Id2 expression during apoptosis and satellite cell activation in unloaded and loaded quail skeletal muscles

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ABSTRACT. Inhibitor of differentiation-2 (Id2) is a basic helix-loop-helix protein that acts as a negative regulator of the myogenic regulatory transcription factor (MRF) family but Id2 has also been implicated in apoptosis in several cell lines. In this study, we tested the hypothesis that Id2 has a role in both in apoptosis associated muscle atrophy and also in muscle hypertrophy. A weight corresponding to 12% of the body weight was attached to one wing of Japanese quail to induce hypertrophy in the patagialis (PAT) muscle. Birds in group 1 were sacrificed after 5 (n=8), 7 (n=10) or 14 days (n=10) of loading. The left wing was loaded for 14 days in group 2 birds, then the weight was removed and the PAT was examined after 7 (n=10), 14 (n=10) or 21 (n=5) days of unloading. A time-released bromodeoxyuridine (BrdU) pellet was implanted subcutaneously upon wing weighting to identify activated satellite cells during loading. The left wing was loaded for 14 days, unloaded for 14 days and then the weight was reattached for a subsequent 7 (n=10) or 14 days (n=10) in group 3 birds. BrdU was implanted upon the second loading phase in this group. Id2 mRNA as measured by kinetic PCR increased by 3.9, 2.7, and 1.6 fold, relative to control levels after 7, 14 and 21 days of unloading (group 2). Id2 protein as estimated by western blots increased by 1.5, 1.4 and 0.75 fold after 7, 14 and 21 days of unloading (group 2). Muscle unloading induced apoptosis, because poly(ADP-ribose) polymerase-(PARP)-positive nuclei increased and caspase 8 levels increased by 2.6 fold and 1.7 fold after 7 or 14 days of unloading respectively (group 2). Although BrdU positive nuclei increased during loading (group 1 and group3), 50% failed to survive during unloading (group 2). Id2 mRNA increased by 2.2 and 1.8 fold after 5 and 7 days of loading, respectively, but decreased to control levels by 14 days of loading in group1. Id2 protein levels increased 2.1 fold after 5 days of loading (group 1). In contrast, Id2 did not increase in reloaded muscles of group 3 birds. These data suggest that Id2 may have a role in apoptosis-associated atrophy of skeletal muscles but its role in muscle hypertrophy is less clear.
INTRODUCTION

Inhibitors of differentiation (Id) proteins are basic helix-loop-helix proteins that act as negative regulators of cell differentiation. However, recent data suggest a wider biological role for Id proteins, including apoptosis. For example, Id proteins possess pro-apoptotic properties in a variety of non-muscle cell lines (25,45) as well as cardiac myocytes (59). It also functions as cooperating or dominant oncoproteins in immortalization of rodent and human cells and in tumor induction in Id-transgenic mice (44). Therefore, it is also possible that the Id family may play a critical role in regulating skeletal muscle atrophy via apoptosis.

We have recently proposed a role for Id repressors in skeletal muscle apoptosis and sarcopenia (9) because Id levels are correlated to muscle wasting (10) and we and others have shown increases in markers of apoptosis in muscles of aged animals (9,22,49). Because we have found high levels of Id2 in sarcopenic muscles of aged rodents (9,10), we were interested in determining if Id2 might be involved in general pathways leading to apoptosis in muscle during periods of unloading, but under conditions were hypertrophied muscle undergoes atrophy to return to control levels of muscle mass.

Limb unloading is a common means to induce atrophy in skeletal muscles. In rodents this is usually achieved by hindlimb unweighting (4,11,24,60) or immobilization (24). Presumably, the rapid loss of muscle fiber cross-sectional area and fiber number during unloading indicates that the atrophying myofibers have activated pathways leading to decreased rates of synthesis and increased degradation of myofibrillar proteins (50,63). Unloading leading to atrophy as compared to control muscles is associated with a decrease in nuclei number (30). Allen and colleagues identified TUNEL positive nuclei in rat skeletal muscle after hindlimb unloading leading to muscle atrophy as compared to control muscles, which suggests that some of the nuclei were lost via apoptosis (4). Nevertheless, hindlimb unloading in rodents results in severe muscle atrophy so
that the mass of unloaded muscles decreases to levels that are well below control muscle mass levels. Reductions in hypertrophied muscles to near control levels and restoration of muscle mass to hypertrophied levels after a period of unloading have not been examined in detail, and this type of stimulus may differ from that where unloading induces atrophy below control levels (6,30,41). The biochemical signals regulating apoptosis during unloading in skeletal muscle have not been well studied and it is important to know if muscle loss, from a beginning hypertrophic state back to control mass levels will invoke apoptotic pathways, or if apoptosis occurs only when muscles atrophy to levels lower than control mass levels. Therefore, in the current study we examined Id2 in experimentally unloaded muscles, which had first achieved hypertrophy to determine if Id2 was involved in unloading-induced atrophy.

In an apparent paradoxical role to apoptosis, Id2 levels are low in control muscles but become elevated in overloaded muscles of young adult rats undergoing hypertrophy (9,10). This is consistent with a dual role for Id repressor genes as has been reported in mouse 32D.3 myeloid cells (25). Furthermore, Id proteins are involved in cell proliferation and in the timing of differentiation during neurogenesis, lymphopoiesis and angiogenesis in various cell lines (38,42,46) including myoblasts (40).

In this study, we tested the hypothesis that Id2 has a dual role in both muscle hypertrophy and muscle atrophy. Since Id repressor levels increase with muscle wasting induced by aging (10), tetrodotoxin and denervation (17), our first hypothesis was that if Id2 repressors were involved in pathways leading apoptosis, Id2 should be elevated as part of a general program involving muscle loss and apoptosis of muscle nuclei. Our second hypothesis was that if Id2 is involved in pathways leading to proliferation of satellite cells, Id2 should be increased during muscle loading, when satellite cell activation is high.

We chose to test these hypotheses using the quail wing loading-unloading model because
in quails, satellite cells or muscle precursor cells (MPCs) are activated during stretch-induced hypertrophy (18) and our pilot studies indicated that the number of muscle nuclei decrease during unloading. This is consistent with observations that the number of myofiber nuclei increases during hypertrophy (5,51) and decreases during muscle atrophy (6,30,41), presumably to maintain a constant nuclear to cytoplasm ratio. Furthermore, the loading conditions of wing weighting can be easily varied in birds, and wing loading does not interfere with eating, or ambulation of the birds. This reduces the potential for conditions other than loading to affect the muscle levels of the genes of interest. Unlike rodent models of surgical ablation or denervation to induce hypertrophy [e.g. reviewed in (36)], stretch overload leading to muscle hypertrophy is induced without surgical intervention, hypertrophy is largely independent of innervation, and the hypertrophy can be easily reversed in the quail model, by simply unloading the wing (7).

These studies were conducted in the quail patagialis (PAT) muscle. The PAT is a twitch muscle composed primarily (~85%-90%) of fibers containing fast myosin (13,33,56) and it shares functional characteristics that are similar to mammalian twitch skeletal muscles (8). The PAT originates on the scapula and inserts onto the ulna and it primarily acts as a flexor of the humeral-ulnar joint. Wing loading stretches the PAT and this results in muscle hypertrophy (8,33,37,56).

In this study, we show that following hypertrophy via wing loading, wing unloading induces PAT muscle loss in part by apoptosis, as measured by increased caspase 8, caspase 3,7,10 and poly(ADP-ribose) polymerase (PARP) staining. Increased Id2 mRNA and protein levels also accompanied PAT muscle loss. The initial wing weighting that resulted in hypertrophy was accompanied by increased satellite cell activation, and elevated Id2 levels. However, the relationship between Id2 and satellite cell activation is unclear, because, satellite cells were activated in the PAT during reloading without significant changes in Id2 expression.
MATERIALS AND METHODS

Animals. Coturnix quail were hatched and raised in pathogen-free conditions at West Virginia University School of Medicine. The birds were provided with food and water ad libitum. They were housed at a room temperature of 22°C with a 12 hour light-dark cycle. Young adult birds ages 8-12 weeks of age were examined. 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 (AAALAC) and fully conformed with the American Physiological Society's "Guiding Principles for Research Involving Animals and Human Beings" (1).

Experimental muscles. The PAT muscle is flexed with the wing on the birds' back at rest, but it is stretched when the wing is extended. In our experimental model of stretch-overload, we place a tube containing 12% of the bird's body weight over the left humeral-ulnar joint (12). This maintains the joint in extension throughout the period of stretch and it also induces some stretch at the origin of the PAT muscle. Wing loading results in hypertrophy of the PAT muscles (12,56). The unstretched right PAT muscle serves as the intra-animal control muscle for each bird.

Weighting, unweighting, and bromodeoxyuridine (BrdU). Three groups of birds were examined. The left wing was loaded in group 1 and birds were sacrificed by an overdose of pentobarbital after 5 days (5W, n=8), 7 days (7W, n=10), or 14 days (14W, n=10) of stretch overload. A subcutaneous time-released BrdU pellet (0.22 µg BrdU.g⁻¹ body mass.day⁻¹; Innovative Research, Sarasota, FL USA) was implanted subcutaneously in each bird while anesthetized with 2% isoflurane, at the point when the wing was first weighted. BrdU is a
thymidine analogue and is incorporated in nuclei during DNA synthesis. BrdU was therefore used to identify activated satellite cells during periods of muscle growth. The left wing was loaded for 14 days in group 2 and then the weight was removed. BrdU was implanted at the point of initial wing weighting for birds in group 2. Birds in group 2 were sacrificed after 7 days, (7U, n=10), 14 days (14U, n=10) or 21 days (21U, n=5) of unloading. Birds in group 3 had the left wing loaded for 14 days, then the weight was removed for 14 days, and finally the wing weight was reattached to the left wing. BrdU was implanted in group 3 birds concurrent with the second loading period (reloading of the limb). The birds in group 3 were sacrificed after either 7 days (7R, n=10) or 14 days (14R, n=10) of reloading.

**Muscle weight and preparation.** The PAT muscles were dissected from the surrounding tissue, removed, blotted, weighed and frozen in isopentane cooled to the temperature of liquid nitrogen then stored at −80°C until used for analyses. Frozen 8 µm tissue cross-sections from left and right muscles were placed on the same glass slide to control for processing differences (e.g. incubation time or temperature, etc.). The remainder of the tissue was processed for isolation of total RNA or total protein.

**Kinetic-polymerase chain reaction (PCR) assays.** Total RNA was extracted from PAT muscles in TriReagent (Molecular Research Company, Cincinnati OH, USA). RNA was treated with DNAAse I (2224, Ambion, Austin TX USA) to remove any DNA contamination and quantified at 260 nm (the 260/280 ratio was ~1.96-2.0). Superscript II reverse transcriptase (8064071, Invitrogen Life Tech., Bethesda, MD USA) was used to make cDNA according to the manufacture’s recommendations. Kinetic (real-time) PCR was conducted using SYBR® Green PCR Reagents kit (4304886, Applied Biosystems Inc., Foster City CA, USA) on a GeneAMP
5700 sequence detector (Applied Biosystems Inc., Foster City CA, USA). Primers (Invitrogen Life Tech., Bethesda, MD USA) were designed against myogenin (forward 5' CGTGCACAGTCCTCCCATGGA 3'; reverse 5' GCAAGCGGGAGCCAGGAAGT 3'), MyoD (forward 5' CGGCCGCCGATGACTTCTATGAC 3'; reverse 5' TCCAGGTCTCAGAAGGATGCTATGT 3'), myosin light chain (MLC, forward 5' CATGCCGTCTCAGAAGGATGCTATGT 3'; reverse 5' GCCGAAGTCACTAAACTCAAG 3'), Id2 (forward 5' GAATTCTCAGATGAAAGCTTTCAGCC 3'; reverse 5' CTCGAGATTCAGCAGACAGCCGCT 3') and cyclophilin (forward 5' GA GGGCCGACGCCGATCTTA 3'; reverse 5' GCCGAAGGACCAGCATGACG 3'). All primers were designed with an annealing temperature of 58-60°C. PCR amplification and sequences were optimized over a range of cDNA templates and primer concentrations, and the PCR products were verified. The threshold for kinetic detection was set to occur over linear amplifications over several ranges of primers and RNA levels. Preliminary experiments were conducted to optimize the conditions (i.e. RNA and primer concentrations, etc.) giving maximum change in fluorescence ($\Delta R_n$), absence of non-specific amplification, and equal amplification efficiencies of the genes of interest and cyclophilin. Absence of non-specific amplification was confirmed by PCR product analysis using agarose gel electrophoresis. All samples were run in duplicate, with control and experimental samples run on the same plate.

The relative quantification of gene expression was calculated using the comparative $C_T$ method as described in detail elsewhere (34). Briefly, $C_T$ value reflects the cycle number at which a significant increase in $\Delta R_n$ (i.e. fluorescence) is first detected. DNA copy number and $C_T$ values are inversely related. Validation methods were conducted over a 10-fold range of cDNA and over a two-fold range of primer concentrations to confirm that, the efficiency of the genes of interest and cyclophilin were equal. A sample was considered positive at the cycle in which the
change in the fluorescence of SYBR® Green exceeds an arbitrary threshold value. The threshold value was set at the midpoint of the run and the cycle number plot. The reproducibility of the assay was tested by analysis of variance (ANOVA) comparing repeat runs of samples, and mean values generated at individual time points were compared by a Student’s t-test. Comparative Cₜ calculations for MyoD, myogenin, MLC and Id2 were expressed relative to the cyclophilin signal generated from the same cDNA sample. To achieve quantitative values, Cₜ values were first averaged from duplicate runs for each sample. The average Cₜ for the gene of interest was subtracted from the corresponding averaged cyclophilin Cₜ value for that sample to give a ∆Cₜ value. ∆∆Cₜ values were achieved by subtracting the control (right) ∆Cₜ value from the experimental (left) ∆Cₜ value. ∆∆Cₜ values that were 2.0 indicated a two-fold difference between experimental and intra-animal control muscles. Therefore, a left-right ∆∆Cₜ >2.0 were considered to represent a significant change in gene expression in the experimental as compared with the control level of gene expression. This provided a reasonable difference in gene expression between intra-animal samples, which, exceeds the potential errors existing with the kinetic PCR quantification method. Setting a higher threshold of differences in gene expression for significance would have decreased the sensitivity for identifying potentially lower differences in gene levels that may have biological relevance during loading or unloading.

Western blot analyses. Western blotting was conducted as reported previously (10) with only minor modifications. 60 µg of soluble protein was loaded on each lane of a 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel and separated for 1.5 h at 20°C (10). 10 µg of an Id2 peptide (Santa Cruz, CA) was loaded on one lane of each gel and this was used as a positive control for Id2 western blots. The gels were blotted to nitrocellulose membranes (1620097, Bio-Rad, Hercules CA, USA), and stained with Ponceau S (P7170, Sigma, Chemical Co. St Louis, MO,
USA) to confirm similar loading and transfers in each lane. The membranes were probed with a polyclonal antibody against Id2, (C-20, Santa Cruz Biotech, Santa Cruz, CA, USA), at a concentration of 3 µg.ml⁻¹ overnight at 4°C. The signals were developed by chemiluminescence (34084, Pierce Biotechnology, Rockford, IL, USA) using peroxidase conjugated secondary antibodies (A132P, Chemicon International, Temecula, CA, USA) and then the membranes were exposed to X-ray film (BioMax MS-1, Eastman Kodak Company, Rochester, NY, USA). The resulting bands were quantified as optical density x band area with Kodak imaging software (Eastman Kodak Company, Rochester, NY) and expressed in arbitrary units.

Assessment of Caspase 3, 7, 10 and 8. Caspase 3,7,10 and caspase 8 were measured by commercial colorimetric apoptosis assay kits (526118, 526126, bioWorld, Dublin, OH, USA), using the free dye of 7-amino-4-trifluromethyl 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 380 nm using a Dynex MRX plate reader controlled through PC software (ThermoLabsystem, Franklin MA, USA). All data were read in duplicate and averaged for each muscle. Samples were incubated in fluromethyl ketone as a negative control. Control and experimental animals were run on the same microplate. Data were calculated from the OD change.min⁻¹ and the data are expressed in arbitrary units.mg protein.min⁻¹.

Immunocytochemistry. Activated satellite cells have incorporated BrdU into their DNA. Immunocytochemistry was used to identify immunopositive BrdU nuclei (555627, BD Biosciences PharMingen, San Diego CA, USA), at a concentration of 10 µg.ml⁻¹, by methods routinely used in our laboratory (18,33,35). BrdU will also label fibroblasts and other mitotically active cells in the interstitium; however, these cells would reside outside of muscle fibers. Labeled nuclei were
quantified if they could clearly be associated with either the periphery or interior of a muscle fiber. All of the nuclei from 6 non-overlapping fields were quantified with light microscopy at an objective magnification of 40X. The BrdU labeling index was expressed as a percent of the total nuclei and determined by: (the number of BrdU positive nuclei associated with muscle fibers) / (labeled + unlabeled nuclei associated with muscle fibers) x 100.

PARP-1 is a zinc-finger nuclear protein activated by DNA breaks that is highly expressed in the nucleus [Reviewed in (55)]. The enzyme poly (ADP-ribose) polymerase (PARP) utilizes β-nicotinamide adenine dinucleotide (βNAD⁺) to form nicotinamide and poly (ADP-ribose) polymers (54). Under basal conditions PARP-1 participates in genome repair (20). During apoptosis, transient stimulation of PARP-1 causes poly(ADP-ribose) accumulation and a depletion of NAD⁺ and ATP and ultimately cell death (29). Caspases cleave PARP-1 into two fragments, of 24kDa and 89kDa. PARP positive nuclei were examined to determine if nuclei in loaded or unloaded muscles had undergone apoptosis. PARP positive nuclei were identified by immunocytochemistry using a polyclonal rabbit, anti-PARP p85 fragment antibody (G7341, Promega, Madison WI, USA) at a dilution of 1:100 according to the manufacturer's recommendations. Color development was via routine immunocytochemistry using avidin-biotin and diaminobenzidine (PK6101, Vector Laboratories Burlingame, CA, USA). The nuclei were counterstained with hematoxylin (CATHE-M, Walnut Creek, CA, USA). The data were expressed as a PARP labeling index. This was determined by counting the number of PARP positive nuclei to total fibers / total number of nuclei x 100. The PARP index for each muscle was calculated from 6 non-overlapping fields taken a microscope objective of x 40.

Statistical Analyses. The data were examined with an ANOVA (time point x experimental condition) using SPSS software, version 10.0. The within subject variable was experimental
induction of hypertrophy/atrophy and the between animal variable was time. Bonferroni post-hoc analyses were conducted when significant time effects were found. Significance level was set at p<0.05. For real-time PCR, $\Delta\Delta CT >2.0$ were represented significant differences from time 0. The data are presented as mean ± SEM.

RESULTS

**Muscle mass.** Left PAT muscle mass was 14 ± 5%, 23 ± 6% and 44 ± 10% greater than the corresponding right (control) muscles in 5W, 7W 14W birds, respectively. In group 2 birds, the left muscle mass was 17 ± 4% (P<0.05) greater than control mass after 7 days of unweighting but it was not different than control mass in 14U and 21U birds. Muscle mass in the left PAT of group 3 was 33 ± 2% (7R) and 34 ± 4% (14R) greater than right muscles (Figure 1).

**BrdU positive nuclei.** BrdU positive nuclei associated with the muscle fibers (i.e., not fibroblasts) were taken to indicate activated satellite cells. BrdU positive nuclei rarely were present in right PAT muscles of any birds. The BrdU labeling index was 5.3% and 7.4% of the total nuclei population in 7W and 14W birds, respectively (Figure 2). The labeling index was 4.7%, 3.9% and 1.8% in the left PAT muscles from 7U, 14U and 21U birds, respectively, in group 2. The BrdU labeling index in left muscles in group 3 birds was 6.4% and 6.9% in 7R and 14R birds, respectively (Figure 2).
Quantification of MyoD, myogenin and MLC mRNA. MyoD, myogenin and MLC mRNA were determined via real-time PCR. A change of 2.0 in $\Delta\Delta C_T$ represents a two-fold difference between the genes expressed in experimental and control muscles. A significant left-right muscle $\Delta\Delta C_T$ value was set at $> 2.0$ to account for potential methodological errors inherent in real-time PCR quantification methods. The $\Delta\Delta C_T$ value for group 3 birds were less than 1.0 for MyoD and MLC at all time points examined (Figure 3). Myogenin $\Delta\Delta C_T$ for group 3 birds was 1.1, 0.5 and 0.9 for 7U, 14U and 21U birds, respectively. Wing unloading in group 3 birds weighting increased MyoD $\Delta\Delta C_T$ to 3.2, 3.1 and 2.2 in 5W, 7W and 14W birds, respectively and 3.4 and 2.2 in 7R and 14R birds, respectively (Figure 3). Myogenin $\Delta\Delta C_T$ was 2.2, 3.4 and 16 in 5W, 7W and 14W birds and 27 and 39 in 7R and 14R birds. MLC $\Delta\Delta C_T$ values did not exceed 2.0 until day 14 of stretch (5W, 1.5; 7W, 0.9; 14W, 2.2). $\Delta\Delta C_T$ was 1.9 and 1.6 in 7R and 14R birds, respectively.

Quantification of Id2 mRNA levels. Id2 $\Delta\Delta C_T$ was 2.2 in 5W birds and 1.8 in 7W birds of group 1 as determined by kinetic-PCR. Id2 levels were similar to control in 14W birds. In group 2, Id2 $\Delta\Delta C_T$ was 3.9, 2.7, and 1.6, in 7U, 14U and 21U birds, respectively. Id2 levels were similar in 21U and 7W birds. Id2 mRNA levels did not change in birds of group 3 (Figure 4A).

Id2 protein levels. The western blot OD x area data for each time point is given in Figure 4B. Left/right Id2 protein levels were 3.1 in 5W birds from group 1. Left/right protein levels in 7W and
14W birds from group 1 were not statistically different from day 0, 7R or 14R. Left/right OD x area for 7U, 14U and 21 U birds in group 2 was 2.5, 2.4 and 1.7.

**Indicators of apoptosis.** *Caspase 3,7,10.* Caspase 3,7,10 was 0.94 ± 4 units.mg protein.min⁻¹ in control muscles at time 0. Left/right caspase levels decreased in muscles from 5W, 7W and 14W birds to 0.55, 0.61 and 0.53, respectively. Left/right caspase levels increased to 2.14 and 1.41 in 7U and 14U days of unloading in group 2 birds but caspase was similar in left and right muscles in 21U birds. Left/right muscle caspase was 0.94 and 0.79 in 7R and 14W birds from group 3 (Figure 5A).

*Caspase 8.* Caspase 8 was 1.12 ± 0.11 units.mg protein.min⁻¹. It was significantly lower in left PAT muscles from 14W birds (69 ± 2) relative to time 0. The ratio of left/right caspase 8 in group 1 was 0.81, 0.72, and 0.61 in 5W, 7W and 14W, respectively. Left/right caspase 8 was 2.43, 1.86 and 1.51 in 7U, 14U and 21U birds from group 2, and 1.08 and 0.71 in 7R and 14R birds from group 3. (Figure 5B). Caspase 8 levels were significantly higher in left muscles from 7U, 14U and 21U birds than time 0 or any loaded conditions.

**PARP labeling.** The 89kDa fragment of cleaved PARP-1 was detected on frozen sections in experimental muscles from group 2 birds but positive cells were not detected in control muscles or muscles from group 3 birds (Figure 6). The PARP labeling index was 0.0 ± 0.1% in control muscles of all groups and in both group 1 and group 3 birds but it was 21.9 ± 4.1%, 11.9± 3.8% and 10.25± 2.4% in 7U, 14U and 21U birds, respectively.

Insert Figure 5

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**Relationship of Id2 and apoptosis.** A strong positive correlation \((r=0.87)\) was found between caspase 8 and Id2 mRNA levels in unloaded muscles (Figure 7). In general, caspase 8 and Id2 levels tended to be highest in 7U and Id2 mRNA declined proportionally to caspase 8 as the duration of unloading increased.

**DISCUSSION**

Loss of muscle under pathological conditions (2,3) and limb unloading is associated with a decrease in nuclei number (6,30,41,51), which occurs at least in part through activation of pathways leading to apoptosis (4). However, because experimental conditions of limb unloading have previously used models that reduce muscle mass well below controls levels, we examined conditions where muscle mass is first increased by overload, then reduced towards control levels by unloading. In this study we report that loss of muscle mass in non-pathological conditions where muscle mass is not reduced below control levels, increases Id2 expression, concomitant with activation of apoptosis. These finding are consistent with the suggestion that one role of Id2 may be in pathways leading to apoptosis of skeletal muscle (9).

**Muscle unloading and apoptosis.** Several pieces of data confirm that apoptosis regulates muscle mass and MPC loss during wing unloading, where hypertrophied muscles lost mass and approached control mass levels. First, the BrdU labeling index declined during unweighting, as compared to the initial 14 days of weighting-induced hypertrophy. This was not the result of loss of BrdU signaling from the nuclei, because we have detected similar levels of BrdU positive nuclei after 1 months and 3 months of constant weighting, where muscle mass does not increase
following 21-30 days of stretch (unpublished data). The decline in the number of BrdU positive nuclei during unweighting must be the result of either apoptosis or degeneration of satellite cells. Although we cannot know for certain if each satellite cell that had been activated during overload (group 1 birds) did not survive as a result of apoptosis, we did find PARP positive nuclei and increased caspase levels in unloaded muscles, indicating that some of the nuclei must die via activation of pathways leading to apoptosis. The elevated caspases may have contributed to increased cleavage of PARP-1 (26), leading to an increased incidence of PARP-1 positive nuclei in unloaded muscles. Our data are consistent with that of Dirks and Leeuwenburgh (22) who have recently shown strong correlations between caspase3 and apoptotic markers in gastrocnemius from old rats, suggesting that mitochondrial pathways may be responsible for part of the apoptosis associated muscle loss leading to sarcopenia. Nevertheless, because PARP-1 activity may trigger apoptosis via apoptosis initiating factor, which promotes apoptosis through a caspase independent pathway (62) we cannot rule out the possibility that mitochondrial associated pathways may not be entirely responsible for the greater incidence of PARP-1 nuclei in unloaded muscles.

The data suggest that unloading may have activated the death domain receptor- tumor necrosis factor (TNF)-α pathway because caspase 8 was increased during unloading-stimulated muscle loss. If TNF-α pathways had not been stimulated, we would not have expected to find elevated caspase 8 levels in unloaded muscle samples (57,58). TNF-α activation is thought to occur in sarcopenic muscles of elderly humans, and this is partially offset by a hypertrophic stimulus such as resistance exercise (27). Additional work is needed to clarify the role of the death domain receptor in regulation of caspases and apoptosis in unloading.

Id2 and apoptosis. Id2 mRNA and protein levels are increased during the onset of unloading-
associated apoptosis and muscle loss. The correlation between Id2 protein levels and caspase 8 levels was 0.87 in unloaded muscles. This is consistent with the idea that Id2 proteins have a role in apoptosis-associated muscle loss. This role may also extend to other members of the Id family, because Id1 has been shown to increase during denervation, which stimulated atrophy (17,28); however, the relationship of Id1 and apoptosis was not examined.

The greatest loss of muscle mass occurred during the first 7 days of wing unloading. This corresponded to the highest expression of Id2 and caspase levels in unloaded muscles as compared with other time points. Id2 expression declined in experimental muscles from 14U and 21U animals relative to 7U animals, nevertheless, Id2 levels were still elevated above control levels throughout unloading. This is consistent with a role for Id2 in apoptosis generated muscle loss, because we would expect Id2 to be high during large mass declines. The decrease in Id2 with prolonged unloading also fits with a role for Id2 in apoptosis because Id2 should decrease as the difference between the current mass and the control levels declines, and we would not expect muscle mass or Id2 in the left PAT to drop any lower than control (right) levels during unloading. We did not measure caspase 9 or Bax/Bcl2, so we do not know if mitochondria initiated apoptosis via caspase pathways or death domain signaling pathways are primarily involved in regulating satellite cell or muscle fiber apoptosis during unloading induced muscle loss as is the case in aging (22,49).

Id2 and satellite cell proliferation. The second purpose of the study was to determine if Id2 had a potential role in regulation of satellite cell proliferation of adult muscle. In this study, we found an increase in BrdU positive nuclei during loading (group 1) and reloading (group 3). Because many of these nuclei presumably died during unloading, new satellite cells were recruited to sustain muscle growth during reloading in group 3 birds. There is evidence that Id proteins have an
important role in the regulation of cell proliferation, and Id gene expression is enhanced in response to mitogenic stimuli (14,19) and is associated with the induction of DNA synthesis (47). In addition, over expression of Id inhibits differentiation and prolongs proliferation of cells of muscle lineages in culture (15,16,31,32) but we do not know if this function is similar in vivo.

52% of the increase in the BrdU index at 14 days of loading, occurred in the first 5 days of loading (Figure 4) and this period corresponded to the greatest elevation in Id2 protein. Thus, these data indicate that Id2 levels are elevated during initial loading of fast quail muscles, when satellite cell activation is the greatest (18). Nevertheless, Id2 quickly returns to control levels during continued loading, during a time that the rate of satellite cell activation also declines. However, it is interesting that reloading (group 3) did not stimulate increases in Id2 mRNA or protein, even though the incidence of BrdU positive nuclei was similar to the labeling index of group 1 birds and myogenin levels were high. Although satellite cell activation was dissociated from Id2 in group 3, we cannot rule out the possibility that Id2 had increased prior to our first time point (7R) that was examined during reloading.

Increases in MyoD and myogenin have been previously reported with loading of the PAT (37). MLC expression was significantly elevated for only 14W birds. In this study, we have extended our previous findings by showing significant increases in MyoD and myogenin during loading (group 1) and reloading (group 3). It is not clear why MLC did not increase in reloaded muscles in group 3 birds, because MLC expression is at least partially controlled by MyoD (61) and MyoD was elevated during reloading. It is possible that the reloaded muscle conditions result in different adaptive patterns than initial loading. Another possibility is that the ∆∆C_T of 2.0 used for significance was too stringent, and the slightly smaller increase in MLC in left muscles of group 3 birds, still represented a biologically important change in the reloaded muscle that was similar to loaded muscles in group 1 birds.
The elevation in myogenin corresponded to the period of decreasing Id2 in PAT muscles from group 1 birds. Id2 was not increased in left PAT muscles from group 3 birds, but myogenin was elevated to even greater levels than found for group 1. Further work is needed to determine if a high level of Id2 is directly linked to lower myogenin expression and delayed differentiation of activated satellite cells during the onset of overload.

**Perspectives.** Our data suggest that in young adult quail, Id2 may play a potential role in apoptosis-induced loss of muscle during unloading. The increases in Id2 were of a similar magnitude and time course as the increases in the caspase and PARP apoptotic markers. Although these results do not prove a causative role for Id2 in apoptosis in skeletal muscle following unweighting, it has been shown to at least partly play such a role in muscle damage and disease (21,48,52). Further experiments are required to establish if Id2 has a regulatory role in apoptosis of adult skeletal muscle via caspase regulation and/or the death domain receptor.

Myonuclei are post-mitotic, and they cannot divide to contribute to muscle growth (39,43). Therefore, satellite cells or their progeny, muscle precursor cells (MPC) provide the only important source for adding new nuclei to initiate muscle regeneration, muscle hypertrophy, and postnatal muscle growth in muscles of both young and aged animals (53). Typically muscles in mammals and birds that have a higher level of fast myosin heavy chain expression appear to be less dependent upon adding new nuclei via MPCs than slow muscles (23,41). This also appears to be the case for the PAT because limited acute hypertrophy can occur without marked activation of satellite cells in PAT quail muscles (33); however, sustained hypertrophy is prevented by irradiation that eliminates proliferation of MPCs (unpublished observations). In this study, we show that satellite cells are activated in modulating muscle remodeling in response to overload in the fast PAT. Nevertheless, the role of Id2 in regulating pathways associated with satellite cell
activation in PAT muscles is less clear. Although Id2 may have a role in early stages of satellite cell proliferation, it appears that increased levels of Id2 are not required for satellite cell activation. Nevertheless, it is possible that Id2 increased transiently, and earlier in reloading (group 3) than the initial loading phase (group 1).
Acknowledgements

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References


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Figure 1

Muscle weight (mg)

- Right
- Left

0 5W 7W 14W 7U 14U 21U 7R 14R
Figure 2

![Graph showing percent of total nuclei (%)](image-url)
Figure 3

Right ΔΔC_{T} - Left ΔΔC_{T}

MyoD
Myogenin
MLC

* * ** ** **
Figure 4

A

![Graph showing Left Id2 \( \Delta C \_T \) vs. Right Id2 \( \Delta C \_T \) for different time points.](image)

B

![Image of Western blot showing Id2 protein at \( \sim 15 \) kd and Ponceau S at 20 kd and 15 kd.](image)

C

![Bar graph showing Id2 protein (OD \( \times \) area, arbitrary units \( \times 10^4 \)) for different time points.](image)
Figure 5

A

Caspase 3,7,10

- Control
- Experimental

B

Caspase 8

- Control
- Experimental
Figure 6

PARP labeling of apoptotic nuclei

Control  7 days of wing unweighting
Figure 7

Id2 vs Caspase 8 in Unloaded Muscles

$y = 0.221 + 1.004x, r = 0.87$
**Figure Legends**

**Figure 1**  Right (control) and left (experimental) muscle weights after 0, 5 (5W), 7 (7W) or 14 days of wing weighting (14W), or 7, 14 or 21 days of unweighting (7U 14U, 21U) or following 14 days of loading and after reloading the wings for 7 (7R) or 14 (14R) days following 14 days of unloading. *, P<0.05, significantly different from the intra-animal control.

**Figure 2**  A subcutaneous BrdU pellet was implanted at the point of wing weighting. BrdU positive nuclei were identified by immunocytochemistry. Hematoxylin counterstaining identified total nuclei. The labeling index was expressed as a percent of the labeled to total (labeled + unlabelled nuclei). The abbreviations for the time points are the same as in Figure 1. *, P<0.05, significantly different from the intra-animal control. **, P<0.05, experimental muscles at 14W, 7R and 14R are significantly different from experimental muscles at other time points.

**Figure 3**  MyoD and myogenin mRNA was measured via real-time PCR. Primers were made for MyoD, myogenin and myosin light chain (MLC). Data are calculated as $\Delta C_T$ by obtaining the average from duplicate runs for the $C_T$ for each gene of interest - cyclophilin $C_T$ for the sample. $\Delta \Delta C_T$ was calculated as left PAT muscle $\Delta C_T$ - control (right) $\Delta C_T$ for duplicate samples. The abbreviations for the time points are the same as in Figure 1. $\Delta \Delta C_T$ levels>2.0 are significantly different from time 0.
A. Id2 expression levels were determined by real-time PCR. Data are calculated as \( \Delta C_T \) by obtaining the average from duplicate runs for the Id2 \( C_T \) - cyclophilin \( C_T \) for the sample. \( \Delta \Delta C_T \) was calculated as left PAT muscle Id2 \( \Delta C_T \) - control (right) Id2 \( \Delta C_T \) for duplicate samples. The abbreviations for the time points are the same as in Figure 1. **, \( P<0.05 \), 7U was significantly greater than all other times. Data having the same letter over the respective bars indicate that they are not different from each other. \( \Delta \Delta C_T \) levels >2.0 are significantly different from time 0.

B. Id2 protein levels as estimated by western blot. 60 µg of protein was separated via a 12% SDS-PAGE for 1.2 hours, and electroblotted to nitrocellulose. Incubation was with an anti-rabbit polyclonal antibody to Id2 (SantaCruz, CA), and the blot was developed with horseradish peroxidase linked chemiluminescence (Pierce, IN). An example of a western blot following incubation with Id2. Ponceau S staining for this same blot is shown on the bottom of the insert. This blot shows that similar protein loading occurred in each lane. The peptide control was not visible with Ponceau S staining at ~15 kDa although other bands were visible. Nevertheless, strong immunopositive reaction occurred on the membrane in the 15 kDa position as shown in the top gel of this insert.

C. The signals in each lane were quantified by Kodak 1D analysis software. The data are expressed as optical density (OD) x the resulting band area, and
expressed in arbitrary units. *P<0.05, unstretched control (0), or 5 (5W)
,7(7W) 14 (24W) days of weighting; 7 (7U), 14, (14U) or 21(21U) days of
unloading following 14 days of weighting; or 7 (7R) or 14 (14R) days of
reloading following 14 days of unloading. P, control protein lysate.

Figure 5  
A. Caspase 3,7,10 levels were estimated from muscle lysates and calculated as
arbitrary caspase units.mg protein.min\(^{-1}\). The ratio of left (experimental) to right
(control) caspase levels is shown in the right panel. Abbreviations for the time
points are the same as in Figure 1. *P<0.05, data are significantly different
from time 0 (unstretched control and intra-animal control data).** P<0.05,
Caspase levels in muscle samples from 7U, and 14 U were greater than all
other time points.

B. Caspase 8 was estimated from muscle lysates and calculated as arbitrary
caspase units.mg protein.min\(^{-1}\). The ratio of left (experimental) to right (control)
caspase levels is shown in the right panel. Abbreviations for the time points are
the same as in Figure 1. *P<0.05, data are significantly different from control
muscles at time 0. **P<0.05, caspase 8 data from 7U was significantly greater
than all other time points.

Figure 6  
Frozen muscle sections were incubated with a polyclonal, rabbit, anti-PARP p85
fragment antibody (Promega, Madison, WI USA). Color development was via
routine immunocytochemistry using avidin-biotin (Vector Laboratories Burlingame,
CA, USA) and diaminobenzidine. The nuclei were counterstained with
hematoxylin. Arrows indicate PARP positive nuclei in muscles unweighted for 7
days, following 14 days of loading. PARP positive nuclei were not found in control
muscles or muscles during reloading.

**Figure 7** The relationship between the increase in Id2 mRNA (as measured by kinetic PCR
and expressed as $\Delta \Delta C_T$) and caspase 8 in lysates from muscles unloaded for 7
(7U), 14 (14U) or 21 (21U) days.