Journal of Cellular Biochemistry 86:21–28 (2002)

Development-Dependent Disappearance of Caspase-3 in Skeletal Muscle Is Post-Transcriptionally Regulated

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Abstract Caspase-3, a major player in apoptosis, engages apoptosis-activated cells into an irreversible pathway leading to cell death. In this article, we report that caspase-3 protein is absent from rat and mouse adult skeletal muscles, despite the abundant presence of its mRNA. During skeletal muscle development, caspase-3 protein is present in neonatal animals, but its expression gradually decreases, and disappears completely by 1 month of age, when there is still abundant caspase-3 mRNA. This discordance between caspase-3 message and protein expression is unique to skeletal muscle, as in all other analyzed tissues the protein presence correlates with the presence of the mRNA. The only circumstance in which caspase-3 protein appears in adults is in regenerating muscles; once regeneration is complete, however, it again becomes undetectable in repaired muscles. We conclude that caspase-3 protein in skeletal muscle is uniquely regulated at the post-transcriptional level, unseen in other tissues such as brain, heart, lung, kidney, thymus, spleen, liver, or testis. The post-transcriptional regulation of caspase-3 might serve as a fail-safe mechanism to avoid accidental cell death. J. Cell. Biochem. 86: 21–28, 2002. © 2002 Wiley-Liss, Inc.

Key words: skeletal muscle; development; caspase-3; apoptosis; mRNA; protein; post-transcriptional regulation; muscle regeneration

Caspases are proteases involved in both cytokine processing and apoptosis, identified in mammals by their homology with the *C. elegans* ced-3 death gene. Among caspases, caspase-8, -9, and -10 are involved in the initiation and amplification process of apoptosis, while caspase-3, -6, -7, and probably -2 are involved in executing the apoptotic program and killing the cells [Nuñez et al., 1998; Thornberry and Lazebnik, 1998; Budihardjo et al., 1999]. Caspases are first synthesized as zymogens; upon activation, they are cleaved into two subunits each that assemble into tetramers to form active caspase enzymes [Rotonda et al., 1996; Nuñez et al., 1998; Budihardjo et al., 1999; Nicholson, 1999; Grütter, 2000].

Caspase-3, by far the best-characterized member of the family and the first caspase directly linked to apoptosis in mammalian cells, is only activated in the late stage of apoptosis [Fernandes-Alnemri et al., 1994; Nicholson et al., 1995; Hale et al., 1996]. Once activated, caspase-3 can cleave numerous substrates: poly(ADP-ribose) polymerase (PARP), an enzyme involved in DNA repair; caspase activated DNase (CAD), involved in DNA cleavage; and many other proteins [reviewed in Nicholson, 1999]. Depending on their genetic background, most caspase-3 knockout mice die perinatally, but some survive a few weeks [Kuida et al., 1996; Woo et al., 1998; Zheng et al., 1999]. Caspase-3 knockout mice show supernumerary neurons, as their neurological development is aberrant due to the absence of apoptosis.

Initially, cultured myotubes were thought to be resistant to apoptosis; they were later found

Grant sponsor: National Institute of Aging of the National Institutes of Health (subproject 4); Grant number: AG16871; Grant sponsor: Doctoral Research Award from the Medical Research Council of Canada (Now Canadian Institutes of Health Research).

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Received 31 January 2002; Accepted 8 April 2002
DOI 10.1002/jcb.10211

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to undergo programmed cell death [Wang and Walsh, 1996; Walsh, 1997; McArdle et al., 1999; Ruest et al., 2002]. In vivo, myofiber apoptosis was first observed during development [Kerr et al., 1972; Webb, 1972; McClearn et al., 1995], and later observed in skeletal muscles under conditions such as muscular dystrophy, burn injury, and denervation [Tidball et al., 1995; Migheli et al., 1997; Tews and Goebel, 1997; Tews et al., 1997; Sandri et al., 1998; Yasuhara et al., 1999, 2000]. Caspase-3 is activated in dying muscular dystrophic muscles, and in muscle cells after chronic heart failure [Libera et al., 1999; Mukasa et al., 1999]. However, little is known about the regulation of caspase-3 protein expression and its functions in skeletal muscle during development, aging, or muscle regeneration. In this report, we show that caspase-3 mRNA is always expressed in skeletal muscle, while the protein is only present in early postnatal development and in regenerating muscles. In healthy adult skeletal muscle, caspase-3 protein is always undetectable, despite the fact that its mRNA is abundant. Tissue survey shows that this post-transcriptional regulation of caspase-3 protein expression is only observed in skeletal muscle, while all other analyzed tissues show concordant presence of this protease's mRNA and the protein.

MATERIALS AND METHODS

Tissue Preparation

Tissues were extracted from rapidly sacrificed animals, and immediately frozen in liquid nitrogen or on dry ice and stored at -90°C. Male Fisher 344 rats of various ages from 1 day to 30 months old were used for the caspase-3 time kinetic analysis in skeletal muscle. For the tissue survey study, 3-month-old male Fisher 344 rats were used to collect brain, heart, spleen, liver, thymus, kidney, lung, and testis; C3H mouse skeletal muscles were also obtained, following the same procedure. Preparation of marcaine-injected (bupivacaine hydrochloride) muscles from 4-month-old rat was performed as described in Khalyfa et al. [1999].

Protein Extraction

The following method was used to prepare *tibialis anterior* muscle extracts from donors of various ages and marcaine-injected animals. Five hundred milligrams of muscle tissue was homogenized in cold protein extraction buffer (300 mM sucrose, 150 mM KCl, 30 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 1.5 mM dithiothreitol, 1 mM EDTA, and 1.5% Triton X-100) complemented with 2 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin, 3 μg/ml leupeptin, and 2 μg/ml pepstatin, using a tissue grinder. Afterwards, 150 μg/ml of DNaseI was added to each extract; samples were incubated for 1 h on ice, then sonicated for 1 min, and centrifuged twice at 3,000 rpm for 15 min in a refrigerated micro-centrifuge. The supernatants were collected and used as protein extracts for Western blotting. Protein extraction from 3-month-old rat brain, heart, skeletal muscle (thigh), thymus, spleen, liver, kidney, lung, and testis was accomplished by homogenizing 300–500 mg of tissue in cold RIPA buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, and 1 mM EDTA), containing fresh protease inhibitor (2 mM PMSF, 10 μg/ml aprotinin, and 2 μg/ml of both leupeptin and pepstatin), using a Polytron homogenizer (Kinematica, Luzern, Switzerland). After 1 min of sonication, extracts were centrifuged at 3,000 rpm for 10 min in a refrigerated microcentrifuge; the resulting supernatant was collected and used as protein extract for immunoblotting analysis. Protein concentration was determined by a modified Bradford method, using Bio-Rad reagents according to the manufacturer's protocol, and compared to a γ-globulin standard curve.

Western Blotting

The same procedure described in Ruest et al. [2002] was used for Western blotting. Polyclonal antibodies HT7 (1:3,000 dilution) and CB5 (1:2,000 dilution), reacting against EF-1α/EF1A-1 and S1/EF1A-2, respectively, were generated in our laboratory [Khalyfa et al., 1999]; caspase-3 H-277 (1:1,500 dilution) and caspase-8 H-134 (1:500 dilution) were purchased from Santa Cruz Biotechnology. Polyclonal caspase-1 (1:500 dilution) was purchased from Upstate Biotechnology, and monoclonal β-actin antibody (1:8,000 dilution) from Oncogene.

RNA Extraction and Semi-Quantitative RT-PCR

Between 300 and 500 mg of tissues were briefly homogenized in 2 ml of Trizol reagent (Life Technologies) using a Polytron (Kinematica). Following homogenization, RNA was extracted from 1 ml aliquots of the homogenates, according to the manufacturer's instructions.

RNA concentration was measured by reading the optical density at 260 nm. DNase treatment of all RNA samples was accomplished prior to reverse transcription by adding 0.2 μg of total RNA, 15 U of RNase inhibitor (Amersham-Pharmacia), 0.8 μl of DNaseI (Life Technologies), and DEPC-treated water to yield a final volume of 12 μl. The reaction was performed at 37°C for 30 min. cDNAs were generated from the treated samples by adding 2 μl of 10 × PCR buffer (10 ×: 200 mM Tris-HCl pH 8.4, 500 mM KCl, 20 mM $MgCl_2$, 1% Triton X-100), 1 mM dNTP final concentration (Amersham-Pharmacia), 0.3 μg random nonamers (Alpha DNA, Montréal, Canada), 15 U RNase inhibitors, 200 U M-MLV reverse transcriptase (Life Technologies), to a final volume of 20 μl. Following incubation at room temperature for 10 min, reverse transcription was carried out at 37°C for 1 h. Both DNase treatment and cDNA generation by reverse transcription were performed in a PCR machine (Perkin-Elmer). To the obtained cDNA, 8 μl of 10 × PCR buffer, 200 ng of each sense and antisense primer, Taq polymerase (laboratory clone), 2 μCi of (α-^{32}P) dCTP (NEN/Perkin-Elmer), and water were added to a final volume of 100 μl. All samples were added in a 94°C pre-warmed PCR machine, and incubated for 3 min before starting amplification cycles as follows: denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and elongation at 72°C for 1.5 min. At the end of the last cycle, temperature was maintained at 72°C for another 7 min before cooling the samples at 4°C. The following primers were used to generate a 300-bp caspase-3 fragment: sense 5′ AGAGGCGACTACTGCCGGAGT and antisense 5′ CGTGGCCACCTTCCGGTTAAC (GenBANK accession number: nm_012922). A classic 18S rRNA primer/competimer set from Ambion was used to generate the 488-bp 18S fragment used as a control. A ratio of 3:7 primer/competimer was used. PCR products were loaded onto 8% polyacrylamide–TBE gels for separation, and amplified fragments were revealed on X-ray films. Two negative controls were generated during the reaction; one sample had all the PCR reagents, but no RNA, while the second had all the reagents and RNA, but no reverse transcriptase.

Northern Blotting

Total RNA was extracted as described above. Fifteen micrograms of total RNA extracted from brain, heart, or skeletal muscle of 3-month-old rat were separated on denaturing formaldehyde–agarose gels. After separation, the gel was soaked for 1 h in 150-mM ammonium acetate (transfer buffer), and RNA were transferred overnight onto a Hybond-N nylon membrane (Amersham-Pharmacia) by capillarity. After transfer, RNA was attached to the membrane using a UV cross-linker, according to the manufacturer's instructions (Bio-Rad). Subsequently, the membrane was blocked for 1 h at 42°C in hybridization buffer (1 M NaCl, 50 mM Tris, pH 7.5, 0.1% tetrasodiumpyrophosphate (PPi), 5 × Denhardt's solution, 10% Dextran sulfate, 50% formamide, and 1% SDS, all final concentration) to which boiled salmon sperm DNA was added to 0.25 mg/ml final concentration. After pre-hybridization, the probe was added to the solution and the membrane was incubated for 18 h at 42°C in a rotating oven. The probe was generated by PCR from a sequenced caspase-3 fragment cloned into PCR II vector (Invitrogen), which was used as a template (see PCR procedures above for fragment generation). PCR amplification was accomplished as described above, with the following modifications: 0.25 μg of vector was used instead of cDNA, and the dNTP mixture was depleted of dCTP, which was supplied separately as (α-^{32}P) dCTP (15 μCi) (NEN/Perkin-Elmer). The probe was separated on a 1.5% agarose/TAE (Tris-acetate–EDTA) gel, and the excised fragment was purified using purification columns following manufacturer's instructions (Qiagen). After boiling, the probe was added to the hybridization solution. The following day, the membrane was washed with 2 × SSC and 0.5% SDS as follows: one time 20 min at room temperature with agitation, and three times 15 min at 65°C in the rotating oven. After washing, the hybridized membrane was processed for autoradiography by exposure to radiographic film (Kodak).

RESULTS

Caspase-3 Protein and mRNA Expression in Skeletal Muscle During Development and Aging

Caspase-3 protein expression during development and aging of rat and mouse thigh skeletal muscle was analyzed by Western blotting. In rat, muscle proteins were extracted from animals aged 1, 7, and 15 days and 1, 3, 6, 9, 15, 21, 27, and 30 months. In addition, embryonic

18-day and postnatal 1-, 7-, 14-, 20-, and 28-day mouse muscles were used for analysis. Caspase-3 protein is present at birth in rat skeletal muscle, then progressively declines and becomes undetectable by 1 month of age. The protein remains absent, thereafter (Fig. 1A). S1/EF1A-2 and EF-1α/EF1A-1 were used to visually confirm the age of the tissues, since in skeletal muscle only EF-1α/EF1A-1 is present at birth, while in mature muscle, only S1/EF1A-2 is expressed [Chambers et al., 1998; Khalyfa et al., 2001]. Similarly, in mouse skeletal muscle, caspase-3 protein cannot be detected 14 days after birth (Fig. 1B). Because of the intriguing nature of caspase-3 protein expression in skeletal muscle, we performed Western blot analysis for caspase-1 and -8 proteins, to ascertain whether or not the same pattern would be observed. Unlike caspase-3, caspase-1 and -8 proteins are present in muscle both at birth and in adulthood (data not shown).

Since caspase-3 protein is not detected by immunoblotting in mature rat skeletal muscle, its mRNA expression was analyzed by RT-PCR to determine whether or not the messenger RNA is present. Total RNA was extracted from rat muscles 0.5, 1, 3, 6, 9, and 15 months of age. We used 28 cycles to amplify 18S rRNA, and 32 cycles to amplify caspase-3; this ensured that both were in their linear range of amplification. As observed in Figure 2, caspase-3 mRNA is always present in skeletal muscle. 18S rRNA was used as a positive control, and RNA extracted from 0.5-month-old rat was used as the negative control without reverse transcription, to show that the amplification did not result from nuclear DNA contamination.

Analysis of Caspsase-3 Expression in Rat Tissues

Since caspase-3 protein is absent from mature rat and mouse skeletal muscle despite the presence of its mRNA, various tissues obtained from 3-month-old rats were analyzed to determine whether this situation is unique to skeletal muscle, or a multi-tissue pattern. Brain, heart, skeletal muscle (thigh), thymus, spleen, liver, kidney, testis, and lung were used for analysis. As revealed by Western blot analysis, caspase-3 protein is detected in all analyzed tissues except skeletal muscle (Fig. 3A). S1/EF1A-2 and EF-1α/EF1A-1 were used as controls to identify the tissues, since only brain, heart, and skeletal muscle express S1/EF1A-2 protein [Lee et al., 1992]. β-actin was used as a control to show the presence of proteins in all samples. By RT-PCR, we confirmed the presence of caspase-3 mRNA in all tissues (Fig. 3B). Once again, the result confirmed that caspase-3 mRNA is present in skeletal muscle, but not the protein. Brain total RNA was used as the negative control without reverse transcriptase,

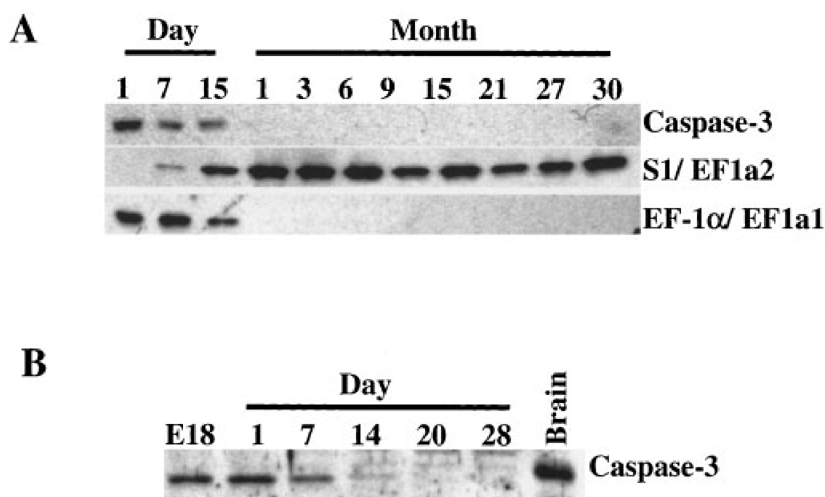

Fig. 1. Caspase-3 protein expression during rat and mouse development and aging. **A**: Immunoblotting analysis of caspase-3 protein expression in rat thigh skeletal muscle during postnatal development and aging. Muscles were taken from 1-, 7-, and 15-day as well as 1-, 3-, 6-, 9-, 15-, 21-, 27-, and 30-month-old animals. S1/EF1A-2 and EF-1α/EF1A-1 were used as controls to demonstrate the age of the donors. **B**: Caspase-3 protein expression in thigh skeletal muscle at embryonic E18 and 1, 7, 14, 20, and 28 days postnatal development in mouse. Brain was used as positive control for caspase-3.

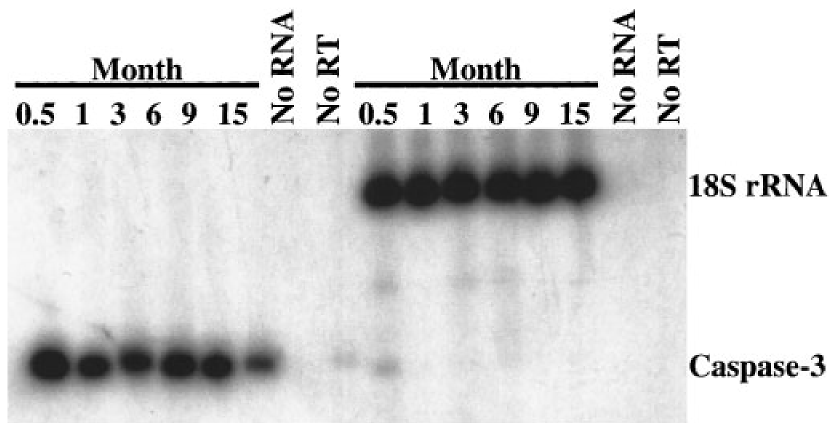

Fig. 2. Detection of caspase-3 mRNA in skeletal muscle. Expression of caspase-3 mRNA in rat skeletal muscle was analyzed by semi-quantitative RT-PCR complemented with (α-^{32}P) dCTP. The acrylamide gels were exposed to X-ray films to reveal the labeled bands. Total RNA was extracted from 0.5, 1, 3, 6, 9, and 15-month-old rat donors. 18S ribosomal RNA was used as control. Amplification of caspase-3 generated a 300-bp fragment, while amplification of 18S generated a 488-bp fragment. Two negative controls were also generated: one sample was depleted of RNA (No RNA), and the second was depleted of reverse transcriptase (No RT), to show that fragment amplifications were generated from RNA transcribed in cDNA.

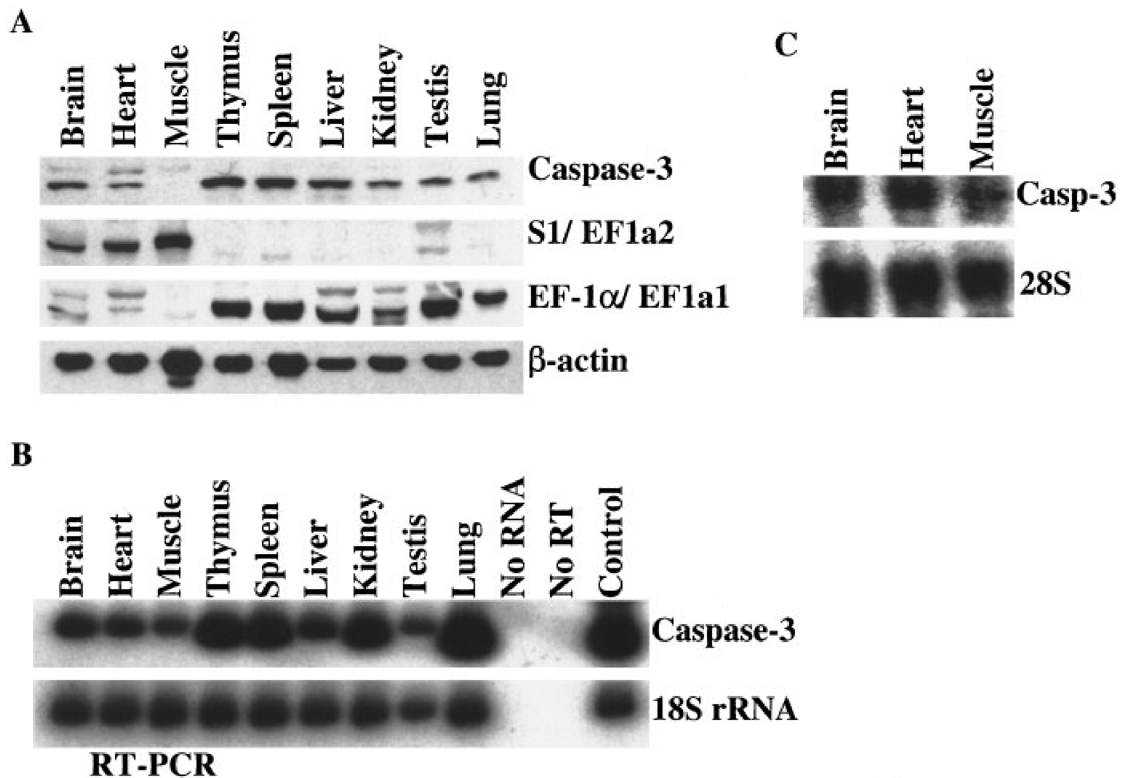

Fig. 3. Caspase-3 analysis in various rat tissues. Caspase-3 protein and mRNA expression was analyzed in 3-month-old Fisher 344 rats. **A**: Caspase-3 protein expression in brain, heart, skeletal muscle, thymus, spleen, liver, kidney, testis, and lung analyzed by immunoblotting after SDS extraction. S1/EF1A-2 and EF-1α/EF1A-1 were used as identification controls, and β-actin was used to demonstrate the presence of proteins in all samples. **B**: Caspase-3 mRNA expression analyzed by RT-PCR in the same tissues. All samples were amplified for 28 cycles using primer/competimer for 18S RNA as a control; caspase-3 samples were amplified for 32 cycles, except for kidney, testis, and lung, where 36 cycles were used. The positive control was generated from rat L6 cells, and the negative controls were generated as described in Materials and Methods section (Brain RNA used in No RT sample). **C**: Caspase-3 mRNA expression analyzed by Northern blotting in 3-month-old rat brain, heart, and skeletal muscle. The PCR-generated probe also unspecifically binds 28S rRNA, shown here as a loading control.

and rat L6 total RNA was used as a positive control. To demonstrate that caspase-3 amplification is not a PCR-generated artifact, Northern blot analysis was performed using brain, heart, and skeletal muscle total RNA from 3-month-old rats. Caspase-3 was detected at about 2.5 kb in all analyzed tissues (Fig. 3C); however caspase-3 mRNA expression was lower in abundance in skeletal muscle. Thus, we confirmed by Northern blot the presence of caspase-3 mRNA in skeletal muscle, as previously published [Juan et al., 1996].

Caspase-3 Protein Expression During Muscle Regeneration

Injection of marcaine (bupivacaine) into skeletal muscle destroys myofibers without affecting innervation [Khalyfa et al., 1999]. Such muscle injury to the existing myotubes induces rapid regeneration by activating satellite cells to divide and fuse, in order to form new myofibers or repair already existing multinucleated muscle cells. Regenerating marcaine-injected rat *tibialis anterior* muscle expresses the 32-kDa caspase-3 zymogen protein, while the *tibialis anterior* muscle from the same animal, but on the other leg (uninjected control) does not (Fig. 4). When the detection reaction is pursued for a longer time, the activated form of caspase-3 (17 kDa) was also detected in marcaine-injected muscle from day 4 to 9 (Fig. 4). The detection of the active form requires a longer exposure to the film; unfortunately this longer exposure increases the background as revealed on the film by the presence of spots. The presence of both caspase-3 zymogen (p32) and activated (p17) form indicates activation of caspase-3 protease in regenerating skeletal muscle.

DISCUSSION

In this report, we show that caspase-3 protein is present at birth in immature rat skeletal muscle, but afterwards its presence gradually decreases. A few weeks after birth, the presence of caspase-3 protein becomes undetectable in skeletal muscle by Western blotting; this is also observed in mouse skeletal muscle. The situation is intriguing, since both caspase-1 and -8 proteins are detected in caspase-3-negative skeletal muscle tissues. Interestingly, in all muscle lacking caspase-3 protein, caspase-3 mRNA is expressed in abundance; caspase-3 mRNA was previously shown in skeletal muscle [Juan et al., 1996]. Thus, in mature skeletal muscle, caspase-3 protein is absent, despite the presence of its mRNA; this suggests that caspase-3 is regulated at the post-transcriptional level. It remains to be established whether the mRNA is translated, or the protein is immediately degraded following biosynthesis. Presence of caspase-3 protein in early postnatal development suggests that the protein is involved in muscle development.

This post-transcriptional regulation of caspase-3 is probably unique to skeletal muscle, since it is the only tissue where the mRNA is detected, but not the protein. The regulation of caspase-3 in skeletal muscle appears to be flexible, since the protein presence reappears in regenerating marcaine-injected muscles (Fig. 4); however, the protein disappears once the regeneration process is completed. The presence of activated caspase-3 in regenerating muscle may be related to the re-sculpting process, to get rid of excess replicating satellite cells or deleting improperly innervated, newly-formed myofibers. Interestingly, this presence of caspase-3 in skeletal muscle correlates with

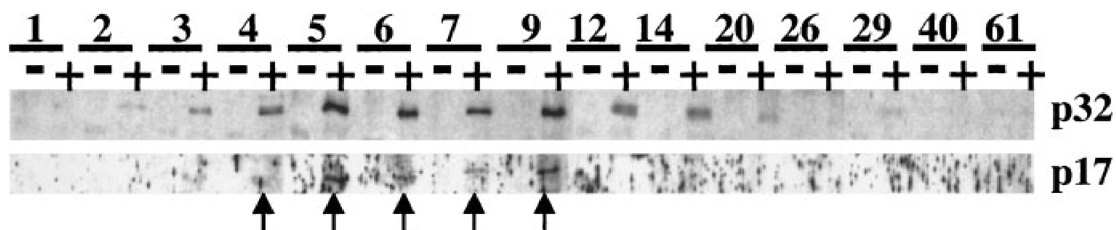

Fig. 4. Caspase-3 protein in regenerating muscle. Caspase-3 32-kDa zymogen protein was detected in regenerating marcaine-injected 4-month-old rat *tibialis anterior* muscle by immunoblotting. The p17 active form of caspase-3 was also detected in regenerating muscles, principally from 4 to 9 days after injection as indicated by the presence of an arrowhead under the corresponding lanes. Numbers represent the number of days post-injection; the minus signs represent the control muscles (left leg of the same animal) and plus signs marcaine-injected muscles (right leg).

the presence of peptide elongation factor EF-1α/EF1A-1, a marker of immature or regenerating muscle, as previously published (Fig. 1A; Khalyfa et al., 1999, for marcaine-injected muscle correlation). Thus, similarly to EF-1α/EF1A-1 [Khalyfa et al., 2001], caspase-3 protein is not expressed in mature skeletal muscle; both proteins follow similar patterns of expression in developing and regenerating skeletal muscle. However, more experiments are needed to unravel the post-transcriptional mechanism regulating caspase-3 translation in skeletal muscle.

Previously, it was found that caspase-3 is involved in muscle cell death, as its activation or presence is observed in muscular dystrophy, after burn injuries, and after heart failure [Libera et al., 1999; Mukasa et al., 1999; Yasuhara et al., 2000]. However, in the light of our results, it appears that the presence of caspase-3 in muscular dystrophy degenerating muscles may be due to the constant regeneration observed in those muscles, since a degeneration/regeneration cycle is completed in about 40 days [McArdle et al., 1995]. The presence of caspase-3 in muscular dystrophic muscles or after burn injury probably renders myotubes susceptible to apoptosis, thus explaining the presence of apoptotic death, as shown by Tidball et al. [1995]. The presence of caspase-3 protein during embryonic development may be important to muscle physiology to remove unwanted myofibers, but its activity is probably later replaced by redundant caspases, since no defects in skeletal muscle organization and structure are observed in caspase-3 knockout mice [Kuida et al., 1996; Woo et al., 1998].

Interestingly, although caspase-3 protein is absent from skeletal muscle, the same does not apply to other caspases. Further studies are needed to determine the mechanism of post-transcriptional regulation of caspase-3 protein in skeletal muscle (whether at the message-processing level after transcription or at the step of message translation). Nevertheless, the post-transcriptional control of caspase-3 serves as a fail-safe mechanism to avoid accidental cell death at the last step of the apoptotic process. This reasoning is based on the fact that caspase-3 acts on the execution of cell death, committing the final decision step for cells to die or survive. Furthermore, the protection against apoptosis in skeletal muscle is also extended by the blockage of the two caspase-3 activation pathways, as caspase-8 inhibitor ARC is expressed, and caspase-9 activator Apaf-1 is absent from skeletal muscle [Koseki et al., 1998; Burgess et al., 1999]. Thus, for healthy muscle, the ultimate protection from accidental death is the inability to produce caspase-3 protein at the final post-transcriptional level.

ACKNOWLEDGMENTS

The authors are grateful to Dr. Bruce M. Carlson for providing marcaine-injected muscles, and to Mr. Alan N. Bloch for proofreading the manuscript. This work was supported by grant #AG16871, subproject 4 from the National Institute of Aging of the National Institutes of Health to EW, and by a Doctoral Research Award from the Medical Research Council of Canada (Now Canadian Institutes of Health Research) to LBR.

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