Effect of formoterol, a long-acting $\beta_2$-adrenergic agonist, on muscle strength and power output, metabolism, and fatigue during maximal sprinting in men

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Kalsen A, Hostrup M, Backer V, Bangsbo J. Effect of formoterol, a long-acting $\beta_2$-adrenergic agonist, on muscle strength and power output, metabolism, and fatigue during maximal sprinting in men. Am J Physiol Regul Integr Comp Physiol 310: R1312–R1321, 2016. First published May 4, 2016; doi:10.1152/ajpregu.00364.2015.—The aim was to investigate the effect of the long-acting $\beta_2$-adrenergic agonist formoterol on muscle strength and power output, muscle metabolism, and phosphorylation of CaMKII Thr287 and FX1D during maximal sprinting. In a double-blind crossover study, 13 males [V\text{O}_2\text{max}: 45.0 ± 2.0 (means ± SE) ml·min\textsuperscript{-1}·kg\textsuperscript{-1}] performed a 30-s cycle ergometer sprint during maximal sprinting. Measurements of phosphorylation status of CaMKII and FX1D were observed in phosphorylation of CaMKII Thr287 and FXYD1 between treatments before the sprint, whereas phosphorylation of CaMKII and FXYD1 was greater ($P < 0.05$) in FOR than in PLA after the sprint. In conclusion, formoterol-induced enhancement in power output during maximal sprinting is associated with increased rates of glycogenolysis and glycolysis that may counteract development of fatigue.

$\beta_2$-agonist; fatigue; muscle metabolism; sprint performance

It is well known that $\beta_2$-agonists signal through the $\beta_2$-adrenergic receptor, activating a cAMP-dependent pathway, but the mechanisms behind acute improvements in human sprint performance have not been elucidated. It has been suggested that acute treatment with $\beta_2$-agonists increase the rate of glycogenolysis and glycolysis (10) and, thereby, counteract development of fatigue through an accelerated rate of ATP resynthesis (1, 54). In support of this idea, $\beta_2$-agonists have been shown to elevate blood lactate following maximal sprinting (21, 25) and to increase muscle glycogen breakdown and lactate accumulation during submaximal exercise (22, 26). However, little is known about the acute effect of $\beta_2$-agonists on muscle metabolism and specifically glycogenolytic and glycolytic rates during maximal sprinting.

A mechanism that may contribute to acute $\beta_2$-adrenergic improvements in sprint performance is an increased release of Ca\textsuperscript{2+} from the ryanodine receptor 1 (RYR1) of the sarcoplasmic reticulum (SR) leading to enhanced force production (6, 7, 8, 18, 22, 44) potentially due to a PKA-dependent phosphorylation on this site occurs rapidly (43) and is completely dependent on Ca\textsuperscript{2+} concentration constantly fluctuates during dynamic muscle transients during exercise in humans. One way to investigate the effect of formoterol on muscle strength and sprint performance is the myoplasmic Ca\textsuperscript{2+} concentration. Studies have shown that acute treatment with $\beta_2$-agonists may also prevent loss of membrane excitability by stimulating the Na\textsuperscript{+}/K\textsuperscript{+}-ATPase activity during maximal sprinting (21, 25) and to increase muscle glycogen breakdown and lactate accumulation during submaximal exercise (22, 26). However, little is known about the acute effect of $\beta_2$-agonists on muscle metabolism and specifically glycogenolytic and glycolytic rates during maximal sprinting.

As $\beta_2$-adrenergic enhancements in sprint performance may be linked to an increased release of Ca\textsuperscript{2+} from the SR, it is highly relevant to measure the myoplasmic Ca\textsuperscript{2+} concentration during maximal sprinting. However, as the myoplasmic Ca\textsuperscript{2+} concentration constantly fluctuates during dynamic muscle contractions, it is difficult to obtain information about changes in Ca\textsuperscript{2+} transients during exercise in humans. One way to evaluate fluctuations in myoplasmic Ca\textsuperscript{2+} transients during exercise is to measure phosphorylation status of Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II (CaMKII) on Thr287, as phosphorylation on this site occurs rapidly (43) and is completely dependent on Ca\textsuperscript{2+}/calmodulin binding. CaMKII Thr287 phosphorylation is both dependent on the frequency and magnitude of Ca\textsuperscript{2+} transients (23), which theoretically makes phosphory-
ulation on this site a good indicator of alterations in myoplasmic Ca$$^{2+}$$ concentrations during exercise.

Thus, the aim of the present study was to investigate the acute effect of the long-acting β2-agonist formoterol on quadriceps muscle strength and power output, muscle metabolism, and phosphorylation of CaMKII Thr287 and FXYD1 during 30 s of maximal sprinting in men.

METHODS

Subjects

Thirteen recreationally active male subjects with a weekly training volume of 1.8 ± 0.3 h took part in the study (32 ± 2 yr, 91.7 ± 3.0 kg, 180.0 ± 1.5 cm, VO$_{2\max}$: 45.0 ± 0.2 ml·min⁻¹·kg⁻¹). Subjects were informed about risks and discomfort related to the different tests and procedures in the study. Written informed consent was collected from all subjects prior to the study. The study was approved by the local scientific ethics committee of Copenhagen (H-4-2012-110) and was performed in accordance with the Helsinki II Declaration.

Experimental Protocol

The study was carried out as a randomized, double-blind crossover study consisting of one screening visit and two identical study visits, where subjects either inhaled formoterol (FOR) or placebo (PLA). On the screening visit, subjects completed an incremental exercise test on a cycle ergometer (Monark 839E, Vansbro, Sweden) to determine performance and maximum oxygen uptake (VO$_{2\max}$). Furthermore, subjects got acquainted with the equipment and the exercise protocol used at the study visits.

At least 2 days after the screening visit and after an overnight fast (~10 h), subjects reported to the laboratory, after which a 20-gauge catheter was inserted into an antecubital vein to allow continuous blood sampling. Under local anesthesia (1 ml of lidocaine, 20 mg/ml without epinephrine), two incisions, spaced by at least 4 cm, were made through the skin and fascia of the right vastus lateralis muscle to allow quick sampling of muscle biopsies. Hereafter, subjects inhaled either 27 μg formoterol (3 puffs) or placebo (3 puffs). Forty minutes later, subjects inhaled the exact same treatment giving a total dose of either 54 μg formoterol or six puffs of placebo. Thirty minutes later, subjects completed a warm-up protocol consisting of three 4-min exercise bouts at an intensity corresponding to 30, 50, and 70% of VO$_{2\max}$. Following 5 min of rest, maximal voluntary contraction (MVC) and contractile properties of quadriceps were measured. After 5 min of rest, a 30-s sprint was performed on a cycle ergometer, and from the previously made incisions, a biopsy was collected before and immediately after the sprint, using a 5-mm Bergström needle with suction. To get the most accurate measurement of the muscular milieu right before the sprint, the biopsy collected before the sprint was taken as close to the time of the start of the sprint. Breath-by-breath measurements were collected 1 min before (baseline) and throughout the sprint, and blood samples were collected before and after 0, 1, 5, 10, and 15 min after the sprint. MVC and contractile properties of quadriceps were measured 30 s after the sprint. 4 ± 1 days after the first study visit, subjects completed the exact same procedures in the same order, but with the opposite treatment. Throughout the study, subjects were instructed to maintain their regular level of physical activity and to abstain from caffeine, alcohol, and strenuous exercise 48 h prior to each study visit. Food and fluid intake were registered 48 h prior to the first study visit and duplicated at the second study visit. The exercise protocol was performed at the same time of the day to ensure minimal differences in hormone concentrations and body temperature.

Experimental Procedures

Determination of VO$_{2\max}$. VO$_{2\max}$ was determined in an incremental cycle ergometer test starting at 150 W and increasing 30 W every min until pedaling frequency fell below 70 rpm for more than 5 s. A respiratory exchange ratio above 1.15 was used as criteria for achievement of VO$_{2\max}$. VO$_{2\max}$ was defined as the highest value averaged in any 30-s period. Pulmonary oxygen uptake was measured using a gas analyzing system (JAEGER MasterScreen CPX; Vivayis Healthcare, Hoechberg, DE). The gas analyzing system was calibrated with a 3-l syringe and with gases of known O$_2$ and CO$_2$ concentrations.

Measurements of MVC and contractile properties. MVC of the quadriceps muscle was measured with the subjects sitting on a table with the right leg fixed and flexed in a knee joint angle of 90°. The ankle was attached to a strain gauge (Tedeau-Huntleigh, Cardiff, UK) just superior to the malleoli. To make sure the subjects remained in the same position during the measurements, velcro strips were tied around the chest, hip, and thighs. To reduce day-to-day variation, the exact body position of the subjects was registered and used throughout the experiment. Before measurements of MVC, the subjects performed five submaximal contractions at 60–80% of MVC. Following 1 min of rest, three 3-s maximal voluntary isometric contractions were performed with 1 min of rest between contractions. Only one MVC measurement was performed after the 30-s sprint. During each MVC, superimposed percutaneus electrical muscle stimulations were delivered to the vastus lateralis muscle and the rectus femoris muscle by two self-adhesive electrodes (PALS Platinum 5 × 9 cm, Axelgaard Manufacturing CO, Lystrup, Denmark), as previously described (22). In brief, electrodes were placed on the skin 25% distal from spina iliaca anterior superior and 25% proximal from the patella covering the vastus lateralis muscle and the rectus femoris muscle. Muscle stimulations were delivered by a constant current stimulator (Digitimer, Stimulator model DS7AH, Hertfordshire, UK) in rectangular pulses of 1 ms. To find the appropriate stimulation intensity to apply during the MVC measurements, stimulation intensity was increased either until a plateau in peak twitch force was observed, until maximal stimulator output (999 mA) was achieved, or if subjects felt pain. The stimulation intensity applied in the intervention elicited a peak twitch force response corresponding to 32 ± 1% of peak MVC, which is similar to that observed by Verges et al. (55), who used electrical and magnetic femoral nerve stimulation. The same stimulation intensity was used for the same subject throughout the intervention. To determine voluntary activation level, a single stimulation was delivered on top of the plateau of each MVC (13, 37). To determine peak twitch force, a single stimulation was delivered 1 s following relaxation of each MVC (13, 40). Potentiated twitches were used since these have been shown to be more sensitive to fatigue than unpotentiated twitches (32, 40). During measurements, subjects received verbal encouragement with no visual feedback. From the three MVC measurements before the sprint, the greatest MVC and the related twitch were selected for data analysis.

The following parameters were determined: MVC, the highest force during the contraction; peak twitch force, highest force measured in response to a single stimulation delivered 1 s after the MVC; twitch half relaxation time, the time from peak twitch force until force reached 50% of peak twitch force; and time-to-peak twitch force, the time from a single stimulation, delivered 1 s after the MVC, until peak twitch force was reached. Voluntary activation level was calculated from single twitches, as described by Crivelli et al. (13). Voluntary activation level = (1 − superimposed twitch force/peak twitch force) × 100, where the superimposed twitch force is the additional force elicited by a single stimulation delivered on top of the plateau of the MVC. A correction was applied to the equation if the superimposed stimulation was delivered slightly before or after the peak MVC (51).

30-s sprint. The protocol for the 30-s sprint was designed in Monark 839E analysis software. Subjects were told to pedal for 30 s with a cadence of 70–80 rpm (against a resistance of 6 N), whereafter,
the load was increased to 0.9 N/kg body mass, and the subjects were told to pedal as fast as possible for 30 s. Subjects remained seated during the test. Verbal encouragement was given during the test. Power output and cadence were recorded with a frequency of 1 Hz. Peak power (highest power output during the test), mean power (average power output during the test), end power (power output the last second of the sprint), and fatigue index: [(peak power − end power)/peak power]·100] were registered. On the basis of unpublished observations and previous studies from our laboratory (20, 21), the coefficient of variation (standard deviation/mean·100) for peak power, and mean power has been shown to be <3% during this type of exercise protocol.

Muscle analysis. Muscle biopsies were analyzed from 11 out of 13 subjects, since two of the subjects refused to have biopsies taken on the second study visit. Biopsies were frozen immediately in liquid nitrogen and stored at −80°C for later analyses. The time delay from cessation of the sprint to freezing of biopsies in liquid nitrogen was not different between the formoterol trial and the placebo trial, being 10.1 ± 0.7 and 10.5 ± 0.8 s, respectively. Biopsies were freeze dried and dissected free of blood, connective tissue, and fat in 18°C with a humidity <30% using a stereo microscope.

Approximately 2 mg dry weight (dw) muscle tissue was extracted in 1 N HCl and hydrolyzed at 100°C for 3 h. Glycogen was determined by the hexokinase method, as previously described (33). Approximately 2 mg dw muscle tissue was extracted in a solution of 1.5 M perchloric acid and 1 mM EDTA, neutralized to pH 7.0 with 2.2 M KHCO3, and stored at −80°C until analyzed fluorometrically for muscle metabolites, as previously described (33).

Two milligrams of dw muscle tissue was homogenized in a freshly made buffer (10% glycerol, 20 mM Na-pyrophosphate, 150 mM NaCl, 50 mM HEPES, 1% Nonidet P-40, 20 mM β-glycerophosphate, 10 mM NaF, 2 mM PMSF, 1 mM EDTA, 1 mM EGTA, 10 g/ml aprotinin, 10 g/ml leupeptin, and 3 mM benzamidine) with a Polytron 3100 (Kinematika) for 30 s. During the homogenization procedure, the samples were kept on ice. Samples were rotated end-over-end for 1 h at 4°C before being centrifuged for 30 min at 17,500 g at 4°C. Lysate was collected, as the supernatant and protein concentrations were determined by ELISA using BSA standards (Pierce Reagents). The lysates were diluted to appropriate protein concentrations in a 6× sample buffer (0.5 M Tris-base, DTT, SDS, glycerol, and bromophenol blue), and for each sample, 10–16 μg of protein were loaded into different wells of 4-15% precasted Criterion TGX stain-free gels (Bio-Rad Laboratories, Hercules, CA). Samples from the same subject were loaded next to each other on the same gel. Gel electrophoresis was carried out for 30–60 min at 60 mA, and a maximum of 250 V/gel followed by semidry transfer of proteins to polyvinylidene difluoride membranes (Turbon PVDF midi, Bio-Rad Laboratories) at 1.8 A and 25 V for 7 min by a Trans Blot Turbo (Bio-Rad Laboratories). Membranes were incubated overnight with 30–35 ml of primary antibody diluted in 2% nonfat milk before being washed briefly in Tris-buffered saline (TWEEN) and incubated with secondary antibody (diluted in 2% nonfat milk) for 1 h at room temperature. Primary antibodies used were αAMPK Thr172 (no. 2531; Cell Signal- ning, Danvers, MA), AMPK α2 (no. 2757; Cell Signaling), total FXYD1 (0.14 μg/ml, rabbit polyclonally, sc-13721-1-AP, Proteintech Group, Chicago, IL), unphosphorylated FXYD1 (kindly provided by Dr. J. Randall Moorman, University of Virginia), pCAMKII Thr287 (no. 3361, Cell Signaling), total CAMKII (sc-9035, Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibodies used were horseradish peroxidase-conjugated antibodies from Dako (Glostrup, Denmark). After primary and secondary antibody treatment, membranes were incubated in chemiluminescent substrate (Immobilon Western Chemilum HRP Substrate; Millipore, Copenhagen, Denmark), and the signal was detected on a Chemidoc MP (Bio-Rad Laboratories). Band intensities were quantified using Image Lab (Image Lab v. 4.0, Bio-Rad Laboratories).

Calculations. In the present study, the difference in muscle metabolite concentration between the biopsy obtained before the sprint and the biopsy obtained immediately after the sprint is denoted by Δ. For lactate, pyruvate and G-6-P, Δ represents a net accumulation during the sprint. For ATP, PCr, and glycogen, Δ represents a net reduction during the sprint. Anaerobic ATP turnover during the sprint was calculated from differences (Δ) in muscle ATP, PCr, lactate, and pyruvate (5): Anaerobic ATP turnover = 2(ΔATP) + ΔPCr + 1.5Δ lactate + 1.5Δ pyruvate.

Mean rates of glycolysis and glycogenolysis during the sprint were estimated from Δlactate, Δpyruvate, ΔG-6-P, and the duration of the sprint (30 s): glycolytic rate = (0.5Δlactate + 0.5Δpyruvate)/30 s and glycogenolytic rate = (0.5Δlactate + 0.5Δpyruvate + ΔG-6-P + 0.33ΔG-6-P)/30 s, where 0.33ΔG-6-P was used as an estimate of glucose-1-phosphate and fructose-6-phosphate accumulation, as previously described (24). It should be noted that the pyruvate oxidized and lactate released to the circulation were not included in the calculations, but because of the short duration of the sprint, the underestimation of the anaerobic ATP turnover, glycolysis, and glycogenolysis is probably less than 20% (9).

Blood analysis. Venous blood samples were collected in heparinized syringes and analyzed immediately for lactate and K+ on a blood gas analyzer ABL 800 Flex (Radiometer, Copenhagen, Denmark). Lactate and K+ were determined for 12 subjects, as one subject refused to have a catheter inserted into his antecubital vein on the second study visit. For determination of epinephrine and norepinephrine, further blood samples were collected in Vacutainers with EDTA and spun at 3,000 rpm for 15 min, after which plasma was collected and stored at −80°C. Plasma epinephrine and norepinephrine were determined before and after the sprint for 10 subjects (randomly selected) with a commercially available ELISA kit (2-CAT Plasma ELISA High Sensitive; Labor Diagnostica Nord, Nordhorn, Denmark), according to the manufacturer’s instructions.

Drugs. Formoterol (Oxis, 9 μg/dose) and placebo (empty turbohaler) were provided by AstraZeneca (London, UK) and were administered by inhalation from identical looking turbohalers. The formoterol particles are very small with no taste, which ensured that the subjects could not identify the treatment. The reason for selecting a β2-agonist (11). Formoterol particles are very small with no taste, which ensured that the subjects could not identify the treatment. The reason for selecting a β2-agonist (11).

Drug administration was carried out by a person not involved in any other parts of the study. Inhalation technique was practiced at the screening visit, and all subjects were familiar with the side effects of formoterol. Five out of thirteen subjects experienced tachycardia and tremor after inhalation of 54 μg formoterol. Seven subjects received placebo at the first visit and six subjects received formoterol at the first visit.

Statistics. The statistical analysis was carried out in SPSS 18.0 (IBM, Armonk, NY). Sample size was determined for a crossover design for the primary outcome “mean power,” as previously described (15). Power was set to 0.8, significance level was set to 0.05, and effect size was based on previous observations of increased mean power during 30 s of maximal sprinting following administration of β2-agonist (11). Sample size calculations revealed that eight subjects were necessary to obtain a power of 0.8. With the risk of dropouts and to ensure a high statistical power, 13 subjects were included. Normality was tested with a Shapiro-Wilk test or Q-Q plots, and all data were normally distributed. Comparisons of mean, peak, and end power, fatigue index, anaerobic ATP utilization, glycogen breakdown, glycololytic rate, glycglycogenolytic rate, and changes (Δ) in muscle metabolites were analyzed with a paired t-test. A two-way repeated-measures ANOVA
(treatment × sampling point) was used to compare MVC, contractile properties, muscle metabolites, venous blood, oxygen uptake, and protein phosphorylation. In case of a significant ANOVA, a Student-Newman-Keuls post hoc test was applied. Significance level was 0.05. The critical value of $P$ was two-tailed.

**RESULTS**

**Power Output**

Power output during the sprint is presented in Fig. 1A. Mean, peak, and end power output during the sprint were $4.6 \pm 0.8$, $3.9 \pm 1.1$, and $9.5 \pm 3.2$ higher ($P < 0.05$) in FOR than in PLA, respectively (Fig. 1B). Fatigue index was higher ($P < 0.05$) in PLA (51 ± 3%) than in FOR (49 ± 3%).

**MVC and Contractile Properties of Quadriceps**

MVC was $3.0 \pm 0.9$% and $9.9 \pm 0.9$% higher ($P < 0.05$) in FOR than in PLA before and after the sprint, respectively (Table 1). Peak twitch force was $8.0 \pm 2.2$% higher ($P < 0.05$) before the sprint and $20.0 \pm 8.8$% higher ($P < 0.05$) after the sprint in FOR than in PLA (Table 1). The relative decline in MVC after the sprint tended ($P = 0.09$) to be smaller in FOR than in PLA (PLA: 31 ± 3 FOR: 27 ± 3%). Before and after the sprint, there was no significant difference in time-to-peak twitch force, twitch half relaxation time, or voluntary activation level between treatments. In PLA, time-to-peak twitch force was longer ($P < 0.05$) after than before the sprint with no difference in FOR (Table 1).

**Muscle Metabolites and Anaerobic ATP Utilization**

Muscle ATP was reduced after the sprint for both treatments ($P < 0.05$) and the reduction in ATP was smaller ($P < 0.05$) in FOR than in PLA ($3.7 \pm 1.5$ vs. $8.0 \pm 1.6$ mmol/kg dw). Muscle PCR and net breakdown of PCR during the sprint were not different between treatments (Table 2).

Muscle glycogen before and after the sprint was not significantly different between treatments, but the net rate of glycogen breakdown was greater ($P < 0.05$) in FOR than in PLA ($2.5 \pm 0.2$ vs. $2.0 \pm 0.2$ mmol·kg$^{-1}$·s$^{-1}$). Before and after the sprint, G-6-P and lactate were higher ($P < 0.05$) in FOR than in PLA. During the sprint, net accumulation of G-6-P ($15.9 \pm 2.1$ vs. $10.0 \pm 1.6$ mmol/kg dw) and lactate ($78.9 \pm 6.8$ vs. $67.3 \pm 7.2$ mmol/kg dw) was higher in FOR than in PLA (Table 2). Before the sprint, pyruvate was not significantly different between treatments, but after the sprint, pyruvate was higher ($P < 0.05$) in FOR than in PLA. Net pyruvate accumulation during the sprint was higher in FOR than in PLA ($2.6 \pm 0.6$ vs. $0.7 \pm 0.2$ mmol/kg dw). During the sprint, the net rate of glycogenolysis and glycolysis were $45.7 \pm 21.0$ and $28.5 \pm 13.4$% higher ($P < 0.05$) in FOR than in PLA, respectively (Fig. 2). During the sprint, anaerobic ATP utilization was $11.4 \pm 4.3$% greater ($P < 0.05$) in FOR than in PLA ($186.1 \pm 14.6$ vs. $170.4 \pm 14.0$ mmol/kg dw; Fig. 3A).

**Oxygen Uptake**

There was no significant difference in oxygen uptake before (baseline) or during the sprint between treatments (Fig. 3B).

**Protein Phosphorylation**

For both treatments, phosphorylation of CAMKIIβ and CAMKIIy/δ on Thr$^{287}$ was higher ($P < 0.05$) after the sprint compared with before the sprint. Before the sprint, phosphorylation of CAMKIIβ and CAMKIIy/δ on Thr$^{287}$ was not significantly different between treatments, but after the sprint, phosphorylation of both CAMKIIβ and CAMKIIy/δ on Thr$^{287}$ was higher ($P < 0.05$) in FOR than in PLA (Fig. 4, A and B). There was no significant difference in unspecific phosphorylation of FXYD1 before the sprint between treatments, but after the sprint, phosphorylation of FXYD1 was higher ($P < 0.05$) in FOR than in PLA (Fig. 4, C). Phosphorylation of AMPK on Thr$^{172}$ was higher ($P < 0.05$) after the sprint compared with before the sprint in FOR, with no difference in PLA. There was no significant difference in AMPK Thr$^{172}$ phosphorylation between treatments (Fig. 4D).

**Plasma Epinephrine, Norepinephrine, Lactate, and K$^+$**

For both treatments, plasma epinephrine and norepinephrine were higher ($P < 0.05$) after the sprint compared with before the sprint. There was no significant difference in plasma epinephrine between treatments, whereas norepinephrine was higher ($P < 0.05$) before the sprint in FOR than in PLA (Table 3). Plasma lactate was higher ($P < 0.05$) in FOR than in PLA at all sampling points except for immediately after the sprint. Plasma K$^+$ was lower ($P < 0.05$) at all sampling points for FOR compared with PLA (Table 3). Blood pH was lower 1, 10, and 15 min after the sprint in FOR than in PLA (Table 3).

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**Fig. 1.** Power output curve (A) and peak power (Peak), mean power (Mean) and end power (End) (B) during 30 s of maximal sprinting after administration of either formoterol (FOR) or placebo (PLA). Values are means ± SE; $n = 13$. *Significantly different from PLA ($P < 0.05$).
The present study is the first to demonstrate increased MVC and the rate of SR Ca2+ release in nonfatigued quadriceps muscle. In the present study, we used changes in phosphorylation status of CaMKII Thr287 as an indicator of alterations in myoplasmic Ca2+ concentrations during maximal sprinting. Phosphorylation of CaMKII Thr287 in both the β and γ/δ isoforms was greater with formoterol than placebo following 30 s of maximal sprinting. The fact that there was no difference in phosphorylation of CaMKII Thr287 between treatments at rest before the sprint, indicates that the myoplasmic Ca2+ concentration may not have been different between treatments before the sprint. Thus, the higher phosphorylation after the sprint in the formoterol trial may potentially be attributed to an increased magnitude and/or frequency of Ca2+ transients during the sprint, which may have caused the greater power output observed in the formoterol trial.

Table 2. Muscle metabolites (nmol/kg dw) before and immediately after 30 s of maximal sprinting following administration of either formoterol or placebo

<table>
<thead>
<tr>
<th></th>
<th>PLA</th>
<th>FOR</th>
<th>After %Δ</th>
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<tbody>
<tr>
<td>ATP</td>
<td>26.2 ± 1.7</td>
<td>25.8 ± 1.0</td>
<td>18.2 ± 2.4#</td>
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<tr>
<td>PCr</td>
<td>77.9 ± 5.3</td>
<td>80.5 ± 5.9</td>
<td>22.1 ± 2.5#</td>
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<tr>
<td>Creatine</td>
<td>66.1 ± 5.9</td>
<td>66.8 ± 6.4</td>
<td>-8.0 ± 1.5</td>
</tr>
<tr>
<td>Total Creatine</td>
<td>144.1 ± 10.6</td>
<td>147 ± 11.7</td>
<td>-3.7 ± 1.6*</td>
</tr>
<tr>
<td>Lactate</td>
<td>5.6 ± 0.6</td>
<td>10.9 ± 1.5*</td>
<td>25.5 ± 5.7#</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>0.9 ± 0.2</td>
<td>0.6 ± 0.2</td>
<td>25.5 ± 5.7#</td>
</tr>
<tr>
<td>Glycogen</td>
<td>396 ± 25</td>
<td>404 ± 39</td>
<td>46.4 ± 5.1#</td>
</tr>
<tr>
<td>G-6-P</td>
<td>0.4 ± 0.1</td>
<td>1.8 ± 0.4*</td>
<td>80.5 ± 10.1</td>
</tr>
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</table>

Values are expressed as means ±SE; n = 11. PCr, phosphocreatine; G-6-P, glucose-6-phosphate. *Significantly different from PLA (P < 0.05). #Significantly different from before the sprint for same treatment (P < 0.05).

Table 1. Maximal voluntary contraction, peak twitch force, time-to-peak twitch force, twitch half-relaxation time, and voluntary activation level of quadriceps before and after 30 s of maximal sprinting following administration of either formoterol (FOR) or placebo (PLA)

<table>
<thead>
<tr>
<th></th>
<th>PLA</th>
<th>FOR</th>
<th>Δ</th>
<th>%Δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>MVC (N)</td>
<td>684 ± 34</td>
<td>704 ± 34*</td>
<td>-8.0 ± 1.5</td>
<td>-3.7 ± 1.6*</td>
</tr>
<tr>
<td>PTF (N)</td>
<td>241 ± 13</td>
<td>260 ± 13*</td>
<td>-121 ± 9</td>
<td>-31 ± 3</td>
</tr>
<tr>
<td>TPTF (ms)</td>
<td>71.5 ± 0.7</td>
<td>72.0 ± 1.5</td>
<td>-4.2 ± 1.6</td>
<td>8 ± 3</td>
</tr>
<tr>
<td>HRT (ms)</td>
<td>38.4 ± 3.5</td>
<td>39.8 ± 3.6</td>
<td>-9.5 ± 2.8</td>
<td>-14 ± 4</td>
</tr>
<tr>
<td>VA (%)</td>
<td>90.5 ± 1.2</td>
<td>90.4 ± 0.9</td>
<td>91.4 ± 3.8</td>
<td>28 ± 17</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE; n = 13. Δ, difference between before and after 30 s of maximal sprinting; %Δ, relative difference between before and after 30 s of maximal sprinting; MVC, maximum voluntary contraction; PTF, peak twitch force; TPTF, time to peak twitch force; HRT, twitch half relaxation time; VA, voluntary activation; FOR, formoterol; PLA, placebo. *Significantly different from PLA at same time point (P < 0.05). #Significantly different from before the sprint for same treatment (P < 0.05).
A limitation in the use of CaMKII Thr\textsuperscript{287} phosphorylation to evaluate myoplasmic Ca\textsuperscript{2+} is that phosphorylation status of CaMKII Thr\textsuperscript{287} does not always parallel changes in frequency/magnitude of Ca\textsuperscript{2+} transients (17, 43), which most likely is due to regulation of phosphorylation status by CaMKII phosphatases. As PKA has been shown to inhibit protein phosphatase-1 in neurons (4), it could be speculated that the greater phosphorylation of CaMKII Thr\textsuperscript{287} after the sprint in the formoterol trial was due to a PKA-dependent inhibition of protein phosphatase-1. However, there was no difference in CaMKII Thr\textsuperscript{287} phosphorylation before the sprint supporting that a PKA-dependent inhibition of protein phosphatase-1 did not contribute to the greater CaMKII Thr\textsuperscript{287} phosphorylation observed after the sprint in the formoterol trial.

The observations of reduced venous K\textsuperscript{+} and greater phosphorylation of FXYD1 after the sprint in the formoterol trial support that increased activity of the Na\textsuperscript{+}/K\textsuperscript{+}-ATPase potentially may have counteracted a reduction in action potential amplitude (46) and, thereby, enhanced power output during the sprint. Interestingly, even though β\textsubscript{2}-agonists are thought to induce hypokalemia by a PKA-dependent phosphorylation on Ser\textsuperscript{68} of FXYD1 (3), we did not observe any effect of formoterol on unspecific phosphorylation of FXYD1 before the sprint despite reduced venous K\textsuperscript{+}. However, the antibody used in the present study detects unspecific phosphorylation of FXYD1 and may, therefore, not be sensitive to detect specific phosphorylation on Ser\textsuperscript{68}. A study by Thomassen et al. (53) confirmed this by showing that unspecific phosphorylation of FXYD1 and phosphorylation of FXYD1 Ser\textsuperscript{68} were not paralleled in either the soleus or extensor digitorum longus muscles of mice. The greater unspecific phosphorylation of FXYD1 after the sprint in the formoterol trial may, therefore, not be induced solely by a PKA-dependent phosphorylation of FXYD1 on Ser\textsuperscript{68}. Instead, the greater unspecific phosphorylation of FXYD1 may be a result of an increased release of Ca\textsuperscript{2+} from RYR1 during the sprint that may have increased the activity of PKC\textsubscript{z}, leading to enhanced phosphorylation on both Ser\textsuperscript{63} and Ser\textsuperscript{68} of FXYD1 (3, 28, 47). Therefore, it could be speculated that the increased unspecific phosphorylation of FXYD1 observed in the formoterol trial after the sprint may be attributed to effects of both PKA and PKC\textsubscript{z}.

It can be ruled out that an increased central motor drive contributed to the formoterol-induced increase in power output, as formoterol had no effect on voluntary activation level measured before and after the sprint.

In the present study, anaerobic ATP utilization was greater in the formoterol trial due to higher rates of glycogenolysis and glycolysis. The higher rates of glycogenolysis and glycolysis may perhaps be important for the greater mean power output observed in the formoterol trial. In support of this, the greater rate of glycogenolysis in the formoterol trial increased the accumulation of G-6-P, thereby reducing the accumulation of P\textsubscript{i}, which may have counteracted development of fatigue (1, 54). Furthermore, as there was no difference in oxygen uptake and breakdown of PCr between treatments, the smaller reduction in ATP content in the formoterol trial should be attributed to a greater rate of glycolysis. Hence, the greater rate of glycolysis in the formoterol trial counteracted a reduction in

Fig. 2. Rate of glycogenolysis and glycolysis calculated from the changes (Δ) in lactate, pyruvate, and glucose-6-phosphate during 30 s of maximal sprinting after administration of either formoterol (FOR) or placebo (PLA). Values are means ± SE; n = 11. *Significantly different from PLA (P < 0.05).

Fig. 3. Anaerobic ATP utilization calculated from changes (Δ) in ATP, PCr, lactate and pyruvate (glycolysis) (A), and oxygen uptake (B) during a 30-s cycle sprint after administration of either formoterol (FOR) or placebo (PLA). PCr, phosphocreatine; VO\textsubscript{2} oxygen uptake; Baseline, VO\textsubscript{2} before the sprint; Sprint, VO\textsubscript{2} during the sprint. Values are expressed as means ± SE; n = 11. *Significantly different from PLA (P < 0.05).
ATP that may have counteracted development of fatigue by reducing accumulation of Pi, free Mg2+/H11001, and AMP (1, 54). The mechanisms behind the higher rates of glycogenolysis and glycolysis observed during maximal sprinting in the formoterol trial may involve an increased activity of the key enzymes glycogen phosphorylase (GP) (31) and phosphofructokinase (PFK) (48) through a PKA-dependent phosphorylation (12, 14, 35, 39). This is supported by higher muscle G-6-P and lactate before the sprint with no difference in ATP or PCr, indicating that the differences in G-6-P and lactate were not a result of accumulation of the activators AMP or Pi (31, 48). The greater phosphorylation of AMPK on Thr172 after 30 s of maximal sprinting in the formoterol trial suggests that elevated concentrations of AMP, through allosteric activation of GP and PFK, may also contribute to the higher rates of glycogenolysis and glycolysis observed during maximal sprinting. However, independent of changes in AMP, AMPK can also be phosphorylated on Thr172 by the Ca2+/calmodulin-activated protein kinase CaMKIIβ (29, 38), which is why the greater AMPK Thr172 phosphorylation observed in the formoterol trial may simply be a result of an increased release of Ca2+ from the SR. This seems plausible as phosphorylation of CaMKII on Thr287 (another Ca2+/calmodulin-activated protein kinase) was greater after the sprint in the formoterol trial. Moreover, the smaller reduction in ATP content during the sprint in the formoterol trial does not support a greater accumulation of AMP, but rather indicates a smaller accumulation of AMP, as accumulation of AMP (estimated from accumulation of IMP) is closely related to the reduction in ATP content (30, 49).

MVC and peak twitch force of quadriceps were higher before and after the sprint in the formoterol trial compared with the placebo trial. While β2-adrenergic enhancements in force is not an uncommon observation in nonfatigued human (21, 22, 25) and animal muscles (6, 8, 18), the finding of a formoterol-induced enhancement in MVC and peak twitch force after 30 s of maximal sprinting is novel and demonstrates that the positive inotropic effect of formoterol is maintained in fatigued skeletal muscles. The observation of greater fatigue resistance (lower fatigue index for power) during the sprint in the formoterol trial along with a tendency ($P = 0.09$) toward a
smaller decline in MVC after the sprint, suggest that formoterol may counteract development of fatigue during 30 s of maximal sprinting. This is supported by the observations of higher ATP, G-6-P, and unchanged PCR after the sprint in the formoterol trial, suggesting a smaller accumulation of Pi, free Mg$^{2+}$ that is known to reduce force by impairing cross-bridge function and by reducing release of Ca$^{2+}$ from the SR (1, 54). Moreover, it cannot be excluded that the greater phosphorylation of CaMKII Thr$^{287}$ in the formoterol trial may have contributed to the greater fatigue resistance, since inhibition of CaMKII has been shown to reduce force production and release of Ca$^{2+}$ from the SR during electrical stimulations in rodents (42, 52). The finding of β$_2$-adrenergic fatigue resistance during 30 s of maximal sprinting is in agreement with observations from animal models, where β$_2$-adrenergic stimulation has been shown to increase fatigue resistance in mouse and rat soleus muscles during 20 s and 5 min of high-frequency stimulations (7, 27). These observations, however, contradict a recent study by Hostrup et al. (22), in which oral terbutaline had no effect on MVC and peak twitch force after 45 s of exhaustive cycling. The contradictory findings may be attributed to differences in the degree of muscle fatigue since β$_2$-agonists have been shown to exert a greater effect on force in fatigued muscles (7, 27). Thus, a ~30% decrease in MVC in the present study vs. a ~20% decrease in Hostrup et al. (22) suggests a greater β$_2$-adrenergic effect on force in the present study.

It could be speculated that the higher concentration of norepinephrine before the sprint in the formoterol trial may have resulted in a greater β$_2$-receptor activation and, thereby, contributed to the positive inotropic effects of formoterol. However, norepinephrine has a very low binding affinity for the β$_2$-receptor (19), which is why the higher concentration before the sprint most likely did not contribute significantly to the positive inotropic effects of formoterol. Moreover, after the sprint, muscle strength was still greater in the formoterol trial compared with the placebo trial, despite no significant difference in the norepinephrine concentrations between treatments. Altogether this indicates that the effects of formoterol most likely are not mediated by elevated norepinephrine.

In conclusion, the long-acting β$_2$-agonist formoterol increased quadriceps muscle strength and power output during 30 s of maximal sprinting. The greater power output was associated with increased rates of glycogenolysis and glycolysis along with greater phosphorylation of CaMKII Thr$^{287}$ and FXYD1. The increased rates of glycogenolysis and glycolysis accelerated the accumulation of G-6-P and counteracted a reduction in ATP content, which may have reduced accumulation of Pi, free Mg$^{2+}$, and AMP and, thereby, perhaps counteracted the development of fatigue. The greater phosphorylation of CaMKII Thr$^{287}$ and FXYD1 (potentially leading to increased activity of the Na$^{+}$/K$^{+}$-ATPase) suggests that the formoterol-induced increase in power output may be associated with increased release of Ca$^{2+}$ from the SR.

**Perspectives and Significance**

According to the World Anti-Doping Agency’s (WADA’s) list of prohibited substances from 2016, athletes are allowed to inhale up to 54 μg formoterol over 24 h. Thus, the present study shows that it is possible to enhance muscle strength and sprint performance legally, which may promote uneven competition among athletes. To avoid misuse of formoterol, WADA should consider adding more restrictions to inhalation of formoterol on the future list of prohibited substances without compromising treatment of athletes with respiratory diseases.

In the present study, formoterol increased mean power and counteracted development of fatigue during 30 s of maximal sprinting, which suggest that formoterol may enhance exercise performance lasting longer than 30 s. This is supported by a recent study in elite swimmers, in which inhalation of β$_2$-agonists enhanced performance in an all-out swim ergometer sprint test lasting ~1 min (25). However, if the exercise duration is further prolonged to an extent where the primary ATP production comes from aerobic metabolism, the performance-enhancing effect of β$_2$-agonists seems to be eliminated (20, 22, 45).

It is not known whether the performance-enhancing effects of formoterol observed in the present study apply to elite athletes. However, enhanced sprint ability and muscle strength have been observed in elite swimmers (25) and endurance athletes (20) following acute administration of β$_2$-agonists, which suggests that the response to β$_2$-agonist is not different between recreationally active subjects and elite athletes. Future studies should investigate the effects of formoterol in elite athletes to confirm that the present findings also apply to this category.
population. Moreover, the underlying mechanisms behind β2-adrenergic enhancements in power output during maximal sprinting should be investigated with special emphasis on the relationship between Ca2+ handling, ATP metabolism, and fatigue development.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

A.K., M.H., V.B., and J.B. conception and design of research; A.K., M.H., V.B., and J.B. performed experiments; A.K., M.H., V.B., and J.B. analyzed data; A.K., M.H., V.B., and J.B. interpreted results of experiments; A.K. prepared figures; A.K., M.H., V.B., and J.B. edited and revised manuscript; A.K., M.H., V.B., and J.B. approved final version of manuscript.

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