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Curcumin treatment prevents increased proteasome and apoptosome activities in rat skeletal muscle during reloading and improves subsequent recovery☆

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Abstract

Immobilization is characterized by activation of the ubiquitin (Ub)–proteasome-dependent proteolytic system (UPS) and of the mitochondrial apoptotic pathway. Increased oxidative stress and inflammatory response occur in immobilized skeletal muscles. Curcumin exhibits antioxidant and anti-inflammatory properties, blocked proteasome activation in intact animals, and may favor skeletal muscle regeneration. We therefore measured the effects of curcumin on immobilization-induced muscle atrophy and subsequent recovery. Rats were subjected to hindlimb immobilization for 8 days (I8) and allowed to recover for 10 days (R10). Fifty percent of the rats were injected daily with either curcumin or vehicle. Proteolytic and apoptotic pathways were studied in gastrocnemius muscles. Curcumin treatment prevented the enhanced proteasome chymotrypsin-like activity and the trend toward increased caspase-9-associated apoptosome activity at I8 in immobilized muscles. By contrast, the increase of these two activities was blunted by curcumin at R10. Curcumin did not reduce muscle atrophy at I8 but improved muscle recovery at R10 and the cross-sectional area of muscle fibers of immobilized muscles. Curcumin reduced the increased protein levels of Smac/DIABLO induced by immobilization and enhanced the elevation of X-linked inhibitory apoptotic protein levels at R10. Ub-conjugate levels and caspase-3 activity increased at I8 and were normalized at R10 without being affected by curcumin treatment. Altogether, the data show that curcumin treatment improved recovery during reloading. The effect of curcumin during the atrophic phase on proteasome activities may facilitate the initiation of muscle recovery after reloading. These data also suggest that this compound may favor the initial steps of muscle regeneration.

Keywords: Ubiquitin–proteasome-dependent pathway; Disuse; Intrinsic apoptotic pathway; Protein breakdown; Reloading

1. Introduction

Muscle wasting is observed in many conditions including immobilization. Skeletal muscle provides power and strength for locomotion and posture but is also the major reservoir of body proteins. Thus, sustained muscle wasting leads to difficulties in performing daily activities and has severe metabolic consequences. The resulting weakness lengthens recovery periods. A major clinical issue is the identification of approaches to limit skeletal muscle atrophy in catabolic situations and/or to improve subsequent muscle recovery.

Muscle protein loss results from an imbalance between protein synthesis and breakdown rates, but also from an imbalance between apoptotic and regeneration processes. The ubiquitin (Ub)–proteasome system (UPS) is involved in the breakdown of the major contractile proteins. In this pathway, a poly-Ub chain is covalently attached to the substrate and then recognized by the 26S proteasome before the subsequent breakdown of the targeted protein [1]. We and others have previously shown that this pathway is activated during disuse muscle atrophy [2–8]. An activation of apoptosis during immobilization has also been previously reported [8–11]. The mitochondrial apoptotic pathway is triggered by the release of killer proteins, such as cytochrome *c* and Smac/Diablo, and is stimulated by oxidative stress [12]. In this pathway, the release of cytochrome *c* from the mitochondria leads to the formation of a complex (called apoptosome) with apoptotic protease activating factor-1 and procaspase-9 and to the activation of caspase-3 [13]. This pathway is believed to be physiologically important in regulating disuse-induced muscle atrophy [8,9,13–15]. In addition, this pathway is concomitantly activated with the UPS during immobilization-induced atrophy and normalized during recovery [8].

The activation of inflammatory processes and oxidative stress pathways in immobilized muscles [16,17] has been suggested to result in muscle damages and in increased proteolysis and apoptosis

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[17,18]. Therefore, using compounds counteracting inflammation and oxidative stress may prove beneficial in disuse atrophy. Curcumin (diferuloylmethane) is a component of the spice turmeric (*Curcuma longa*), which exhibits anti-inflammatory and antioxidant properties [19]. Treatment of cultured myotubes with curcumin prevented the increase in protein degradation caused by a cachectic factor purified from experimental tumors in mice [20]. Furthermore, in other experiments, intraperitoneal (ip) administration of curcumin prevented the loss of muscle mass and protein in lipopolysaccharide (LPS)-injected mice [21]. This compound blocked the induction of the UPS in several catabolic conditions [19–22]. Curcumin could also prevent the induction of some signaling pathways (i.e., nuclear factor κB, p38 MAP (mitogen-activated protein) kinase, ERK kinase (extracellular signal-regulated kinase), xanthine oxidase, β-catenin …) [21,23–27]. These pathways are activated during disuse [16,17,28–30] and associated with the induction of the UPS and the mitochondrial apoptotic pathway [11,31–33]. Finally, curcumin stimulated muscle regeneration after traumatic injury in mice [24]. In addition, curcumin is well tolerated and did not exhibit detrimental effects on muscle recovery after myotoxic injury at low doses (<2 mg/kg; ip) [34]. Therefore, we hypothesized that ip curcumin treatment may (1) limit immobilization-induced atrophy and/or favor muscle recovery and (2) modulate the activity of the UPS and the mitochondrial apoptotic pathway.

2. Methods and materials

2.1. Experimental animals

The present study was approved by the Animal Care and Use Committee of the Institut National de la Recherche Agronomique and adhered to the current legislation on animal experimentation in France. Male Wistar rats, 8 months old (Charles River Laboratories, L'Arbresle, France), were housed individually under controlled environmental conditions (temperature, 22°C; 12 h dark period starting at 8 h), fed a basal diet ad libitum, and given free access to water. The macronutrient composition of the basal diet was (in g/kg diet) as follows: 470 wheat flour, 152 casein, 100 sucrose, 134 lactose, 30 colza oil, 27 peanut oil, 3 sunflower oil, 35 AIN93 mineral mix, 10 AIN93 vitamin mix and 35 cellulose. After a 3-week adaptation period, 32 rats were anesthetized with forene inhalation and subjected to unilateral hindlimb immobilization via an orfit-soft plaque (Gibaud, France) for 8 days. The foot was positioned in plantar extension to induce maximal atrophy of the gastrocnemius and the soleus muscles [5]. We previously reported that the gastrocnemius muscle mass from the nonimmobilized contralateral leg of the casted rats is not significantly different from the gastrocnemius mass of noncasted rats [8]. This indicates that the muscles from the contralateral leg did not hypertrophy because of overloading and that the muscle atrophy at I8 reflects a loss of muscle mass in the immobilized leg [8]. Therefore, the contralateral noncasted leg served as the control in all experiments. At the end of the immobilization period, casts were removed, and animals were allowed to recover for 10 days. During immobilization and recovery periods, animals were daily injected (ip) with either 1 mg/kg body weight of curcumin diluted in dimethyl sulfoxide/0.9% NaCl (1:1000) (curcumin group) or dimethyl sulfoxide/0.9% NaCl alone (vehicle group). At the end of the immobilization (I8) and the recovery periods (R10), rats (n=8/group) were sacrificed under pentobarbital sodium anesthesia (50 mg/kg body weight, ip). Hindlimb gastrocnemius muscles were carefully excised and weighed. The central part of gastrocnemius samples was frozen in cooled isopentane (−160°C), at resting length, and stored at −80°C until histochemical analysis (see below). The remaining was finely pulverized in liquid nitrogen and stored at −80°C until further analyses.

2.2. Histochemical analyses of muscle

Cross 10-μm-thick cryosections were performed using a cryostat (HM560 Microm) and stained using picro-Sirius red coloration [35], which reveals the collagen of perimysium and endomysium. Observations and image acquisitions were performed using a photonic microscope in bright field mode (Olympus BX 71) coupled to a high-resolution cooled digital camera (Olympus DP 71) and Cell F software (Olympus Soft Imaging Solutions). For each muscle section, 15–20 images, depending on the total area of the muscle, were acquired using a 10× objective. Image analysis was performed using the Visilog 5.4 software (Noesis, France). The green component, in gray level, of initial color image was used for higher contrast. For a given optical field, automatic thresholding on gray level allowed segmentation of the connective tissue network (perimysium and endomysium). Measurement of the area of this network was performed by counting the number of pixels. Additional segmentation procedure of the binary images of the connective network with the "watershed" method results in separating objects corresponding to muscle cells. Fiber boundaries were manually corrected when necessary. Cross-sectional area (CSA) of each cell was automatically calculated.

2.3. Measurement of proteasome and apoptosome activities

Powder (150 mg) of gastrocnemius muscles from control and immobilized hindlimbs at I8 and R10 was homogenized in an ice-cold buffer (pH 7.5) containing 50 mM Tris, 250 mM sucrose, 10 mM ATP, 5 mM $MgCl_2$, 1 mM DTT (dithiothreitol) and protease inhibitors (10 $\mu g \cdot ml^{-1}$ of antipain, aprotinin, leupeptin and pepstatin A, and 20 μM PMSF (phenylmethylsulfonyl fluoride)). Apoptosome complexes exhibit molecular weight ranging from ~700 to ~1,500 kDa and therefore cosediment with proteasomes [36]. Accordingly, caspase 9-linked apoptosome activity coelutes with peptidase activities of the proteasome on 10%–40% sucrose gradients [8,36]. Apoptosome complexes and proteasomes were therefore concomitantly isolated by three sequential centrifugations as described previously [37]. Briefly, homogenates were first centrifuged at 10,000*g* for 20 min at 4°C. The resulting supernatant was then centrifuged at 100,000*g* for 1 h at 4°C. This last supernatant was then centrifuged at 100,000*g* for 5 h at 4°C. The final pellet was resuspended in a buffer containing 50 mM Tris (pH 7.5), 5 mM $MgCl_2$ and 20% glycerol. The protein content was determined according to Lowry et al. [38]. The proteasome chymotrypsin-like and the apoptosome-linked caspase-9 activities were determined by measuring the hydrolysis of the fluorogenic substrates succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (LLVY-AMC) (Sigma) and the *N*-acetyl-Leu-Glu-His-Asp-7-amino-4-methylcoumarin (LEHD-AMC) (Biomol), respectively. To measure the chymotrypsin-like activity of the proteasome, 15 μl of each pellet was added to 60 μl of medium containing 50 mM Tris (pH 8.0), 10 mM $MgCl_2$, 1 mM DTT, 2 U apyrase and 300 μM of LLVY-AMC with or without the proteasome inhibitor MG132 (40 μM; Affiniti). To measure the apoptosome-linked caspase-9 activity, 25 μl of each pellet was diluted to 50 μl with buffer A [20 mM HEPES (pH 7.4), 10 mM NaCl, 1.5 mM $MgCl_2$, 20% glycerol, 0.1% Triton X-100 and 1 mM DTT]. Fractions were then incubated with 50 μl of the reaction buffer B [50 mM PIPES, 0.1 mM EDTA, 10% glycerol, 10 mM DTT] containing 50 μM of LEHD-AMC. Reactions were performed with or without the caspase-9 inhibitor LEHD-CHO (50 μM; Biomol). Both activities were determined by measuring the accumulation of the fluorogenic cleavage product (methylcoumaryl-amide, or AMC) using a fluorescence spectrometer FLX800 (Biotek) during 45 min at 380-nm excitation wavelength and 440-nm emission wavelength. The proteasome chymotrypsin-like and the apoptosome-linked caspase-9 activities were measured by calculating the difference between arbitrary fluorescence units recorded with or without the specific inhibitors in the reaction medium. The final data were corrected by the amount of protein in the reaction medium. The time course for the accumulation of AMC after hydrolysis of the substrate was analyzed by linear regression to calculate activities, that is, the slopes of best fit of accumulating AMC versus time.

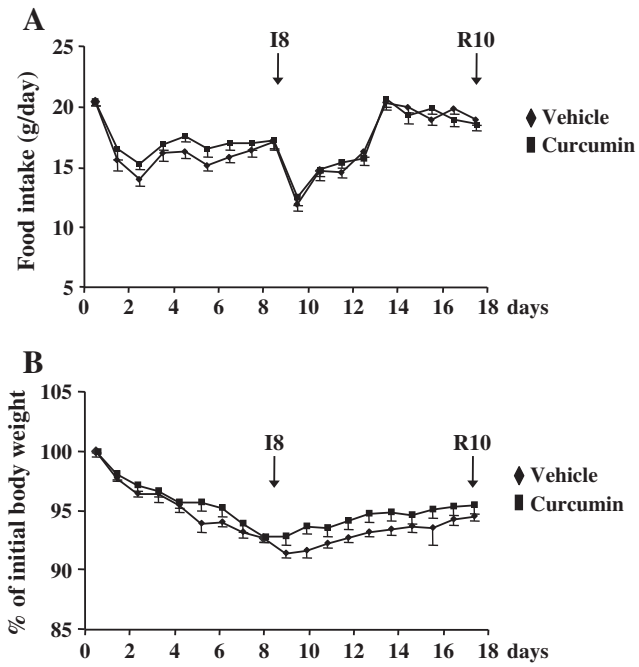

Fig. 1. Effect of curcumin treatment on food intake and body weight during immobilization and recovery. Food intake (A) and body weight (B) are expressed in g/day and in percent of initial body weight, respectively. Data are means±S.E.M. (vertical bars) for n=8 rats per group.

2.4. Caspase-3 activity measurements

Caspase-3 activity was assessed on total cytosolic protein extracts. Powder (150 mg) of gastrocnemius muscles was homogenized in an ice-cold buffer A (see above) as described by Siu et al. [10]. Extracts were centrifuged at 1,500*g* for 5 min at 4°C, and the resulting supernatants were subjected to three successive centrifugations at 3,500*g* for 5 min at 4°C. The last supernatants were stored as total cytosolic protein extracts at −80°C. A protease inhibitor cocktail containing 104 mM 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSE), 0.08 mM aprotinin, 2 mM leupeptin, 4 mM bestatin, 1.5 mM pepstatin A and 1.4 mM E-64 (Sigma) was added to a fraction of the supernatants, which were then stored at −80°C for subsequent determination of X-linked inhibitory apoptotic protein (XIAP) and Smac/Diablo protein levels (see below). Protein concentration was determined according to Lowry et al. [38]. To determine caspase-3 activity, 25 µl of the total cytosolic protein extracts was diluted to 50 µl with buffer A and incubated with 50 µl of buffer B (see above) containing 50 µM of Ac-Asp-Glu-Val-Asp-AMC (Calbiochem). Reactions were performed with or without 50 µM of the inhibitor of caspase-3 (Ac-Asp-Met-Gln-Asp-CHO [PMQD-CHO] Calbiochem). Caspase-3 activity was determined by measuring the accumulation of AMC using a fluorescence spectrometer FLX800 (Biotek) during 1 h at 380-nm excitation wavelength and 460-nm emission wavelength. The caspase-3 activity was calculated as described for the apoptosome and the proteasome activities.

2.5. Western blots analyses

The accumulation of Ub conjugates was assessed by immunoblotting on myofibrillar protein extracts. Powder (150 mg) of gastrocnemius muscles was homogenized in an ice-cold buffer (buffer C) containing 5 mM Tris–HCl (pH 7.5), 5 mM EDTA, pH 8, 1 mM PMSF, 0.25 mM tosyl-lysylchloromethane), 5 mM *N*-ethyl-

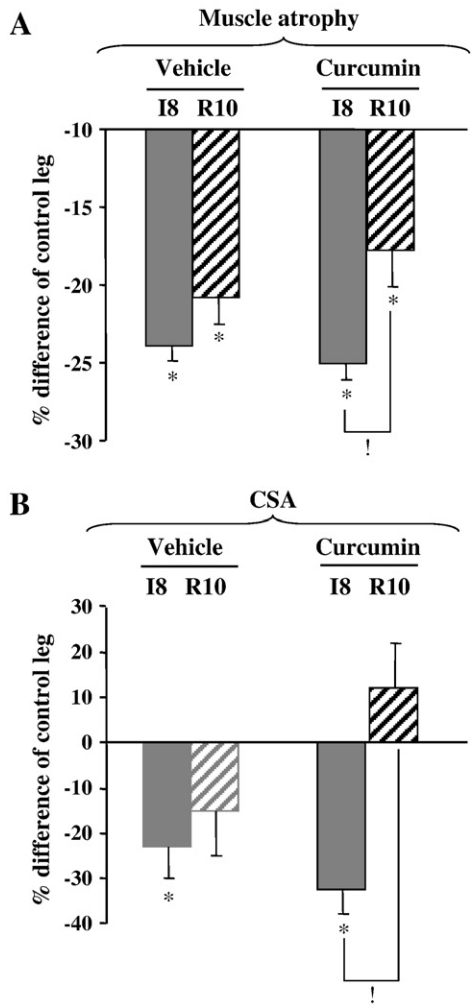

Fig. 2. Curcumin treatment improved muscle recovery after immobilization. (A) Gastrocnemius muscle atrophy is expressed in percent difference of the contralateral noncasted control leg. (B) Cross-sectional fiber area (CSA) is expressed in percent difference of the contralateral noncasted control leg. CSA (µm^2) was measured on 987 to 1,725 control and immobilized muscle fibers from vehicle- and curcumin-treated rats. Means were calculated per muscles. CSAs of control muscle fibers from vehicle and curcumin-treated rats were 3,512±274 and 5,152±401 (I8), and 3,619±493 and 2,760±283 (R10), respectively. CSAs of immobilized muscle fibers from vehicle and curcumin-treated rats were 2,549±143 and 3,396±250 (I8), and 2,848±196 and 3,000±226, respectively (R10). The difference between immobilized and control muscle fibers CSA was calculated, and values are means±S.E.M. (vertical bars) for n=8 rats per group. Differences between control and immobilized muscles were assessed by the Student's t test (*P<.05); Changes in the percent difference observed in immobilized muscles compared with control muscles were assessed by two-way ANOVA (curcumin effect and recovery effect). When significant differences were detected by ANOVA, a post hoc Fisher's test was performed to compare the variations between groups. $^{!}P$<.05, I8 versus R10 in curcumin group.

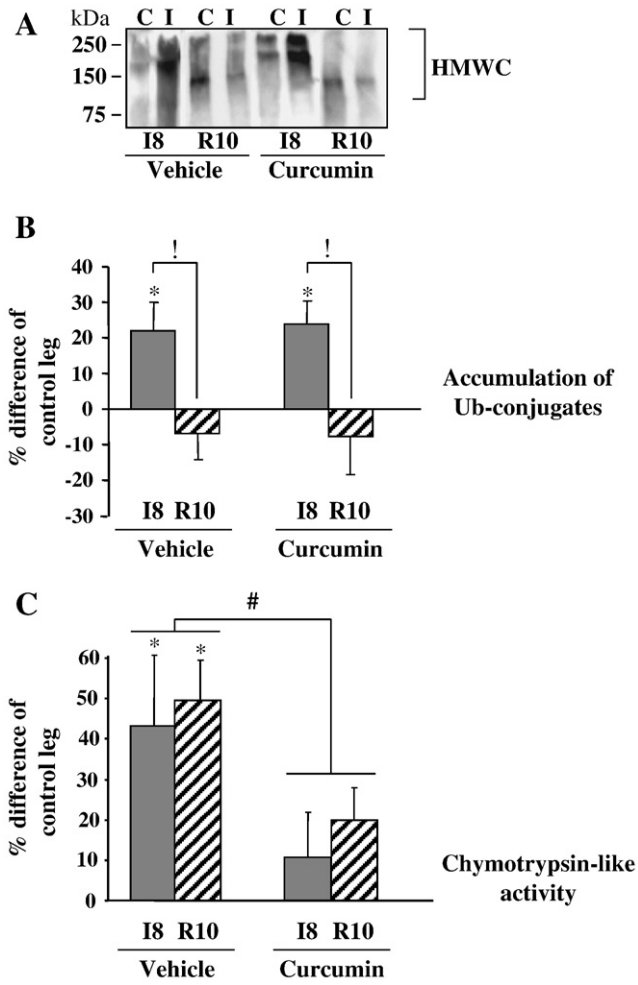

Fig. 3. Curcumin treatment blocked increased proteasome activity but not Ub-conjugate accumulation during immobilization and recovery. (A) High-molecular-weight Ub conjugates (HMWC) were measured on 25 µg of myofibrillar proteins from control (C) or immobilized (I) gastrocnemius muscles from vehicle- and curcumin-treated rats by Western blotting using an antibody that recognizes specifically poly-Ub chains. (B) Signals were quantified in each lanes by using the Image J software and normalized against the amount of proteins (determined following Ponceau Red staining) to correct for uneven loading. Changes of Ub-conjugate accumulation in immobilized muscles compared with control muscles were calculated, and data were expressed in percent variation from control leg. (C) The chymotrypsin-like activity of the proteasome was measured by using a fluorogenic substrate on partially purified proteasome extracts from gastrocnemius muscles as described in experimental procedures. All data are expressed as percent difference of control leg and are means±S.E.M. (vertical bars) for n=8 rats per group. Differences between control and immobilized muscles were assessed by the Student's t test (*P<.05). Changes in the percent difference observed in immobilized muscles compared with control muscles were assessed by two-way ANOVA (curcumin effect and recovery effect). When significant differences were detected by ANOVA, a post hoc Fisher's test was performed to compare the variations between groups. $^{\#}P$<.05, curcumin versus vehicle group; $^{!}P$<.05, I8 versus R10 in vehicle or curcumin group.

maleimide, 5 μg/ml leupeptin and 5 μg/ml soybean trypsin inhibitor [8]. Homogenates were centrifuged for 5 min (1,500*g*, 4°C) to pellet myofibrillar proteins, which were then washed three times in buffer C containing 1% Triton X-100. Myofibrillar proteins were then resuspended in 8 M urea/5 mM Tris–HCl (pH 7.5). Protein concentration was determined as described [38]. The accumulation of Ub conjugates was measured on 25 μg of myofibrillar proteins separated on 7.5% (wt/vol) acrylamide gels and transferred onto PVDF (polyvinylidene difluoride) membranes. The FK1 antibody (Affiniti), which recognizes poly-Ub chains, was used at a 1:1000 dilution.

The amount of protein levels for the antiapoptotic protein XIAP and for the proapoptotic protein Smac/Diablo (Second mitochondrial-derivated activators of caspases) was assessed by immunoblotting on the cytosolic proteins extracted for the caspase-3 activity measurements and stored in the presence of protease inhibitors (see above). Fifty micrograms of protein was separated on a 15% (wt/vol) polyacrylamide gels and transferred onto PVDF membranes (GE Healthcare). Antibodies (Cell Signalling) against XIAP and Smac/DIABLO were used at 1:1000 and 1:4000 dilution, respectively. Signals were detected using the ECL+ detection kit (GE Healthcare) after exposition onto radiographic films (Hyperfilm ECL; GE Healthcare), quantified by using the Image J software, and normalized against the amount of proteins (determined following Ponceau Red staining) to correct for uneven loading.

2.6. Statistical analyses

All data are expressed as means±S.E.M.. Differences between control and immobilized muscles at each time point were assessed using the Student's *t* test. The effect of curcumin treatment (vehicle vs. curcumin-treated rats) and of recovery by itself (immobilization vs. recovery periods) were assessed by two-way analysis of variance. When significant differences were detected by ANOVA, comparisons between groups were made using the post hoc Fisher's test. Significance was defined at the .05 level. All tests were performed by using Statview (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Animal characteristics

Food intake decreased by 20% in both curcumin- and vehicle-injected rats after 8 days of immobilization (Fig. 1A), so that their body weight decreased by ~8% (Fig. 1B). After cast removal, food intake increased progressively and was rapidly normalized (about 20 g of dry matter intake) in both groups. Consequently, body weight increased progressively, but rats did not recover their initial body weight 10 days after cast removal (R10).

3.2. Muscle atrophy and recovery

Hindlimb gastrocnemius muscles atrophied in vehicle-injected and curcumin-treated rats, respectively, by 24% and 25% ($P<.0001$) after 8 days of immobilization (I8) (Fig. 2A). Similarly, the muscle fiber CSA decreased by 23% (vehicle) and 32% (curcumin) at I8 ($P<.05$) (Fig. 2B). Two-way ANOVA analysis showed that muscle atrophy and CSA reduction were not modified by curcumin treatment but between immobilization and recovery ($P<.05$). Ten days after cast removal, gastrocnemius muscles mass from the casted leg remained significantly lower compared with control muscles ($P<.0001$) in both groups of rats (Fig. 2A). However, an improvement of muscle atrophy and CSA reduction was observed between I8 and R10 only in curcumin-treated rats ($P<.05$) (Fig. 2A and B).

3.3. Regulation of the Ub–proteasome-dependent pathway

This pathway (also called UPS) involves two distinct steps, that are the ubiquitination of proteins and their subsequent degradation by the 26S proteasome. We have investigated both steps, by (1) measuring the accumulation of Ub conjugates, which are the final products of the ubiquitination step, and (2) the chymotrypsin-like activity of the proteasome.

Fig. 3A and B shows that Ub conjugates accumulated to the same extent at I8 in immobilized gastrocnemius muscles from vehicle- (+43%, $P<.05$) and curcumin-treated (+24%, $P<.05$) rats. The amount of poly-Ub conjugates was normalized at R10 in both groups. The

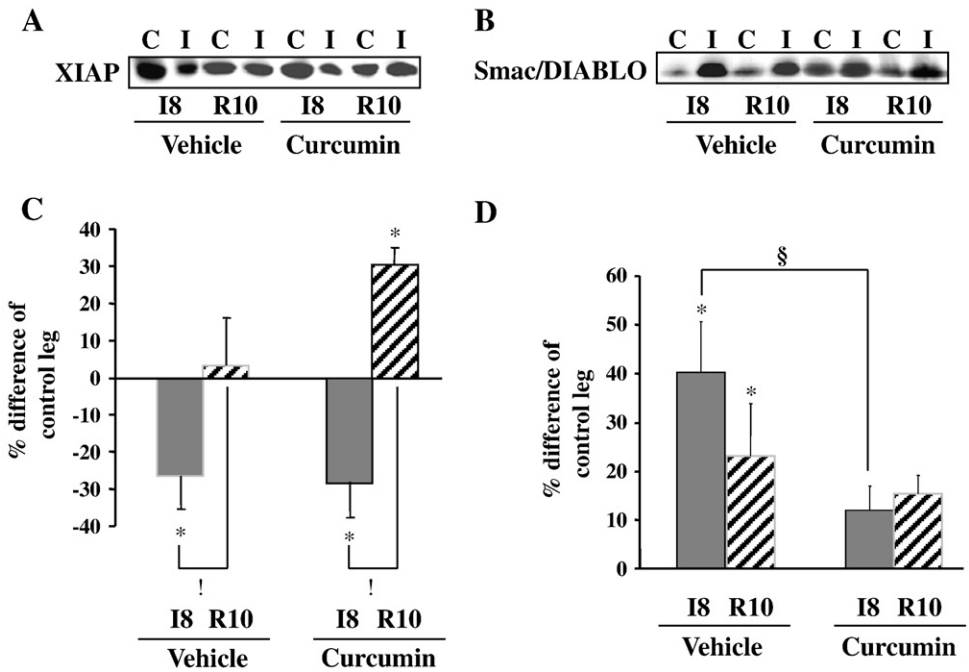

Fig. 4. Curcumin treatment increased XIAP protein content during recovery. Levels for the antiapoptotic protein XIAP (A) and for the proapoptotic protein Smac/DIABLO (B) were assessed on control (C) or immobilized (I) gastrocnemius muscles by Western blotting as described in experimental procedures. Signals recorded for XIAP (C) and Smac/DIABLO (D) were quantified by using the Image J software and normalized against the amount of proteins (determined following Ponceau Red staining) to correct for uneven loading. All data are expressed as percent difference of control leg and are means±S.E.M. (vertical bars) for $n=5$ rats per group. Differences between control and immobilized muscles were assessed by the Student's *t* test (*$P<.05$); Changes in the percent difference observed in immobilized muscles compared with control muscles were assessed by two-way ANOVA (curcumin effect and recovery effect). When significant differences were detected by ANOVA, a post hoc Fisher's test was performed to compare the variations between groups. [!]$P<.05$, I8 versus R10 in vehicle or curcumin group; [§]$P<.05$, vehicle versus curcumin group at I8.

chymotrypsin-like activity of the 20S proteasome increased in muscles from the immobilized leg compared with the control leg in the vehicle-treated group at both I8 and R10 (+43% and +50%, respectively; $P<.05$) (Fig. 3C). This increased activity did not prevail in curcumin-treated rats during immobilization or the subsequent recovery ($P<.05$, two-way ANOVA). Thus, at the end of immobilization period (I8), muscles from curcumin-treated rats likely exhibit higher ubiquitination rates, because Ub conjugates accumulate without change in proteasome chymotrypsin-like activity. Similarly, ubiquitination rates are also likely enhanced at R10 in muscles from untreated rats, because normalization of Ub conjugates occurs during recovery, whereas proteasome chymotrypsin-like activities are still elevated. It is noteworthy that muscle recovery was initiated only in muscles from curcumin-treated rats at R10, when both chymotrypsin -like and accumulation of Ub conjugates were normalized. This further indicates that normalization of both steps of the Ub–proteasome-dependent pathway is crucial for muscle mass gain.

3.4. Regulation of the apoptotic mitochondrial pathway

We have previously reported a role for the apoptotic mitochondrial pathway during immobilization-induced atrophy and muscle recovery [8]. The antiapoptotic XIAP protein level (Fig. 4A and C) was reduced in immobilized gastrocnemius muscles compared with control muscles from vehicle- (−27%, $P<.05$) and curcumin-treated group (−28%, $P<.05$). These changes were improved during recovery ($P<.05$, two-way ANOVA): between I8 and R10, the protein levels for XIAP were normalized in vehicle-treated rats, but increased above basal levels in immobilized muscles from curcumin-treated animals (+30%, $P<.01$) (Fig. 4C). The proapoptotic Smac/DIABLO protein level (Fig. 4B and D) increased in immobilized muscles compared with control muscles at I8 (+40%, $P<.05$) and R10 (+23%, $P<.05$) in the vehicle-injected group but were not different in the curcumin-treated rats. Curcumin treatment prevented the increased Smac/DIABLO protein levels in immobilized muscles at I8 ($P<.05$).

The regulation of the mitochondrial apoptotic pathway was next assessed by measuring the caspase-3 and the apoptosome-linked caspase-9 activities. The apoptosome-linked caspase-9 activity increased in muscles from the immobilized leg compared with control leg in vehicle-treated group at R10 (+26%, $P<.05$), but only tended to increase at I8 (+43%, $P=.07$) (Fig. 5A). Conversely, this activity was unchanged in immobilized muscles from curcumin-treated rats compared with control muscles at I8 or at R10. Fig. 5B shows that caspase-3 activity increased at I8 in immobilized gastrocnemius muscles from vehicle- (+51%, $P<.0001$) and curcumin-treated (+66%, $P<.0001$) rats. This activity was normalized at R10 in both groups.

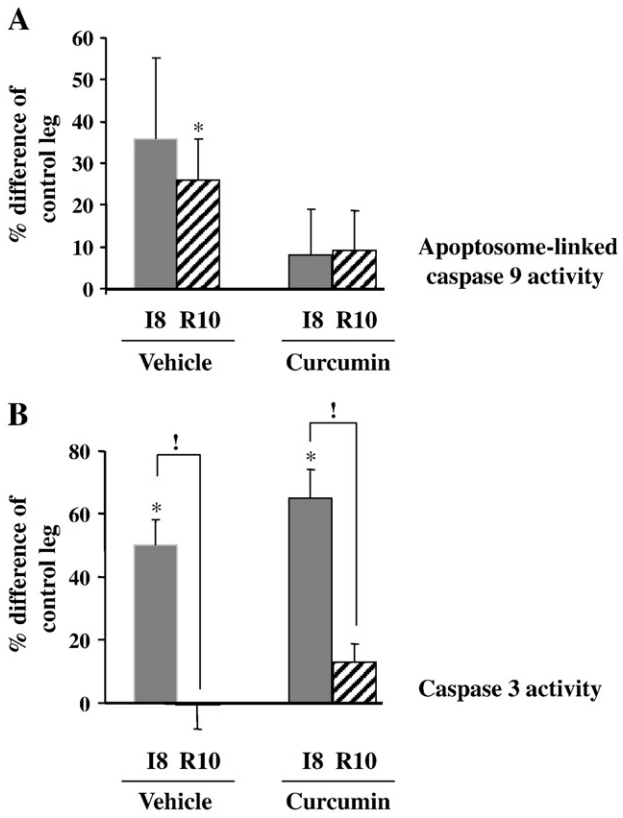

Fig. 5. Curcumin blocked increased apoptosome activity but not increased caspase-3 activity during immobilization and recovery. The caspase-9 associated apoptosome (A) and the caspase-3 (B) activities were measured using fluorogenic substrates on gastrocnemius protein extracts as described in experimental procedures. All data are expressed as percent difference of control leg and are means±S.E.M. (vertical bars) for $n=8$ rats per group. Differences between control and immobilized muscles were assessed by the Student's t test (*$P<.05$); Changes in the percent difference observed in immobilized muscles compared with control muscles were assessed by two-way ANOVA (curcumin effect and recovery effect). When significant differences were detected by ANOVA, a post hoc Fisher's test was performed to compare the variations between groups. [!]$P<.05$, I8 versus R10 in vehicle or curcumin group.

4. Discussion

This work suggests that curcumin had beneficial effects during skeletal muscle recovery following immobilization. After cast removal, curcumin treatment resulted in an increased expression of the antiapoptotic XIAP protein and improved muscle recovery, through increased CSA and muscle mass.

As previously reported [8], all processes measured in the UPS and the mitochondria-associated apoptotic pathway were concomitantly activated during immobilization. Curcumin administration prevented the elevation of proteasome activity in immobilized skeletal muscles but failed to suppress the accumulation of poly-Ub conjugates, suggesting that ubiquitination rates were enhanced. This effect is in agreement with previous studies showing that curcumin inhibited 20S proteasome activities in muscles from septic rats or MAC16 tumor-bearing mice [39,40] but did not alter the amount of poly-Ub conjugates in muscles from septic or LPS-treated animals [21,39]. We further demonstrate here that curcumin prevented the increased expression of the proapoptotic protein Smac/DIABLO without any effect on the depressed expression of the antiapoptotic protein XIAP. This selective effect of this anti-inflammatory compound on Smac/DIABLO protein levels increased the ratio of XIAP to Smac/DIABLO in immobilized muscles. Previous work showed that mynocycline, which also exhibit anti-inflammatory properties, increased this ratio and decreased caspase 9 activation in the intact heart exposed to ischemia/reperfusion [41]. Altogether, this suggests that inflammation could trigger the entry into apoptosis through modulating this ratio. Our data also indicate that curcumin did not prevent all processes of the mitochondrial apoptotic pathway during immobilization-induced muscle atrophy: curcumin prevented the trend toward enhanced apoptosome-linked caspase-9 activity in immobilization-induced muscle atrophy, but not the increased caspase-3 activity. Therefore, our work also suggests that the extrinsic pathway of apoptosis, which also leads to caspase-3 activation, is presumably enhanced during immobilization-induced muscle atrophy.

Previous reports indicate that the CSA decreased during immobilization [42,43], hindlimb unloading [44–46] or bed-rest [47]. The present study shows that muscle atrophy and reduced CSA observed after 8 days of immobilization were not prevented by a daily administration of curcumin. However, the efficacy of curcumin on

skeletal muscle atrophy may depend on the route of administration, its bioavailability and/or the catabolic model used [21,39,40]. For example, an ip administration of curcumin (as in the present study) prevented the muscle wasting of LPS-treated mice [21] or septic rats [39]. Similarly, the administration of high doses of the curcumin c3 complex (100 mg/kg body weight) did prevent muscle wasting in MAC16 tumor-bearing mice [40]. This effect on muscle mass was associated with a protective effect of curcumin on fiber CSA in muscles from these cachectic cancer mice [40]. By contrast and in accordance with our data, the administration of curcumin *per os* did not prevent skeletal muscle dysfunction and atrophy in unloaded mice [45], another model of immobilization. In addition to the effect of curcumin on the chymotrypsin-like activity of the proteasome during immobilization, we show here that this compound prevented increased apoptosome-linked caspase-9 and chymotrypsin-like proteasome activities during recovery. This resulted in a beneficial effect on muscle mass and CSA during reloading after cast removal. Altogether, our data suggest for the first time that curcumin treatment is potentially useful for improving subsequent recovery in a model of immobilization. These effects of curcumin, which exhibits anti-inflammatory properties, suggest also that inflammatory processes play key roles in alterations of proteolytic and apoptotic processes during immobilization-induced atrophy and the subsequent recovery.

The p38 MAP kinase and nuclear factor kB pathways are enhanced in immobilized muscles [16,17]. In our work, we showed that curcumin did not prevent muscle atrophy, suggesting that it does not regulate these two pathways in our model. However, we showed that curcumin improved muscle recovery, although the p38 MAP kinase has been reported to be normalized very early during recovery (i.e., 3 day after cast removal) [48]. Altogether, these observations suggest that this compound may act through other pathways to improve muscle mass gain during recovery. Indeed, pathways involving the ERK kinase, the xanthine oxidase or the β-catenin are activated during disuse muscle atrophy [29-31] and are regulated by curcumin [25–27,49]. Moreover, some of them are involved during muscle regeneration such as ERK [50] and β-catenin [51]. Altogether, curcumin may modulate one or several of these pathways during immobilization and recovery and thereby exert its beneficial effect on muscle mass gain.

Curcumin administration at low doses in this study concomitantly inhibited both proteasome chymotrypsin-like and apoptosome-linked caspase-9 activities after cast removal and most importantly improved muscle recovery. Our data further show that curcumin treatment increased muscle XIAP protein levels during reloading above basal levels. This antiapoptotic protein has been recently reported to restrict the apoptotic pathway in skeletal muscle under differentiation [52]. In addition, curcumin stimulated muscle regeneration in vivo and restored the normal tissue architecture after local muscle injury in vivo [24]. Finally, in vitro studies indicate that curcumin can act directly on myoblasts to stimulate cell proliferation as well as fusion and differentiation [24]. Altogether, these observations suggest that curcumin may improve muscle recovery and differentiation/regeneration processes following immobilization by regulating the antiapoptotic XIAP protein of the mitochondrial apoptotic pathway.

In conclusion, our work shows that curcumin treatment during immobilization prevented increased proteasome chymotrypsin-like activity and proapoptotic smac/DIABLO protein levels. During reloading, curcumin treatment blocked the elevated proteasome chymotrypsin-like and apoptosome-linked caspase-9 activities, increased XIAP protein levels and improved the recovery of muscle mass and CSA. This suggests that curcumin may modulate the entry into apoptosis during immobilization and stimulate initial steps of muscle regeneration, which are highly important for muscle function (total strength/fatigability).

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References

[1] Attaix D, Ventadour S, Codran A, Bechet D, Taillandier D, Combaret L. The ubiquitin–proteasome system and skeletal muscle wasting. Essays Biochem 2005; 41:173–86.
[2] Bodine SC, Latres E, Baumhueter S, Lai VKM, Nunez L, Clarke BA, et al. Identification of ubiquitin ligases required for skeletal muscle atrophy. Science 2001;294:1704–8.
[3] Gomes MD, Lecker SH, Jagoe RT, Navon A, Goldberg AL. Atrogin-1, a muscle-specific F-box protein highly expressed during muscle atrophy. Proc Natl Acad Sci U S A 2001;98:14440–5.
[4] Ikemoto M, Nikawa T, Kano M, Hirasaka K, Kitano T, Watanabe C, et al. Cysteine supplementation prevents unweighting-induced ubiquitination in association with redox regulation in rat skeletal muscle. Biol Chem 2002;383:715–21.
[5] Krawiec BJ, Frost RA, Vary TC, Jefferson LS, Lang CH. Hindlimb casting decreases muscle mass in part by proteasome-dependent proteolysis but independent of protein synthesis. Am J Physiol Endocrinol Metab 2005;289:E969–980.
[6] Taillandier D, Aurousseau E, Meynial-Denis D, Bechet D, Ferrara M, Cottin P, et al. Coordinate activation of lysosomal, Ca^{2+}-activated and ATP–ubiquitin-dependent proteinases in the unweighted rat soleus muscle. Biochem J 1996;316:65–72.
[7] Tawa Jr NE, Odessey R, Goldberg AL. Inhibitors of the proteasome reduce the accelerated proteolysis in atrophying rat skeletal muscles. J Clin Invest 1997;100: 197–203.
[8] Vazeille E, Codran A, Claustre A, Averous J, Listrat A, Bechet D, et al. The ubiquitin–proteasome and the mitochondria-associated apoptotic pathways are sequentially downregulated during recovery after immobilization-induced muscle atrophy. Am J Physiol Endocrinol Metab 2008;295:E1181–1190.
[9] Borisov AB, Carlson BM. Cell death in denervated skeletal muscle is distinct from classical apoptosis. Anat Rec 2000;258:305–18.
[10] Siu PM, Pistilli EE, Alway SE. Apoptotic responses to hindlimb suspension in gastrocnemius muscles from young adult and aged rats. Am J Physiol Regul Integr Comp Physiol 2005;289:R1015–1026.
[11] Siu PM, Alway SE. Mitochondria-associated apoptotic signalling in denervated rat skeletal muscle. J Physiol 2005;565:309–23.
[12] Siu PM, Wang Y, Alway SE. Apoptotic signaling induced by H_2O_2-mediated oxidative stress in differentiated C2C12 myotubes. Life Sci 2009;84:468–81.
[13] Kujawa M, Baran W, Jankowska-Steifer E. Morphometric ultrastructural analysis of satellite cells in denervated rat soleus muscle. Exp Mol Pathol 2004;76:166–72.
[14] Allen DL, Linderman JK, Roy RR, Bigbee AJ, Grindeland RE, Mukku V, et al. Apoptosis: a mechanism contributing to remodeling of skeletal muscle in response to hindlimb unweighting. Am J Physiol 1997;273:C579–587.
[15] Charge SB, Rudnicki MA. Cellular and molecular regulation of muscle regeneration. Physiol Rev 2004;84:209–38.
[16] Powers SK, Kavazis AN, Mcclung JM. Oxidative stress and disuse muscle atrophy. J Appl Physiol 2007;102:2389–97.
[17] Bar-Shai M, Carmeli E, Ljubuncic P, Rezmck AZ. Exercise and immobilization in aging animals: the involvement of oxidative stress and NF-Kappa B activation. Free Radic Biol Med 2008;4:202–14.
[18] Juo P, Kuo CJ, Reynolds SE, Konz RF, Raingeaud J, Davis RJ, et al. Fas activation of the p38 mitogen-activated protein kinase signalling pathway requires ICE/CED-3 family proteases. Mol Cell Biol 1997;17:24–35.
[19] Alamdari N, O'Neal P, Hasselgren PO. Curcumin and muscle wasting: a new role for an old drug? Nutrition 2009;25:125–9.
[20] Wyke SM, Russell ST, Tisdale MJ. Induction of proteasome expression in skeletal muscle is attenuated by inhibitors of NF-kappaB activation. Br J Cancer 2004;91: 1742–50.
[21] Jin B, Li YP. Curcumin prevents lipopolysaccharide-induced atrogin-1/MAFbx upregulation and muscle mass loss. J Cell Biochem 2007;100:960–9.
[22] Jana NR, Dikshit P, Goswami A, Nukina N. Inhibition of proteasomal function by curcumin induces apoptosis through mitochondrial pathway. J Biol Chem 2004; 279:11680–5.
[23] Carter RS, Pennington KN, Ungurait BJ, Arrate P, Ballard DW. Signal-induced ubiquitination of I kappaB Kinase-beta. J Biol Chem 2003;278:48903–6.
[24] Thaloor D, Miller KJ, Gephart J, Mitchell PO, Pavlath GK. Systemic administration of the NF-kappaB inhibitor curcumin stimulates muscle regeneration after traumatic injury. Am J Physiol 1999;277:C320–329.
[25] Zhou H, Li X, Gao M. Curcumin protects PC12 cells from corticosterone-induced cytotoxicity: possible involvement of the ERK1/2 pathway. Basic Clin Pharmacol Toxicol 2009;104:236–40.
[26] Souchard JP, Limasset B, Michel F, Crastes de Paulet A, Labidalle S, Nepveu F. Substituted methoxyphenol with antioxidative activity: correlation between physicochemical and biological results. C R Seances Soc Biol Fil 1995;189(6): 1171–81.
[27] Aggarwal BB. Targeting inflammation-induced obesity and metabolic diseases by curcumin and other nutraceuticals. Annu Rev Nutr 2010;30:173–99.

[28] Choi I, Lee K, Kim M, Lee M, Park K. Differential activation of stress-responsive signalling proteins associated with altered loading in a rat skeletal muscle. J Cell Biochem 2005;96:1231–43.
[29] Kondo H, Nakagaki I, Sasaki S, Hori S, Itokawa Y. Mechanism of oxidative stress in skeletal muscle atrophied by immobilization. Am J Physiol 1993;265: E839–844.
[30] Cifuentes-Diaz C, Goudou D, Mège RM, Velasco E, Nicolet M, Herrenknecht K, et al. Distinct location and prevalence of alpha-, beta-catenins and gamma-catenin/-plakoglobin in developing and denervated skeletal muscle. Cell Adhes Commun 1998;5:161–76.
[31] Cai DQ, Lee KKH, Li M, Tang MK, Chan KM. Ubiquitin expression is up-regulated in human and rat skeletal muscles during aging. Arch Biochem Biophys 2004;425: 42–50.
[32] Li YP, Chen YL, John J, Moylan J, Jin BW, Mann DL, et al. TNF-alpha acts via p38 MAPK to stimulate expression of the ubiquitin ligase atrogin1/Mafbx in skeletal muscle. Faseb J 2005;19:362–70.
[33] Zheng B, Ohkawa S, Li H, Roberts-Wilson TK, Price SR. FOXO3a mediates signaling crosstalk that coordinates ubiquitin and atrogin-1/MAFbx expression during glucocorticoid-induced skeletal muscle atrophy. FASEB J 2010;24: 2660–9.
[34] Vignaud A, Cebrian J, Martelly I, Caruelle JP, Ferry A. Effect of anti-inflammatory and antioxidant drugs on the long-term repair of severely injured mouse skeletal muscle. Exp Physiol 2005;90:487–95.
[35] Flint FO, Pickering K. Demonstration of collagen in meat products by an improved picro-Sirius red polarisation method. Analyst 1984;109:1505–6.
[36] Cain K, Brown DG, Langlais C, Cohen GM. Caspase activation involves the formation of the aposome, a large (approximately 700 kDa) caspase-activating complex. J Biol Chem 1999;274:22686–92.
[37] Fang CH, Li BG, Fischer DR, Wang JJ, Runnels HA, Monaco JJ, et al. Burn injury upregulates the activity and gene expression of the 20 S proteasome in rat skeletal muscle. Clin Sci (Lond) 2000;99:181–7.
[38] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265–75.
[39] Poylin V, Fareed MU, O'Neal P, Alamdari N, Reilly N, Menconi M, et al. The NF-kappaB inhibitor curcumin blocks sepsis-induced muscle proteolysis. Mediators Inflamm 2008;2008:317851 (13 pp).
[40] Siddiqui RA, Hassan S, Harvey KA, Rasool T, Das T, Mukerji P, et al. Attenuation of proteolysis and muscle wasting by curcumin c3 complex in MAC16 colon tumour-bearing mice. Br J Nutr 2009;102:967–75.
[41] Scarabelli TM, Stephanou A, Pasini E, Gitti G, Townsend P, Lawrence K, et al. Minocycline inhibits caspase activation and reactivation, increases the ratio of XIAP to smac/DIABLO, and reduces the mitochondrial leakage of cytochrome *C* and smac/DIABLO. J Am Coll Cardiol 2004;43:865–74.
[42] Nicks DK, Beneke WM, Key RM, Timson BF. Muscle fibre size and number following immobilisation atrophy. J Anat 1989;163:1–5.
[43] Tomanek RJ, Lund DD. Degeneration of different types of skeletal muscle fibres. II. Immobilization. J Anat 1974;118:531–41.
[44] Desplanches D, Kayar SR, Sempore B, Flandrois R, Hoppeler H. Rat soleus muscle ultrastructure after hindlimb suspension. J Appl Physiol 1990;69:504–8.
[45] Farid M, Reid MB, Li YP, Gerken E, Durham WJ. Effects of dietary curcumin or *N*-acetylcysteine on NF-kappaB activity and contractile performance in ambulatory and unloaded murine soleus. Nutr Metab (Lond) 2005;2:20.
[46] Thomason DB, Booth FW. Atrophy of the soleus muscle by hindlimb unweighting. J Appl Physiol 1990;68:1–12.
[47] Berg HE, Larsson L, Tesch PA. Lower limb skeletal muscle function after 6 wk of bed rest. J Appl Physiol 1997;82:182–8.
[48] Childs TE, Spangenburg EE, Vyas DR, Booth FW. Temporal alterations in protein signaling cascades during recovery from muscle atrophy. Am J Physiol Cell Physiol 2003;285:C391–398.
[49] Shishodia S, Sethi G, Aggarwal BB. Curcumin: getting back to the roots. Ann N Y Acad Sci 2005;1056:206–17.
[50] Liang KW, Ting CT, Yin SC, Chen YT, Lin SJ, Liao JK, et al. Berberine suppresses MEK/ERK-dependent Egr-1 signaling pathway and inhibits vascular smooth muscle cell regrowth after in vitro mechanical injury. Biochem Pharmacol 2006; 71:806–17.
[51] Kim KI, Cho HJ, Hahn JY, Kim TY, Park KW, Koo BK, et al. Beta-catenin overexpression augments angiogenesis and skeletal muscle regeneration through dual mechanism of vascular endothelial growth factor-mediated endothelial cell proliferation and progenitor cell mobilization. Arterioscler Thromb Vasc Biol 2006;26:91–8.
[52] Smith MI, Huang YY, Deshmukh M. Skeletal muscle differentiation evokes endogenous XIAP to restrict the apoptotic pathway. PLoS One 2009;4:e5097.