroxide exposure, i.e., 100% force did not differ between groups. Data are from Place et al. (2009).

(10 μM) and at high temperature (43°C; Place et al., 2009). Thus, these studies do not support an ability of antioxidants to prevent the reductions in SR Ca^{2+} release that occur during fatigue. Accordingly, if effects are seen, antioxidant supplementation must exert its beneficial effects on exercise performance via some other mechanism.

The changes occurring during fatiguing stimulation of skeletal muscle fibers often include an elevation of baseline $[Ca^{2+}]_i$, which can be due to impaired SERCA function (Westerblad and Allen, 1991, 1993). Studies on muscle biopsies taken after exercise in humans have shown impaired SR Ca^{2+} uptake into the SR (Booth et al., 1997; Duhamel et al., 2007). Scherer and Deamer (1986) found that administration of oxidants to SR microsomes reduced SERCA function and Ca^{2+} transport. Moreover, prolonged exposure to high concentrations of oxidants resulted in impaired SR Ca^{2+} uptake and an elevation in baseline $[Ca^{2+}]_i$ in isolated intact muscle fibers (Andrade et al., 1998a, 2001). Accordingly, antioxidant supplementations may improve performance by preventing adverse effects on SERCA function, but we are not aware of any studies where this potential mechanism has been shown to occur.

Impairment in the ability of the contractile elements to respond to Ca^{2+} (myofibrillar Ca^{2+} sensitivity) is a common feature of fatigue (Allen et al., 2008). In this case, force can become depressed in the absence of any impairment of SR Ca^{2+} handling. Prolonged exposure to oxidants reduces myofibrillar Ca^{2+} sensitivity in unfatigued fibers (Andrade et al., 1998a, 2001). In addition, Andrade et al. (1998b) showed that nitric oxide (NO) donors reduce myofibrillar Ca^{2+} sensitivity in unfatigued fast-twitch fibers. Pye et al. (2007) used dissociated fast-twitch skeletal muscle fibers from mice and fluorescent indicator to measure NO production during contractions and observed a marked increase after 5 min of contractions. Accordingly, the production of NO, leading to the production of RNS in contracting skeletal muscle, may contribute to the decrease in myofibrillar Ca^{2+} sensitivity during fatiguing stimulation. Thus, NAC and other antioxidant treatments could potentially enhance fatigue resistance by counteracting any ROS/RNS-induced decrease in myofibrillar Ca^{2+} sensitivity.

Taken together, the experimental evidence regarding a positive role for antioxidant supplementation during exercise indicates that, whereas diaphragm muscle fibers display beneficial effects, these effects have not been observed in muscles involved in locomotion. Accordingly, the positive effects of NAC observed in experiments on exercising humans seem not to be due to direct antioxidant effects on limb muscle fibers.

ANTIOXIDANTS AND RECOVERY

Depending on the nature of exercise, the time for recovery may vary between minutes to days. An increased rate of recovery is beneficial, e.g., by allowing bouts of exercise to be performed at short intervals. In this section, we will discuss the role of ROS in the recovery process and whether antioxidants can help improve recovery of force.

In humans, there is a rapid rate of recovery of maximum voluntary contraction force (Baker et al., 1993; Allman and Rice, 2001), and force at high frequencies (50–100 Hz) of electrical stimulation within the first 5 min after fatigue (Edwards et al., 1977; Allman and Rice, 2001). However, force is not fully restored by 5 min, and it can take several hours to recover maximal, and especially submaximal, force generating capacity (Edwards et al., 1977; Allman and Rice, 2001; Hill et al., 2001).

Edwards et al. (1977) were the first to report a pronounced delay in the recovery of force at low stimulation frequencies (10–20 Hz) lasting several hours or even days after repeated voluntary isometric contractions performed in humans. This delayed recovery was initially named "low-frequency fatigue," but this name has unfortunately been used to describe many different situations with decreased force production and therefore it was recently re-defined as prolonged low-frequency force depression (PLFFD; Allen et al., 2008; Bruton et al., 2008; Lamb and Westerblad, 2011; Westerblad and Allen, 2011). The slow recovery of force at low frequencies could explain the sensation of muscle weakness at submaximal levels of voluntary effort that appears to last for a similar duration as PLFFD. Results from isolated muscles of rodents have determined that the primary mechanisms causing PLFFD in fast-twitch fibers are decreased SR Ca^{2+} release and decreased Ca^{2+} sensitivity (Bruton et al., 2008). In intact muscle fibers of wild-type mice it appears that the ROS superoxide induces impairments in SR Ca^{2+} release that can explain PLFFD in intact single fibers (Bruton et al., 2008). In contrast, a similar PLFFD was observed in intact single fibers of wild-type rats, but in this case it was due to decreased Ca^{2+} sensitivity (Bruton et al., 2008). Superoxide dismutase (SOD) converts superoxide into hydrogen peroxide, and hydrogen peroxide exposure has been shown to decrease myofibrillar Ca^{2+} sensitivity in rested fibers even at very low concentrations in muscle (Andrade et al., 2001; Murphy et al., 2008), and blunt the recovery of 50 Hz force in fatigued amphibian single fibers (Oba et al., 2002). Wild-type rat muscles displayed higher SOD activity and thus would produce more hydrogen peroxide during fatigue than wild-type mouse fibers (Bruton et al., 2008). Furthermore, **Figure 2** shows a similar PLFFD in muscle fibers of wild-type and SOD2 overexpressing mice, but the underlying mechanism differs: in wild-type fibers, where superoxide would dominate, PLFFD is due to decreased SR Ca^{2+} release, whereas in SOD2 overexpressing fibers, where hydrogen peroxide would dominate, PLFFD is due to decreased myofibrillar Ca^{2+} sensitivity (Bruton et al., 2008). Decreased myofibrillar Ca^{2+} sensitivity might be related to ROS-induced oxidation of the contractile proteins since 0.5–1 mM dithiothreitol, a non-reversible reducing agent, has been shown to restore low frequency force in intact rat (Diaz et al., 1998; Bruton et al., 2008) and mouse intact skeletal muscle (Moopanar and Allen, 2006) without affecting SR Ca^{2+} release (Moopanar and Allen, 2006; Bruton et al., 2008).

To sum up, there is clear-cut experimental evidence supporting important effects of oxidants generated during fatiguing contractions on the recovery process. However, there are also many puzzling results in this respect. For instance, studies have shown that only the antioxidant NAC attenuates the low-frequency force decline during fatigue (Shindoh et al., 1990; Reid et al., 1994), but a consistent finding is that NAC does not improve the recovery after fatigue (Shindoh et al., 1990; Reid et al., 1994; Bruton et al., 2008). Thus, many questions need to be addressed in future investigations of the recovery processes, including the need to identify the specific site of action of ROS, and the extent to which different ROS

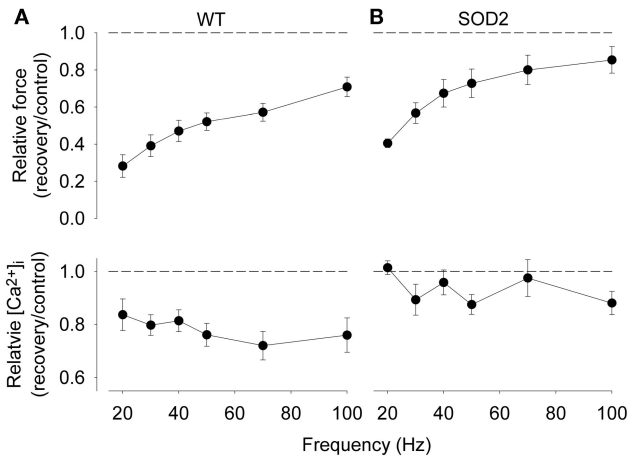

FIGURE 2 | Both wild-type (WT) and superoxide dismutase 2 (SOD2) overexpressing fibers display marked PLFFD but the underlying mechanism differs. Mean data (±SEM) of the relative change in tetanic force (top) and $[Ca^{2+}]_i$ (bottom) obtained in mouse WT **(A)** and SOD2 overexpressing **(B)** fibers ($n = 4$). Relative changes were calculated as ratio 30 min after (recovery) to before (control) fatiguing stimulation; dashed lines indicate no change. Contractile force and tetanic $[Ca^{2+}]_i$ before fatigue did not differ between WT and SOD2 overexpressing fibers. Data are from Bruton et al. (2008).

are potentially responsible for the prolonged impairment in force during recovery.

CONCLUSION

Experimental evidence does not support the "common knowledge" that antioxidant treatment greatly improves exercise performance and recovery. On the contrary, studies with antioxidant supplementations generally show no effect on muscle function during and after exercise. The exception is NAC treatment, which has been found to improve performance during submaximal exercise. The limited effects of ROS/RNS and antioxidants during exercise are unexpected in that increases in ROS/RNS are likely to occur and these are potentially harmful. It appears that muscle fibers are in some way protected against deleterious effects of oxidants during exercise and fibers are generally much more sensitive to exposure to oxidants in the rested state than during fatigue. For instance, experiments on single mouse muscle fibers have shown that application of 10 μM hydrogen peroxide did not affect fatigue development (Place et al., 2009), whereas concentrations as low as 100 pM hydrogen peroxide affected contraction and Ca^{2+} handling in rested fibers (Andrade et al., 2001). Thus, numerous questions remain to be answered in relation to the effects of oxidants during and after exercise. In order to do this, improved methods to measure ROS/RNS are essential, since the effects of these highly reactive substances are likely to strongly depend on both their temporal and spatial distribution. Furthermore, the effects are likely to show marked differences between slow-twitch fatigue resistant and fast-twitch easily fatigued muscle fibers due to a major difference in ROS/RNS production, endogenous oxidant levels and sensitivity of Ca^{2+} handling and contractile properties.

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Muscle fatigue and cognition: what is the link?

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A commentary on

Endurance factors improve hippocampal neurogenesis and spatial memory in mice *by Kobilo, T., Yuan, C., and van Praag, H. (2011). Learn Mem. 18, 103–107.*

Increasingly, exercise is recognized as an important means to enhance cognition, improve mood, and reduce the probability of neurodegenerative diseases (Hillman et al., 2008). The beneficial effects of exercise are considered to be mediated at least in part by increased neurogenesis, elevated trophic factors, and vascularization in the hippocampus (Cotman and Berchtold, 2002; Hillman et al., 2008; van Praag, 2008). In addition, exercise has been suggested to increase the brain's resistance to oxidative stress (Radak et al., 2001).

However, high intensity exercise may have damaging central and peripheral effects. In mice, this type of exercise may result in increased brain lipid peroxidation (Rosa et al., 2007; see however Ozkaya et al., 2002; Acikgoz et al., 2006) and impaired performance in spatial learning and memory tasks (Rosa et al., 2007; Aguiar et al., 2010). In humans, high intensity exercise and overtraining (OTS) can have detrimental effects on mood. It was proposed that OTS and major depression have similar etiologies (Armstrong and VanHeest, 2002). Staleness, characterized by reduction in athletic performance and mood disturbance (O'Connor, 2007) could possibly be caused by overtraining and may be induced by changes in brain circuits involved in depression. Marathons and other forms of heavy exertion exercise may increase susceptibility to inflammatory processes during the period of training. During 1- to 2-weeks afterward muscle fatigue and stress responses are observed. Indeed, exercise concurrent with inadequate recovery, can cause performance decrements and chronic maladaptations (Appell et al., 1992; Akerström and Pedersen, 2007), such as disturbance of the contractile apparatus of the gastrocnemius muscle. The mitochondria in skeletal muscle fibers can show focal swelling and crystalline inclusions. Cell necrosis is evident 7 days after the race (Appell et al., 1992). Furthermore, as little as 10 days of increased training may result in over-reaching (Armstrong and VanHeest, 2002).

What are the mechanisms underlying effects of overtraining on CNS? We propose that there is a strong link between factors mediating muscle endurance and brain function that may help explain the consequences of muscle fatigue on cognition. We recently demonstrated that peripheral endurance related factors lead to improved spatial memory in sedentary mice, suggesting that skeletal muscle activation by exercise or pharmacological agents underlies cognitive effects of aerobic activity (Kobilo et al., 2011; **Figure 1**). Our work was based on the findings that peroxisome proliferator activated receptor δ (PPARδ) regulates muscle fiber contraction and metabolism (Wang et al., 2004; Narkar et al., 2008). PPARδ is regulated by AMP-activated protein kinase (AMPK), a master metabolic regulator important for exercise physiology (Hardie, 2004). Treatment with AMPK agonist 5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) enhanced endurance in sedentary mice (Narkar et al., 2008). We showed that the effects of activation of AMPK and PPARδ extend from the periphery to brain. AICAR had robust effects on memory, cell proliferation, and hippocampal neurogenesis, consistent with its endurance phenotype (**Figure 1**). The PPARδ agonist GW501516 was less effective than AICAR reflecting its modest "exercise" phenotype (Kobilo et al., 2011). Narkar et al. (2008) showed that GW treatment increased running endurance only when it was paired with training.

Interestingly, the effects of AICAR were dependent on the duration of administration, as 7 days of treatment enhanced adult neurogenesis and memory function, but 14 days had no beneficial effects (**Figure 1**), and may have caused muscle fatigue. In fact, the control group showed a trend toward better performance, suggesting that long-term injection of AICAR may have bi-directional effects on cognition, similar to exercise (Akerström and Pedersen, 2007). These results are supported by finding that short-term AICAR treatment promoted sirtuin 1 protein expression in skeletal muscle, whereas 14 days did not (Suwa et al., 2011). Given the fact that intracerebral infusion of AICAR impaired memory function (Dash et al., 2006) and that AICAR has a very low ability to cross the blood brain barrier (BBB), estimated at <1% (Marangos et al., 1990), it is likely that the beneficial effects of AICAR on the brain are indirect. Indeed, when administered peripherally, AICAR and GW may lead to release of factors from muscle into circulation that can cross the BBB and enhance cell genesis. Thus, similar to differential effects of moderate and high intensity exercise, AICAR improved memory and neurogenesis in a dose dependent way. Prolonged administration of AICAR may result in harmful neural responses, similar to overtraining.

We hypothesize that mechanisms contributing to muscle damage after strenuous exercise may be the same as those that could be caused by high doses of AICAR. Free oxygen radicals are generated during exercise as a side product of oxidative metabolism. In particular, increased production of nitric oxide (NO) derivates is a desired consequence of exercise for proper muscle function but higher levels of NO can cause contractile dysfunction, resulting in muscle fatigue. Strenuous exercise can accelerate the generation of NO to levels that result in oxidative stress (Nikolaidis et al., 2008), sustained for days after exercise (Appell et al., 1992). NO induces mitochondria biogenesis in skeletal muscle via upregulation of PGC1α, and interacts with AMPK. Pharmacological activation of AMPK with AICAR and the subsequent induction of GLU4 are blunted by inhibition of NO

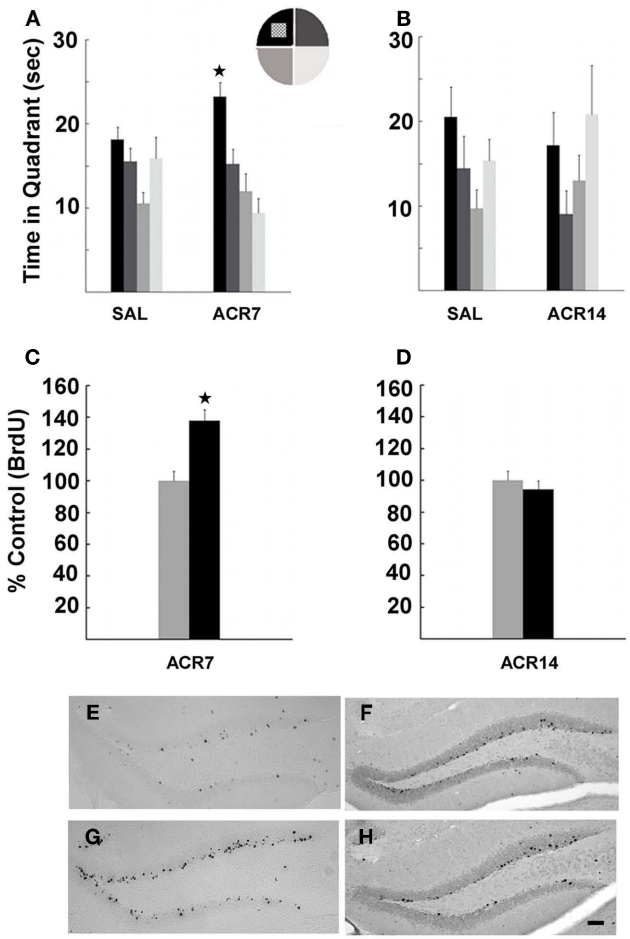

FIGURE 1 | Water maze performance and neurogenesis in mice treated with saline (SAL) or AICAR (500 mg/kg) for 7 (ACR7) or 14 days (ACR14). Mice were trained to find the hidden platform in the Morris water maze over 1 week of daily training trials. The ACR7 mice performed better than SAL7 mice in probe trials. **(A)** Significant retention of platform location in ACR7 mice was observed 4 h (*$P < 0.006$) after the last training session. **(B)** ACR14 mice showed no retention of spatial memory in the 4-h probe trial. In fact, the control group showed a trend toward better performance then ACR14 mice. In parallel, ACR7 significantly enhanced new cell survival **(C)**, while there was no effect of ACR14 on $BrdU^+$ cell number **(D)**. Photomicrographs of $BrdU^+$ cells surviving 4 week after the last of a series of 7 BrdU (50 mg/kg) and AICAR injections in SAL7 **(E)**, ACR7 **(G)** mice, and SAL14 **(F)**, ACR14 **(H)** mice. Scale bar, 50 μm.

production. AMPK phosphorylates and activates eNOS and nNOS, and is necessary for NO-dependent increase in the expression of PGC1α, mitochondrial gene expression, and respiration in skeletal muscle cells. It was proposed that NO and AMPK interact through a positive feedback loop in skeletal muscle (Lira et al., 2010). Moreover, in neurons NO production is elicited by AMPK, and in turn, increases AMPK activity (Murphy et al., 2009). Altogether, high doses of AICAR may be harmful for body and brain.

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Hemodialysis fatigue: just "simple" fatigue or a syndrome on its own right?

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INTRODUCTION

In this opinion article we discuss whether fatigue symptoms and signs often noticed in the hemodialysis (HD) patients should be collectively viewed and addressed as a "syndrome" and not in usual polarities such as peripheral vs. central fatigue, or, physical vs. mental fatigue etc. Our rationale follows the example of "Chronic Fatigue Syndrome (CFS)" to help us substantiate our argument. It is important to stress that "Sign" and "Symptom" are terms with different meanings; symptoms are problems that a patient perceives while signs are whatever features an observer can objectively detect or measure. In practice a syndrome combines symptoms, signs, and phenomena that the health practitioner recognizes, being alerted in that if some are observed then more are expected. In the case of what is being increasingly described as "HD fatigue" symptoms and signs may not have a very clear association resulting in ineffective or no action to remedy, partly because we have not yet identified/labeled them, as a scientific or health care community, as a group, i.e., as a syndrome.

Chronic renal disease is a "silent epidemic" affecting up to 10% of the population in the USA, and Asian countries, with some sufferers progressing in to end-stage renal failure (Stenvinkel, 2010). Renal disease patients are characterized by progressively worsening muscle weakness and muscle atrophy due to both a metabolic and a disuse component collectively described as uremic myopathy. While various interventions in stable HD patients have helped these patients improve their functionality, they still have not proven enough to bring their muscle quality and quantity up to the levels of a healthy sedentary person (Sakkas et al., 2003b; Johansen et al., 2006). Moreover, patients present with sleep problems (Sakkas et al., 2008a), neurological and quality of life issues (Giannaki et al., 2011), anxiety, and/or symptoms of depression but most notably they complain of chronic fatigue and "lack of energy" (McCann and Boore, 2000).

WHAT IS FATIGUE?

The traditional approach in identifying, researching, and assessing signs of fatigue has a dual nature: central vs. peripheral, brain vs. muscle, physical vs. mental etc.

Mental fatigue is a psychobiological state caused by prolonged and intensive cognitive activity and is expressed by the lack of concentration and the inability of staying focused under certain conditions (Marcora et al., 2009). Physical (muscle) fatigue on the other hand is accepted mainly as an inability to exert or sustain muscle force or power output for a given task (Edwards, 1975). Moreover, there is heated discussion in the literature on the origins and modulation of physical fatigue (Shephard, 2009; Noakes, 2011a,b), beyond the scope of this article.

Likewise in chronic disease patients, it is been suggested that the symptoms of fatigue relate to two components: the mental that encompasses emotional and cognitive qualities and the physical, encompassing sleepiness, lack of energy, and muscle weakness (Hardy and Studenski, 2010).

How we view fatigue is of course important as it dictates how we identify signs, how we interpret or link symptoms and how we implement rehabilitation interventions and overall patient care. We can conclude that "*fatigue is the inability of sustaining an effort either mentally or physically or both while signs and symptoms may be interconnected in a way not always clearly defined.*"

FATIGUE IN HEMODIALYSIS PATIENTS

While HD *per se* is a life saving procedure, it cannot substitute for a healthy kidney, it taxes the patient and HD related fatigue symptoms significantly affect patients' quality and way of life as suggested by many (McCann and Boore, 2000; Caplin et al., 2011; Gordon et al., 2011; Jhamb et al., 2011). Muscle status is gravely affected with HD patients demonstrating severe atrophy, fat infiltration, and other anomalies (Sakkas et al., 2008b). Overall, HD patients exhibit low levels of physical activity and functional capacity while they suffer from generalized weakness, exercise intolerance, and muscle atrophy, all leading to generalized sense of fatigue (Johansen and Painter, 2012). The causes of fatigue in HD patients are not well understood but it is been shown that these should include both muscular and central activation failures (Johansen et al., 2005).

Moreover, correction of anemia (Jhamb et al., 2011), exercise training (Koufaki et al., 2002; Sakkas et al., 2003b) with or without anabolic steroid (Johansen et al., 2006), and/or nutritional supplements (Kalantar-Zadeh et al., 2011) do improve the clinical condition of the HD patients, however, despite such improvements, we have observed that HD patients' functional capacity cannot equal that of a sedentary aged matched healthy counterpart.

A cause of the observed minimal levels of physical activity is probably that the HD procedure *per se* [e.g., duration of dialysis sessions (Caplin et al., 2011) etc.,] contributes to fatigue. One third of the patients report that they feel worse in the immediate hours after the dialysis session while one out of four reports severe or very severe intensity of fatigue after dialysis (Gordon et al., 2011). The severity of "Post-dialysis

Fatigue" symptoms could range from mild to severe and can last from a few hours after the dialysis procedure up to until the next day (Lindsay et al., 2006) or for a "very long time" (Gordon et al., 2011). Thus many HD patients may spent a large proportion of their time in a state of fatigue (Caplin et al., 2011; Gordon et al., 2011), and since they perceive fatigue (whether in dialysis or in non-dialysis days) as an important barrier (Delgado and Johansen, 2012), this adversely affects their physical activity levels.

Other factors that contribute to the excessive fatigue area lack of restorative sleep (Sakkas et al., 2008b), excess predialysis weight (Sklar et al., 1999), poor nutritional status (Jhamb et al., 2011), restless legs syndrome (Giannaki et al., 2011), and the overall mental status of the patients (Jhamb et al., 2011). Evidently, of all of these factors can contribute to a self-exacerbating process, a vicious circle, of fatigue due to inactivity and further inactivity due to fatigue. This sensation of an enduring fatigue interferes with physical and social activities and feeds perceptions of increased restrictions and barriers (Delgado and Johansen, 2012), and leads to a significant reduction of physical activity and functional capacity, which in turn contributes to the increased cardiovascular risk and a high mortality rate among these patients (Sarnak et al., 2003).

The state of current knowledge regarding the differences between generalized fatigue and HD treatment related fatigue is not well understood however many variables have been implicated in the severity and the prevalence of the symptoms. The variables can be divided into: (a) social and demographic including age, dialysis vintage, gender, and race, (b) clinical variables including anemia, anorexia, nutritional status, chronic inflammation, sleep disorders, physical inactivity, comorbidities, and depression, and (c) some laboratory variables including Kt/V, serum creatinine and urea, and levels of parathyroid hormone (Bossola et al., 2011). The etiology of fatigue in HD patients is not a simple "one stop" investigation. It involves many aspects of patients' health as well as various social and behavioral factors that depend on patients' health characteristics and mental attitude (McCann and Boore, 2000).

WHAT WE CAN LEARN FROM CHRONIC FATIGUE

A disorder called "CFS" is generally defined by persistent mental and physical fatigue accompanied by other specific symptoms (Arnett et al., 2011). While its causes are undefined, it is managed as a syndrome (given some accepted "operational" diagnostic criteria; Wessely, 2001) and sufferers are offered multidisciplinary care.

Patients on maintenance HD therapy share many similarities to those suffering by CFS since they experience generalized weakness (Johansen et al., 2003), exercise in tolerance (Koufaki et al., 2002), and disturbed sleep (Sakkas et al., 2008a) all leading to a sense of generalized fatigue and "lack of energy" (McCann and Boore, 2000; Kovacs et al., 2011). This chronic state of "HD Fatigue" among HD patients satisfies one major requirement for the diagnosis of CFS which is persistent fatigue present at least during 50% of the time over a period of at least 6 months (Jason et al., 2003). However, as renal failure is present, the second requirement for the diagnosis of CFS, which is the absence of disease, is contradicted. So far the single symptom approach of fatigue in HD patients did not succeed to ameliorate patients' sense of fatigue (Letchmi et al., 2011) and therefore, by viewing signs and symptoms of fatigue in a holistic approach would at least allow practitioners and scientists to address the problem as efficiently as in CFS patients. Such an approach will be challenging, given the variety in intensity and the causes of these symptoms, but not impossible, and can hold large benefits to the patients' quality of life as the CFS treatments have shown so far in other populations.

HYPOTHESIS OF "HEMODIALYSIS FATIGUE SYNDROME"

The prevalence of general undefined fatigue in HD patients ranges from 30 to 80% depending on the assessment tools and the dialysis modality (Bossola et al., 2011). The average score of fatigue in HD patients is the worst of all chronic disease patients (Ware et al., 1993) including those with severe depression (Yatham et al., 2004), cancer patients undergoing chemotherapy (Adamsen et al., 2009), and lupus patients (Jolly, 2005). In addition, the majority of HD patients complain of various "non-specific" symptoms that are very often considered by their health care providers as "irrelevant" to fatigue. However, if those "irrelevant" symptoms (see below) could be seen under the prism of a "syndrome" (as in the case of CFS), it is possible that the final diagnosis and treatment of symptomatology would be much different.

For example, in the diagnosis of CFS, various complementary criteria play an important role in the final decision such as pain in multiple joints, headaches, nausea, chest pain, shortness of breath, difficulties in maintaining upright position, and various psychological problems such as depression, irritability, mood swings, and other (Burton et al., 2009). Those are evident in HD patients however, the clinical significance of those symptoms changes when viewed in relation to fatigue.

The realization of any physical activity requires an efferent action to recruit the proper number of motor units in the active muscles. At the beginning of the activity, the extent of muscle recruitment will depend mostly on the physical health of the person (Hettinga et al., 2011), the mental and emotional health, the extent of mental fatigue (Marcora et al., 2009), the level of sleep deprivation (Skein et al., 2011), the quality of rest, and recovery from a previous activities (Eston et al., 2007) as well as the level of motivation and self-esteem (Micklewright et al., 2010).

Hypothetically, when the same consideration applies to a HD patient, one should consider that a lower number of motor neurons would be activated due to uremic neuropathy (Krishnan and Kiernan, 2007), that the HD patient may be experiencing muscle atrophy and/or fat infiltration (Sakkas et al., 2003a, 2008b; thus will be weaker than an aged matched control), in addition to various emotional and mental distresses that seem to affect the level of exercise tolerance (Kouidi et al., 2010). Furthermore, the majority of HD patients complain of "brain fog" and lethargy (Caplin et al., 2011) especially in the hours post dialysis (Tryc et al., 2011), while one out of two have very low quality of sleep and suffer from daily sleepiness (Sakkas et al., 2008a; Giannaki et al., 2011). Moreover, the HD procedure *per se* filters away many small molecules involved in metabolism (glucose, amino acids, minerals, hormones etc.) leaving the patient feeling drained and exhausted, with inadequate time to recover, since the next

dialysis will take place in less than 48 h (Lim and Flanigan, 1989). Finally, the high hospitalization rate (Collins et al., 2009), the proneness to infection (Kallen et al., 2010), and the feeling of being hospital-bound and stuck to a dialysis machine reduces significantly the level of motivation and the degree of self-esteem (Hedayati et al., 2012). Eventually for an HD patient a simple physical activity, such as a walk around the block assumes a totally different meaning!

CAN A "HEMODIALYSIS FATIGUE SYNDROME" EXIST?

The diagnosis of "CFS" is based on Fukuda's criteria (Fukuda et al., 1994) and includes: unexplained persistent or intermittent chronic fatigue that is of new or definable onset, fatigue not the result of an identified ongoing exertion, not ameliorated by rest and that results in reduced participation in personal, social, or professional activities (Fukuda et al., 1994; Arnett et al., 2011).

According to this definition and diagnosis, a significant proportion of the HD patients may be considered to suffer from a "type of" CFS that remains undiagnosed and untreated. Even though there is not yet scientific evidence suggesting that HD patients' fatigue symptoms are related to the CFS, anecdotal reports from patients, and clinical practitioners suggest that the sense of fatigue experienced by HD patients is more a syndrome than a single symptom secondary to other causes.

PERSPECTIVE AND RESEARCH DIRECTIONS

We believe that the current descriptions of HD fatigue point to a "syndrome." In current practice, treatment for fatigue symptoms and signs, if offered, and dependent on the country and type of care facility, is given mostly by the nephrologists and sometimes by the nursing staff. We believe that to address such complex symptomatology necessitates a more holistic approach which should include other specializations such as neurologists, psychiatrists, exercise physiologists, pain management specialists, and others. It is difficult to effectively treat symptoms such as "lack of energy" and signs such as "muscle weakness" as it is obvious that they cannot be treated fragmentarily, e.g., only with pain killers or antidepressants. A multidisciplinary approach is needed and we propose that while challenging, to formulate, it will be rewarding for patients, health care providers, and scientists.

Future research needs to focus on clinical and functional examination of those HD patients with severe and persistent fatigue vs. those with no or minimal signs and symptoms and clarifying whether or not HD Fatigue may be "equivalent" to CFS. This could lead to better care for HD patients.

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Fatigue is a brain-derived emotion that regulates the exercise behavior to ensure the protection of whole body homeostasis

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An influential book written by A. Mosso in the late nineteenth century proposed that fatigue that "at first sight might appear an imperfection of our body, is on the contrary one of its most marvelous perfections. The fatigue increasing more rapidly than the amount of work done saves us from the injury which lesser sensibility would involve for the organism" so that "muscular fatigue also is at bottom an exhaustion of the nervous system." It has taken more than a century to confirm Mosso's idea that both the brain and the muscles alter their function during exercise and that fatigue is predominantly an emotion, part of a complex regulation, the goal of which is to protect the body from harm. Mosso's ideas were supplanted in the English literature by those of A. V. Hill who believed that fatigue was the result of biochemical changes in the exercising limb muscles – "peripheral fatigue" – to which the central nervous system makes no contribution. The past decade has witnessed the growing realization that this brainless model cannot explain exercise performance. This article traces the evolution of our modern understanding of how the CNS regulates exercise specifically to insure that each exercise bout terminates whilst homeostasis is retained in all bodily systems. The brain uses the symptoms of fatigue as key regulators to insure that the exercise is completed before harm develops. These sensations of fatigue are unique to each individual and are illusionary since their generation is largely independent of the real biological state of the athlete at the time they develop. The model predicts that attempts to understand fatigue and to explain superior human athletic performance purely on the basis of the body's known physiological and metabolic responses to exercise must fail since subconscious and conscious mental decisions made by winners and losers, in both training and competition, are the ultimate determinants of both fatigue and athletic performance.

Keywords: fatigue, central nervous system, central governor model, anticipation, feedback, feedforward, brain, skeletal muscle

INTRODUCTION

More modern attempts to understand the factors that determine fatigue and superior athletic performance can be traced to European studies beginning in the late nineteenth century. An influential book (Mosso, 1915) written by Italian physiologist A. Mosso, Professor of Physiology at the University of Turin was one of the first to consider the biological basis for the fatigue that develops during exercise. From his observations of a range of natural performances by animals and birds and of experimental muscle fatigue in human subjects, Mosso concluded that: "In raising a weight we must take account of two factors, both susceptible to fatigue. The first is of central origin and purely nervous in character – namely, the will; the second is peripheral, and is the chemical force which is transformed into mechanical work" (pp. 152–153). He made a number of other observations that were prescient including: "On an examination of what takes place in fatigue, two series of phenomena demand our attention. The first is the diminution of the muscular force. The second is fatigue as a sensation" (p. 154); and "If we regard the brain and the muscles as two telegraph offices, we can understand that the nerves which join them do not suffer from fatigue. But the central or psychical station may influence the peripheral or muscular station, even if the latter is not doing work, seeing that both brain and muscles are irrigated by the blood" (p. 281). He also understood that fatigue that "at first sight might appear an imperfection of our body, is on the contrary one of its most marvelous perfections. The fatigue increasing more rapidly than the amount of work done saves us from the injury which lesser sensibility would involve for the organism" (p. 156). He realized too that the brain is unique as it is the only organ protected from the effects of starvation: "If the brain is the organ in which the most active change of material takes place, how can one explain the fact that it does not diminish in weight when all the rest of the body is wasting?" (p. 282). But he is best remembered for being one of the first to propose that "nervous fatigue is the preponderating phenomenon, and muscular fatigue also is at bottom an exhaustion of the nervous system" (Bainbridge, 1919, p. 177).

It has taken studies of "fatigue" more than a century (Di Giulio et al., 2006) to rediscover what Mosso believed to be obvious – that both the brain (Marcora et al., 2009) and the skeletal muscles (Amann et al., 2006; Amann and Dempsey, 2008) alter

their function during exercise; that the change in skeletal muscle function is characterized by a slowing of the force and speed of contraction (Jones et al., 2009); and that fatigue is principally an emotion (St Clair Gibson et al., 2003), part of a complex regulation (Noakes et al., 2004; Noakes, 2011b), the goal of which is to protect the body from harm part. So fatigue is indeed one of the human body's "most marvelous perfections."

Interestingly Mosso's ideas did not gain immediate purchase in the exercise sciences but lay dormant until rediscovered more recently (Di Giulio et al., 2006). Instead they were supplanted after 1923 by a different and more simplistic interpretation promoted by English Nobel Laureate Archibald Vivian Hill.

The studies that would become perhaps the most influential in the history of the exercise sciences were performed by Hill and his colleagues at University College, London between 1923 and 1925 (Hill and Lupton, 1923; Hill et al., 1924a,b,c). But Hill's personal beliefs of what causes fatigue predetermined his interpretation of the results of his quite simple experiments. Thus his conclusions and, as a result, the intellectual direction down which his ideas channeled the exercise sciences were determined by Hill's preconceptions even before he undertook his first experiment (Noakes, 1997, 2008a,b). His personal beliefs were fashioned by at least three factors.

Firstly since he was principally a muscle physiologist, it was naturally that Hill's theories would begin from that perspective.

Secondly were a series of studies performed at Cambridge University by another Nobel Laureate Frederick Gowland Hopkins. The crucial 1907 study (Fletcher and Hopkins, 1907) that influenced Hill's thinking had been designed to develop a novel method accurately to measure muscle lactate concentrations in recently killed laboratory animals, specifically frogs. By plunging excised frog muscles into ice-cold alcohol, Fletcher and Hopkins were able to show that lactate concentrations were elevated in muscles that had been stimulated to contract until failure. We now know that ice-cold alcohol denatures the glycolytic enzymes activated by ischemia and anoxia and the activation of which cause muscle lactate concentrations to increase in ischemia and hypoxia. Fletcher and Hopkins also showed that skeletal muscle lactate concentrations fell in muscles stored in a high oxygen concentration and conversely rose when stored in nitrogen.

As a result Fletcher and Hopkins concluded that: "Lactic acid is spontaneously developed, under anaerobic conditions, in excised muscle" so that "the accumulation of lactic acid in muscle occurs only in the conditions of anaerobiosis. With a proper oxygen supply it fails to accumulate at all." They also wrote that: "Fatigue due to contractions is accompanied by an increase of lactic acid."

But Hill's interpretation of these results was more doctrinaire specifically (a) that lactic acid is produced *only* under conditions of muscle anaerobiosis, and (b) that muscle fatigue is *caused* by increased muscle lactate concentrations. These ideas would form the twin pillars of Hill's nascent theory of the factors that cause fatigue and determine human athletic performance.

Thirdly were studies published in 1909 and 1910 (Hill and Mackenzie, 1909; Hill and Flack, 1910) apparently showing that the inhalation of oxygen significantly improved performance during exercise. This led to the conclusion that "this limit (to muscular work) is imposed by the supply of oxygen to the muscles and brain rather than by the function of the skeletal muscles" (Bainbridge, 1919, p. 133) so that "the supply of oxygen to the body is the decisive factor in setting the limit to exercise" (Bainbridge, 1919, p. 136).

As a result of studies conducted on himself when he ran at 10, 12, and 16 km/h around an 84.5 m track near the Physiological Laboratory, Manchester, Hill concluded that increasing muscle lactate (lactic acid) concentrations secondary to the development of skeletal muscle anaerobiosis, caused the fatigue he experienced when running at 16 km/h. Accordingly he developed a model of human exercise physiology (**Figure 1**) that has dominated teaching and research in the exercise sciences ever since (Mitchell et al., 1958; Mitchell and Blomqvist, 1971; Bassett Jr. and Howley, 1997, 2000; Mitchell and Saltin, 2003; Levine, 2008).

Hill's model predicts that shortly before the termination of maximal exercise the oxygen demands of the exercising muscles exceed the (limiting) capacity of the heart to supply that oxygen. This causes skeletal muscle anaerobiosis with the accumulation of "poisonous" lactate (lactic acid) in the muscles. So Hill believed that the heart's capacity to pump a large volume of blood to the active skeletal muscles was the single factor determining the human's ability to perform maximal exercise since the higher the blood supply to muscle, the greater the exercise intensity that could be achieved before the onset of anaerobiosis and fatigue.

Remarkably the most interesting component of Hill's model is that which has been (conveniently) ignored for the past 90 years. For his model invites the really important question: if the heart's capacity to produce a maximum cardiac output indeed limits maximum exercise performance, then what limits the maximal cardiac output? This is the key question that has been systematically ignored by all who have favored Hill's theory for the past 90 years.

Hill believed that the answer was obvious – specifically the development of myocardial ischemia the instant the maximum

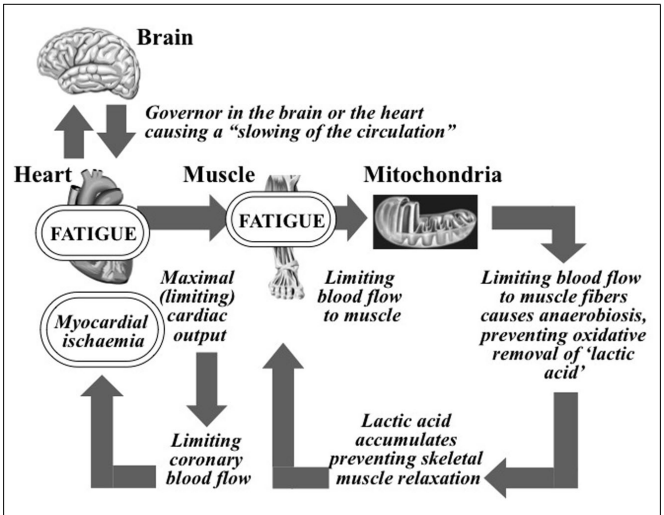

FIGURE 1 | The complete A. V. Hill Cardiovascular/Anaerobic/ Catastrophic Model of Human Exercise Performance. The governor component causing a "slowing of the circulation" was lost from the model some time after the 1930s.

(limiting) cardiac output was reached. Indeed this would be the modern conclusion since it is established that the development of myocardial ischemia during exercise impairs cardiac function, producing a progressive left ventricular dilatation as a result of impaired myocardial contractility (Rerych et al., 1978).

So Hill's complete model theorized that maximal exercise is limited by the development of myocardial failure consequent to the development of myocardial ischemia. This model is "catastrophic" since it predicts that exercise is limited by a failure of homeostasis, in this case in the regulation of cardiac function.

This model soon became the standard teaching in the textbooks of the day (Bainbridge, 1931): "The blood supply to the heart, in many men, may be the weak link in the chain of circulatory adjustments during muscular exercise, and as the intensity of muscular exertion increases, a point is probably reached in most individuals at which the supply of oxygen to the heart falls short of its demands, and the continued performance of heavy work becomes difficult or impossible" (pp. 175–176).

Mosso's concept that the nervous system could also be the site of fatigue was not entirely abandoned. For the 1931 edition (Bainbridge, 1931) of Bainbridge's original monograph (Bainbridge, 1919), edited at A. V. Hill's request by the American physiologists A. V. Bock and D. B. Dill, includes the following statement: "There appear, however, to be two types of fatigue, one arising entirely within the central nervous system, the other in which fatigue of the muscles themselves is superadded to that of the nervous system" (p. 228). But this concept of central fatigue, perhaps borrowed from Mosso, would soon disappear from the teaching of the exercise sciences as the idea became entrenched that peripheral fatigue, situated exclusively in the skeletal muscles, explains all forms of exercise fatigue.

But Hill had not completed his model; he added one final and decisive embellishment to his model. He concluded that some mechanism must exist to protect the ischemic heart from damage whilst it continues to contract until the "poisoning" of the skeletal muscles causes the exercise finally to terminate. So he proposed that a "governor" either in the heart or brain reduces the pumping capacity of the heart immediately this inevitable myocardial ischemia develops. By causing a "slowing of the circulation" (Hill et al., 1924a) this governor would protect the ischemic myocardium from damage in this critical period before the exercise terminated.

But sometime after World War II, Hill's concept of a "governor" mysteriously disappeared from the next generation of exercise physiology textbooks, perhaps because the introduction of electrographically monitored maximal exercise testing established that the healthy heart does not become ischemic even during maximal exercise (Raskoff et al., 1976). Instead the presence of electrocardiographic evidence of ischemia soon became an important diagnostic tool for the detection of coronary artery disease; the absence of these signs of ischemia was interpreted as evidence that the heart is healthy (Lester et al., 1967).

But instead of concluding that the absence of myocardial ischemia during maximal exercise disproves the Hill model, succeeding generations of exercise physiologists simply removed this inconvenient component from their adopted model. Instead they have continued to preach, as fact, the original Hill hypothesis that a limiting cardiac output is the sole important regulator of human exercise performance.

Indeed the special 2008 Olympic Games edition of the influential *Journal of Physiology* includes the statement that: "(2) the primary distinguishing characteristic of elite endurance athletes that allows them to run fast over prolonged periods of time is a large, compliant heart with a compliant pericardium that can accommodate a lot of blood, very fast, to take maximal advantage of the Starling mechanism to generate a large stroke volume" (Levine, 2008, p. 31).

Like the Hill model, this explanation continues to interpret fatigue as a "catastrophic" event that occurs only after skeletal muscle function has failed, specifically "severe functional alterations at the local muscle level." Overlooked is Mosso's conclusion that fatigue is "one of its (the human body's) most marvelous perfections."

But Levine does acknowledge that his description cannot adequately explain why athletes ultimately choose to stop exercising. So he adds that which Hill did not: "(3) athletes stop exercising at $V_{O_2\,max}$ because of severe functional alterations at the local muscle level due to what is ultimately a limitation in convective oxygen transport, which activates muscle afferents leading to cessation of central motor drive and voluntary effort" (p. 31). This explanation differs from the original Hill model that hypothesizes that some form of central motor command slows the functioning of the heart not the skeletal muscles. It is however entirely compatible with the action of a central governor (Noakes, 2011b). Paradoxically one aim of Levine's article was to discredit the concept of such a governor.

PROBLEMS WITH THE TRADITIONAL A. V. HILL EXPLANATION OF HOW HUMAN EXERCISE PERFORMANCE IS "LIMITED"

Hill's original explanation poses a number of significant problems. First, it seems improbable that human athletic performance can be reduced to a single variable and especially one that allows no role for psychological factors such as motivation and self-belief that most agree clearly play some role in human athletic performance. Although scientists may not believe that such factors are important for performance, this is not a belief shared by many coaches and athletes.

For if exercise is regulated purely by a failure of the cardiac output to provide the muscles with an adequate oxygen supply, then psychological factors cannot play any role in human exercise performance. Yet even those who vigorously defend the Hill model, still acknowledge that by providing "motivation," the brain is indeed involved in determining a maximal effort. Hence: "There is no doubt that motivation is necessary to achieve $V_{O_2\,max}$" (Levine, 2008, p. 26). But the Hill model in which the skeletal muscles "limit" the exercise performance, specifically excludes any such interpretation.

For if exercise is regulated purely by a failure of first the heart and then of skeletal muscle function, then there is no need for any special motivation to reach that inevitable state of biological failure; one simply continues to move the legs until they fail. Like the proverbial dead horse, no amount of beating (motivation) can force muscles with "severe functional alterations" to keep working.

Nor is any beating required to achieve that catastrophic state. A painful beating will enhance performance only if there is a biological control system that prevents a truly maximal effort (but which can be partially over-ridden or distracted by a "beating").

Indeed if exercise is "limited" solely by an inevitable catastrophic skeletal muscle failure, then is there no need for the symptoms of fatigue whose principal function must be to forestall homeostatic failure (St Clair Gibson et al., 2003). So the presence of the noxious symptoms of fatigue must indicate that exercise cannot be regulated solely by an inevitable and unavoidable failure of skeletal (and or cardiac) muscle function. Rather fatigue symptoms must play a significant biological role as foreseen by Mosso.

Secondly, according to the Levine interpretation, the best athletes must have the largest hearts and the greatest capacity to transport and consume oxygen. But this has never been shown (Coetzer et al., 1993; Billat et al., 2003). Neither is the $V_{O_2\,max}$ – a surrogate measure of peak cardiac function according to this theory – a good predictor of athletic ability (Snell and Mitchell, 1984; Coetzer et al., 1993; Lucia et al., 1998) nor even of the changes in performance that occur with training (Jones, 1998, 2006; Legaz Arrese et al., 2007; Vollaard et al., 2009; Robertson et al., 2010).

Thirdly, if exercise performance is limited solely by the function of the heart, then one would expect the cardiac output always to be maximal during all forms of exercise. But this is clearly not the case.

Improbably, these significant logical arguments have not prevented the global acceptance of this theory as the sole correct explanation (Bassett Jr. and Howley, 1997, 2000; Levine, 2008; Shephard, 2009).

REPLACING THE HEART ALONE "LIMITATIONS" MODEL OF HUMAN EXERCISE PERFORMANCE

Replacing Hill's cardiovascular/anaerobic/catastrophic model of exercise performance with a novel model began with the realization that the Hill model is unable to explain two of the most obvious characteristics of human exercise performance. The first is that athletes begin exercise at different intensities or paces depending on the expected duration of the planned exercise bout – a bout of short duration is begun at a much faster pace than is one of longer duration. Furthermore athletes will tend to run harder in competition than in training confirming that physiology alone cannot explain performance. The point is that athletes always show an anticipatory component to their exercise performance and that this anticipatory component can be influenced by neural mechanisms relating to motivation. Since as far as we currently know human skeletal muscle probably does not have the capacity to anticipate what is to happen in the future and especially the demands to which it will be exposed (by the brain), the Hill model of peripheral exercise regulation cannot explain this phenomenon.

The second inexplicable observation is that humans also speed up near the end of exercise, the so-called end spurt. This finding significantly disproves the popular belief that fatigue increases progressively and inexorably during prolonged exercise so that athletes reach their most fatigued state immediately prior to the termination of exercise. Were this so, the end spurt could not occur.

In addition to these two rather obvious logical limitations to the predictions of the Hill model, are also a number of significant problems with certain physiological predictions of this model. These include (Noakes and St Clair Gibson, 2004): an absence of evidence that muscle become "anaerobic" during exercise; the absence of a "plateau" in oxygen consumption or cardiac output at exhaustion during maximal exercise; the failure to identify metabolites that explain why muscles "fatigue" during exercise (Jones, 2010) so that "Metabolic causes for these changes (in fatigued skeletal muscle) are hard to identify" (p. 2985); and the absence of evidence for any catastrophic failure of organ function at exhaustion. Rather exercise always terminates with the maintenance of cellular homeostasis.

But the most compelling evidence is the finding that skeletal muscle is never fully recruited during any form of exercise (Noakes and St Clair Gibson, 2004). For the Hill model predicts that as (peripheral) fatigue develops in the exercising muscle fibers so the brain must compensate by recruiting additional fresh fibers in order to assist those fatiguing fibers to sustain the work rate. This process would continue progressively until all the available motor units in the active muscles had been recruited. Once all recruited fibers had each begun to fail, the work rate would fall and "fatigue" would become apparent.

Yet it is now established that fatigue in all forms of exercise develops *before* there is complete skeletal muscle recruitment. Indeed only between 35 and 50% of the active muscle mass is recruited during prolonged exercise (Tucker et al., 2004; Amann et al., 2006); during maximal exercise this increases to only about 60% (Sloniger et al., 1997a,b; Albertus, 2008).

These findings suggest that the Hill model is too simple properly to explain how human exercise performance is truly regulated.

THE EVOLUTION OF A COMPLEX MODEL OF HUMAN EXERCISE REGULATION

Inspired by Hill's concept of a governor regulating human exercise performance, my colleagues and I have proposed a complex model of human exercise regulation in which human exercise performance is not *limited* by a failure of homeostasis in key organs like the skeletal muscles but is rather *regulated in anticipation* specifically to insure that no such biological failure can ever occur, at least in healthy humans. This complex regulation originates within the central nervous system; hence we have termed it the Central Governor Model to honor A. V. Hill's original concept that a "governor" ultimately protects the body from damage during maximal exercise. This model finally re-integrates a body of evidence provided by the neuroscientists that has largely been ignored by those, principally cardio-respiratory physiologists, who have been responsible for sustaining the Hill model for the past 90 years.

THE CONTRIBUTION OF NEUROSCIENTISTS TO THE STUDY OF EXERCISE FATIGUE

Whilst most exercise scientists have embraced the "brainless" Hill model as the defining explanation for the factors determining human exercise performance (Bassett Jr. and Howley, 1997, 2000; Bassett Jr., 2002; Joyner and Coyle, 2008; Levine, 2008; Shephard, 2009), a large body of research has been conducted independently by neuroscientists interested in the mechanisms explaining the

development of fatigue during exercise. Whilst the original focus was predominantly on sustained isometric contractions, in time the research methodologies advanced to be able to study also voluntary dynamic exercise of different durations and intensities. The most complete review (Gandevia, 2001) of these studies establishes that "muscle fatigue . . . may arise not only because of peripheral changes at the level of the muscle but also because the central nervous system fails to drive the motoneurons adequately." As a result "human muscle fatigue does not simply reside in the muscle" (p. 1725).

This conclusion suggests that any model attempting to explain exercise performance and the development of fatigue purely on the basis of peripheral changes in the exercising muscles as does the "brainless" Hill model (Noakes, 2008c), cannot provide a completely satisfactory explanation of all these complex phenomena (Noakes, 2011b).

THE CENTRAL GOVERNOR MODEL OF EXERCISE REGULATION

The key components of this model and the body of published evidence that it can explain are shown in **Figure 2**. This model places the brain firmly at the center of this regulation in keeping with the conclusions of the work reviewed by Gandevia (2001).

According to this model exercise begins with feedforward motor output to recruit the appropriate number of motor units in the exercising muscles. The extent of this recruitment will be determined by a host of factors including, but not exclusively, the biological state of the athlete at the start of exercise (Hettinga et al., 2011) including the emotional state (Renfree et al., 2011), the extent of mental fatigue (Marcora et al., 2009), or sleep deprivation (Martin, 1981), the state of recovery from a previous exercise bout (Eston et al., 2007), the level of motivation and prior experience (Corbett et al., 2009; Foster et al., 2009; Mauger et al., 2009; Swart et al., 2009a; Micklewright et al., 2010), the degree of self-belief (Micklewright et al., 2010) including superstitious beliefs (Damisch et al., 2010). Factor specific to the event that alter performance include monetary reward (Cabanac, 1986), prior knowledge of the exercise end-point (Ansley et al., 2004a,b; Wittekind et al., 2011), and the presence of competitors (Wilmore, 1968) especially if they are of similar ability (Corbett et al., 2012). A number of chemical agents including the stimulants – amphetamine (Swart et al., 2009b), caffeine (Del et al., 2008; Foad et al., 2008; Hogervorst et al., 2008), pseudoephedrine (Gill et al., 2000; Hodges et al., 2006; Pritchard-Peschek et al., 2010), modafinil (Jacobs and Bell, 2004), and the dopamine/noradrenaline reuptake inhibitor bupropion (Roelands et al., 2008; Roelands and Meeusen, 2010; Watson et al., 2010) – as well as the analgesic, acetaminophen (Mauger et al., 2010), or the analgesic naloxone (Surbey et al., 1984; Sgherza et al., 2002), or the cytokines interleukin-6 (IL-6; Robson-Ansley et al., 2004), or brain IL-1β (Carmichael et al., 2006) have all been shown to alter exercise performance as do placebos (Clark

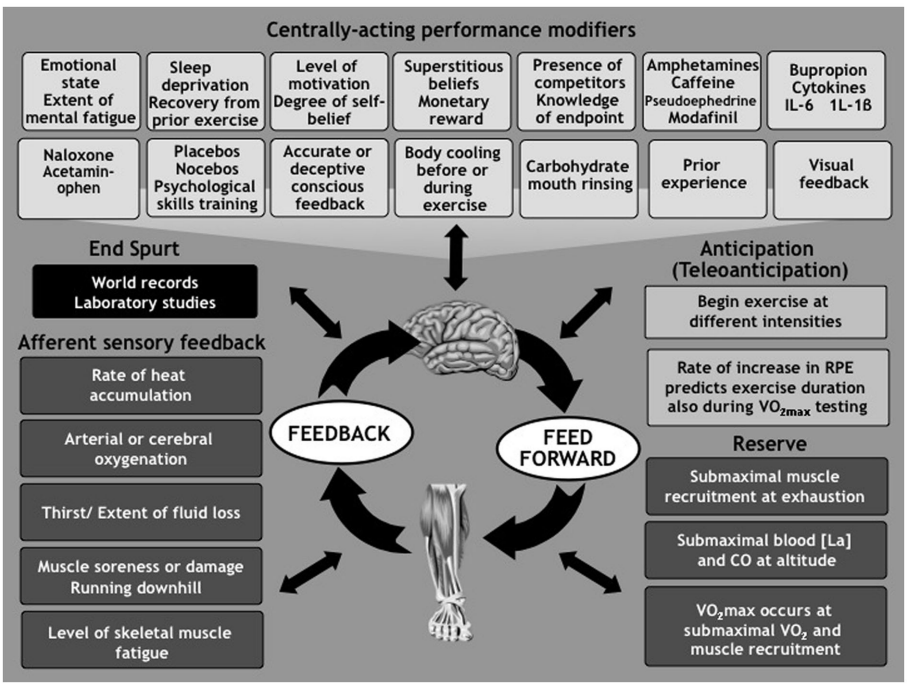

FIGURE 2 | The Central Governor Model of Exercise Regulation proposes that the brain regulates exercise performance by continuously modifying the number of motor units that are recruited in the exercising limbs. This occurs in response to conscious and subconscious factors that are present before and during the exercise, and those which act purely during exercise. The goal of this control is to insure that humans always exercise with reserve and terminate the exercise bout before there is a catastrophic failure of homeostasis. The brain uses the unpleasant (but illusory) sensations of fatigue to insure that the exercise intensity and duration are always within the exerciser's physiological capacity. This model therefore predicts that the ultimate performances are achieved by athletes who best control the progression of these illusory symptoms during exercise. (For more details see St Clair Gibson et al., 2003; Noakes et al., 2004, 2005; St Clair Gibson and Noakes, 2004; Tucker, 2009; Tucker and Noakes, 2009; Noakes, 2011a,b).

et al., 2000; Benedetti et al., 2007; Pollo et al., 2008; Trojian and Beedie, 2008). Psychological skills training (Barwood et al., 2008) or pre-exercise whole body cooling (Booth et al., 1997) can also improve subsequent exercise performance.

Exercise then begins at an intensity that the brain has determined can be sustained for the expected duration of the exercise bout. As a result all forms of exercise are submaximal since there is always a reserve of motor units in the exercising limbs (Amann et al., 2006; Swart et al., 2009b; Marcora and Staiano, 2010; Ross et al., 2010) that is never fully utilized even during maximal exercise (Sloniger et al., 1997a,b; Albertus, 2008) especially when undertaken at altitude (Kayser et al., 1994; Noakes, 2009). Indeed recent studies show that the conventional testing of the maximum oxygen consumption produce submaximal values for oxygen consumption (Beltrami et al., 2012; Mauger and Sculthorpe, 2012), a finding which seriously challenges the foundation finding on which Hill based his model.

An interesting challenge occurs when exercise is open-ended, that is when the athlete has no idea of the expected duration of the exercise bout in which he or she is participating. This typically occurs during the maximal exercise test used to measure the $V_{O_2\,max}$ (Noakes, 2008c) but also occurs when the athlete is unaccustomed to the demands of the specific exercise bout. Under these conditions athletes pace themselves conservatively throughout the exercise bout, increasing their effort only when they are certain how close they are to the finish (Swart et al., 2009a). This uncertainty is associated with a slower rate of rise of the ratings of perceived exertion (RPE).

Once exercise begins, the pace is continuously modified contraction-by-contraction (Tucker et al., 2006a) by continuous feedback from conscious sources including accurate information of the distance covered (Faulkner et al., 2011) and of the end-point (Swart et al., 2009a; Billaut et al., 2011; de Koning et al., 2011). Allowing the pace to change during exercise reduces the physiological effort required to perform a constant amount of work (Lander et al., 2009). Conscious deceptions that improve performance include using the Ramachandran mirror to observe the non-fatigued arm when working with the opposite arm (Tanaka et al., 2011), listening to music (Barwood et al., 2009; Lim et al., 2009; Schneider et al., 2010), the provision of inaccurate information provided by a clock that runs slowly (Morton, 2009) or of the actual distance to be covered (Paterson and Marino, 2004), or of the pace of a prior performance that had been deceptively increased by 2% (Stone et al., 2012), or of the true environmental conditions in which the exercise is being performed and the athlete's real core body temperature response (Castle et al., 2012). Factors that influence performance and which are likely sensed subconsciously include the degree of arterial (Noakes and Marino, 2007) or cerebral oxygenation (Nybo and Rasmussen, 2007; Rupp and Perrey, 2008, 2009; Johnson et al., 2009; Seifert et al., 2009; Billaut et al., 2010; Rasmussen et al., 2010a,b), the size of the muscle glycogen stores (Rauch et al., 2005; Lima-Silva et al., 2010), the extent of fluid loss or thirst (Edwards et al., 2007; Edwards and Noakes, 2009), and variables relating to the rate of heat accumulation (Marino et al., 2000; Tucker et al., 2004, 2006c; Morante and Brotherhood, 2008; Altareki et al., 2009; Flouris and Cheung, 2009; Schlader et al., 2011). A variety of cooling techniques including to the lower body (Castle et al., 2006; Duffield et al., 2010), the upper body (Arngrimsson et al., 2004), the neck (Tyler et al., 2010; Tyler and Sunderland, 2011a,b), or palms (Kwon et al., 2010) all improve performance presumably by altering the nature of the sensory feedback to the control regions in the brain. Rinsing the mouth with carbohydrate (Rollo et al., 2008, 2010, 2011; Chambers et al., 2009; Gant et al., 2010) improves performance perhaps by acting on specific brain areas. Running downhill (Baron et al., 2009; Townshend et al., 2010) and the presence of muscle damage (Marcora and Bosio, 2007) or muscle soreness (Racinais et al., 2008) are all associated with reduced performance further suggesting the presence of specific sensory pathways subserving these functions. The exercise intensity may also be regulated to insure that a critical level of fatigue is not reached (Amann et al., 2008, 2009, 2010; Amann, 2011). If true this requires a muscle sensor able to detect the level of fatigue in individual motor units.

Finally the presence of the end spurt in which the athlete is able to increase her pace for the last 10% of the exercise bout (Kay et al., 2001; Tucker et al., 2004, 2006b, 2007; Amann et al., 2006; Noakes et al., 2009) confirms the submaximal nature of all exercise performances. More importantly it raises the intriguing questions: Exactly what is fatigue? For how can an athlete speed up near the end of exercise when she is the most tired and should therefore be slowing down according to the traditional definition which describes fatigue as an inability of the contracting muscles to maintain the desired force. According to this definition the athlete who speeds up near the end of exercise cannot be fatigued, regardless of how she feels.

The prediction of this model is that potentially "everything," not just those factors identified in **Figure 2**, can potentially affect athletic performance. But that the most important of these effects begin and end in the brain.

THE ROLE OF THE SENSATIONS OF FATIGUE IN THE REGULATION OF THE EXERCISE PERFORMANCE IN ORDER TO PROTECT HOMEOSTASIS

A key component of the CGM is its proposal that fatigue is not a physical event but rather an emotion (St Clair Gibson et al., 2003) that is used by the brain to regulate the exercise performance (Tucker, 2009). This occurs through changes in the RPE which rise as a linear function of the percentage of the planned exercise bout that has been completed or which remains (Noakes, 2004, 2008b; Tucker, 2009) and which always reach a maximum value at the termination of any truly maximal physical effort. Since the RPE rises as a linear function of the exercise duration, then it must be pre-set either before the exercise bout begins or shortly after its initiation.

Accordingly Tucker (2009) has proposed a model of exercise regulation which "incorporates anticipatory/feedforward as well as feedback components, using an expectation of exercise duration to set an initial work rate and to generate what has been termed a subconscious 'template' for the rate of increase in the RPE. During exercise, afferent feedback from numerous physiological systems is responsible for the generation of the conscious RPE, which is continuously matched with the subconscious template by means of adjustment in power output. The subjective rating is biologically linked, allowing the pacing strategy to be adjusted

to prevent catastrophic changes in the monitored physiological variables (homeostats)" (p. 400).

More recently Swart et al. (2012) have advanced our understanding of the manner in which two separate sets of fatigue symptoms interact to determine the exercise performance. These authors wished to distinguish between the symptoms that develop during exercise, specifically the physical sensations produced by exercise as distinct from the sensations produced by the physiological/psychic effort required to continue performing a task at a chosen intensity. They note that in his original description Dr. Gunnar Borg described the RPE as a measure of an "individual's total physical and psychic reaction to exertion" (Borg, 1962).

Thus they wished to separate the physical sensations produced by the actual performance of the work from those psychic or psychological sensations that represent the neural effort of maintaining a given level of physical work. They loosely defined this later group of sensations – the sense of effort – as the subjective sensations not based on any known physiological changes induced by exercise but which are generated by the brain in response to as yet unidentified specific components of the exercise bout. They further postulated that the sense of effort would serve a biological purpose – in particular the maintenance of homeostasis – so that it would rise only when the exercise was of such an intensity or duration that it threatened homeostasis. A rising sense of effort would then force the subject to reduce the exercise intensity in order to prevent a catastrophic biological failure.

To distinguish changes in the physical symptoms produced by exercise from those measuring the sense of effort, they studied subjects who had been carefully instructed to use the Borg RPE scale to measure only the physical symptoms they experienced during exercise. To quantify their sense of effort –the effort of maintaining the work rate – they were instructed in the use of a novel scale – the task effort and awareness (TEA) scale.

Subjects then completed two 100 km cycling bouts, one at a maximal and the other at a submaximal effort. A series of all-out 1 km sprints were included in both exercise bouts. The key was that subjects were instructed to perform all these sprints with an absolutely maximal effort.

The findings showed that whereas RPE rose progressively during exercise in both trials and was lower in the submaximal trial, it reached a maximal value of 19 only in the final sprint in both trials. In contrast, the TEA score was maximal at the end of each sprint even during the submaximal trial in which each sprint began at a lower TEA (and RPE) score.

Thus this study confirms that the brain uses two distinct and separate sets of fatigue symptoms to insure that homeostasis is maintained during all forms of exercise. The first set are the physical sensations induced by exercise and which are adequately captured by Borg's original RPE scale. These sensations rise as a linear function of the exercise duration and reach a maximum value only at the point of exercise termination. Maintaining an exercise intensity that produces this linear increase in RPE produces the optimum pacing strategy.

The second group of symptoms measured by the TEA quantifies the psychic effort of sustaining the effort that produces a specific RPE. Provided the rate of increase in RPE matches the predetermined template, the sense of effort remains low and is not consciously perceived. But attempting to maintain a pace that causes an inappropriate increase in the RPE will produce an increase in the conscious sense of effort. Thus: "The direct consequence of the increasing sense of effort will be an altered behavior, specifically a voluntary reduction in the exercise intensity. Conversely, exercise intensities that do not pose a threat to homeostatic control produce no or little sense of effort." As a result the authors conclude: "the conscious decision of whether to maintain, increase or decrease the current workload or indeed to terminate the exercise altogether may be the outcome of a balance between motivation and affect and the sensation that is defined as the sense of effort."

It is indeed as Bainbridge wrote in 1919: ". . .the sense of fatigue is often a very fallacious index of the working capacity of the body. . .there is not necessarily any correspondence between the subjective feelings of fatigue and the capacity of the muscles to perform work . . . it is a protective feeling, which tends to restrain a man from continuing to perform muscular work when this would react injuriously upon his whole system" (Bainbridge, 1931, pp. 176–177).

POSSIBLE BRAIN AREAS ASSOCIATED WITH THE FEEDBACK REGULATION OF THE EXERCISE RESPONSE IN HUMANS

A series of early studies have found evidence for activation of the insular cortex, the anterior cingulate cortex (ACC) or medial prefrontal region as well as thalamic regions in the brain in response to increased perception of effort during exercise (Williamson et al., 2006). Williamson and colleagues suggest that different areas in the insular cortex appear to respond to inputs from skeletal muscle afferents and from "central command" whereas the anterior cingulate gyrus "may work in conjunction with portions of the insular cortex as a 'central command network' functioning to interpret an individual's sense of effort and then eliciting appropriate autonomic adjustments to affect cardiovascular responses" (p. 56). Thalamic regions are considered to be involved in the regulation of blood pressure by baroreflex mechanisms. More recent studies have further advanced these ideas.

Thus the brain responses to a form of pedaling exercise studied with fMRI found activation of the medial primary sensory and motor cortices, premotor cortex, supplementary area, and anterior cerebellum associated with the task (Mehta et al., 2009). Studying brain areas involved in the decision to terminate exercise, Hilty et al. (2011a) found activation of the mid/anterior insular region immediately prior to the termination of fatiguing isometric handgrip contractions. Since this area is involved in the evaluation of other homeostatic threats, the authors suggest that activation of this brain region may alert the organism to "urgent homeostatic imbalances." More recently the same group (Hilty et al., 2011b) found evidence for increased communication between the mid/anterior insular and the motor cortex during fatiguing exercise indicating "a fatigue-induced increase in communication between these regions" (p. 6). They propose that the mid/anterior insular region "might not only integrate and evaluate sensory information from the periphery, but also be in direct communication with the motor cortex" (p. 1). The effect of this could be to act as a central regulator of motor output to the exercising limbs in keeping with the concept of

a central governor mechanism responding to afferent sensory feedback.

Studying the response of trained athletes and untrained volunteers to an aversive activity, Paulus et al. (2011) reported "profound" activation of the right and left insula, the dorsolateral prefrontal cortex, and the anterior cingulate gyrus in response to the unpleasant task. But trained athletes showed an attenuated response of the right insular cortex compared to non-athletes suggesting that attenuating the right insular cortex response may be an important adaptation favoring superior athletic performance.

Studies of drugs injected directly into areas of the rat brain show that exposure of the ventromedial hypothalamic (VMH) nuclei to muscarinic blockade substantially reduced exercise performance (Guimaraes et al., 2011). Thus the authors conclude: "muscarinic transmission within the VMH modulates physical performance, even when the effects of the thermoregulatory responses on fatigue are minimal" (p. 9).

Summarizing the current evidence Tanaka and Watanabe (2012) have proposed that physical fatigue is regulated by the balance between inhibitory and facilitatory influences on the motor cortex. Thus "sensory input from the peripheral system to the primary motor cortex (M1) decreases the motor output (supraspinal fatigue), and a neural pathway that interconnects the spinal cord, thalamus (TH), secondary somatosensory cortex, medial insular cortex, posterior insular cortex, ACC, premotor (PM) area, supplementary motor area (SMA), and M1 constitutes the inhibition system. In contrast, a facilitation system . . . that interconnects the limbic system, basal ganglia (BG), TH, orbitofrontal cortex, prefrontal cortex, ACC, PM, SMA, and M1 constitutes the facilitation system and a motivational input to this facilitation system enhances SMA and then M1 to increase the motor output to the peripheral system" (p. 730).

SO IS IT REALLY MIND OVER MUSCLE?

For decades physiologists have searched for a single biological variable – a biological silver bullet – that would explain why some athletes are better than all others. Usually this has focused on the heart and circulation (Bassett Jr. and Howley, 2000; Levine, 2008), reflecting the dominance that the Hill model has exerted in this field. But already Bean and Eichna (1943) warned that: ". . . physical fitness cannot be defined nor can differences be detected by means of a few simple physiological measurements . . .. obtained during limited tests . . .. To do so results in focusing attention on some erroneous concept. Man is not a pulse rate, a rectal temperature, but a complex array of many phenomena. . .. Into performance enters the baffling yet extremely important factor of motivation, the will-to-do. This cannot be measured and remains an uncontrollable, quickly fluctuating, disturbing variable which may at any time completely alter the performance regardless of physical or physiologic state" (p. 157).

Similarly Dr. Roger Bannister, the first man to run the mile in less than 4 min wrote in 1956 (Bannister, 1956) that: "The human body is centuries in advance of the physiologist, and can perform an integration of heart, lungs, and muscles which is too complex for the scientist to analyse" (p. 48). Later he continued: "It is the brain not the heart or lungs, that is the critical organ, it's the brain" (Entine, 2000, p. 13). Future generations of exercise scientists would be well advised to head the words of these most observant scientists.

Indeed elite athletes, like Sir Roger Bannister, believe that something more complex than the heart is the ultimate determinant of their performances.

Thus Paavo Nuurmi, perhaps the greatest distance runner of all time since he won nine gold and three silver medals in the Olympic Games wrote that: "Mind is everything. Muscles are pieces of rubber. All that I am, I am because of my mind."

Franz Stampfl who coached Roger Bannister to become the first human to run the mile in less than 4 min also wrote that: "The great barrier is the mental hurdle" (Stampfl, 1955).

One of the greatest mile runners of all time, Australian Herb Elliott has also written that: "To run a world record, you have to have the absolute arrogance to think you can run a mile faster than anyone who's ever lived; and then you have to have the absolute humility to actually do it" (Elliott, 2011, p. 110). Of Elliott and his coach, a contemporary runner Derek Ibbotson who was unable to beat Elliott wrote admiringly: "Together Cerruty and Elliott have brought athletics to the threshold of a new era. They have proved conclusively that not only the body but also the mind must be conquered" (Ibbotson, 1960). Another Australian, former world marathon record holder Derek Clayton wrote: "The difference between my world record and many world class runners is mental fortitude. I ran believing in mind over matter" (Clayton, 1981).

But how might the CGM help us to understand their meaning. I am particularly interested in what the CGM predicts about the athlete who finishes second in a close event.

According to the traditional Hill model the athlete who finished a close second in any event must have had either higher muscle lactate concentrations or lower muscle glycogen concentrations so that his "poisoned" or "depleted" muscles were simply unable to close that 3-s gap. But simple logic exposes the error in this explanation.

For in the final stages of any race, perhaps as many as 65% of the muscle fibers in both the leading athletes' legs are inactive and do not contribute to the physical effort. Surely the second runner could have activated just a few more of those fibers in order to achieve everlasting sporting glory? What prevented that choice?

The CGM predicts that brain-generated sensations of fatigue unique to each individual and influenced by a host of currently unknown individual factors (**Figure 2**), insure that athletes will complete all exercise bouts without risking a catastrophic failure. In the case of a close finish the CGM was clearly successful – neither athlete died. But if the second runner did not die, why did he not run just a little faster and so approach death a little closer? For surely he could have sped up by just a fraction without dying? Yet he did not. Why not?

My unproven hypothesis is that in the case of a close finish, physiology does not determine who wins. Rather somewhere in the final section of the race, the brains of the second, and lower placed finishers accept their respective finishing positions and no longer choose to challenge for a higher finish. Once each runner consciously accepts his or her finishing position, the outcome of the race is decided. So just as a single athlete must "decide" to win, so too must the rest of the top finishers decide the opposite – specifically that they are not going to win.

Furthermore the CGM suggests that this outcome will be strongly influenced by the manner in which the brains of the respective runners generate the sensations of fatigue during exercise. Recall that these symptoms of fatigue are entirely self-generated by each athlete's brain and so are unique to each individual. As such they are illusionary.

According to this model, the winning athlete is the one whose illusionary symptoms interfere the least with the actual performance – in much the same way that the most successful golfer is the one who does not consciously think when playing any shot.

In contrast athletes who finish behind the winner may make the conscious decision not to win, perhaps even before the race begins. Their deceptive symptoms of "fatigue" may then be used to justify that decision. So the winner is the athlete for whom defeat is the least acceptable rationalization.

How athletes and coaches achieve this winning mental attitude is the great unknown. But if the study of the purely physiological basis of fatigue has taught us anything, it is that such studies will never provide an adequate answer.

Rather that future lies in identifying the manner in which the brains of different athletes generate these illusory symptoms. Especially interesting would be studies of the performance of athletes competing in events in which they do not have any experience nor any knowledge of the quality of the opposition. In a close finish under these conditions, how does each athlete decide where she or he will finish? For surely under those specific conditions the uncertain mind will be an even more important determinant of the outcome?

And why they are suppressed in the winning athletes even as they exercise more vigorously than all others.

"The fight," wrote Muhammad Ali "is won or lost far away from witnesses, behind the lines, in the gym, out there on the road, long before I dance under the lights" (De Rond, 2009, p. 154).

Vince Lombardi, the great American football coach, once wrote that: "Fatigue makes cowards of us all." But he was wrong. For his arrow of causation points in the wrong direction.

It is cowardice that exacerbates the sensations of fatigue, not the reverse.

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