Formoterol decreases muscle wasting as well as inflammation in the rat model of rheumatoid arthritis

Ana Belén Gómez-SanMiguel,1* Carolina Gomez-Moreira,1* María Paz Nieto-Bona,2 Carmen Fernández-Galaz,1 María Ángeles Villanúa,1 Ana Isabel Martín,1 and Asunción López-Calderón1

1Department of Physiology, Faculty of Medicine, Complutense University, Madrid, Spain; and 2Department of Basic Sciences in Health, Faculty of Health Sciences, Rey Juan Carlos University, Madrid, Spain

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Formoterol decreases muscle wasting as well as inflammation in the rat model of rheumatoid arthritis. Am J Physiol Endocrinol Metab 310: E925–E937, 2016; doi:10.1152/ajpendo.00503.2015.—Adjuvant-induced arthritis is an experimental model of rheumatoid arthritis that is associated with body weight loss and muscle wasting. β2-adrenergic receptor agonists are powerful anabolic agents that trigger skeletal muscle hypertrophy and have been proposed as a promising treatment for muscle wasting in human patients. The aim of this work was to determine whether formoterol, a selective β2-adrenoreceptor agonist, is able to ameliorate muscle wasting in arthritic rats. Arthritis was induced in male Wistar rats by intradermal injection of Freund’s adjuvant. Control and arthritic rats were injected daily with 50 μg/kg sc formoterol or saline for 12 days. Body weight change, food intake, and arthritis index were analyzed. After euthanasia, in the gastrocnemius mRNA was analyzed by PCR, and proteins were analyzed by Western blotting. Arthritis decreased gastrocnemius weight, cross-sectional area, and myofiber size, whereas formoterol increased those variables in both arthritic and control rats. Formoterol decreased the external signs of arthritis as well as NF-κB(p65) activation, TNFα, and COX-2 levels in the gastrocnemius of arthritic and control rats. Those effects of formoterol were associated with a decreased expression of myostatin, atrogin-1, and MuRF1 and in LC3b lipidation. Arthritis increased the expression of MyoD, myogenin, IGF-I, and IGFBP-3 and -5 in the gastrocnemius. In control and in arthritic rats, treatment with formoterol increased Akt phosphorylation and myogenin levels, whereas it decreased IGFBP-3 expression in the gastrocnemius. These data suggest that formoterol has an anti-inflammatory effect and decreases muscle wasting in arthritic rats through increasing Akt activity and myogenin and decreasing myostatin, the p-NF-κB(p65)/TNF pathway, and IGFBP-3.

cachexia; adjuvant-arthritis; β2-adrenoreceptor; myogenin; insulin-like growth factor-binding protein-3

CHRONIC INFLAMMATORY ILLNESSES such as cancer, sepsis, and obstructive pulmonary disease are associated with a decrease in body weight and cachexia. Similarly, patients with rheumatoid arthritis have rheumatoid cachexia, which increases morbidity and mortality (33). Adjuvant-induced arthritis is an experimental model of rheumatoid arthritis that induces inflammatory cachexia. On days 10 and 11 after adjuvant injection, rats stop gaining body weight and develop chronic inflammation, polyarthritis, anorexia, and cachexia due to whole body wasting, including muscle and fat tissue loss (6, 33). Skeletal muscle loss in arthritic rats is secondary to systemic inflammation and not to anorexia (7) or the reduction of spontaneous locomotion (15). Muscle wasting in several inflammatory illnesses is due to hyperactivation of the ubiquitin-proteasome pathway and autophagy lysosomal proteases, which are involved in protein degradation in skeletal muscle (22, 35). The two E3 ubiquitin ligases muscle RING-finger protein-1 (MuRF-1) and atrogin-1 are sensitive markers for muscular atrophy (35), and both are upregulated in arthritic rats (6). Moreover, arthritis also increased autophagic gene expression in muscle (18).

In addition to activating muscle proteolysis, the increased release of cytokines during chronic inflammation also promotes satellite cell activation and myogenesis (62). In this sense, we have reported that arthritis increases muscle regulatory factors and markers of satellite cell activation, myogenic differentiation factor D (MyoD), and myogenin (6), which play key roles in muscle plasticity and regeneration. The insulin-like growth factor-I (IGF-I)-insulin growth factor-binding protein (IGFBP) system in the skeletal muscle plays an important role in the maintenance of muscle mass (59). Adjuvant-induced arthritis is associated with alterations in the IGF-IGFBP system, and IGF-1 administration to arthritic rats decreases muscle wasting as well as arthritis-induced increase in muscle IGFBP-3 mRNA (40, 41).

β2-Adrenergic receptors are the main adrenergic receptors expressed in muscle cells, and their stimulation has well-known anabolic actions (for review, see Ref. 58). Activation of β2-adrenergic receptors have also been shown to have anti-inflammatory effects in various inflammatory conditions, such as bronchial asthma, chronic obstructive pulmonary disease, and carrageen-induced paw oedema (14, 28, 63), as well as in several experimental models of sepsis (34). However, the role played by the sympathetic nervous system on arthritis development is not well known. It has been described that the timing of adrenergic drug administration is a critical factor in determining disease progression (21, 42). Sympathectomy or administration of β-adrenergic antagonist before the onset of arthritis decreases the severity of the illness in experimental arthritis (37, 38). However, when adrenergic therapy is given after disease onset, it decreases joint inflammation and bone destruction in the arthritic hind limbs (42). β2-adrenoreceptor receptors are expressed in immune cells, and their stimulation decreases production of proinflammatory cytokines such as TNFα, whereas it increases the release of the anti-inflammatory interleukin-10 (10, 49). The anti-inflammatory effect of β2-adrenergic stimulation was also evidenced in mice lacking β2-adrenergic receptors, where there was decreased survival and increased inflammation in the endotoxemia setting (64).

* A. B. Gómez-SanMiguel and C. Gomez-Moreira contributed similarly to the study.

Address for reprint requests and other correspondence: A. López-Calderón, Departamento de Fisiología, Facultad de Medicina, Universidad Complutense, 28040, Madrid, Spain (e-mail: ALC@ucm.es).

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Formoterol is a highly β2-selective adrenergic receptor agonist that is capable of producing skeletal muscle hypertrophy at microgram doses (55). Its hypertrophic effect is mediated both by stimulating skeletal muscle synthesis and by decreasing muscle proteolysis (3, 25, 36). Accordingly, formoterol has been proposed as a possible pharmacological therapy for muscle loss (3, 26). Therefore, the purposes of this study were to 1) analyze the possible anti-inflammatory effect of formoterol administration to arthritic rats and 2) elucidate whether its effect on skeletal muscle is mediated by a decrease in proteolysis or/and by changes in myogenic regulatory factors and the IGF-IGFBP system.

MATERIALS AND METHODS

Animals. Arthritic and control male Wistar rats weighing 160 g (6 wk old) were purchased from Charles River Laboratories (Barcelona, Spain). Arthritis was induced in rats by an intradermal injection of 0.1 ml of heat-inactivated Mycobacterium butyricum (40 mg/ml in parafin oil) in the right hindpaw under isoflurane anesthesia (6). Control animals were injected with vehicle. After arrival (day 3 after adjuvant injection), rats were housed three per cage and maintained under standardized conditions of temperature (20–22°C) and light (lights on from 7:30 AM to 7:30 PM). The procedures followed the guidelines recommended by the European Union for the care and use of laboratory animals and were approved by the Complutense University Animal Care Committee.

In the first experiment, the effect of formoterol administration on arthritic rats was examined. On day 11 after adjuvant injection, both control and arthritic rats were randomly divided into four groups, each with 20 rats. Control rats were subcutaneously (sc) injected daily with 250 μl of saline at 9 AM for 6 or 12 days and divided into 1) control rats (C) fed ad libitum and 2) pair-fed rats (PF) that received the same amount of food (g/100 g body wt) eaten on the previous day by AA rats treated with formoterol. AA-form rats had higher body weight gain than PF rats. Arthritis decreased and formoter treatment for 12 days increased gastrocnemius weight, cross-sectional area, and mean fast fiber area (P < 0.01). AA rats treated with formoterol had higher body weight gain than AA rats injected with saline and similar or higher body weight gain than PF rats. Arthritis decreased and formoterol treatment for 12 days increased gastrocnemius weight, cross-sectional area, and mean fast fiber area (P < 0.01). Data are expressed as means ± SE for n = 7–10 rats/group. **P < 0.01 vs. control rats injected with saline; *P < 0.05, **P < 0.01 vs. PF rats; +P < 0.05, ++P < 0.01 vs. AA rats injected with saline. Least significant difference (LSD) multiple comparison test.

Fig. 1. A–D: effect of arthritis and formoterol administration (50 μg/kg sc) for 12 days on cumulative body weight gain (A), gastrocnemius weight (B), average cross-sectional area (C), and mean fast and slow fiber area (D). E: representative transverse sections from fast fiber region (top) and mixed region (bottom) of gastrocnemius muscle. Slow fibers (filled in gray resulting from slow type I MHC immunofluorescence) are mixed with fast fibers. All fibers are outlined by wheat germ agglutinin (black). Amplification of the inserts marked on each cross-section. Bar, 50 μm. C, control; PF, pair-fed; AA, arthritic rats; AA-form, arthritic rats treated with formoterol. AA rats had lower body weight gain than control and PF rats (P < 0.01). AA rats treated with formoterol had higher body weight gain than AA rats injected with saline and similar or higher body weight gain than PF rats. Arthritis decreased and formoterol treatment for 12 days increased gastrocnemius weight, cross-sectional area, and mean fast fiber area (P < 0.01). Data are expressed as means ± SE for n = 7–10 rats/group. **P < 0.01 vs. control rats injected with saline; *P < 0.05, **P < 0.01 vs. PF rats; +P < 0.05, ++P < 0.01 vs. AA rats injected with saline. Least significant difference (LSD) multiple comparison test.
Liquid nitrogen and stored at the left hindpaw was removed, dissected, weighed, and frozen in liquid nitrogen and stored at −80°C until analyses were performed. Food intake per cage was calculated by measuring the difference between the initial and the remaining amount of pellets in the feeder and expressed as grams per 100 grams body weight per day.

In a second experiment, we tested the effect of formoterol administration on control rats. Rats were euthanized by decapitation 2.5 h after the last injection, after being injected with 50 µg/kg sc or saline (250 µl) over 2, 6, or 12 days. The gastrocnemius muscle of the left hindpaw was removed, dissected, weighed, and frozen in liquid nitrogen and stored at −80°C until analyses were performed.

Muscle morphology and immunohistochemistry. The gastrocnemius from the left hindpaw of the rats treated over 12 days was weighed, and the medial part was dissected, placed on a transparency film, glued at one end to a cork with gum tragacanth (Fibraguard; Fardi, Madrid, Spain), frozen in isopentane, cooled by liquid nitrogen, and stored at −80°C. Ten-micrometer cryostat sections taken from the midbelly region were fixed with 100% acetone and stained with hematoxylin-eosin. Parallel sections were kept at −80°C until further processing for immunohistochemical analysis. Four to six muscle hematoxylin-eosin stained sections were used to determine the whole cross-sectional area. Sections were scanned by transparency and converted to JPEG images (Epson scanner 4990). The area of each section was measured with ImageJ software, using the measure of the slide width (25 mm) to set the scale.

We used muscle fiber cross-sectional size as an index of type-specific fiber atrophy. Slow muscle fibers were detected with a monoclonal antibody against slow myosin heavy chain form (1:80; NCL-MHCs-Novocastra, Newcastle upon Tyne, UK) and secondary Alexa fluor 488 Goat antimouse IgG (A11001-Invitrogen, 1:100; Invitrogen, Madrid, Spain). The extracellular matrix was detected by wheat germ aglutinin labeled with Texas Red (W849, 1 µg/ml; Invitrogen). Sections were mounted with Prolong-Gold antifade reagent combined with DAPI (P36931; Invitrogen). Digital images were taken with a Leica DMI300 microscope. Several ×10 photomicrographs were used to compose fast fiber and mixed fiber regions with GIMP software. Complete sections were reconstructed in some cases. Compositions were made with at least two layers: WGA and slow myosine images. The WGA layer was used to border detection using Difference of Gaussians and inversion. After selecting the white WGA lines by tone, they were filled with black. Gaps on the

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Assessment of arthritis was performed by measuring the arthritis index of each animal, which was clinically scored by grading each paw from 0 to 4. Grading was determined as follows: 0, no erythema or swelling; 1, slight erythema or swelling of one or more digits; 2, swelling of paw; 3, swelling of entire paw and ankle; 4, ankylosis, incapacity to bend the ankle. The severity score was the sum of the clinical scores of each limb, the maximum value being 16 (6). Body weight, food intake, and arthritis index scores were examined daily.

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boundaries and connective tissue were corrected manually. Slow fibers on mixed areas were selected and filled with red (f00000). The JPEG image with scale was first made binary to analyze particles with ImageJ software. This procedure allows measuring of the fast-fiber area. To analyze the area of the slow fibers from the mixed JPEG image, black boundaries were converted to white by color threshold and red fibers changed to black. At least two images from each region were selected for counting.

RNA extraction and real-time PCR. Gastrocnemius (100 mg) was homogenized, and total RNA was extracted using Ultraspec (Biotecx Laboratories, Houston, TX), following the manufacturer’s protocol. The final concentration of RNA was determined (260 nm) with a BioPhotometer (Eppendorf, Germany), and the integrity of the RNA was confirmed by agarose gel electrophoresis. First-strand cDNA synthesis was performed using 1 µg of total RNA with a Quantiscript Reverse Transcription kit (Qiagen, Valencia, CA).

Real-time PCR for quantification of mRNA was performed on a SmartCycler (Cepheid, Sunnyvale, CA), using a SYBR Green protocol on the fluorescence temperature cycler. Each real-time PCR reaction consisted of 10 ng of total RNA equivalents, 1× Takara SYBR Green Premix Ex Taq (Takara Bio, Otsu, Shiga, Japan), and 300 nM forward and reverse primers in a reaction volume of 25.5 µl. Primers for real-time PCR were obtained from Roche (Madrid, Spain). The thermal cycling profile consisted of a preincubation step at 95°C for 10 s followed, by 40 cycles of 95°C denaturation steps for 15 s, 60°C annealing steps for 30 s, and 72°C extension steps for 30 s. Results were expressed relative to the control animals, where the relative mRNA abundance has been arbitrarily set to 1, using the cycle threshold (ΔΔCT) method, with 18S as reference gene. PCR products were separated using agarose gel electrophoresis to confirm product presence and size.

Fig. 4. Effect of formoterol (form) treatment (50 µg/kg body wt sc daily) to arthritic rats for 6 (A) or 12 (B) days on TNFα mRNA, NF-κB(p65) phosphorylation, and COX-2 in the gastrocnemius. C, control; PF, pair-fed; AA, arthritic rats; AA-f, arthritic rats treated with formoterol. TNFα mRNA was measured by PCR; p-NF-κB(p65), NF-κB(p65), and COX-2 were measured by Western blot. Representative Western blots are shown in A and B, top. Boxes through immunoblots represent spliced images to follow group and treatment order. Arthritis increased TNFs expression, NF-κB(p65) phosphorylation, and COX-2 levels in the gastrocnemius. Formoterol treatment for 6 or 12 days prevented arthritis-induced increase in TNFα and NF-κB(p65) phosphorylation and decreased COX-2 in the gastrocnemius (P < 0.01). Data represent means ± SE (n = 6–10). *P < 0.05, **P < 0.01 vs. control rats; †P < 0.05, ††P < 0.01 vs. PF rats; +P < 0.05, ++P < 0.01 vs. AA rats injected with saline. LSD multiple comparison test.

E928 FORMOTEROL DECREASES INFLAMMATION AND CACHEXIA IN ARTHRITIS
**Western blot.** Gastrocnemius was homogenized in RIPA buffer (10 μl/mg) with protease inhibitor cocktail of 12.5 mM sodium deoxychlorate, 100 mM phenylmethane sulfonfyl fluoride, and 12.5 mM sodium orthovanadate and phosphatase inhibitors (Sigma-Aldrich, Madrid, Spain). The homogenate was later centrifuged at 13,000 rpm at 4°C for 30 min to remove tissue debris. Protein concentration was determined using the Bradford protein assay, with bovine serum albumin as standard. The protein extract was boiled for 5 min with a 1:1 volume of Laemmlli loading buffer. Proteins (100 μg) were resolved by electrophoresis on 14% polyacrylamide gels under reducing conditions and then transferred onto nitrocellulose membranes that were blocked by incubation in 5% nonfat dry milk and 0.1% Tween (Sigma-Aldrich) in Tris-buffered saline. Ponceau S staining was performed to ensure equal transfer prior to blocking. Membranes were probed overnight at 4°C with antibodies against p-AktSer(473), Akt, p-NF-κB(p65) Ser536, NF-κB/H9260 (LC3b) (Cell Signaling Technology Inc, Boston, MA), Akt, p-NF-κB(p65) Ser536, NF-κB/H9260, COX-2 (D5H5), and microtubule-associated protein-1 light chain 3b (LC3b) (Santa Cruz Biotechnology, Santa Cruz, CA), and light chain 3b (LC3b) mRNA (Santa Cruz Biotechnology), and peroxidase activity was detected using enhanced chemiluminescent reagent (Amersham Biosciences). Following ECL detection of phosphoproteins, membranes were stripped with stripping buffer (Restore Western Blot Stripping Buffer; Thermo Scientific Rockford, IL) and reprobed for total protein. Band intensities were quantified by densitometry using a PC-Image VGA24 program for Windows. The density of the protein band in each lane was expressed as the percentage of the mean density of control rats after load normalization using α-tubulin.

**Statistical analysis.** Results were compared using the statistics program Statgraphics plus for Windows. Normal distribution of data was assessed using a Shapiro-Wilk W-test. Data are presented as means ± SE. Statistical evaluation of the data was performed by one-way analysis of variance; post hoc comparisons were made using the least significant difference multiple range test. Statistical significance was set at $P < 0.05$.

**RESULTS**

As expected, arthritis decreased body weight gain ($P < 0.01$) because arthritic rats treated with saline had lower body weight gain than control and pair-fed rats from days 2 and 4 of treatment, respectively (Fig. 1A). Formoterol administration to
arthritic rats increased body weight gain, where these rats had higher body weight gain than pair-fed rats, on days 4 and 5 of treatment. The stimulatory effect of formoterol treatment on body weight gain was not due to changes in food intake, since formoterol did not modify the inhibitory effect of arthritis on food intake (data not shown). Formoterol administration to control rats for 12 days also increased gastrocnemius mass from 69 ± 2.3 g in rats treated with saline to 82 ± 1.4 g in control rats treated with formoterol (P < 0.01), but it did not modify food intake (data not shown).

As expected, the decrease in body weight gain observed in arthritic rats was associated with muscle wasting. Evolution of gastrocnemius muscle wet mass on days 6 and 12 of formoterol treatment is shown in Fig. 1B. Arthritis decreased gastrocnemius mass on day 6 (P < 0.01) compared with both control and pair-fed rats. Gastrocnemius mass decreased further on day 12 in arthritic rats treated with saline, having lower values than on day 6 (P < 0.01). This decrease in skeletal muscle mass is not secondary to the decrease in food intake, since pair-fed rats have gastrocnemius mass similar to that of control rats fed ad libitum, and is higher than that of arthritic rats. Formoterol administration to arthritic rats for 6 days tended to increase gastrocnemius mass, but this increase was not statistically significant. However, formoterol treatment for 12 days increased gastrocnemius mass (P < 0.01) and prevented the decrease in gastrocnemius mass observed between days 6 and 12 in arthritic rats treated with saline. Formoterol administration to control rats for 12 days increased gastrocnemius mass (1,257 ± 34 mg in rats treated with formoterol over 12 days vs. 1,066 ± 22 in rats treated with saline, P < 0.01).

Fig. 1, C–E, shows that arthritis induced a dramatic decrease in gastrocnemius cross-sectional area and in mean fast and slow fiber area (P < 0.01). As we have observed previously, myofiber atrophy was higher in fast than in slow fibers (8). Six days of treatment with formoterol did not significantly increase gastrocnemius mass or cross-sectional or fiber area (data not shown). However, the stimulatory effect of 12 days of formoterol treatment on gastrocnemius mass in arthritic rats was associated with an increase in gastrocnemius cross-sectional area (P < 0.01) and mean fast fiber area (P < 0.05). In control rats, 12 days of formoterol treatment increased gastrocnemius cross-sectional area as well as mean fast and slow fiber area (Fig. 2, A and B).

As shown in Fig. 3, formoterol treatment decreased arthritis score index (P < 0.01), where the difference was significant from day 4 of treatment with formoterol. Arthritis increased TNFα mRNA in the gastrocnemius muscle on both day 6 and day 12 of treatment (P < 0.01; Fig. 4, A and B). Formoterol prevented the stimulatory effect of arthritis on TNFα expression in the gastrocnemius muscle on both day 6 and day 12 (P < 0.01), where the arthritic rats treated with formoterol for 6 days had lower TNFα mRNA values than those of arthritic rats treated with saline and pair-fed rats. The inhibitory effect of formoterol on TNFα expression in the gastrocnemius was also observed in control rats from the 2nd day of treatment with formoterol (P < 0.01; Fig. 5A). Arthritis increased NF-
κB(p65) phosphorylation on both day 6 and day 12 (P < 0.05; Fig. 4, A and B), but the increase was higher on day 6 of treatment (and 16 days after adjuvant injection) than on day 12 of treatment (and 22 days after adjuvant injection). Arthritic rats treated with formoterol showed lower NF-κB(p65) phosphorylation in skeletal muscle on both days (P < 0.01). Formoterol was also able to decrease NF-κB(p65) phosphorylation in the gastrocnemius of control rats on all days analyzed (Fig. 5B). The arthritis-induced increase in COX-2 levels in the gastrocnemius was decreased by formoterol treatment on both day 6 (P < 0.05; Fig. 4A) and day 12 of treatment (P < 0.01; Fig. 4B). However, COX-2 levels in arthritic rats treated with formoterol were higher than in pair-fed rats. Pair-feeding the rats did not modify TNFα, COX-2, or pNF-κB(p65)/H9260B(p65) levels in rats. Formoterol was also able to decrease NF-κB(p65) phosphorylation in skeletal muscle on both days (P < 0.01). Formoterol administration to control rats for 12 days decreased the expression of myostatin, the adenovirus E1B 19-kDa protein-interacting protein-3 (Bnip-3), and atrogin-1, MuRF1 in the gastrocnemius muscle, since gastrocnemius of arthritic rats treated with saline had Akt phosphorylation similar to control rats treated with formoterol. Atrogin-1 and MuRF1 mRNA expression in the gastrocnemius of arthritic rats was higher than in control and in pair-fed rats on both day 6 and day 12 of treatment (P < 0.01; Fig. 6, A and C), where the increase was higher on day 6 than on day 12. Formoterol treatment prevented arthritis-induced increase in atrogin-1 and MuRF1 mRNA, but to higher levels than those of pair-fed rats. Arthritis also increased MuRF1 protein in arthritic rats treated with saline (P < 0.01) but not in rats treated with formoterol (Fig. 6, B and D).

As shown in Fig. 5A, formoterol administration to control rats for 12 days decreased the expression of myostatin, atrogin-1, MuRF1, and the autophagic marker gene BCL2/adenovirus E1B 19-kDa protein-interacting protein-3 (Bnip-3) as well as MuRF1 protein (P < 0.05; Fig. 5C). Lipidation, modification of LC3b from from LC3-I to the lipidated form (LC3b-II), has been widely used to indicate the occurrence of autophagy. Formoterol administration to control rats for 12 days decreased lipidation of LC3b, measured as the LC3b-II/LC3b-I ratio (P < 0.05; Fig. 5D). However, formoterol was not able to decrease LC3b expression in control rats (Fig. 5A).

The effect of arthritis and formoterol treatment on myostatin, atrogin-1, and MuRF1 in the gastrocnemius is shown in Fig. 6. Although myostatin mRNA was not modified by arthritis or pair-feeding the rats (Fig. 6, A and C), expression was decreased dramatically on days 6 and 12 (P < 0.01) in arthritic rats treated with formoterol. Atrogin-1 and MuRF1 mRNA expression in the gastrocnemius of arthritic rats was higher than in control and in pair-fed rats on both day 6 and day 12 of treatment (P < 0.01; Fig. 6, A and C), where the increase was higher on day 6 than on day 12. Formoterol treatment prevented arthritis-induced increase in atrogin-1 and MuRF1 mRNA, but to higher levels than those of pair-fed rats. Arthritis also increased MuRF1 protein in arthritic rats treated with saline (P < 0.01) but not in rats treated with formoterol (Fig. 6, B and D). Figure 7 shows the effect of arthritis and formoterol on the autophagic gene expression Bnip-3 and LC3b in control rats. Percent of control means are ± SE (n = 6 -10). **P < 0.01 vs. control rats; *P < 0.05, **P < 0.01 vs. PF rats; ++P < 0.05, +++P < 0.01 vs. AA injected with saline. LSD multiple comparison test.

**Fig. 7. Effect of formoterol (form) administration (50 μg/kg body wt sc daily) for 6 or 12 days to arthritic rats on Bnip-3 and LC3b mRNA (A and C) and on LC3b lipidation (B and D) in the gastrocnemius. C, control; PF, pair-fed; AA, arthritic rats. Bnip-3 and LC3b mRNA were measured by PCR and are presented as increase of the mean value in control rats. LC3b lipidation was represented as the LC3b II/LC3b I ratio measured by Western blot. Form treatment prevented arthritis-induced increase in Bnip-3 mRNA in LC3b lipidation and attenuated the increase in LC3b mRNA levels. Data represent means ± SE (n = 6 -10). **P < 0.01 vs. control rats; *P < 0.05, **P < 0.01 vs. PF rats; ++P < 0.05, +++P < 0.01 vs. AA injected with saline. LSD multiple comparison test.**
Differences between MyoD and myogenin expression in arthritic rats (Fig. 8, A and C). In contrast, in arthritic rats treated with formoterol for 6 or 12 days there was an increase in p-Akt levels as well as in the p-Akt/Akt ratio (P < 0.01). Similarly, formoterol increased p-Akt and p-Akt/Akt ratio after 6 and 12 days of treatment in control rats (Fig. 9A). On day 6 of treatment there was an increase in MyoD expression in the gastrocnemius of arthritic rats regardless of whether they were treated with saline or formoterol (P < 0.01; Fig. 8B). However, the stimulatory effect of arthritis on MyoD expression was not seen on day 12, since MyoD levels were similar in all groups of rats (Fig. 8D). As shown in Fig. 9B, formoterol treatment did not significantly modify MyoD protein levels in the gastrocnemius of control rats. Arthritis increased myogenin levels on days 6 and 12 (P < 0.01; Fig. 8, B and D). As has been reported (60), increased MyoD in muscle suggests that satellite cells are in a proliferative phase in arthritic rats on day 6. However, on day 12 (22 after adjuvant injection), MyoD levels in the gastrocnemius were not increased in arthritic rats. In contrast, myogenin levels were increased on both days. Differences between MyoD and myogenin expression in arthritic rats on day 12 of treatment can be due to the fact that MyoD acts on early stage of muscle regeneration, whereas myogenin is involved at a later stage of differentiation (30). Formoterol treatment for 12 days further increased myogenin in the gastrocnemius of arthritic rats (P < 0.01). Formoterol treatment for 6 and 12 days was also able to increase myogenin levels in control rats (Fig. 9B), but these increases were lower than those induced by arthritis.

The expression of IGF-I and IGFBPs mRNA in the gastrocnemius was modified by arthritis and formoterol treatment but not by pair-feeding the rats (Fig. 10, A and B). IGFBP-3 mRNA levels in the gastrocnemius of the arthritic rats treated with saline were markedly increased on both day 6 and day 12 (P < 0.01). In contrast, IGF-I mRNA levels were increased in arthritic rats treated with saline on day 12 of treatment. Arthritis increased gastrocnemius IGFBP-5 levels mRNA in rats treated with saline on day 6 (P < 0.01), and they increased further on day 12 of treatment. The time course expression of IGFBP-5 in arthritic rats was modified in a pattern similar to that of IGF-I mRNA and myogenin, with their expression possibly involved in the regulation of muscle growth and differentiation.

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**Fig. 8.** Effect of formoterol (form) administration (50 μg/kg body wt sc daily) for 6 or 12 days to arthritic rats on Akt phosphorylation (p-Akt; A and C) and MyoD and myogenin levels (B and D) in the gastrocnemius. A, control; PF, pair-fed; AA, arthritic rats. Akt, p-Akt, myogenin, and MyoD were measured by Western blot. Representative Western blots are shown at the top. Arthritis increased MyoD levels only on day 6 and myogenin on days 6 and 12 (P < 0.01). Formoterol increased Akt phosphorylation on days 6 and 12 and myogenin on day 12 of treatment (P < 0.01). PF rats have a higher p-Akt/Akt ratio than control rats on day 12 (P < 0.05). Data represent means ± SE (n = 6–10). **P < 0.01 vs. control rats; *P < 0.05, ++P < 0.01 vs. PF rats; *+P < 0.01 vs. AA injected with saline. LSD multiple comparison test.
discrepancy in regenerating muscle (65), since knockdown of this IGFBP inhibits myogenic differentiation (54).

Formoterol administration to arthritic rats decreased IGFBP-3 expression ($P < 0.01$). Similarly, in control rats formoterol administration was able to decrease IGFBP-3 expression in the gastrocnemius ($P < 0.01$; Fig. 9C). Arthritic rats treated with formoterol had higher gastrocnemius IGFBP-5 than pair-fed rats but lower gastrocnemius IGFBP-5 than arthritic rats treated with saline on day 6 ($P < 0.05$). However, on day 12 of treatment, the two groups of arthritic rats had similar IGFBP-5 mRNA (Fig. 10B). In control rats, formoterol treatment did not modify IGF-I or IGFBP-5 mRNA in the gastrocnemius (Fig. 9C).

**DISCUSSION**

The present study indicates that formoterol administration to arthritic rats decreases inflammation and prevents inflammatory cachexia. The protective effect of formoterol on skeletal muscle mass is associated with decreased inflammation, myostatin, atrogenes, autophagic gene and IGFBP-3 expression, and LC3b lipidation together with Akt and myogenin activation.

Our data show that formoterol treatment decreases the severity of arthritis. These data are in accord with those reported previously in experimental models of arthritis, in which $\beta_2$-adrenergic stimulation inhibits inflammation during the chronic symptomatic phase of arthritis (for review, see Ref. 31). The anti-inflammatory effect of adrenergic stimulation has also been reported in rheumatoid arthritis patients who lose sympathetic nerve fibers, where catecholamine-producing cells in the synovial tissue start to replace sympathetic nerve fibers and have a strong anti-inflammatory effect (5). Formoterol has an anti-inflammatory effect on skeletal muscle of arthritic as well as of control rats, since it decreases NF-$\kappa$B(p65) phosphorylation and the expression of TNF$\alpha$ and COX-2 in the gastrocnemius in both groups of rats. The anti-inflammatory effect of $\beta_2$-adrenergic stimulation has been reported in different cell types such as leukocytes, lung smooth muscle, and endothelial cells (28). In the skeletal muscle, it has been found that $\beta$-adrenergic blockade by propranolol treatment exacerbated sepsis-induced increase in muscle IL-6 and TNF$\alpha$ expression (34), suggesting an anti-inflammatory action of $\beta$-adrenergic activation in muscle.

In addition to its anti-inflammatory effect, formoterol treatment increased body and gastrocnemius weights. These data are in accord with the well-known antiatrophic actions of $\beta_2$-adrenergic agonist on skeletal muscle (26, 32). To our
knowledge, this is the first study directly showing the role of β2-adrenergic activation in arthritis-induced muscle wasting. As we have reported previously (6, 7), myostatin expression was not increased in arthritic rats. The lack of modification of myostatin in arthritic rats is in agreement with data reported in other conditions of muscle atrophy such as dexamethasone administration (23), muscle disuse (46), and some types of cancer (4). Formoterol was able to decrease myostatin expression in arthritic rats after 6 and 12 days of treatment and in control rats after 12 days of treatment. The inhibitory effect of formoterol on myostatin is well known (52), and it has been proposed that certain anabolic actions of formoterol in cancer-induced cachexia may be mediated via the myostatin system (4). In this sense, myostatin downregulation can contribute to the inhibitory effects of formoterol on muscle proteolysis, since myostatin upregulates atrogin-1 and MuRF1 expression, through the inhibition of Akt phosphorylation, thereby increasing the levels of active FoxO1 (47).

Arthritis increased atrogin-1, MuRF1, Bnip3, and LC3B expression and LC3B-I conversion to LC3B-II by lipidation, suggesting an activation of the ubiquitin-proteasome and autophagic-lysosomal proteolytic pathways. Taking into account that arthritis does not modify Akt/FoxO3 phosphorylation (7), atrogenes and autophagy in arthritis can be induced through NF-kB activation, as it has been reported in hepatocytes after LPS administration (9). Formoterol significantly decreased arthritis-induced atrogin-1, MuRF1, Bnip3, and LC3b expression in the gastrocnemius as well as LC3b lipidation. Similarly, β2-adrenergic agonists have been reported to prevent cancer-induced muscle wasting and denervation-induced atrogin-1 and MuRF1 upregulation in muscle (3, 12, 19). Furthermore, chronic depletion of epinephrine exacerbates fasting-induced muscle proteolysis, increases atrogin-1, MuRF1, and LC3b expression and LC3b lipidation, and decreases p-Akt levels (20). The inhibitory effect of epinephrine on muscle proteolysis seems to be mediated by cAMP (50). Therefore, formoterol-induced MuRF1, atrogin-1, Bnip3, and LC3b repression can be mediated, as mentioned above, through the activation of AMPc/Akt and/or through downregulation of inflammation.

As reported previously, arthritis did not modify Akt phosphorylation in muscle (8). These data differ from those observed in other acute wasting conditions such as lipopolysaccharide (LPS) or glucocorticoid administration, which decrease Akt phosphorylation, whereas the transcription factors Forkhead box-containing protein O subclass (FoxO)-1 and -3a activities are increased (13, 44). In cachexia induced by chronic illnesses, the activity of the Akt/FoxO signaling pathway is not well known. It has been reported that FoxO1 and FoxO3 protein activity were unchanged in human cancer cachexia (61). Furthermore, in humans with chronic obstructive pulmonary disease, muscle atrophy is associated with an increase in Akt activation (17, 57). These and several other data indicate that under certain conditions Akt/FoxO and atrogenic activity can be independent of each other. A possible explanation for these discrepancies can be that cachexia induced by a chronic process is not as rapid and severe as the acute muscle wasting observed in acute experimental models of muscle wasting. Accordingly, the physiological regulators may not be exactly the same. The fact that arthritis increased muscle TNFα expression and NF-kB(p65) phosphorylation suggests that this pathway can be responsible for arthritis-induced increased expression of atrogin-1 and MuRF1.

As reported previously with β2-adrenergic receptor agonists (29), 6 and 12 days of formoterol treatment also increased Akt activation in arthritic and control rats. It has been postulated that this stimulatory effect is mediated by an IGF-1-independent growth pathway through the Gα/βγ/phosphatidylinositol 3-kinase signaling pathway (29, 56). Although some authors found that the stimulatory effect of formoterol on Akt phosphorylation decreases after 10 days of treatment (25), others reported that administration of formoterol is able to increase Akt phosphorylation after 28 days (31). Our data and those of Koopman et al. (31) suggest that some of the effects of formoterol on skeletal muscle mass are exerted via Akt signaling.

As we have reported previously, arthritis increases the myogenic regulatory factors MyoD and myogenin (6, 8). Upregulation of MyoD and myogenin together with an increase in inflammatory markers has also been reported in burn-induced muscle atrophy (53). Moreover, collagen-induced arthritis and immobilization increase muscle proteolysis and decrease muscle mass (15). However, whereas collagen-induced arthritis stimulated inflammation and myogenin expression, immobili-
zation did not modify the inflammatory response, and it decreased MyoD (15). These data are in accord with those showing that inflammation, although it induces muscle wasting, is also able to trigger muscle regeneration by activating satellite cell proliferation and differentiation, as indicated by Tidball (62). Stimulation of genes that regulate muscle development and growth, such as myogenin, has been reported in rats submitted to 5–14 days of an experimental intensive care unit model (39). These authors interpreted that result as a compensatory mechanism to reduce the excessive down-regulation of sarcomeric proteins and upregulation of different proteolytic pathways.

A stimulatory effect of formoterol on muscle regeneration and on muscle regulatory factors has also been reported (11). The effects of formoterol on gastrocnemius do not seem to be mediated by MyoD, since MyoD expression in the gastrocnemius was not modified by formoterol administration to arthritic or to control rats. However, formoterol treatment was able to increase the expression of myogenin in the gastrocnemius of both arthritic and control rats. Similarly, in immobilized rat plantaris muscle, clenbuterol upregulated myogenin, whereas MyoD expression was unaffected (16). It has been reported previously that the inhibition of NF-κB activity increases Akt phosphorylation and promotes skeletal muscle regeneration by limiting inflammatory response and fibrosis (48). Therefore, it is possible that formoterol stimulates Akt and myogenin expression through its inhibitory action on NF-κB.

The effect of β2-adrenoreceptor stimulation on the IGF-I system in skeletal muscle is not well known. Gongalves et al. (19) found that clenbuterol did not change IGF-I levels in the soleus muscle. In contrast, clenbuterol has been reported to induce overexpression of IGF-I mRNA in the soleus and masseter muscles (1, 45). Arthritis increased IGFBP-5 in a way similar to myogenin, where the highest expression was observed on day 12. This could be explained by the fact that IGFBP-5 and myogenin are related to the differentiation process in regenerating muscle (65), since knockdown of this IGFBP inhibits myogenic differentiation (54). However, the present study shows that formoterol administration increases Akt activity, but it does not significantly modify IGF-I or IGFBP5 in control or arthritic rats. Despite the aforementioned discrepancies, β2-adrenoreceptor stimulation does not increase serum IGF-I levels but actually decreases circulating IGF-I (1, 2). These and our data are in accord with the second IGF-I-independent growth pathway involving the activation of β2-adrenoreceptors and subsequent Akt activation that has been mentioned above (29).

In contrast to the lack of effect of formoterol on IGF-I, formoterol decreased the arthritis-induced increase in gastrocnemius IGFBP-3 mRNA as well as IGFBP-3 expression in control rats. An inhibitory effect of clenbuterol on IGFBP-3 expression has been reported previously in the masseter muscle (45). IGFBP-3 is able to suppress myoblast proliferation through an IGF-I-independent mechanism (51). In different tissues, local IGFBP-3 is an inhibitor of cell growth and/or promoter of apoptosis by a non-IGF-dependent mechanism (27). Furthermore, IGFBP-3 upregulation is mediated by pro-apoptotic and growth inhibitory factors such as TNFα (27). Therefore, it is not surprising that muscle wasting induced by inflammation is associated with increased IGFBP-3 levels, as we have observed not only in arthritis but also after endotoxin injection (44). Taking into account that Akt is a negative regulator of IGFBP-3 (24), the inhibitory action of formoterol treatment on IGFBP-3 in the gastrocnemius can be mediated by the increase in p-Akt and/or the decrease in TNFα. Therefore, downregulation of IGFBP-3 levels in the gastrocnemius by formoterol treatment can contribute to skeletal muscle growth, development, and hypertrophy.

In summary, formoterol has an anti-inflammatory and hypertrophic action in skeletal muscle through increasing pAkt activity and myogenin and decreasing the p-NF-κB/p65/TNFα pathway, myostatin, and IGFBP-3 in both arthritic and control rats. This study shows for the first time the effects of β2-adrenergic agonist on muscle atrophy and transcription factors involved in muscle protein degradation in arthritis. Our observations suggest that formoterol treatment could be useful in reducing arthritis symptoms and in preserving muscle mass in rheumatoid arthritis patients. Further histological and functional studies are required to clarify the potential effect of formoterol on muscle regeneration and myogenesis.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


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