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The lumbrical muscle: a novel in situ system to evaluate adult skeletal muscle proteolysis and anticatabolic drugs for therapeutic purposes

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Bergantin LB, Figueiredo LB, Godinho RO. The lumbrical muscle: a novel in situ system to evaluate adult skeletal muscle proteolysis and anticatabolic drugs for therapeutic purposes. *J Appl Physiol* 111: 1710–1718, 2011. First published September 15, 2011; doi:10.1152/japplphysiol.00586.2011.—The molecular regulation of skeletal muscle proteolysis and the pharmacological screening of anticatabolic drugs have been addressed by measuring tyrosine release from prepubertal rat skeletal muscles, which are thin enough to allow adequate in vitro diffusion of oxygen and substrates. However, the use of muscle at accelerated prepubertal growth has limited the analysis of adult muscle proteolysis or that associated with aging and neurodegenerative diseases. Here we established the adult rat lumbrical muscle (4/hindpaw; 8/rat) as a new in situ experimental model for dynamic measurement of skeletal muscle proteolysis. By incubating lumbrical muscles attached to their individual metatarsal bones in Tyrode solution, we showed that the muscle proteolysis rate of adult and aged rats (3–4 to 24 mo old) is 45–25% of that in prepubertal animals (1 mo old), which makes questionable the usual extrapolation of proteolysis from prepubertal to adult/senile muscles. While acute mechanical injury or 1- to 7-day denervation increased tyrosine release from adult lumbrical muscle by up to 60%, it was reduced by 20–28% after 2-h incubation with β-adrenoceptor agonists, forskolin or phosphodiesterase inhibitor IBMX. Using inhibitors of 26S-proteasome (MG132), lysosome (methylamine), or calpain (E64/leupeptin) systems, we showed that ubiquitin-proteasome is accountable for 40–50% of total lumbrical proteolysis of adult, middle-aged, and aged rats. In conclusion, the lumbrical model allows the analysis of muscle proteolysis rate from prepubertal to senile rats. By permitting eight simultaneous matched measurements per rat, the new model improves similar protocols performed in paired extensor digitorum longus (EDL) muscles from prepubertal rats, optimizing the pharmacological screening of drugs for anticatabolic purposes.

protein degradation; tyrosine release; muscle atrophy; ubiquitin-proteasome; calpain

IN VERTEBRATES, skeletal muscle functions as an important regulator of body energy homeostasis, providing amino acids generated from protein breakdown (5, 30, 54). However, increased protein breakdown is the major determinant of muscle atrophy associated with pathological conditions, such as diabetes, cancer cachexia, or neuromuscular dysfunction (25, 29, 30). By studying the molecular mechanisms underlying the loss of muscle mass it is possible to define new targets for development of therapeutic interventions to counteract or reverse muscle atrophy.

In general, these mechanisms as well as the pharmacological screening of drugs that interfere with muscle atrophy have been addressed by measuring the ex vivo release of tyrosine from muscles (4, 26, 32, 50, 55), an amino acid that is neither synthesized nor metabolized by this tissue (3, 17).

Tyrosine can be easily measured by colorimetric or fluorometric assay (52). However, because incubation procedures demand intact muscles sufficiently thin to allow an adequate diffusion of nutrients and oxygen (3), until now almost all muscle proteolysis studies have been performed in skeletal muscle from sexually immature (30–40 day old) rats (4, 14, 19, 26, 32, 50). At prepubertal age, skeletal muscle exhibits important metabolic characteristics that differ from those of adulthood, such as accelerated growth (10), increased insulin-stimulated glucose uptake (42), and reduced levels of anabolic sexual hormones (45). Therefore, it is not accurate to expand the conclusions obtained with prepubertal rat muscle to adulthood. In addition, the effect of aging or neurodegenerative diseases on muscle protein breakdown cannot be assessed directly in this model, requiring the use of cultured myotube models (53) or the alternative analysis of muscle static markers, such as MAFbx/Atrogin-1 and MuRF1 (6, 22, 40). However, recent findings concerning MAFbx/Atrogin-1 have shown unexpected problems of interpretation, since MAFbx/Atrogin-1 might also influence protein synthesis (2, 33).

To overcome the limitation of measuring protein breakdown rate in skeletal muscles from sexually immature rats, we evaluated whether lumbrical muscles from adult rats would satisfy the requirements for proteolysis experiments. In addition, taking into account the presence of four small lumbrical muscles in each hindpaw (8 muscles/rat), the use of lumbrical muscle as a proteolysis experimental model would allow the simultaneous analysis of eight matched samples compared with the current protocol that uses paired extensor digitorum longus (EDL) muscles from prepubertal rats.

MATERIALS AND METHODS

Animals. Experiments were performed in lumbrical and EDL muscles isolated from prepubertal (1 mo old), adult (3–4 mo old), middle-aged (12 mo old), and aged (24 mo old) Wistar rats from the institutional animal care facility. Rats were maintained on a 12:12-h light-dark cycle with free access to water and standard lab diet. All procedures were in accordance with the *Guide for the Care and Use of Laboratory Animals* prepared by the National Academy of Sciences and published by the National Institutes of Health and were approved by the Institutional Ethics Committee (protocols no. 1123/07 and no. 1431/08).

Muscle morphological studies. Animals were killed by decapitation, and lumbrical and EDL muscles from both hindlimbs were removed, weighed, dehydrated in starch, and embedded in Tissue-Tek OCT compound (Miles Laboratories, Naperville, IL). After fixing in corks, muscles were immediately frozen in liquid nitrogen and stored at −80°C. To determine muscle fiber diameter, cryostat cross sections (5 μm) of the lumbrical and EDL muscles were stained with hema-

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toxylin and eosin (H & E), as described by Fukushima et al. (16). Images of stained myofibers (7 fields/section) were acquired under light microscope (Nikon Eclipse E800, Melville, NY; ×40 objective), analyzed with HL Image 97 software (Western Vision Software, Layton, UT), and processed with Adobe Photoshop CS 4.0 (Adobe Systems, Mountain View, CA). Frequency histograms of lumbrical fiber diameter from adult male rats were constructed and compared with those obtained from EDL muscles.

Muscle proteolysis analysis. Rats were killed by decapitation, and lumbrical (8/rat) and EDL (2/rat) muscles were removed and carefully dissected. To avoid tissue damage, lumbrical muscles were kept attached by tendons to their own metatarsal bones at exact resting length, while EDL muscles were pinned by their tendons on inert plastic supports at approximately resting length. Each lumbrical/bone or EDL system was transferred to 1.5-ml tubes containing carbogen-saturated Tyrode solution (in mM: 135 NaCl, 5 KCl, 1 $MgCl_2$, 15 $NaHCO_3$, 2 NaH_2PO_4, 2 $CaCl_2$, 11 glucose, pH 7.4, at 37°C) plus 0.5 mM cycloheximide (Tyrode-C) to prevent protein synthesis and reincorporation of tyrosine back into proteins. After a 30-min equilibration period, tissues were transferred to microtubes and incubated with drugs, in a 1.5-ml final volume of fresh Tyrode-C, in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. After 1–5 h, 500-μl aliquots were collected and immediately used for proteolysis determination. The muscles were disconnected from the metatarsal bones, carefully dissected to remove tendons, and subsequently weighed.

Experimental design. To validate the lumbrical muscle as a model to evaluate skeletal muscle proteolysis of adult rat, we conducted several experiments including measurement of basal tyrosine release from control, denervated, or acutely injured lumbrical muscles from 3- to 4-mo-old rats.

The contribution of ubiquitin-proteasome, calpain, and lysosomal systems to muscle proteolysis was evaluated in lumbrical muscles from 4-mo-old rats in the presence of 20 μM MG132 (26S-proteasome inhibitor) (26, 50), 10 mM methylamine (lysosome inhibitor) (19, 38), or 25 μM E64 plus 50 μM leupeptin (calpain inhibitors) (39, 55). Age-dependent changes in the total and proteasome-dependent proteolysis were followed in lumbrical muscle from 1-, 4-, 12-, and 24-mo-old rats. In addition, the involvement of the β_2-adrenergic/cAMP signaling pathway in the regulation of muscle proteolysis was assessed in muscles from adult rats incubated with 10–100 μM isoproterenol (nonselective β-adrenoceptor agonist), 1–10 nM formoterol (β_2-adrenoceptor agonist), 100–1,000 μM IBMX (nonselective phosphodiesterase inhibitor), and 10–100 nM forskolin (adenylyl cyclase activator). Finally, results obtained with 3 nM formoterol and 100 nM forskolin in lumbrical muscle of adult rats were compared with those obtained in EDL and lumbrical muscles of prepubertal rats.

Fluorometric determination of tyrosine release. Muscle proteolysis was determined by measuring the rate of tyrosine release into the incubation medium. The method initially developed by Waalkes and Udenfriend in 1957 (52) has been adapted to the 96-well microplate as described here in detail. Samples (500 μl) were incubated for 30 min at 55°C with 200 μl of a 1:1 (vol/vol) reaction mixture: 1% (wt/vol) 1-nitroso-2-naphthol diluted in ethanol and 72.5 mM sodium nitrite in 20% nitric acid. After cooling for 10 min, extraction of unreacted

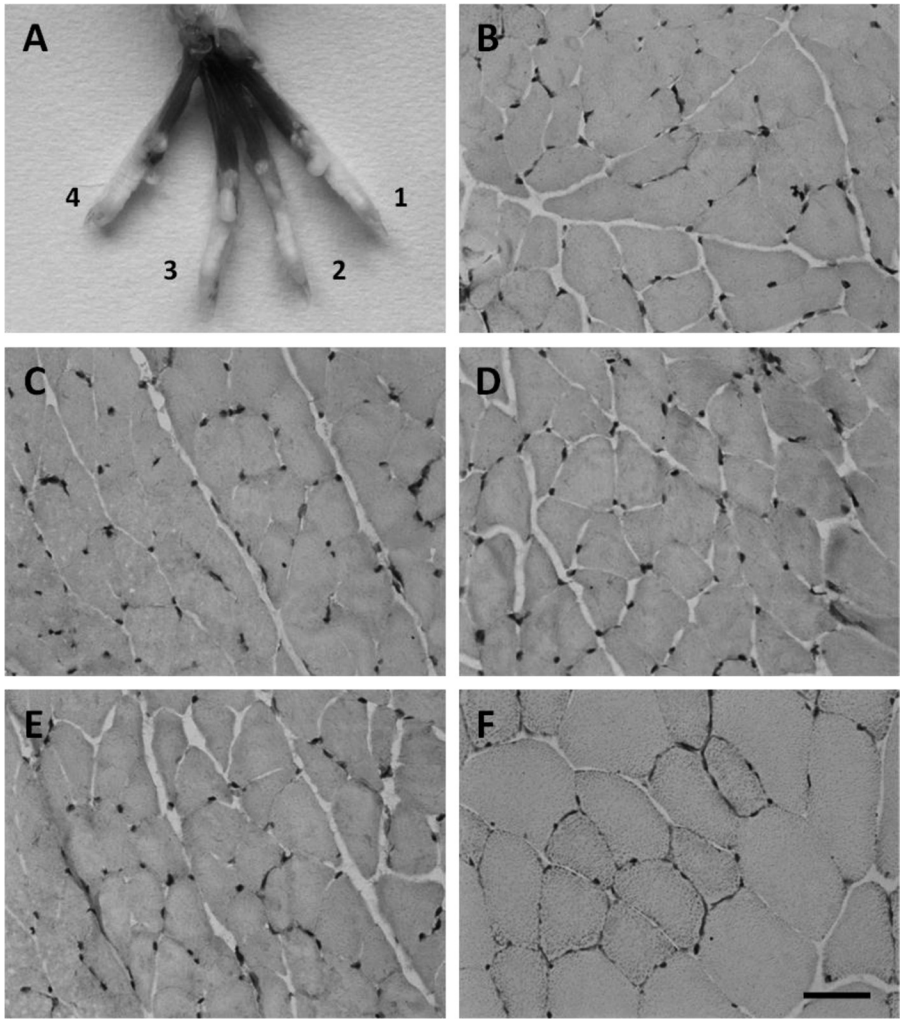

Fig. 1. Lumbrical muscles of adult rat. *A*: right paw of a 4-mo-old male rat showing the 4 lumbrical muscles, numbered from *1* (medial) to *4* (lateral) according to anatomic position. *B–F*: cross sections (5 μm) of 4 lumbrical (*B–E*) and extensor digitorum longus (EDL; *F*) muscles stained with hematoxylin and eosin (H & E). Bar, 20 μm.

1-nitroso-2-naphthol was performed by adding 1 ml of 1,2-dichloroethane. This extraction procedure increased by 200 times the sensitivity of tyrosine measurement previously proposed by Lutke-Eversloh and Stephanopoulos (35). The samples were then vigorously mixed and centrifuged for 10 min at 800 *g*, and tyrosine was quantified from the upper aqueous phase by fluorometric assay in 96-well microplates with excitation/emission wavelengths of 485 nm/590 nm. Rates of proteolysis were expressed as nanomoles of tyrosine per milligram of wet mass per hour.

To measure total muscle tyrosine content, lumbrical muscles were homogenized with 10 vol of 5% perchloric acid (PCA) and centrifuged at 10,000 *g* for 10 min (29). Total muscle protein content was measured by Bradford protein assay. The fractional degradation rate (FDR) of total protein was calculated as follows:

$$\text{FDR} = \frac{\text{rate of tyrosine}\left[\text{nmol}/\left(\text{h} \times \text{g}\right)\right] \times 24\text{h}}{\dfrac{\text{total tyrosine content}}{\text{g muscle protein}}}$$

Mechanical injury and denervation of lumbrical muscles. To investigate whether the novel model could detect changes in tyrosine release induced by acute mechanical injury, concentric cuts in the medial region of lumbrical muscles were performed immediately before the proteolysis analysis with rat-tooth forceps.

Denervation of hindlimb muscles from adult rats was carried out under ketamine (48 mg/kg) and xylazine (6 mg/kg) anesthesia by cutting the tibial nerve. To prevent muscle reinnervation, a 2- to 3-mm distal segment of nerve end was removed. After 1–7 days, at the time of the experimental procedure, lumbrical and EDL muscle denervation was confirmed by the absence of contraction in response to repetitive stimulation of the nerve stump.

Statistical analysis. Results are expressed as means ± SE or ± 95% confidence limits (CLs). Differences in muscle mass (from EDL of prepubertal vs. adult rats or from injured vs. control lumbrical muscles of adult rats) or tyrosine release rate (from injured vs. control lumbrical muscles) were analyzed by two-tailed unpaired Student's *t*-test. Nonparametric Kruskal-Wallis followed by Dunn's multiple comparison tests were used to evaluate the statistical significance of EDL and/or lumbrical fiber diameter results. The effects of *1*) forskolin and formoterol, *2*) leupeptin/E64 and MG132 ± leupeptin/E64, and *3*) methylamine and MG132 ± methylamine on muscle proteolysis were compared with one-way ANOVA followed by Tukey's multiple comparison test. One-way ANOVA followed by Newman-Keuls multiple comparison tests were used to test the effect of aging or of multiple concentrations of isoproterenol, forskolin, formoterol, or IBMX on muscle proteolysis. The level of significance was set at 5% ($P < 0.05$), and all analyses were performed with GraphPad Prism for Windows 5.01.

RESULTS

Morphometric analysis of lumbrical muscles. Figure 1 shows lumbrical muscles from adult rat, numbered *1–4* according to their anatomic position (Fig. 1*A*), and representative images of H & E-stained lumbrical (Fig. 1, *B–E*) and EDL (Fig. 1*F*) muscles.

Morphometric analysis of muscle cross sections (Fig. 1, *B–E*) did not detect any statistically significant difference between fiber diameter of *lumbrical muscles 1*, *2*, and *4* [26.39 μm (CL = 25.92–26.87) to 27.18 μm (CL = 26.69–27.67); $n = 432–464$], which represented 95–98% of *lumbrical muscle 3* (Fig. 2*C*; $n = 402$). However, the EDL fiber diameter [34.69 μm (CL = 33.86–35.51); $n = 344$; Fig. 2, *B* and *C*] was 25–31% higher than those of lumbrical muscles (Fig. 2, *C* and *D*).

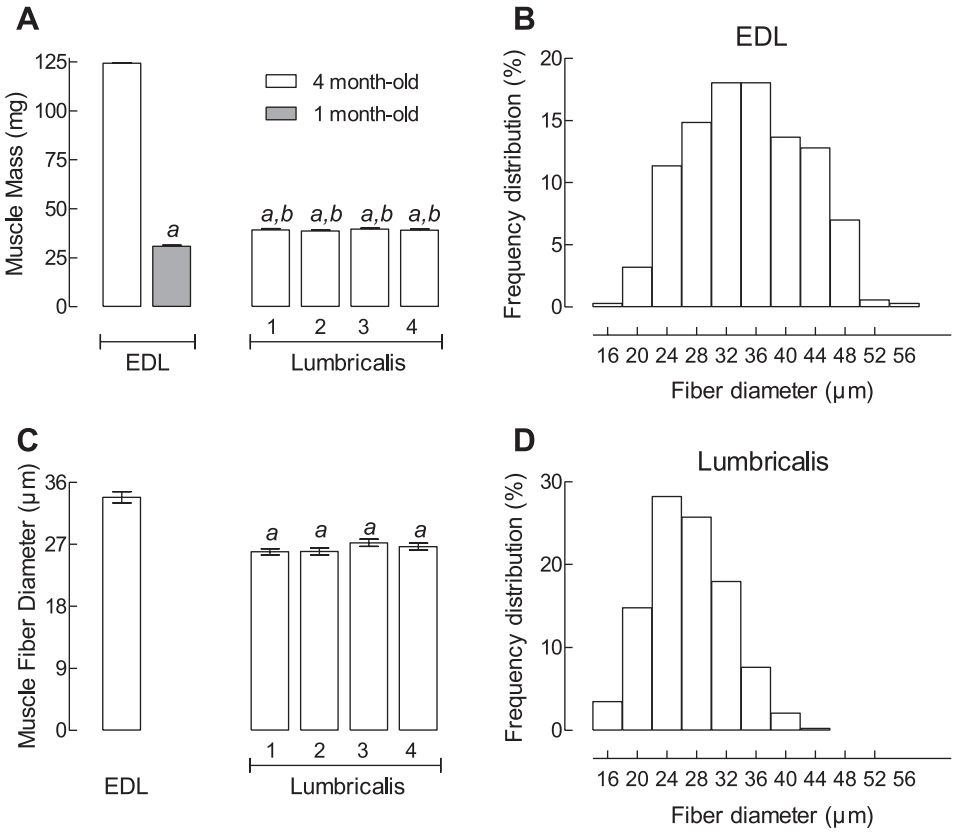

Fig. 2. Mass and fiber diameter of lumbrical and EDL muscles from prepubertal and adult rats. *A* and *C*: wet mass ($n = 4–8$ muscles; *A*) and fiber diameter (*C*) of lumbrical (numbered according to anatomic position; $n = 402–464$ muscle fibers) and EDL muscles from adult or prepubertal rats ($n = 344$ muscle fibers). *B* and *D*: frequency distribution of EDL (*B*) and lumbrical (*D*) fiber diameters. Each bar represents mean ± SE (*A*) or confidence limits (*B*) of values obtained from 4–8 rats. [a]Significantly different from EDL of adult rat; [b]significantly different from EDL of prepubertal rat ($P < 0.05$; *A*, ANOVA followed by Tukey's multiple comparison test; *C*, Kruskal-Wallis followed by Dunn's multiple comparison test).

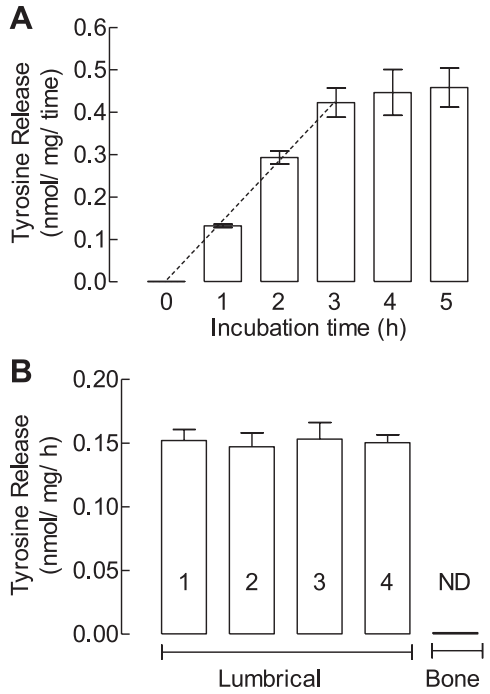

Fig. 3. Tyrosine release from adult rat lumbrical muscles. *A*: muscles of 4-mo-old male rats were incubated in Tyrode solution with cycloheximide (Tyrode-C) for 1–5 h. *B*: lumbrical muscles, numbered according to their anatomic position, and metatarsal bones were incubated in Tyrode-C for 2 h. Tyrosine released into the medium was measured by fluorometric assay. Each bar represents mean ± SE of 3–9 muscles from 3–5 rats. ND, not detected.

There was no statistical difference in mass values among the four adult lumbrical muscles (38.6 ± 0.6 to 39.5 ± 0.7 mg; $n = 4$). While lumbrical muscle mass from adult rats was one-third of the EDL value (124.3 ± 0.3 mg; $n = 4$), it was approximately equal to EDL mass from 1-mo-old rats (30.9 ± 0.7 mg; $n = 16$; Fig. 2*A*).

Basal tyrosine release from lumbrical muscles and its relationship with muscle anatomic position. To evaluate the rate of muscle proteolysis, lumbrical muscles from adult rats were incubated for 1–5 h in Tyrode-C at 37°C. As shown in Fig. 3*A*, the basal release of tyrosine from lumbrical muscle into the medium increased linearly from 0.125 ± 0.04 to 0.42 ± 0.03 nmol/mg muscle ($n = 6$–9), reaching a plateau after 3 h. Considering the total amount of tyrosine (482.2 ± 10.4 nmol/g protein) and the basal rate of tyrosine release (1.25 nmol tyrosine·g protein^{-1}·h^{-1}), the daily basal FDR of adult rat lumbrical muscle was 6.21 ± 0.14%.

It is important to note that no significant difference in tyrosine release rate was detected among the four lumbrical muscles, indicating that basal proteolysis is equivalent in the four muscles. Furthermore, the release of tyrosine from metatarsal bone alone was negligible (Fig. 3*B*), indicating that skeletal muscle is the main source of tyrosine detected in the medium. On the basis of these data, in subsequent experiments determination of tyrosine release was performed after 2-h incubation with individual lumbrical muscle/metatarsal bone ($n = 8$ muscles/rat).

Effect of mechanical injury and denervation in lumbrical muscle proteolysis. To assess whether the lumbrical model could be used to detect changes in protein breakdown induced by wound or disuse, we evaluated the effect of mechanical injury or surgical denervation of lumbrical muscles on the release of tyrosine. As shown in Fig. 4*A*, acute injury increased by 60% the basal lumbrical proteolysis rate (0.14 ± 0.01 nmol·mg^{-1}·h^{-1}), which in the prepubertal muscle model has been associated with intense influx of Ca^{2+} and subsequent increase of Ca^{2+}-dependent protein breakdown (19).

Likewise, denervation of lumbrical muscle for 3 and 7 days increased by 28% and 64% the basal protein breakdown rate (0.14 ± 0.01 nmol·mg^{-1}·h^{-1}; Fig. 4*B*), which was accompanied by 10% and 25% reduction of muscle mass (control = 38.5 ± 1.2 mg; Fig. 4*C*), validating the adult rat lumbrical muscle as a model to study skeletal muscle proteolysis.

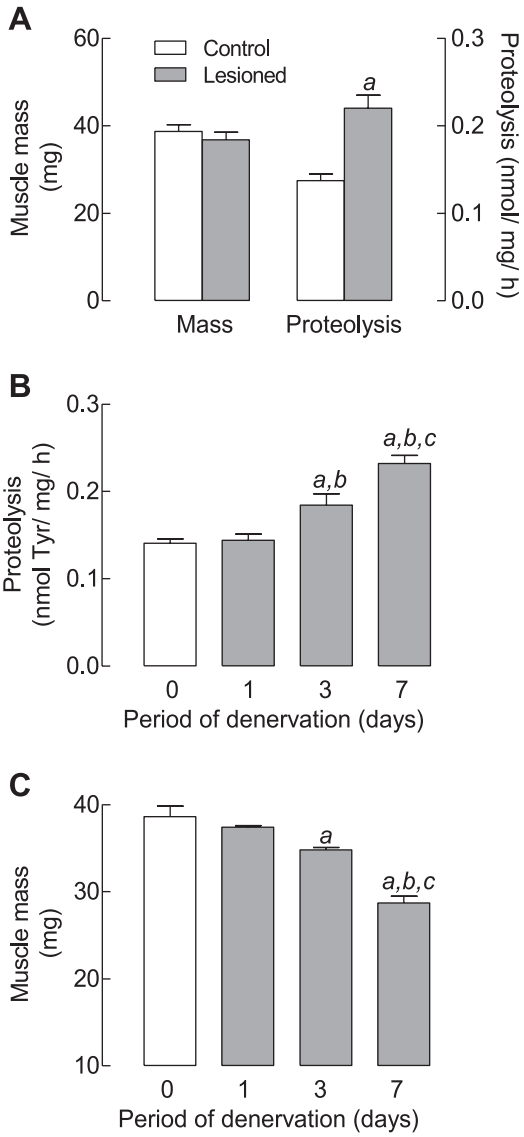

Fig. 4. Effect of acute mechanical injury (*A*) or 1- to 7-day denervation (*B* and *C*) of lumbrical muscles on wet mass (*A*, *C*) and on rate of proteolysis (*A* and *B*). Each bar represents mean ± SE of values obtained from 3–4 adult male rats. [a]Significantly different from control group; [b]significantly different from 1-day denervated group; [c]significantly different from control 3-day denervated group ($P < 0.05$; *A*, Student's *t*-test; *B* and *C*, ANOVA followed by Newman-Keuls multiple comparison test).

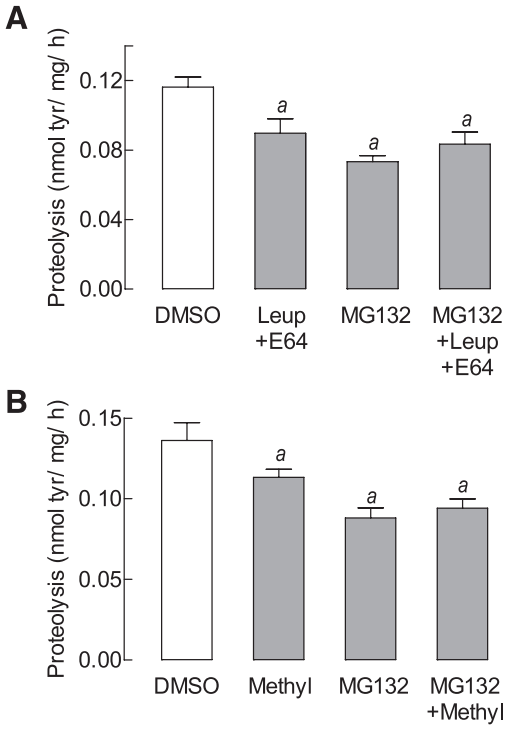

Fig. 5. Contribution of ubiquitin-proteasome and calpain systems on lumbrical muscle proteolysis. The rate of lumbrical muscle proteolysis was measured in the presence or absence of proteasome inhibitor MG132 (20 μM) and calpain inhibitors E-64/leupeptin (Leup) (25 μM/50 μM) (*A*) or lysosomal inhibitor methylamine (Methyl, 10 mM) (*B*). After 2 h, tyrosine released into the medium was measured by fluorometric assay. Each bar represents mean ± SE of values obtained from 8 muscles of 4 adult male rats. [a]Significantly different from control group ($P < 0.05$; ANOVA followed by Tukey's multiple comparison test).

Contribution of ubiquitin-proteasome, calpain, and lysosomal systems to lumbrical muscle proteolysis. The major pathways of skeletal muscle protein degradation involve ATP-dependent ubiquitin-proteasome and Ca^{2+}-dependent calpain systems (25). To assess the contribution of these pathways to the basal proteolysis of lumbrical muscles from adult rats, tyrosine release was evaluated in muscles preincubated with 20 μM MG132, an inhibitor of the ubiquitin-proteasome system (26, 50), 25 μM E64 plus 50 μM leupeptin to inhibit the calpain system (19, 38, 39), or 0.2% DMSO as vehicle. MG132 and E64/leupeptin reduced by 39% and 25% the proteolysis obtained in the presence of DMSO (0.12 ± 0.01 $nmol \cdot mg^{-1} \cdot h^{-1}$, Fig. 5*A*). To examine the role of lysosome in the degradation of lumbrical muscle proteins, we measured the release of tyrosine in the presence of 10 mM methylamine. The lysosomal inhibitor reduced the basal rate of tyrosine release by 17% (Fig. 5*B*). Combination of MG132 with E64/leupeptin (Fig. 5*A*) or with methylamine (Fig. 5*B*) had no additive effect, which confirms the current theory that the ubiquitin-proteasome pathway is the rate-limiting step in the proteolytic process of skeletal muscle catabolism (25). Incubation of muscles with DMSO alone did not modify the basal release of tyrosine (data not shown).

Effect of modulators of cAMP signaling pathway in skeletal muscle proteolysis. Considering the crucial role of stimulatory G (G_s) protein/adenylyl cyclase (AC)/cAMP in the maintenance of muscle mass (31), established exclusively in the prepubertal skeletal muscle model (4, 26, 32), we analyzed the effect of activators of β-adrenoceptor/cAMP signaling on lumbrical muscles from adult rats. The nonselective β-adrenoceptor agonist isoproterenol (30–100 μM) decreased by 14–20% basal tyrosine release (0.16 ± 0.01 $nmol \cdot mg^{-1} \cdot h^{-1}$; Fig. 6*A*). Isoproterenol's effect was mimicked by the selective β_2-adrenoceptor agonist formoterol (3–10 nM), which reduced by

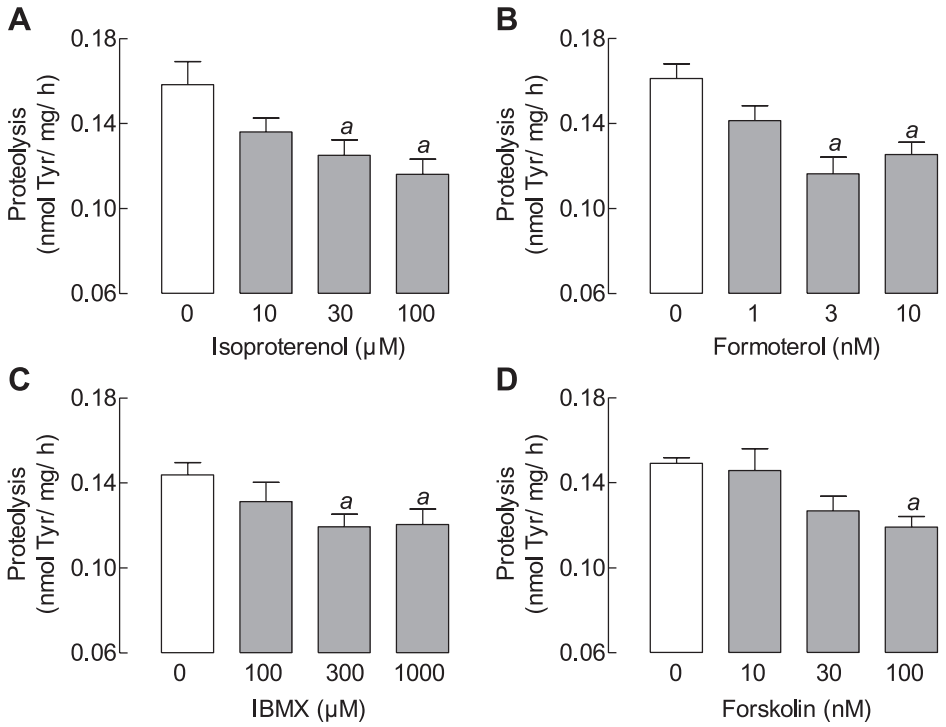

Fig. 6. Inhibitory effect of the receptor-activated G_s protein (GsPCR)/cAMP signaling system on lumbrical muscle proteolysis. The rate of lumbrical muscle proteolysis was measured in the presence or absence of β-adrenoceptor agonists isoproterenol (10–100 μM; *A*) and formoterol (1–10 nM; *B*), phosphodiesterase inhibitor IBMX (100–1,000 μM; *C*), and adenylyl cyclase activator forskolin (10–100 nM; *D*). After 2 h, tyrosine released into the medium was measured by fluorometric assay. Each bar represents mean ± SE of values obtained from 4–16 muscles of 3–6 adult male rats. [a]Significantly different from control group ($P < 0.05$; ANOVA followed by Newman-Keuls multiple comparison test).

22–27% basal proteolysis (0.16 ± 0.01 nmol·mg^{-1}·h^{-1}; Fig. 6*B*). Furthermore, incubation of lumbrical muscle with the nonselective phosphodiesterase inhibitor IBMX (0.1–1.0 mM) reduced by up to 20% the basal proteolysis (0.14 ± 0.01 nmol·mg^{-1}·h^{-1}; Fig. 6*C*). Finally, direct activation of AC with 100 nM forskolin reduced by 20% the basal tyrosine release from muscle (0.15 ± 0.01 nmol·mg^{-1}·h^{-1}; Fig. 6*D*). By showing the inhibitory effect of the AC/cAMP cascade on protein breakdown of adult skeletal muscle, our results support the idea that AC/phosphodiesterases are potential pharmacological targets for anticatabolic therapy, previously demonstrated in muscles from prepubertal rats (37).

Age-dependent changes in lumbrical muscle proteolysis. To assess whether the lumbrical experimental model allows direct analysis of age-related changes in proteolysis muscle, we compared the rate of proteolysis of lumbrical muscles from prepubertal (1 mo old), adult (4 mo old), middle-aged (12 mo old), and senile (24 mo old) rats. As shown in Fig. 7*A*, total muscle proteolysis was maximal in the prepubertal rat (0.31 ± 0.010 nmol·mg^{-1}·h^{-1}), being reduced by 55% in adult (0.14 ± 0.005 nmol·mg^{-1}·h^{-1}) and by 71–74% in middle-aged (0.08 ± 0.004 nmol·mg^{-1}·h^{-1}) and aged (0.09 ± 0.008 nmol·mg^{-1}·h^{-1}) rats. From prepuberty to adulthood, changes in protein breakdown were inversely proportional to the gain of muscle mass (prepubertal = 22.2 ± 0.8 mg, adult = 37.1 ± 0.7 mg; Fig. 7*B*). In contrast, lumbrical muscle from aged rats atrophied (25.9 ± 0.9 mg), reaching mass values close to those of prepubertal rats. The sarcopenia detected in 24-mo-old rats was accompanied by 16% loss of body mass compared with the 4-mo-old group (Fig. 7*C*).

To investigate whether the magnitude of decrease in muscle proteolysis was correlated to age-dependent changes in proteasome activity, we determined the tyrosine release from lumbrical muscles in the absence or presence of 20 μM MG132. As shown in Fig. 7*A*, MG132 reduced proteolysis in muscle by 40–50% from prepubertal to aged rats, validating the use of the lumbrical model to analyze age-dependent changes in the rate of muscle protein breakdown.

Comparison between lumbrical muscle model system and current methodology. Finally, the efficiency of the new ex vivo model proposed here was compared with the current methodology, which uses EDL muscles from prepubertal rats. By analyzing the effect of forskolin and formoterol, our results showed that either direct or receptor-dependent activation of AC reduced by ~20% the proteolysis of lumbrical (0.32 ± 0.01 nmol·mg^{-1}·h^{-1}) and EDL (0.10 ± 0.01 nmol·mg^{-1}·h^{-1}) muscles from prepubertal rats (Fig. 8, *A* and *B*). More important, by using lumbrical muscle we were able to show that activation of AC also reduces proteolysis in muscles of adult rats (Fig. 8*C*). On the other hand, the evaluation of muscle proteolysis could not be performed in EDL muscles from adult rats because of methodological limitations related to muscle thickness.

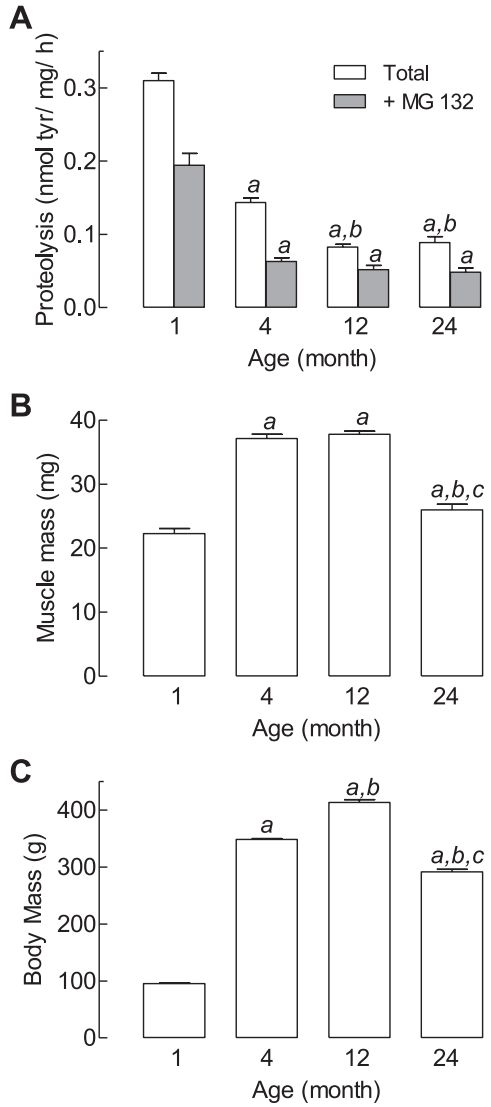

Fig. 7. Age-related changes in lumbrical muscle proteolysis. *A*: rate of proteolysis was measured in lumbrical muscle from 1-, 4-, 12-, and 24-mo-old male rats. Lumbrical muscles were incubated in Tyrode-C for 2 h in the presence or absence of proteasome inhibitor MG132 (20 μM). Tyrosine released into the medium was measured by fluorometric assay. *B*: muscle mass. *C*: body mass. Each bar represents mean ± SE of 6–16 muscles from 3–4 rats. [a]Significantly different from 1-mo group; [b]significantly different from 4-mo group; [c]significantly different from 12-mo group ($P < 0.05$; ANOVA followed by Newman-Keuls multiple comparison test).

DISCUSSION

In the present study, we established and validated the rat lumbrical muscle as a new ex vivo experimental model for the dynamic measurement of skeletal muscle proteolysis in prepubertal, adult, and/or aged rats. All four adult rat lumbrical muscles have similar tyrosine release rate (Fig. 3*B*) with a daily basal FDR of 6.21 ± 0.14%, which is not different from that obtained in vivo in perfused adult rat hindquarters (6.8 ± 0.12%) (29).

Adult rat lumbrical muscles have contraction properties similar to those of EDL muscles (20), but their major advantage is that they are thin enough to allow a suitable measurement of tyrosine release (Fig. 2), which is not possible with EDL muscles because of the inevitable necrosis of inner muscle fibers (3). Moreover, taking into account the similar rate of tyrosine release of all four lumbrical muscles (Fig. 3*B*), this model has a numerical advantage (8 muscles/rat) over the established experimental model, paired EDL muscles. By using eight lumbrical muscles per animal, it is possible to compare seven matched experimental groups with a control value ob-

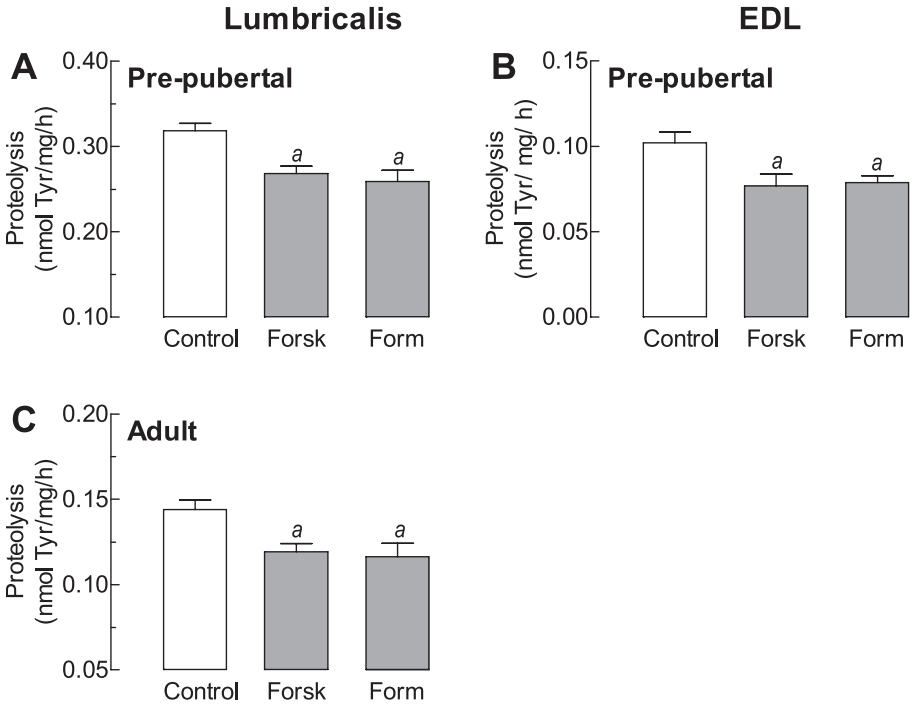

Fig. 8. Effect of modulators of β_2-adrenoceptor/cAMP signaling pathway on proteolysis of lumbrical muscles (*A* and *C*) and EDL (*B*) from prepubertal and adult male rats. The rate of proteolysis of lumbrical muscle from 1 (*A*)- and 4 (*C*)-mo-old rats was measured in the presence or absence of β_2-adrenoceptor agonist formoterol (Form, 3 nM) and adenylyl cyclase activator forskolin (Forsk, 100 nM) and compared with those of EDL from prepubertal rats. Because of methodological limitations, it was not possible to evaluate the rate of proteolysis of EDL muscles from adult rats. Each bar represents mean ± SE of values obtained from 4–16 muscles of 3–6 adult male rats. [a]Significantly different from control group ($P < 0.05$; ANOVA followed by Newman-Keuls multiple comparison test).

tained in the same animal (e.g., 1 control vs. 7 different drugs or vs. 7 different concentrations of a drug). In contrast, it would be necessary to use at least 8 EDL muscle (4 rats) to perform unpaired analysis or 14 EDL (7 rats) to perform paired analysis (one muscle as control and the contralateral one as experimental muscle). Thus the use of the lumbrical model might substantially reduce the number of animals assigned to ex vivo proteolysis experiments. In addition, here we adapted for the 96-well microplate the fluorometric macromethod for tyrosine measurement described by Waalkes and Udenfriend (52), which reduced by ~90% the amount of chemical waste produced.

Furthermore, the lumbrical model here presented was able to detect changes in tyrosine release induced by either acute mechanical injury or muscle denervation (Fig. 4), which involve Ca^{2+}-dependent calpain (19) and ATP-dependent proteasome (25) mechanisms, validating the use of adult rat lumbrical muscle for evaluation of the major proteolytic systems responsible for skeletal muscle protein breakdown.

In fact, the relative contribution of ATP, Ca^{2+}-dependent, and lysosomal proteolytic systems to adult skeletal muscle proteolysis was assessed by incubation of lumbrical muscles with inhibitors of proteasome (MG132) (27), calpain (leupeptin and E64) (55), and lysosome (methylamine) systems. Our results clearly showed that the proteasome system is the major route of protein degradation in adult skeletal muscle (Fig. 5). Interestingly, association of MG132 with calpain (Fig. 5*A*) or lysosome (Fig. 5*B*) inhibitors had no additive effect, which supports the current theory that the ubiquitin-proteasome pathway is the limiting step of skeletal muscle proteolysis (43).

The lumbrical model also allows a strict quantitative analysis of signal transduction pathways involved in the regulation of muscle proteolysis. It is well accepted that the cAMP signaling pathway mediates the biological action of many endogenous substances or synthetic drugs (e.g., catecholamines and β_2-adrenoceptor agonists) via receptor-activated G_s proteins (GsPCR) (1, 7, 15, 18, 21, 23, 46, 51).

The anticatabolic effect of β_2-adrenoceptor agonists involves distinct cAMP-dependent pathways, such as those associated with activation of PKA, which increases expression and activation of calpastatin, an endogenous regulator of m-calpain (9, 48). Many in vivo and ex vivo studies have confirmed the therapeutic potential of β_2-adrenoceptor agonists for attenuating or reversing muscle wasting (for review, see Ref. 36). Some of these have been based on ex vivo proteolysis experiments performed with prepubertal rat muscles (12, 26, 28, 32, 37, 47, 50). Here we found that β-adrenergic agonists isoproterenol (nonselective) and formoterol (β_2-selective) also reduced proteolysis of adult rat skeletal muscle by 12–20%. Similar effects were obtained by using drugs that increase intracellular cAMP levels, via activation of AC (forskolin) or inhibition of phosphodiesterases (IBMX). These observations highlight the GsPCR/AC/cAMP system as a pharmacological target for anticatabolic therapies and attenuation of adulthood and age-dependent skeletal muscle protein breakdown.

More importantly, by comparing the tyrosine release from lumbrical muscle of prepubertal, adult, and aged rats, we showed that overall skeletal muscle proteolysis decreases with maturation and aging (Fig. 7*A*). This observation alone makes questionable the direct extrapolation of results obtained from prepubertal muscles to adult or senile muscles. In fact, Reynolds et al. in 2002 made similar observations with 2- to 4-mo-old to 12- to 17-mo-old male B6C3F1 mice, showing that skeletal muscle proteolysis is lower with maturity (44). In addition, the decline in total and proteasome-dependent muscle proteolysis observed during aging (Fig. 7*A*) is supported by previous studies showing a marked reduction of Atrogin-1/MAFbx and MuRF1 levels (13) and of chymotrypsin and trypsin-like activities of proteasome in senescent muscles (8,

24, 41), which might contribute to accumulation of damage proteins in skeletal muscle during senescence (34).

Finally, a high rate of protein synthesis is observed in skeletal muscles of newborn rats, but as maturation progresses this rate declines rapidly (11). As consequence, in adult life, the basal fractional synthesis rate (FSR) of rat fast-twitch muscles reaches values similar to those of the basal FDR (6.8%/day) (29, 49), which is quite similar to lumbrical FDR values (6.2%/day) obtained in the present study. Altogether, these data indicate an appropriate balance between basal protein synthesis and degradation in adult lumbrical skeletal muscle.

In summary, we have established an in situ experimental model—the lumbrical muscle—for dynamic measurement of proteolysis in skeletal muscles from prepubertal to aged rats, which overcomes the methodological limitation of using only sexually immature rats. This is of special relevance considering that muscle proteolysis in the adult rat is ~50% of that in the 1-mo-old animal, which limits the direct extrapolation of results obtained from prepubertal muscles to adult or senile muscles. Finally, the use of eight lumbrical muscles per rat and the adjustment of the tyrosine assay to a 96-well microplate format definitively may reduce the number of animals and chemical waste in the experimental analysis of skeletal muscle proteolysis, optimizing the pharmacological screening of anticatabolic drugs for treatment and prevention of specific adult-onset diseases or those associated with aging.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: L.B.B. and R.O.G. conception and design of research; L.B.B. and L.B.F. performed experiments; L.B.B. and R.O.G. analyzed data; L.B.B., L.B.F., and R.O.G. interpreted results of experiments; L.B.B. and R.O.G. drafted the manuscript; L.B.B. and R.O.G. edited and revised the manuscript; L.B.B., L.B.F., and R.O.G. approved the final version of the manuscript; R.O.G. prepared figures.

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