

# Denervation Stimulates Apoptosis But Not Id2 Expression in Hindlimb Muscles of Aged Rats

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Inhibitors of differentiation (Id) proteins are repressors of myogenic regulatory factors and have been implicated in apoptosis and muscle atrophy during aging. Indeed, we have previously found that Id levels are elevated in muscles from old rodents, possibly as a consequence of loss of  $\alpha$ -motoneurons during senescence. To determine if Id2 proteins increase after denervation and if this is accompanied by increased apoptosis in aged as compared with adult animals, the gastrocnemius and soleus muscles were denervated in 1 limb of Fischer 344  $\times$  Brown Norway rats aged 9 months (adult,  $n = 12$ ) and 33 months (aged,  $n = 9$ ), while the contralateral limb served as the intra-animal control. After 14 days, the muscles in each limb were removed. The levels of Id1, Id2, and Id3 mRNA and protein were significantly greater in muscles of old as compared with young adult rats. Denervation, however, did not significantly increase Id1, Id2, and Id3 mRNA in soleus or gastrocnemius muscles from either young or old rats. Also Id2 protein levels were similar in denervated and control muscles from young adult and old rats. In young adult rats only, denervation induced an increase in Id1 and Id3 protein levels in both the soleus (Id1 113%; Id3 900%) and gastrocnemius (Id1 86%; Id3 80%). Denervation induced a significant increase in caspase 8 in both soleus and gastrocnemius muscles from young (101% and 147%, respectively) and old rats (167% and 190%, respectively). Bax protein levels, as estimated by western blots, increased by 726% and 1087% after denervation in the soleus and by 368% and 49% in the gastrocnemius muscles of young and old rats, respectively. The data suggest that the denervation-induced muscle loss was at least partly due to apoptosis as indicated by elevated caspase 8 and Bax levels in denervated muscles. While Id2 may have a role in aging-induced sarcopenia, Id2 does not appear to directly regulate apoptosis during denervation. The elevated Id expression in muscles from aged animals is therefore not a direct consequence of loss of  $\alpha$ -motoneurons during senescence.

**A**GING is accompanied by fiber atrophy and a decrease in fiber number in skeletal muscles; this condition is called sarcopenia (1,2). A potential mechanism underlying sarcopenia is apoptosis (3,4), which also takes place during muscle unloading (5), heart failure (6), muscular dystrophies (7), spinal cord injury (8), and denervation (9,10). The possibility that the age-related loss of  $\alpha$ -motoneurons (11,12) mediates apoptosis, which occurs during aging, has not been studied.

Apoptosis may be initiated by oxidative damage in mitochondria in heart and skeletal muscles during aging (13,14). Alternative mechanisms have been suggested by Florio and colleagues (15), who provided evidence for a role of inhibitors of differentiation (Id) proteins in apoptosis. Correspondingly, Id protein levels have been shown to be elevated in muscles of old rats (16), and to correlate positively to markers of apoptosis during aging (4,16). Nevertheless, it is not clear if Id proteins play a role in regulating apoptosis in skeletal muscle of aged animals via activation of Bax/Bcl-2 apoptotic pathways, as occurs at least in part in muscle damage and disease (6,17–19).

During denervation, the expression of myogenic regulatory factors (MRFs) and Id proteins have been reported to

increase in muscles from young animals (20–22). The denervation-associated increase in myogenin is attenuated in muscles of old animals (23); however, it is not known, if the elevated Id levels at old age (16) might attenuate an increase in myogenin following denervation in muscles from old rats as compared with muscles in young rats. Because elevated Id1 and Id2 levels are associated with denervation (20–22) and neuronal death (24), respectively, we hypothesized that denervation results in a more pronounced increase in Id levels and apoptosis in muscles of aged animals as compared with muscles of young adults. Furthermore, the elevated level of Id proteins at old age might hamper the denervation-related increase in MRFs. In this study, we report that denervation was associated with apoptosis in fast and slow muscles, but there was no significant increase in Id2 in denervated muscles in either young adult or old animals.

## METHODS

### Animals

Experiments were conducted on young adult (aged 9 months;  $n = 12$ ) and aged (33 months;  $n = 9$ ) Fischer 344  $\times$  Brown Norway F1 hybrid male rats (Harlan, Indianapolis,

IN). The rats were housed separately in specific pathogen-free conditions at 20–22°C with a 12-hour light/dark cycle. They were fed rat chow and water ad libitum.

#### *Denervation of Soleus and Gastrocnemius Muscles*

Rats were placed under a general anesthesia of 2% isoflurane and 1.5 L of oxygen/minute. After reflex activity had disappeared, the tibial nerve was dissected proximal to the cranial border of the gastrocnemius muscle. Care was taken to avoid disruption of blood vessels and the tibial nerve. The medial and lateral branches of the tibial nerve that innervate the medial and lateral heads of the gastrocnemius and soleus muscles were transected as close to their entry point in the muscle belly as possible (25). The sectioned nerves were reflected proximally and sutured to a segment of the hamstring muscles with 4–0 silk sutures to ensure that the nerve stumps did not reinnervate the plantar flexor muscles. Innervation to the plantaris and deep toe flexor muscles was left intact so that the animal could ambulate normally around their cages. Following surgical denervation, the hamstring muscle layers were closed with reabsorbable suture and the skin incision was closed with 9-mm wound clips. The contralateral limb served as the intra-animal control. Animals received 0.3 mg of buprenex (s.c.) as an analgesic at the end of the surgery. The animals recovered quickly and were alert and walking within ~45 minutes postsurgery.

Preliminary studies showed that significant atrophy occurred in soleus and gastrocnemius muscles 14 days postdenervation. Furthermore, this was beyond the earlier time-points when muscles could have been affected by surgery or the immediate responses to denervation. Because we also wanted to avoid slowed atrophy with aging reported with long-term denervation (26), and investigate potential mechanisms regulating the rapid loss of muscle mass, we chose to examine muscles after 14 days of denervation. The soleus and gastrocnemius muscles in each limb were removed 14 days postoperatively while the animals were heavily anesthetized (ketamine hydrochloride, 9 mg/100 g body wt, and xylazine hydrochloride 1 mg/100 g body wt). The muscles were quickly weighed, frozen in liquid nitrogen, and then stored at –80°C. Animals were subsequently euthanized with an overdose of pentobarbital sodium (60 mg/kg, i.p.). All experiments carried approval from the institutional animal use and care committee from West Virginia University School of Medicine. The animal care standards were followed by adhering to the recommendations for the care of laboratory animals as advocated by the American Association for Accreditation of Laboratory Animal Care and following the policies and procedures detailed in the *Guide for the Care and Use of Laboratory Animals*, as published by the U.S. Department of Health and Human Services and proclaimed in the Animal Welfare Act (PL89-544, PL91-979, and PL94-279).

#### *Isolation of RNA and Generating cDNA*

Semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis was conducted as described in detail elsewhere (16,27). Briefly, total RNA was extracted from muscles, treated with Dnase I (Ambion, Austin TX) to remove any contamination by DNA, and reverse-transcribed

(RT) with oligo dT primers according to standard methods (Invitrogen, Bethesda, MD). Control RT reactions were done in which the RT enzyme was omitted.

#### *Semiquantitative RT-PCR for Id and MRF Genes*

PCR was conducted for myogenin, MyoD, Id1, Id2, and Id3 using primers that were generated against published Gene Bank sequences as previously described (4,16,27). One µl of cDNA was then amplified by PCR using 100 ng of each primer, 250 µM dNTPs, 1× PCR buffer, and 2 units *Taq* polymerase (Sigma Chemical Co., St. Louis, MO) in a final volume of 50 µl. The number of PCR cycles was determined for each gene from RNA isolated from both young and old animals so that analyses were done in the linear range of amplification. Amplification of cyclophilin was used as a negative control because cyclophilin levels do not differ between muscles of aged and young adult rats (28). We also repeated the RT-PCR analyses using 18S as an internal control. For these experiments, we made cDNA from total RNA using random primers and amplified 18S primer pairs and competitor to the primers in the PCR reaction along with the gene of interest according to the manufacture's protocols (Ambion). The gene of interest was expressed as a ratio to the 18S signal in the same PCR product. Although the signal ratios of a gene normalized to cyclophilin differed from the same gene normalized to 18S under these conditions, the relative aging-associated and denervation-induced differences between muscles of aged and young adult rats was similar when the RT-PCR signals were normalized to either cyclophilin or 18S. Therefore, we have reported the PCR signals normalized to cyclophilin from the same PCR product. The cDNA from all muscle samples were amplified simultaneously. Following amplification, 10 µl of each reaction was electrophoresed on 1.5% agarose gels. Gels were stained with ethidium bromide, the images were captured, and the signals were quantified in arbitrary units (AU) as optical density × band area, using Kodak 1D image analysis system (Eastman Kodak Company, Rochester, NY). PCR signals were normalized to the cyclophilin signal of the corresponding RT product to provide a semiquantitative estimate of gene expression.

#### *Western Blot Analyses*

Muscle samples were minced on ice and homogenized in ice-cold T-PER tissue protein extraction buffer (Pierce Biotechnology, Rockford, IL) containing 1 mM each of protease inhibitors aprotinin, leupeptin, and phenylmethanesulfonyl fluoride. Solubilized protein extracts were quantified in duplicate by using bicinchoninic acid reagents (Pierce Biotechnology) and bovine serum albumin standards. Forty µg of soluble protein was loaded on each lane of a 10%–12% polyacrylamide gel and separated by routine SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) for 1.5 hours at 20°C (16). The gels were blotted to nitrocellulose membranes (Bio-Rad, Hercules, CA), blocked in 5% milk, and probed with either antimyogenin clone FD5 (Hybridoma Bank, Iowa University, IA), at a concentration of 3 µg/ml in Tris-buffered saline containing 0.5% Tween 20, or anti-MyoD, Id1, Id2, Id3, and Bax (BD PharMingen, San Diego, CA), at a concentration of 2 µg/ml in Tris-buffered

Table 1. Body Weight and Muscle Mass Characteristics

	Soleus				Gastrocnemius			
	Control		Denervated		Control		Denervated	
	Young Adult	Aged Adult	Young Adult	Aged Adult	Young Adult	Aged Adult	Young Adult	Aged Adult
Muscle wet weight/ body weight (mg/g)	0.49 ± 0.01	0.29 ± 0.03*	0.29 ± 0.03 <sup>†</sup>	0.20 ± 0.02* <sup>†</sup>	4.51 ± 0.24	1.82 ± 0.17*	2.72 ± 0.11 <sup>†</sup>	1.49 ± 0.10* <sup>†</sup>
Wet weight (mg)	218 ± 7	140 ± 13*	122 ± 8 <sup>†</sup>	96 ± 7* <sup>†</sup>	1982 ± 130	926 ± 112*	1212 ± 74 <sup>†</sup>	742 ± 55* <sup>†</sup>

Data represent denervated soleus and gastrocnemius muscle data and the contralateral control limb muscles obtained from 12 young adult and 9 aged rats. Values are mean ± SEM (standard error of mean).

\* Significantly different from young adult at  $p < .05$ .

<sup>†</sup> Significantly different from control muscle.

saline, 0.5% Tween 20 in 2% milk. Secondary antibodies were conjugated to horseradish peroxidase (Chemicon International, Temecula, CA) and the signals were developed by chemiluminescence (Bio-Rad). The signals were visualized by exposing the membranes to x-ray film (BioMax MS-1, Eastman Kodak Company), and digital records of the films were captured with a Kodak 290 camera. The bands were quantified as optical density × band area by a 1D-image software package (Eastman Kodak Company). A subset of protein samples was run on SDS-PAGE gels and stained with coomassie blue to confirm that protein loads were similar in each lane. The membranes were also stained with Ponceau S (Sigma Chemical Co.) to confirm similar loading and transfer in each lane.

#### Assessment of Caspase 3,7,10 and Caspase 8

Caspase 3,7,10 and caspase 8 were measured using commercial colorimetric apoptosis assay kits (bioWorld, Dublin, OH), using the free dye of 7-amino-4-trifluoromethyl coumarin (AFC) as a standard, according to the procedures outlined by the manufacturer. Briefly, 10 mg of tissue was homogenized in the lysis buffer supplied with the kit. Lysates were incubated in 50 μM of the AFC-conjugated substrate at 22°C and read at 308 nm using a Dynex MRX plate reader controlled through PC software (Revelation, ThermoLabsystem, Franklin, MA). All analyses were done in duplicate and averaged for each muscle. Samples were incubated in fluormethyl ketone as a negative control. Control and experimental animals were run on the same microplate. Data were given as units/μg soluble protein/minute. The data were expressed as folds increase of the experimental muscle versus the intra-animal control muscle.

#### Statistical Analyses

A two-way analysis of variance was used to examine aging and denervation differences across normalized PCR signals, and western blot and caspase levels for each muscle using SPSS software, version 10.0 (SPSS, Inc., Chicago, IL). Bonferroni post hoc analyses were conducted when significant age effects were found. Significance level was set at  $p < .05$ .

## RESULTS

#### Body Weight and Muscle Characteristics

Aged rats were 28% heavier and had lower muscle to body/weight ratios as compared with old animals before

denervation. Absolute muscle mass and muscle mass expressed relative to body weight was significantly less in both soleus and gastrocnemius of old rats as compared with young adult rats. The denervation-induced loss of soleus muscle weight was greater in young adult as compared with aged rats ( $42 \pm 5\%$  vs  $31 \pm 3\%$ ). Similarly, the denervation loss of gastrocnemius weight was greater in young adult ( $47 \pm 10\%$ ) as compared with aged rats ( $18 \pm 4\%$ ) (Table 1). Relative to body weight, the decrease in muscle mass with denervation was larger in both the soleus and the gastrocnemius muscles of young animals ( $40 \pm 2\%$ ,  $27 \pm 13\%$ , respectively) compared with aged animals ( $31 \pm 4\%$ ,  $17 \pm 5\%$ , respectively).

#### Semiquantitative RT-PCR of MyoD and Myogenin in Denervated Muscles

MyoD and myogenin mRNA levels were significantly greater in control muscles of aged rats as compared with young adult rats. In young adult rats, MyoD and myogenin mRNA were significantly increased by ~53% and 823%, respectively, in the denervated soleus muscles, and both significantly increased by ~21% and 170%, respectively, in the denervated gastrocnemius muscles, as compared with control muscles (Figure 1). The absence of an age-by-denervation interaction for MyoD expression indicates that the denervation-associated increase in MyoD in the soleus (53%) or gastrocnemius (15%) of old rats was similar to that in young rats. In contrast, there was a significant ( $p < .05$ ) age-by-denervation interaction for myogenin, which was reflected as a blunted denervation-induced increase in myogenin mRNA at old age; myogenin mRNA was elevated by only 55% (young 823%) and 22% (young 170%) after denervation in the soleus and in the gastrocnemius, respectively, in aged rats (Figure 1).

#### Myogenin and MyoD Protein Levels

MyoD and myogenin were identified in western blots as immunoreactive bands of ~45 kDa and ~34 kDa, respectively. MyoD and myogenin were detectable in all muscles, and MyoD levels were greater in the soleus of old versus young adult rats. In young adult animals, denervation significantly increased soleus MyoD and myogenin by ~166% and 304%, whereas in the gastrocnemius, MyoD and myogenin increased significantly by ~156% and 531%. Aging markedly suppressed the denervation-associated increase in MyoD and myogenin protein levels in the

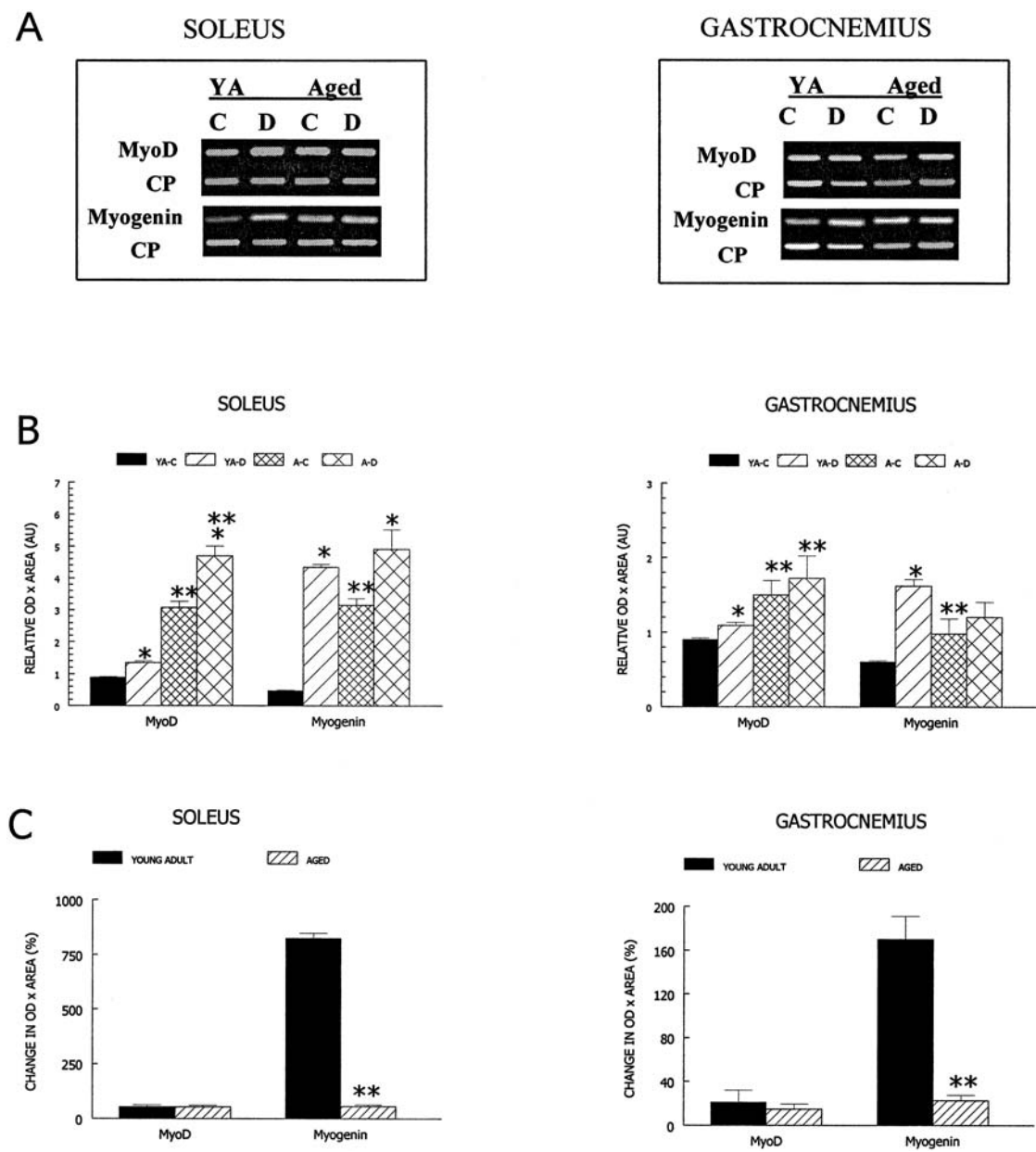


Figure 1. **A**, Representative gels for the soleus (left panel) and gastrocnemius (right panel) from contralateral control [C] and denervated [D] muscles from young adult (YA;  $n = 12$ ) and aged rats (A;  $n = 9$ ). Polymerase chain reaction (PCR) products were visualized with ethidium bromide. Samples were run in duplicate on different gels for each gene. Semiquantification of PCR products was obtained by densitometric analysis of the signal product area  $\times$  optical density. **B**, Results of reverse transcriptase-PCR for MyoD and Myogenin normalized to cyclophilin in young adult control [YA-C], young adult denervated [YA-D], aged control [A-C], and aged denervated [A-D] rat soleus and gastrocnemius muscles. **C**, Percent change in MyoD and myogenin mRNA levels after denervation. All data are presented as mean  $\pm$  standard errors. \*  $p < .05$ , control versus denervated muscles of the same age group; \*\*  $p < .05$ , young adult versus aged adult muscles from the same experimental condition.

gastrocnemius as compared with young adult muscle samples as shown by an age-by-denervation interaction for MyoD ( $p < .02$ ). In the soleus muscle, a significant ( $p < .05$ ) age-by-denervation interaction was found for myogenin but not for MyoD, which was evident as an attenuated denervation-induced rise in myogenin at old age (Figure 2).

RT-PCR of Id Genes

The signals obtained for the repressor genes were all normalized to the corresponding cyclophilin signal. Semi-

quantitative RT-PCR analysis indicated that the levels of Id1, Id2, and Id3 mRNA were significantly greater in muscles of old as compared with young adult rats. Id1, Id2, and Id3 did not change significantly in the soleus muscle following denervation in either young or old rats (Figure 3). Similarly, in the gastrocnemius muscle, Id1, Id2, and Id3 mRNA levels did not change in denervated muscles from either young adult or aged rats as compared with the corresponding intra-animal control muscles.

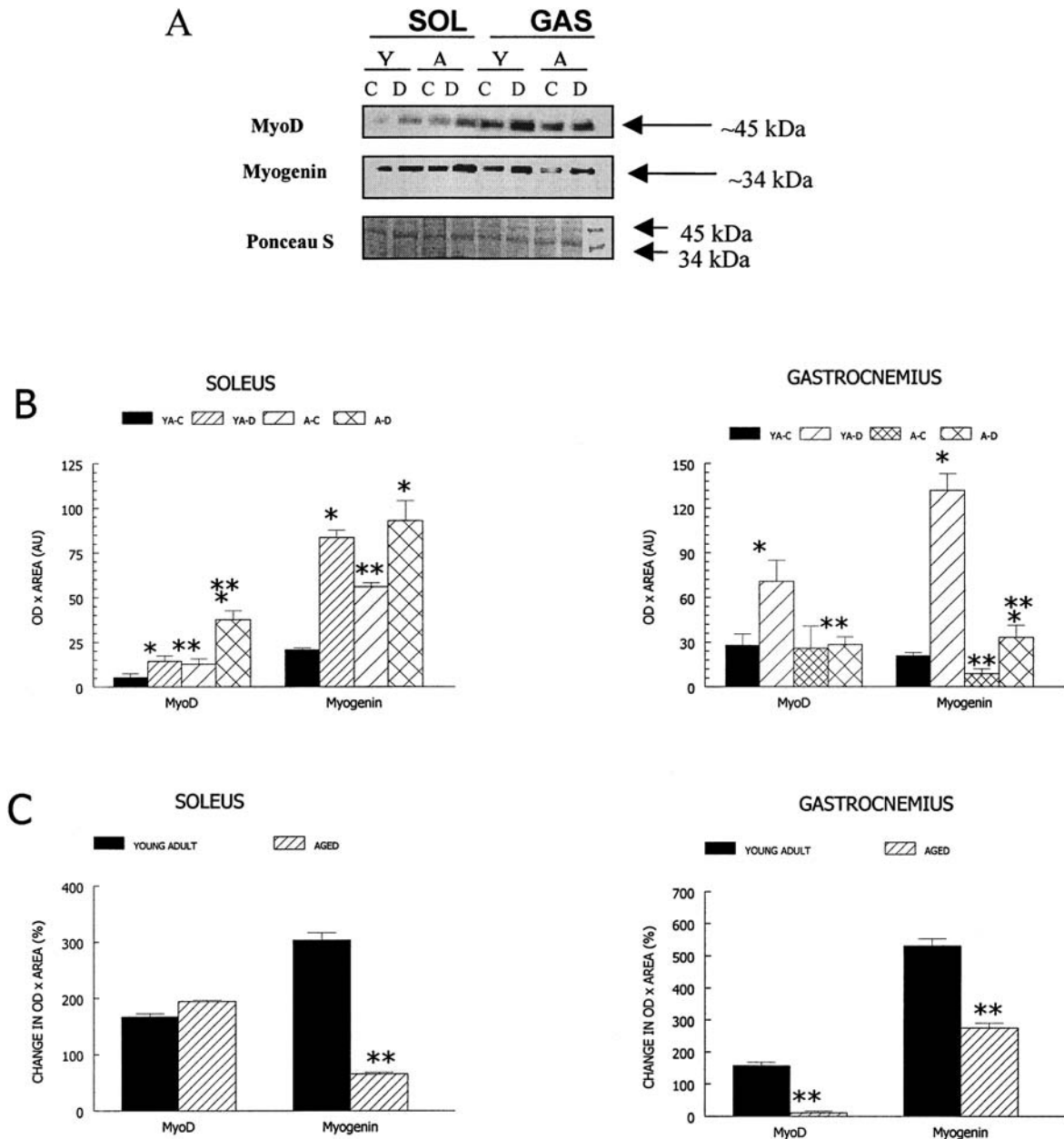


Figure 2. **A**, Representative western blots for MyoD and myogenin from control [C] and denervated [D] soleus (SOL) and gastrocnemius (GAS) muscles of young adult (Y;  $n = 12$ ) and aged (A;  $n = 9$ ) rats. Forty  $\mu$ g of soluble protein extracts was loaded on each lane, separated by SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis), transferred to PVDF (polyvinylidene difluoride) membranes or nitrocellulose membranes, and incubated with either antimyogenin or anti-MyoD primary antibodies. The signals were developed by chemiluminescence. The resulting bands were quantified as band optical density  $\times$  band area (OD  $\times$  area), as determined by Kodak 1D-image software. The membrane was stained with Ponceau S after electrotransfer to confirm that protein loads were similar in each lane. **B**, MyoD and myogenin protein levels in young adult control [YA-C], young adult denervated [YA-D], aged control [A-C], and aged denervated [A-D] rat soleus and gastrocnemius muscles. **C**, The denervation-induced change in MyoD and myogenin protein levels are expressed as percent change in OD  $\times$  area from control muscles. Data are expressed as mean  $\pm$  SEM (standard error of mean). \*  $p < .05$ , control vs denervated muscles of the same age group; \*\*  $p < .05$ , young adult versus aged muscles from the same experimental condition.

### Id Repressor Protein Levels

Aging-associated increases in Id proteins were observed in both the gastrocnemius and soleus muscle. Id2 in rat soleus muscles was ~1300% greater in old as compared with young adult soleus muscles, and 340% greater in gastrocnemius muscles of old versus young adult rats. Id1 was 260% greater in soleus of old rats versus muscles of

young adult rats, and 370% greater in gastrocnemius samples of old rats versus young adult rats. Id3 was 1900% greater in soleus of old rats compared with young adult rats and 126% greater in gastrocnemius of old rats compared with young adult rats.

Id2 was similar in denervated and control muscles from young adult and old rats. In young adult rats, protein levels

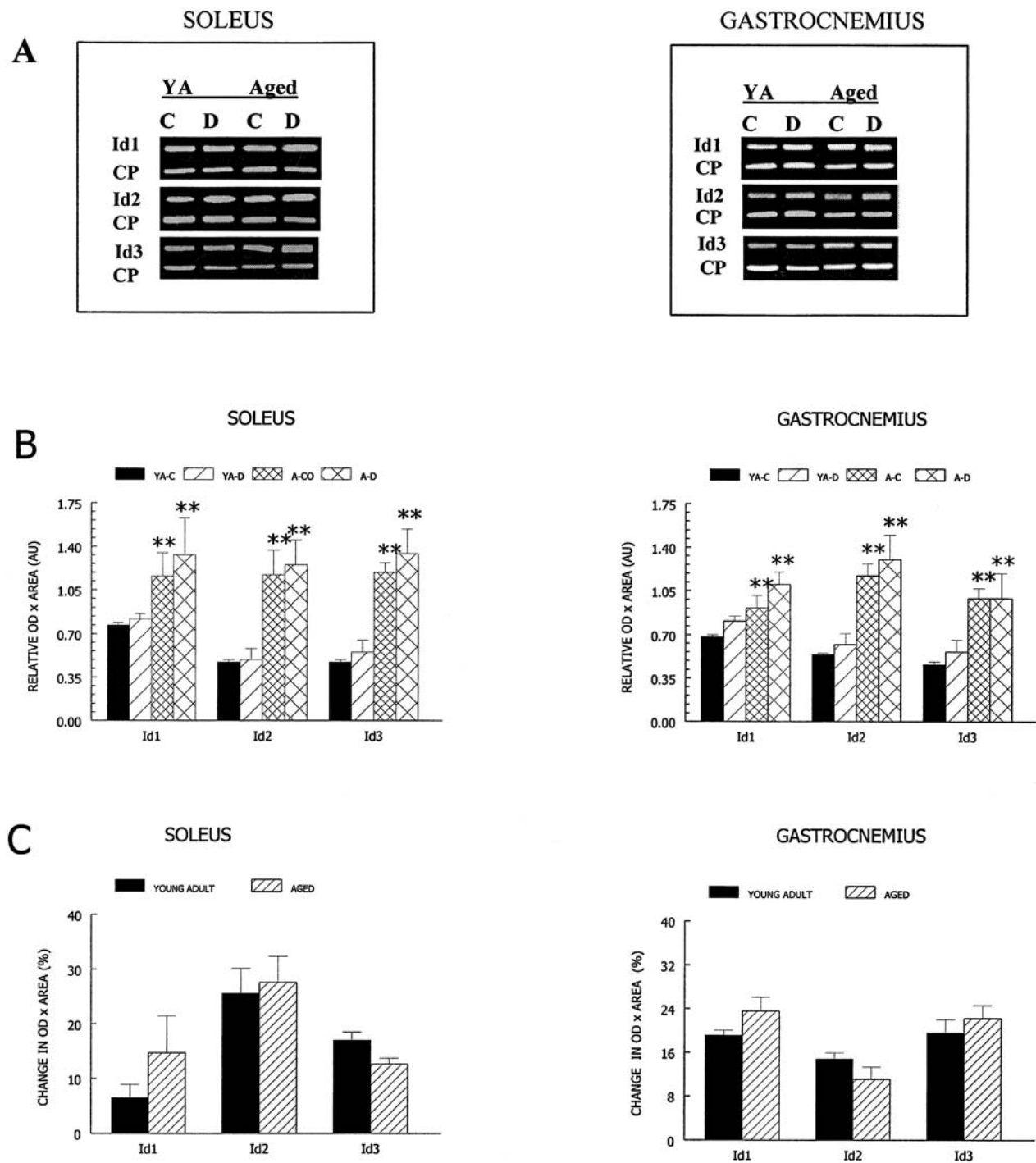


Figure 3. **A**, Representative agarose gels for Id1, Id2, and Id3 are shown with the corresponding cyclophilin product for control [C] and denervated [D] soleus (left panel) and gastrocnemius muscles (right panel) for young adult (YA;  $n = 12$ ) and aged (A;  $n = 9$ ) rats. **B**, Semiquantitative expression of Id1, Id2, and Id3 mRNAs in young adult control [YA-C], young adult denervated [YA-D], aged control [A-C], and aged denervated [A-D] rat soleus and gastrocnemius muscles as determined by reverse transcriptase-polymerase chain reaction (RT-PCR) amplification. The expression of each PCR product was normalized to the cyclophilin signal of the corresponding RT product. The resulting bands were quantified as band optical density  $\times$  band area (OD  $\times$  area), as determined by Kodak 1D-image software. **C**, The denervation-induced changes in Id1, Id2, and Id3 protein levels are expressed as percent change in OD  $\times$  area from control muscles. Data are expressed in arbitrary units (mean  $\pm$  SEM [standard error of mean]). \*\*  $p < .05$ , young adult versus aged muscles from the same experimental condition.

of Id1 and Id3 were estimated to be increased significantly by  $\sim 113\%$  and  $900\%$  in the denervated soleus and by  $86\%$  and  $80\%$  in denervated gastrocnemius relative to control muscles (Figure 4). The age-by-denervation interactions for

Id1 and Id3 ( $p < .05$ ) were apparent as a more-pronounced denervation-induced increase in Id1 ( $136\%$ ) in the gastrocnemius muscle and an absence of a rise in Id3 in the soleus muscle of old rats.

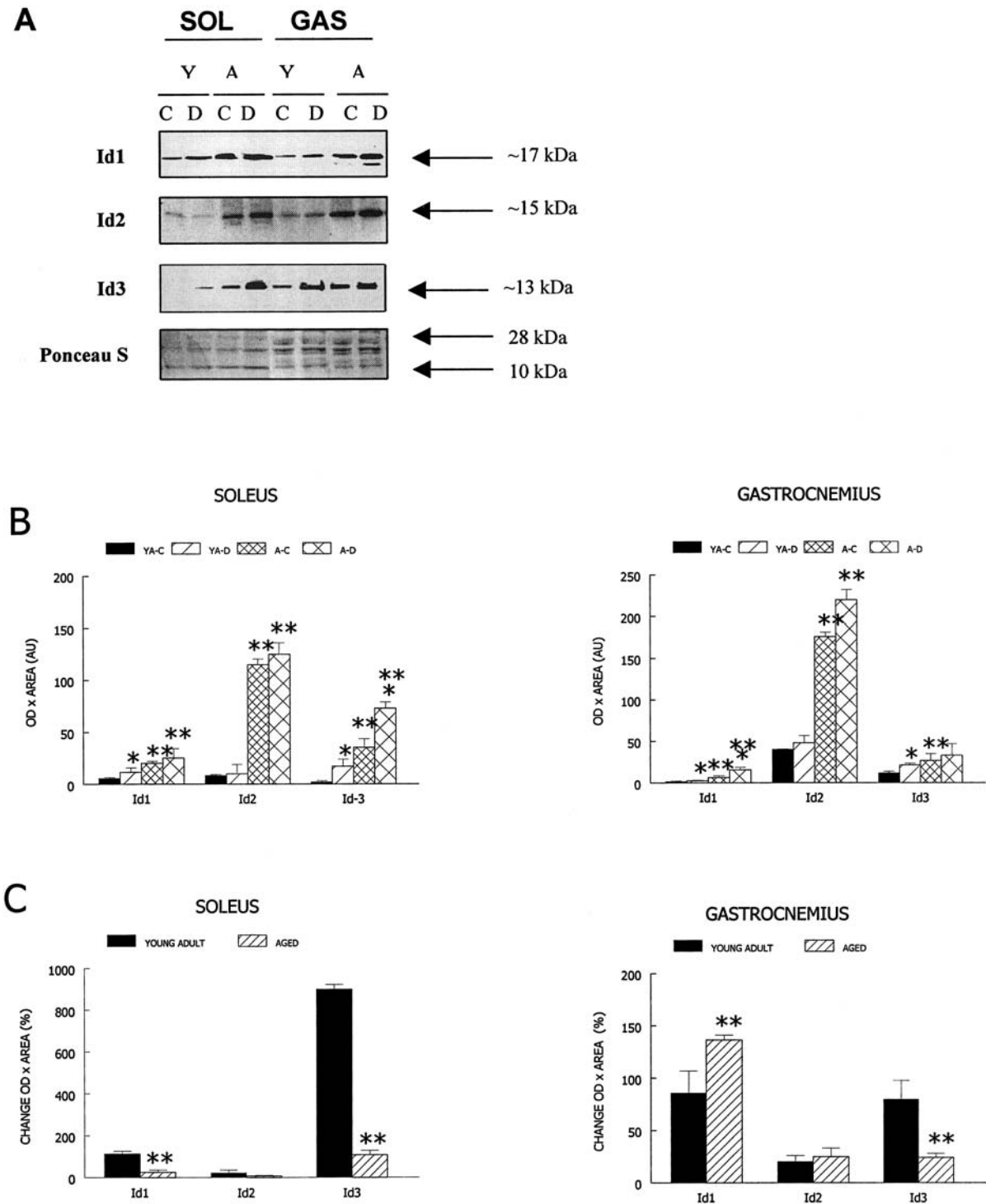


Figure 4. **A**, Representative western blots for Id1, Id2, and Id3 from control [C] and denervated [D] soleus (SOL) and gastrocnemius (GAS) muscles of young adult (Y;  $n = 12$ ) and aged (A;  $n = 9$ ) rats. Forty  $\mu$ g of soluble protein extracts was loaded on each lane, separated by SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis), transferred to PVDF (polyvinylidene difluoride) membranes or nitrocellulose membranes, and incubated with the appropriate primary antibody. Incubation was conducted with secondary antibodies conjugated to alkaline phosphatase, and the resulting signals were developed by chemiluminescence and exposed to x-ray film. Ponceau S staining of membranes after electrotransfer of proteins shows that similar quantities of protein were loaded in each lane. **B**, Summary of western blot data for Id1, Id2, and Id3 protein levels in young adult control [YA-C], young adult denervated [YA-D], aged control [A-C], and aged denervated [A-D] rat soleus and gastrocnemius muscles. The resulting bands were quantified as band optical density  $\times$  band area (OD  $\times$  area), as determined by Kodak 1D-image software. **C**, The denervation-induced changes in Id1, Id2, and Id3 protein levels are expressed as percent change in OD  $\times$  area from control muscles. Data are expressed as mean  $\pm$  SEM (standard error of mean). \*  $p < .05$ , control versus denervated muscles of the same age group; \*\*  $p < .05$ , young adult versus aged adult muscles from the same experimental condition.

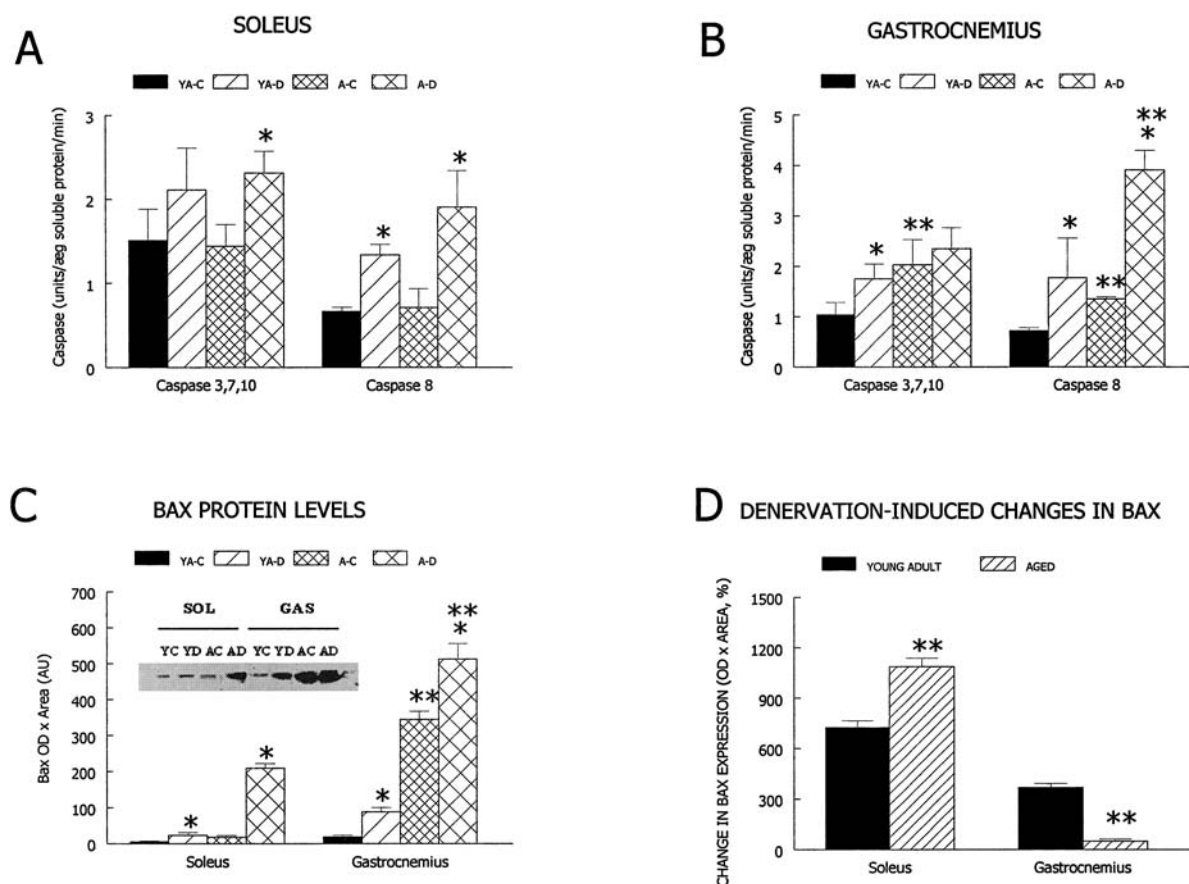


Figure 5. Caspase 3,7,10 and caspase 8 were measured in soluble muscle extracts from the soleus (A) and gastrocnemius (B) muscles of young adult control [A-C], young adult denervated [YA-D], aged control [A-C], and aged denervated [A-D] muscles. Data are mean  $\pm$  SEM (standard error of mean) of young adult ( $n = 12$ ) and aged ( $n = 9$ ) rats. C, Bax protein western blot analysis of soleus and gastrocnemius muscle samples. The resulting bands were quantified as band optical density  $\times$  band area (OD  $\times$  area), as determined by Kodak 1D-image software. Data are expressed in arbitrary units (mean  $\pm$  SEM [standard error of mean]). The insert shows a representative western blot for Bax for young adult control [YC], young adult denervated [YD], aged control [AC], and aged denervated [AD] rat soleus (SOL) and gastrocnemius (GAS) muscles. Forty  $\mu$ g of soluble protein extracts was loaded on each lane, separated by SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis), transferred to PVDF (polyvinylidene difluoride) membranes or nitrocellulose membranes, and incubated with the appropriate primary antibody. Incubation was conducted with secondary antibodies conjugated to horseradish peroxidase, and the resulting signals were developed by chemiluminescence and exposed to x-ray film. D, The percent change in Bax protein levels as estimated by western blot analysis is shown for the control and denervated samples obtained from soleus and gastrocnemius muscles from young adult and aged rats. \*  $p < .05$ , control versus denervated muscles of the same age group; \*\*  $p < .05$ , young adult versus aged adult muscles from the same experimental condition.

### Markers of Apoptosis

Caspase 3,7,10 and caspase 8 were measured using commercial colorimetric apoptosis assay kits, and data were expressed as arbitrary units/ $\mu$ g soluble protein/minute. The age-by-denervation interaction for the caspase 3,7,10 in the soleus ( $p < .05$ ) was apparent as similar caspase 3,7,10 levels in young soleus muscles, and a significant denervation-induced 60% rise in old soleus muscles. In the gastrocnemius, on the other hand, the age-by-denervation interaction ( $p < .05$ ) was revealed by a 68% greater level of caspase 3,7,10 in denervated gastrocnemius muscles of young rats and no change in gastrocnemius samples from old rats. Caspase 8 increased significantly in denervated soleus and gastrocnemius of young (101% and 147%, respectively) and old muscles (167% and 190%, respectively). Caspase 8 levels were significantly higher in the gastrocnemius of old versus young rats in both control and denervated conditions (Figure 5).

Bax protein levels were significantly elevated in muscles of old animals. Bax protein levels were 520% and 1700% greater in control soleus and gastrocnemius muscles of old versus young adult rats. Bax levels were 790% and 480% greater in denervated soleus and gastrocnemius muscles of old as compared with young adult rats. Bax protein levels as estimated by western blots increased by 726% and 1087% in denervated soleus and by 368% and 49% in denervated gastrocnemius muscles of young and old rats, respectively, as compared with the corresponding intra-animal control muscles (Figure 5).

### DISCUSSION

#### Id Repressor Expression

We hypothesized that Id2 expression is sensitive to the innervation status of a muscle and that denervation induces an upregulation of Id2 expression. Here we show, however,



that denervation does not result in increased Id2 mRNA and protein levels in muscles from both young adult and old rats. The absence of an elevated Id2 expression cannot be explained by reinnervation as the severed nerve stumps were sutured to inappropriate muscles preventing reinnervation of the original muscles. Therefore, we conclude that Id2 transcription in skeletal muscle is not regulated by the innervation status of the muscle. Thus, the elevated Id2 expression in old muscles, consistent with our previous observations in rats (4,16) and birds (29), is most likely not the result of loss of  $\alpha$ -motoneurons that accompanies aging (11,12). Yet, it lends further support to the notion that Id2 may have a role in aging-associated muscle atrophy but not in the denervation–reinnervation process.

### Apoptosis

Our results are consistent with other studies showing evidence for apoptosis in postmitotic skeletal muscle with aging (3,4,30) and muscle atrophy and denervation (10,31). The denervation-associated loss of muscle weight was quite similar for both age groups. Also, the denervation-induced changes in protein composition were qualitatively similar, as reflected by comparable changes in myosin heavy-chain composition in muscles from young adult and old rats (data not shown).

The denervation-induced atrophy was at least partly due to apoptosis as indicated by the increase in the proapoptotic proteins Bax, caspase 3,7,10, and caspase 8 in both soleus and gastrocnemius muscles from young adult and old rats following denervation. These results are consistent with reports of denervation-induced apoptosis, as shown by an elevation in caspase 8 and lower Bcl2 levels (32) in rat forelimb muscles with a severed brachial plexus and in denervated muscle fibers associated with spinal muscle atrophies (31,33). The apoptosis might be mediated by tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) signaling via the death domain receptor, since it has been reported that this pathway contributes to muscle loss in elderly humans, where TNF- $\alpha$  suppression by resistance exercise attenuated muscle wasting (34). The increase in the mitochondrial-specific apoptotic protein Bax suggests that the denervation-induced apoptosis was also regulated via mitochondrial pathways. Because the soleus muscle has primarily type I myosin heavy chain and therefore more mitochondria than the gastrocnemius, it is not surprising that the relative increase in Bax following denervation was larger in the soleus than in the gastrocnemius muscle. Bax may also contribute to muscle loss via inhibiting muscle signaling for differentiation and repair but using pathways that are independent of caspase (35). Further experiments are required to determine if members of the Bcl2 family are differentially involved in denervation-induced atrophy in muscles of old rats as compared with muscles in young rats, or if caspase-independent pathways involving apoptosis-inducing factor (36) or PW1 (35) may also be involved in the regulation of denervation-induced apoptosis.

### Id2 and Apoptosis

It is generally thought that if signals for proliferation are too strong, apoptosis will occur (37,38). Indeed, over-

expression of Id proteins has been shown to induce apoptosis in cultured myeloid cells (15), fibroblasts (39), neonatal cardiomyocytes (40), smooth muscle cells (41), and myoblasts (42). Recent data, however, indicate that enhanced levels of Id2 were related to proliferation of epithelial cells in Id2-deficient mice (43) and satellite cells in stretch-overloaded quail skeletal muscles (29). Together, these data suggest that Id over-expression may, depending on the cell environment and status, initiate either apoptosis (15,29,44) or proliferation (29,45–47).

Previously, we provided evidence that the elevated Id levels in skeletal muscle at old age may have a role in pathways leading to apoptosis, and thereby contribute to the age-related loss of muscle mass (4). Consistent with the preferential atrophy of fast fibers during aging (1) is our observation that caspase 3,7,10 and caspase 8 increased in the gastrocnemius, but not in the slow soleus muscle during aging. The age-related increase in Id proteins, however, occurred in both fast and slow muscles (4,16), indicating that the increase in muscle Id proteins is not fiber-type specific, but rather an age-specific feature of skeletal muscle.

In young animals, the elevated caspase 8 in denervated muscles was accompanied by an increased expression of both Id1 and Id3, hinting to a role of these proteins in denervation-induced apoptosis. At old age, however, the elevated caspase 8 in the denervated gastrocnemius muscle was not accompanied by an increase in any Id protein. This suggests that, in contrast to their role in apoptosis at young age, Id proteins play no major role in the denervation-induced apoptosis at old age. Alternatively, it is possible that Id1 and Id3 may have redundant roles, being involved in denervation-induced apoptosis of slow (soleus) and fast (gastrocnemius) muscle, but aging modulates this. Our results generally agree with the previously reported role of Id1 in regulating muscle atrophy (21,22). The absence of a denervation-induced change in Id2 mRNA or protein expression indicates that Id2 plays no major role in denervation-induced apoptosis. Furthermore, the similar Id2 expression in normal and denervated muscles rules out the possibility that aging-associated elevations in Id2 result from loss of  $\alpha$ -motor neurons at old age (11,12).

It is notable that the denervation-induced changes in Id protein levels did not parallel the increase in their mRNA levels. Apparently, the level of Id proteins is not only controlled transcriptionally, but is also post-transcriptionally regulated. It is possible that part of the post-transcriptional control occurs via enhanced activity of the ubiquitin–proteasome pathway as is observed during aging (48) and some conditions of muscle wasting (49).

### Regulation of Muscle Transcription by Id Proteins

In addition to apoptosis, the elevated Id repressor levels during aging and denervation might cause muscle wasting by sequestration of E proteins. The limited availability of E proteins for heterodimerization with MRFs may reduce MRF binding to E boxes on muscle genes (50), thereby reducing transcription of these genes. Since MRFs also positively regulate their own expression, the attenuated denervation-induced increase in myogenin and MyoD at old age might also be due to the sequestration of E proteins by

the elevated Id expression. The observation that MRF protein levels in muscles from old rats were lower despite higher transcript levels as compared with young adult rats (16) indicates also that post-transcriptional control of MRF protein levels contributes to age-related muscle wasting. Finally, we cannot rule out the possibility that Id proteins are elevated, at least in part, to offset the denervation-induced atrophy by enhancing muscle growth (51) via increasing satellite cell proliferation (29,52,53) and recapitulation of the developmental process (44,54). It is unlikely, however, that satellite cell proliferation occurred in the denervated muscles, as we observed increased rather than reduced Bax, which has been reported to accompany satellite cell activation (55).

### Conclusion

Sarcopenia has, in part, been attributed to loss of  $\alpha$ -motoneurons (11,12) and subsequent denervation. The present study and other recent reports (3,4,10,30,31) suggest that muscle cell apoptosis may also contribute to sarcopenia-induced muscle atrophy. However, the pathways leading to apoptosis in skeletal muscle have not been elucidated. In this study, we show that Id2 levels and markers for apoptosis are elevated with aging in rat skeletal muscles, and denervation induces increases in markers of apoptosis in muscles from both young and old animals. Nevertheless, we cannot rule out the possibility that proapoptotic proteins might increase concurrent with atrophy rather than acting as a primary cause of denervation-induced atrophy.

In young and aged animals, the increases in indicators of apoptosis might be related to an increased expression of Id1 and Id3 proteins. The absence of rise in Id2 expression indicates that Id2 does not mediate the denervation-induced increase in the muscle apoptotic markers caspase 8 and Bax and/or atrophy in the muscles of old rats. This study does not address the possibility that the age-associated elevation in Id2 could have an indirect role in sarcopenia by contributing to the loss of  $\alpha$ -motoneurons, which occurs with aging.

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