Contribution of Oxygenation to BOLD Contrast in Exercising Muscle

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The potential physiological and therapeutic applications of functional MRI (fMRI) in skeletal muscle will depend on our ability to identify factors that may contribute to fluctuations in the BOLD signal. Until now, interpretations of signal changes in fMRI studies of muscle have mostly relied on the increase in muscle T_2 associated with osmotically driven fluid shifts. However, recent studies have documented increases in BOLD signal intensity (SI) after single contractions, coinciding with increases in muscle hemoglobin saturation. In this study, the factors that contribute to variations in the intensity of the BOLD signal in exercising muscle are further addressed. For this purpose, BOLD imaging was performed during and after a moderate electrical stimulation was applied to the sciatic nerve in mice. In addition, oxygen pressure (pO_2), blood flow, and skeletal muscle T_2 (fast and slow components: T_2 and T_2', respectively) were monitored. A comparison between mice lacking eNOS (eNOS$^{-/-}$ mice) and their wild-type (WT) littermates was performed. In WT mice, the BOLD SI, as well as muscle oxygenation and T_2', were significantly increased for a prolonged time in response to this moderate exercise protocol. Blood flow immediately dropped after the electrical stimulation was stopped. In eNOS$^{-/-}$ mice, the high BOLD SI did not persist after the exercise protocol ended. This finding correlates well with the evolution of muscle oxygenation, which progressively decreases after stimulation in eNOS$^{-/-}$ mice. However, T_2' remained high for a prolonged time after stimulation. We therefore concluded that the maintenance of BOLD SI in moderately exercising skeletal muscle depends mainly on changes in pO_2, rather than on blood flow or T_2 effects. Magn Reson Med 52: 391–396, 2004. © 2004 Wiley-Liss, Inc.

Key words: skeletal muscle; exercise; BOLD fMRI; T_2; EPR oximetry

Functional MRI (fMRI) studies have shown that the blood oxygenation level-dependent (BOLD) contrast mechanism can be sensitive to small changes in oxygenation state and blood volume (1). While this method is now widely used to study functionality in the brain, and is beginning to be used in tumors (2–6), only a few studies have focused on fMRI of skeletal muscle (7–9). Measuring the dynamics of blood flow and oxygenation in skeletal muscle during exercise could be important for elucidating aspects of muscle physiology and pathology.

In the last few years, the feasibility of observing time-resolved BOLD effects in exercising human muscles has been demonstrated in several studies (10–12). These studies showed that transient increases in the signal intensity (SI) in skeletal muscle occur after single, short-duration contractions, and that this effect is prolonged for several tens of seconds (as opposed to the on/off kinetics in the brain). This effect coincided with the time course of transient increases in muscle hemoglobin saturation, as shown by near infrared spectroscopy (11), and was further demonstrated to be field-dependent (12). These observations suggest that the transients are due to a positive BOLD effect.

Other investigators have studied the relaxation behavior of skeletal muscle in vivo using T_2 relaxation times. It is now well established that the T_2 of skeletal muscle water increases during exercise (13,14). Although the underlying mechanism is not fully understood, it has been used to map the location and relative intensity of muscle recruitment during various motor tasks (15,16). The assumed mechanism is that the T_2 increase is related to osmotically driven shifts of fluid between intra- and extracellular compartments (13,17).

The usefulness of fMRI in muscle will depend on our ability to identify the factors that contribute to the BOLD signal fluctuations. This issue was first investigated in the brain (18), and later in tumors (4,6,19). The aim of the present study was to characterize the parameters that can contribute to the BOLD contrast in exercising mouse skeletal muscle. For this purpose, BOLD imaging was performed during and after application of an electrical stimulation protocol to the sciatic nerve. In addition, hemodynamic parameters (such as partial oxygen pressure (pO_2) and blood flow) were monitored. We also investigated whether the T_2 parameter of skeletal muscle could be modified by this exercise protocol. To discriminate between these factors, we compared control mice with mice lacking endothelial nitric oxide synthase (eNOS$^{-/-}$). Indeed, NO is able to inhibit oxygen consumption in muscle, and eNOS$^{-/-}$ mice may have a different sensitivity to the exercise protocol in terms of oxygenation (20,21).

MATERIALS AND METHODS

Animal Models

Male C57/BL6J wild-type (WT) (Elevage Janvier, Le Genest-St-Isle, France) and age-matched eNOS knockout mice (eNOS$^{-/-}$) were studied. C57BL/6J eNOS$^{-/-}$ mice were originally from the Jackson Laboratory (JAX® GEMM® Strain) and were inbred at the FATH Laboratory (UCL,

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Brussels, Belgium). Each procedure was approved by the local authorities according to national animal-care regulations.

Experimental Protocol

Anesthesia was induced with an i.p. injection of ketamine (80 mg/kg)/xylazine (8 mg/kg), and maintained with ketamine alone (30 mg/kg). The body temperature of the mice was maintained at 37°C by warm air blown into the magnet for the MR experiments, or by a homeothermic blanket for the flow and oxygenation measurements. Platinum electrodes were placed around the right sciatic nerve. A 15-min exercise protocol was achieved by electrical stimulation at 5 Hz, with 0.2-ms duration pulses (22). The pulse voltage was 0.5 V in the moderate exercise protocol generally applied in this study, and ~3 V in a more intense protocol used in some individual cases (see Discussion).

MRI Experiments

MRI acquisition was performed with a 4.7 Tesla (200 MHz, ^{1}H) bore system (40-cm inner diameter; Bruker Biospec, Ettlingen, Germany). A birdcage radiofrequency (RF) coil with an inner diameter of 70 mm was used. For both MR techniques, a preliminary anatomical T_2 image (rapid acquisition with relaxation enhancement (RARE) sequence: TR = 1500 ms, TE = 11.5 ms, six averages, echo train length = 8, slice thickness = 2 mm, and acquisition time = 2 min 32 s) was acquired to define a first region of interest (ROI) encompassing the muscle of the stimulated leg (about 40 pixels), and a second ROI encompassing the nonstimulated muscle (control).

BOLD Imaging

BOLD-sensitive images were acquired with the use of a regular gradient-echo (GE) pulse sequence. The raw data acquisition parameters were TR = 200 ms, TE = 15 ms, α = 45 °, FOV = 6 cm, matrix = 128 × 128, six averages, and slice thickness = 2 mm. The total data acquisition time was 2 min 33 s. We first acquired five BOLD images to establish the baseline, and then acquired five images during the 15-min exercise protocol. The acquisitions were continued every 3 min for 30 min following stimulation. To avoid effects from external sources of variation, we normalized the relative change in BOLD SI in the stimulated leg with the values from the control leg.

T$_2$ Decay

Multi-echo T_2 images were also acquired to follow the evolution of the T_2 parameter in stimulated and nonstimulated muscles. The imaging parameters were TR = 3000 ms, TE = 6.65 ms, matrix size = 64 × 64, FOV = 6 cm, one average, slice thickness = 2 mm, 30 echoes, and total acquisition time = 6 min 49 s. Acquisitions were performed every 7 min before, during, and after the exercise protocol was applied. We analyzed the T_2 decay using a biexponential fit in order to determine the fast (T_2) and short (T_2') T_2 components of the skeletal muscle. An arbitrary noise threshold was fixed at three times the SNR, below which the values were not taken into account in the fitting process.

pO$_2$ and Flow Measurements

Local muscle oxygenation measurements were carried out with the use of two independent techniques: electron paramagnetic resonance (EPR) oximetry, and fiber-optic probes (OxyLite™). We used OxyFlo™ probes to assess the blood flow inside the muscle.

EPR Oximetry

EPR spectra were recorded with an EPR spectrometer (Magnettech, Germany) with a low-frequency microwave bridge operating at 1.2 GHz, and an extended loop resonator. Charcoal (CX0670-1; EM Science, Gibbstown, NJ) was used as the oxygen-sensitive probe in all of the experiments. We obtained the calibration curves by measuring the EPR line width as a function of pO$_2$. The charcoal suspension (100 mg/ml, 50 µl injected, 1–25 µ particle size) was injected into the mice in the center of the gastrocnemius muscle. The EPR measurements were initiated 2 days after the injection. We placed the muscle under study in the center of the extended loop resonator (the sensitive volume of which extended 1 cm into the muscle mass) using a previously described protocol (23,24).

OxyLite/OxyFlo™ Technique

We used Oxylite™ in conjunction with Oxyflo™ (Oxford Optronix, Oxford, UK) to simultaneously and continuously monitor tissue blood flow, oxygenation, and temperature at the same location. Fiber-optic microprobes combining a laser Doppler system, an oxygen-sensor, and a thermocouple were inserted into the muscle (25,26). We inserted the probes with a 26G needle in order to make a track inside the tissue. We performed back-scatter measurements to validate that movement artifacts did not influence the flow measurements. A baseline of 15 min of stable readings was obtained before the stimulation protocol was initiated. Data were collected continuously at a sampling frequency of 20 Hz before, during, and after electrical stimulation.

Data Analysis

Data are reported as mean ± SEM. We used a Student's t-test or analysis of variance (ANOVA) with a Tukey's multiple-comparison post-hoc test when appropriate.

RESULTS

The relative BOLD SI in mouse muscle increased directly when the stimulation protocol began. During the stimulation period, the average SI showed a mean (±SEM) increase of 6.4% ± 0.7% (N = 4, P < 0.01) for control C57BL6 mice, and 3.3% ± 0.4% (N = 4, P < 0.01) for eNOS knockout mice (Fig. 1a). While the SI remained high in the post-stimulation period for control mice (4.9% ± 0.6%, P < 0.01), it slowly decreased in eNOS$^{-/-}$ mice (1.4% ± 0.6%, P > .05) and returned to the basal level after 15 min (Fig. 1a).
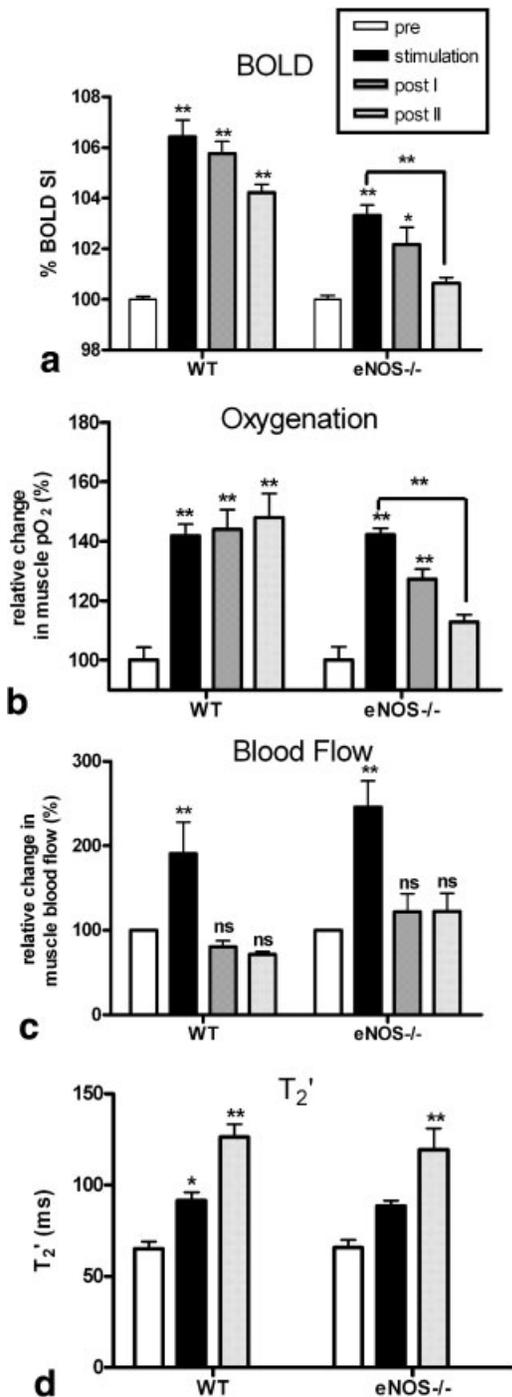
FIG. 1. Comparison between WT mice and eNOS$^{-/-}$ mice. **a:** Evolution of the relative increase in the BOLD SI before (pre), during (stimulation), and after (0–15 min: post I; 15–30 min: post II) electrical stimulation of the sciatic nerve (5-Hz, 0.5-V pulses). **b:** Relative increase in muscle pO$_2$ (EPR measurements) before (pre), during (stimulation), and after (0–15 min: post I; 15–30 min: post II) electrical stimulation of the sciatic nerve. **c:** Relative change in muscle blood flow before (pre), during (stimulation), and after (0–15 min: post I; 15–30 min: post II) electrical stimulation of the sciatic nerve. **d:** Evolution of the T_2' (slow T_2 component) parameter before (pre), during (stimulation), and after (0–15 min: post I; 15–30 min: post II) electrical stimulation of the sciatic nerve. Data are reported as mean ± SEM; **$P < 0.01$; *$P < 0.05$; ns: not significant (ANOVA, Tukey's multiple-comparison post-hoc test).
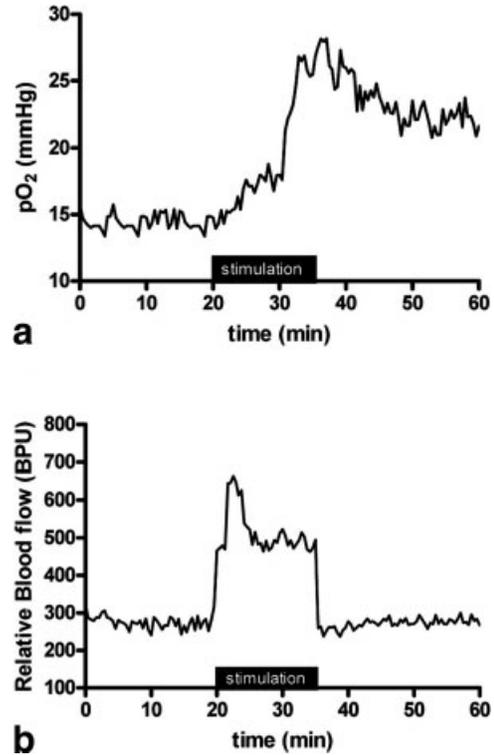
FIG. 2. **a:** Typical monitoring of WT mouse skeletal muscle oxygenation (mmHg) before, during, and after application of a 15-min electrical stimulation protocol, using OxyLite™ probes. **b:** Typical monitoring of WT mouse skeletal muscle blood flow (arbitrary relative units) before, during, and after application of a 15-min electrical stimulation protocol, using OxyFlo™ probes.

We used EPR oximetry, an in vivo non-oxygen-consuming technique, to monitor fluctuations in muscle tissue oxygenation. Muscle oxygenation increased during the exercise protocol (Fig. 1b). The mean increases in pO$_2$ were 41.8% ± 3.9% ($N = 3$, $P < 0.01$) for C57BL6 mice, and 42.2% ± 2.1% ($N = 4$, $P < 0.01$) for eNOS$^{-/-}$ mice. This effect was prolonged during the post-stimulation period in WT mice; however, in mice lacking eNOS, muscle oxygenation significantly decreased after the end of the exercise protocol (down to 20.0% ± 2.9% of the increase). These results were confirmed by OxyLite™ probes (see typical result in Fig. 2a). OxyLite measurements were acquired simultaneously with Doppler measurements (see below), and hence could be compared with changes in blood flow.

Relative changes in muscle blood flow were monitored by laser Doppler probes (OxyFlo™), continuously before, during, and after the stimulation protocol. The OxyFlo™ technique allows relative measurements of blood flow in arbitrary units (blood perfusion unit) to be obtained. Both groups presented a similar pattern, namely an immediate increase (an approximately twofold increase) in muscle blood flow at the onset of the exercise protocol, and a relatively fast decrease after electrical stimulation stopped. The level of increase in the blood flow was quite variable from one mouse to another. The mean changes in muscle blood flow are presented in Fig. 1c, and a typical monitoring is shown in Fig. 2b.

Table 1
T_2 Relaxation Times of the Fast (T_2) and Slow (T_2') Component
Measured in Mouse Skeletal Muscle, Before, During, and After
Application of an Exercise Protocol

Relaxation time	Littermate	Pre (ms)	Stimulation (ms)	Post (ms)
T_2	WT	18.5 ± 0.6	20.1 ± 0.3	21.3 ± 0.5
	eNOS$^{-/-}$	19.9 ± 0.9	19.0 ± 2.0	18.8 ± 0.4
T_2'	WT	65.0 ± 3.9	$91.4 \pm 4.7*$	$126.3 \pm 7.0**$
	eNOS$^{-/-}$	65.7 ± 4.2	88.4 ± 3.0	$119.3 \pm 11.6**$

Comparison between wild type (WT) and eNOS knockout mice
(eNOS$^{-/-}$).
Results are presented as mean $\pm$ sem. *$P < 0.05$, **$P < 0.01$
(ANOVA, Tukey's Multiple Comparison post-hoc test).

Finally, the T_2 relaxation time was evaluated with the
use of a regular spin-echo (multi-echo, 6.7 ms echo-spac-
ing) sequence. First, we measured T_2 relaxation times of
muscle from C57BL6 mice before and after intramuscular
injection of a saline solution (0.9% NaCl) in order to val-
idate the feasibility of this study. Osmotic effects are
known to modulate T_2 decay of muscle in vivo by elevat-
ing the extracellular water component in muscle (13,17).
Two relaxation components (a fast one (T_2) and a slower
one (T_2')), which are assigned to intra- and extracellular
water protons, are usually observed in skeletal muscle.
This setting allowed us to observe osmotically induced
changes in T_2 in C57BL6 mice (data not shown). The same
acquisitions were repeated before, during, and after elec-
trical stimulation of the sciatic nerve in eNOS$^{-/-}$ and WT
mice. When the decay was analyzed with a biexponential
fit, a significant increase in the slow T_2 component (T_2')
was observed in the stimulated leg, whereas no changes
were found in the control muscle (Fig. 1d, Table 1). Inter-
estingly, both the eNOS$^{-/-}$ and WT mice showed a sus-
tained increase in T_2' after stimulation. The fast T_2 com-
ponent was not modified by the exercise protocol in any of
the groups (Table 1).

DISCUSSION

We document here that the BOLD SI increases in response
to an exercise protocol achieved by electrical stimulation
of the sciatic nerve. Both eNOS-knockout and WT mice
were responsive during the stimulation period. This in-
crease was maintained in the post-stimulation period in
WT mice, but not in mice lacking eNOS. Those observa-
tions correlate with the evolution of muscle oxygenation:
pO$_2$ increased during electrical stimulation, and progres-
sively returned to initial levels in eNOS-knockout mice
(contrary to the WT mice). We observed an immediate
increase in blood flow at the onset of the exercise protocol,
which explains the initial modification in muscle oxygen-
ation. Muscle blood flow decreased rapidly in the post-
stimulation period. Accordingly, the persistence of a high
BOLD SI in the post-stimulation period has to be related to
oxygenation, rather than to blood flow. However, the in-
crease in BOLD SI at the onset of exercise could be related
to both oxygenation and blood flow.

Our flow data are in accord with previous reports that
blood flow in human muscles rapidly increases at the
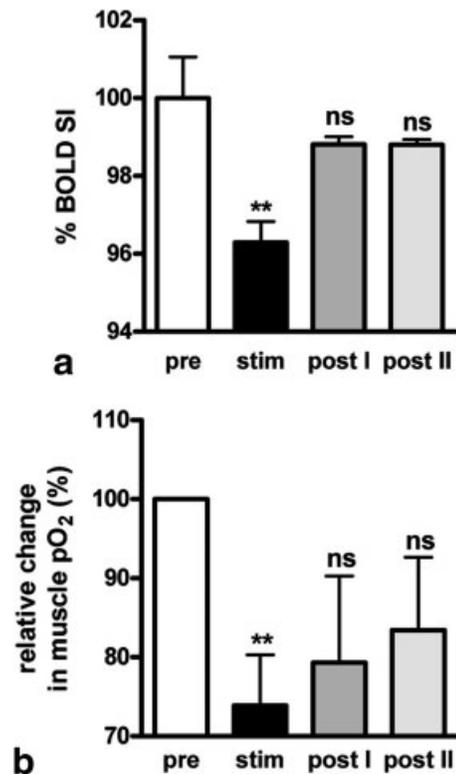
FIG. 3. **a:** Evolution of the relative BOLD SI (mean $\pm$ SEM) before,
during, and after (0–15min: post I; 15–30 min: post II) a high-
intensity (3-V) electrical stimulation protocol of the sciatic nerve in
WT mice ($N = 3$). **b:** Relative change in muscle oxygenation (EPR
measurements) (%, mean $\pm$ SEM) before, during, and after (0–
15 min: post I; 15–30 min: post II) a high-intensity (3-V) electrical
stimulation protocol of the sciatic nerve in WT mice ($N = 4$). **$P <
0.01$; ns: not significant (ANOVA, Tukey's multiple-comparison
post-hoc test).

onset of exercise, and linearly in relation to work intensity
(21,27–31). Therefore, blood flow is precisely regulated to
match the oxygen demand and the local metabolic require-
ments of skeletal muscle. The control mechanisms that are
engaged in exercise-induced skeletal muscle vascular reg-
ulation include muscle mechanical, metabolic, myogenic,
neural, and endothelial-derived relaxing factors (e.g., NO).
It has been documented that NO is formed in skeletal
muscle in response to contraction (21,29).

However, our data are valid only for a moderate exercise
protocol, where the oxygen supply (improved by the in-
crease in blood flow) largely compensates for the oxygen
demand during exercise. Meyer et al. (11) also observed a
significant BOLD response in muscle during "light" exer-
cise. In experiments with higher-intensity exercise (3-V
pulses instead of 0.5-V pulses), a drop in pO$_2$ and BOLD SI
were observed (Fig. 3). Muscle deoxygenation during in-
tense exercise has often been described (32,33). These
results reinforce the notion that oxygenation is related to
the changes observed in T_2^*-GRE imaging of skeletal mus-
cle.

We observed that muscle oxygenation remained high
even when the blood flow decreased to basal levels in the
post-stimulation period. This effect is likely due to a de-

crease in oxygen consumption, mediated by NO. Indeed, there is much evidence that NO regulates mitochondrial respiration of muscle cells by virtue of reversible interactions with cytochrome *c* oxidase (reversible inhibition of mitochondrial respiration by NO competing with O_2 for the active site of cytochrome oxidase) (20,21,34–36). Higher NO levels or more prolonged exposure to NO may also involve the permanent inhibition of complexes I and II. NO donors have been shown to decrease oxygen consumption of tissue slices from canine limb muscle and inhibition of NOS increased oxygen consumption (21). It has been demonstrated that skeletal muscle can produce NO, and that endogenous NO modulates muscle function. The physiological importance of endogenous NO as a mitochondrial modulator in intact muscle remains controversial, however. In this regard, we observed here that mice lacking eNOS showed a decrease in muscle oxygenation after the exercise protocol ended, contrary to the WT mice. This finding further supports the hypothesis that NO is produced in skeletal muscle in response to exercise, and can inhibit mitochondrial respiration and hence the oxygen consumption rate in muscle cells, which can then maintain a high pO_2. In the absence of eNOS, muscle oxygenation decreases in the post-stimulation period, concomitant with blood flow. As stated above, this hypothesis is only applicable in the case of a moderate exercise protocol, since we observed that pO_2 was decreased during exercise of higher intensity.

In addition to hemoglobin, the rate of myoglobin saturation/desaturation with oxygen could also account for changes in BOLD SI. Nevertheless, at physiological muscular pO_2, myoglobin is always saturated (33), and accordingly is not involved in the changes observed with fMRI.

In order to discriminate between an effect of oxygenation and an involvement of T_2 changes on the BOLD SI, we evaluated T_2 before, during, and after exercise in control and eNOS$^{-/-}$ mice. Muscle T_2 has been reported to be modified during exercise (13–17), and may therefore be involved in BOLD SI changes. In this study, the slow component (T_2') of T_2 decay was shown to be modified by the exercise protocol. The observed increase in T_2' is in good agreement with previous studies (13,14,16). Nevertheless, T_2' remained high in the post-stimulation period in both C57Bl6 (WT) and eNOS$^{-/-}$ mice. Consequently, the evolution of the BOLD SI is better correlated with muscle oxygenation than with T_2' changes, since only pO_2 decreased in the post-stimulation period for eNOS$^{-/-}$ mice, similarly to the BOLD intensity.

CONCLUSIONS

This study documents that increases in the BOLD SI can be observed in mouse skeletal muscle during a moderate and prolonged (15-min) exercise protocol. We demonstrated that the BOLD response likely depends on oxygenation and blood flow effects at the onset of exercise, and on the single effect of oxygenation after electrical stimulation stops. Blood flow and T_2 changes were not correlated with the sustained increase in the BOLD SI after exercise, in contrast to muscle pO_2. These data present new possibilities for elucidating physiological and pathological phenomena in skeletal muscle by fMRI.

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