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Parkin Protects against Mitochondrial Toxins and β-Amyloid Accumulation in Skeletal Muscle Cells*

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Mutations in the ubiquitin ligase-encoding *Parkin* gene have been implicated in the pathogenesis of autosomal recessive Parkinson disease. Outside of the central nervous system, Parkin is prominently expressed in skeletal muscle. We have found accumulations of Parkin protein in skeletal muscle biopsies taken from patients with inclusion body myositis, a degenerative disorder in which intramyofiber accumulations of the β-amyloid peptide are pathognomonic. In comparing primary cultures of skeletal muscle derived from parkin knock-out and wild-type mice, we have found the absence of parkin to result in greater sensitivity to mitochondrial stressors rotenone and carbonyl cyanide 3-chlorophenylhydrazone, without any alteration in sensitivity to calcium ionophore or hydrogen peroxide. Utilizing viral expression constructs coding for the Alzheimer disease and inclusion body myositis-linked β-amyloid precursor protein and for its metabolic byproducts Aβ42 and C100, we found that parkin knock-out muscle cells are also more sensitive to the toxic effects of intracellular Aβ. We also constructed a lentiviral system to overexpress wild-type Parkin and have shown that boosting the levels of parkin expression in normal skeletal muscle cultures provides substantial protection against both mitochondrial toxins and overexpressed β-amyloid. Correspondingly, exogenous Parkin significantly lowered Aβ levels. These data support the hypothesis that in myocytes parkin has dual properties in the maintenance of skeletal muscle mitochondrial homeostasis and in the regulation of Aβ levels.

The Parkin protein is considered an E3 [3] ubiquitin ligase and when mutated has been linked to the development of autosomal recessive Parkinson disease (1–3). In overexpression (or in cellular) models it functions to modify specific target proteins by ubiquitination, earmarking them for proteasomal degradation (3). Putative targets of Parkin include, among others, Pael-R (Parkin-associated endothelin-like receptor) (4), synphilin-1 (5), a modified form of α-synuclein (6), CDC-rel1 (7), and p38/JTV-1, an aminoacyl-tRNA synthetase cofactor (8). Previous reports have indicated that the cellular stress response promotes the formation of a complex between Parkin, the chaperone Hsp70, the C terminus of Hsc70 interacting protein CHIP, and Pael-R (9). Recently, Parkin has also been shown to interact with the α4 subunit of the 19 S proteasome, although the latter does not appear to serve as a substrate for parkin-directed ubiquitination (10). Substantial accumulations of Parkin are found associated with α-synuclein and ubiquitin bearing Lewy bodies in Parkinson disease and dementia with Lewy bodies (11), suggesting a role in the pathologic sequestration of proteins in these disorders. Although parkin mRNA is expressed throughout the central nervous system, it is also prominently expressed in skeletal and cardiac muscle, tissues with sustained levels of protein turnover (1).

Although Parkin can be found in both cytosolic and membrane-associated compartments of the cell, it has been localized to the outer mitochondrial membrane where it has been shown to protect against mitochondrial-dependent cell death (12). parkin deficiency in *Drosophila* results in marked loss of selected muscle groups and spermatocytes (13). Thus, inactivation of parkin by P-element-mediated insertion caused degenerative changes and dysfunction involving the indirect flight muscles. Substantial mitochondrial abnormalities were found associated with this selective cell death. Similarly, Pesah *et al.* (14) showed that the absence of parkin in *Drosophila* led to an increased sensitivity to the mitochondria-specific stressor, paraquat, while also identifying ultrastructural alterations in skeletal muscle. These *Drosophila* studies suggested the possibility of an important role for parkin in the normal physiology of vertebrate skeletal muscle. The link between parkin function and mitochondrial performance was further strengthened by data from an unbiased proteomic approach that showed alterations in mitochondrial function in the ventral midbrain of parkin-null mice and revealed systemic changes due to oxidative stress (15). Recent data using *Caenorhabditis elegans* devoid of parkin have reinforced the importance of this protein to mitochondrial function (16).

Inclusion Body Myositis (IBM), the most common myopathy in aging humans, occurs in both sporadic and familial forms (17). The pathologic hallmarks in affected muscle are intramyofibrillar inclusions bearing the β-amyloid peptide, as well as other fragments of the β-amyloid precursor protein (βAPP), and cytoplasmic tubulofilaments, composed of hyperphosphorylated neurofilament and microtubule-associated tau proteins. Studies in cultured muscle have shown that the accumulation of these proteins ultimately leads to myofibrillar dysfunction and subsequent death (18–20). IBM skeletal muscle biopsies reveal the accumulation of additional proteins, including the β-amyloid-converting enzyme (BACE-1 and 2, β-secretase) (21), the serine/threonine kinase cdk-5 (22), proteins related to endoplasmic reticulum stress and the unfolded protein response (23), and notably, increased ubiquitin levels (24). The accumulation of ubiquitin in affected IBM muscle has raised the likelihood that alterations in proteasomal function could play a part in the etiology of this disorder.

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[3] The abbreviations used are: E3, ubiquitin-protein isopeptide ligase; IBM, inclusion body myositis; βAPP, β-amyloid precursor protein; CCCP, carbonyl cyanide 3-chlorophenylhydrazone; HSV, herpes simplex virus; MES, 4-morpholineethanesulfonic acid; CTF, C-terminal-derived fragment; AD, adenovirus; Lv-Park, lentiviral-parkin.

The early accumulation of multiple subfragments of βAPP in IBM muscle and the ability of directed overexpression of full-length βAPP (25, 26), its C-terminal fragment, C99 (27), or Aβ itself (20) to recapitulate seminal features of IBM in transgenic animals and in cultured muscle suggest a causal role for dysregulated βAPP metabolism in its pathogenesis. The relationship between βAPP, its amyloidogenic byproducts, and the proteasome-dependent degradation pathway has not yet been explored in IBM. Against this backdrop, we investigated the impact of the absence of parkin on β-amyloid-induced and mitochondria-directed toxic insults in cultured mouse primary skeletal muscle. Here, we report that primary myotubes from parkin-null mice show selective vulnerability to mitochondrial toxins and to β-amyloid peptide expression as compared with wild-type skeletal muscle cells; accordingly, we find that human Parkin overexpression affords added protection against these insults in normal muscle.

EXPERIMENTAL PROCEDURES

Primary Muscle Culture—The generation of mice harboring a targeted germ line disruption of exon 3 of the *parkin* gene was described previously (28). Hind limb muscle from parkin knock-out and wild-type newborn mice (postnatal day 1 to 2) was dissected, minced, and then trypsinized for 30 min at 37 °C. Trypsinized tissue was triturated in complete muscle growth medium (Dulbecco's modified Eagle's medium; 1 mM pyruvate; penicillin/streptomycin; 2 mM glutamine plus 20% fetal bovine serum). Following passage through a 70-μm filter, the cell suspension was preplated for 15 min on uncoated tissue culture dishes to allow for the adherence of fibroblasts, allowing for enrichment of the myoblast population in the cell suspension. Cells were counted and adjusted to 3×10^5/ml in muscle growth medium prior to plating on dishes precoated with Matrigel basement membrane preparation (BD Biosciences). Cultures were grown until confluent and the muscle induced to differentiate by switching to Dulbecco's modified Eagle's medium containing 2% adult horse serum (muscle differentiation medium, MDM). Muscle cultures were used for various treatments as described under "Results." Tissue culture medium and sera were from Invitrogen.

Chemical Agents—A23187 (calcium ionophore), 30% hydrogen peroxide (H_2O_2), and the mitochondrial toxins rotenone and carbonyl cyanide 3-chlorophenylhydrazone (CCCP) were purchased from Sigma. When myotube cultures were tested for sensitivity to H_2O_2, pyruvate-free medium was used in order to eliminate its peroxide-associated protective effects.

Antibodies—Monoclonal antibody 6E10 against β-amyloid, and Prk8 against Parkin, were from Signet Pathology (Dedham, MA). Affinity-purified, rabbit anti-Parkin antibody HP2A was used as described previously (11). Chicken anti-human myosin heavy chain antibody was purchased from Orbigen (San Diego, CA). Anti-βAPP (22C11) was from Chemicon (Temecula, CA). Rabbit polyclonal antibody C8 against the C terminus of βAPP was a gift from Dr. Dennis Selkoe (Harvard Medical School). Monoclonal anti-ubiquitin (P4D1) was from Santa Cruz Biotechnology (Santa Cruz, CA).

Immunofluorescence Staining—10-μm cryostat sections of skeletal muscle biopsies were fixed in 100% methanol at −20 °C for 20 min. After washing in phosphate-buffered saline, sections were blocked in phosphate-buffered saline containing 5% normal goat serum and 0.4% bovine serum albumin (w/v) for 1 h. Antibodies were applied in the blocking buffer overnight at 4 °C. After washing in phosphate-buffered saline, sections were incubated with fluorescently labeled secondary antibodies (Jackson ImmunoResearch; West Grove, PA) in the blocking buffer. After final washes, samples were mounted in antifade solution and viewed using a Nikon TE200 epifluorescence microscope. Staining comparisons were made between IBM ($n = 3$, mean age 68 ± 10 years), polymyositis (PM) ($n = 2$, mean age 52 ± 16 years), and normal ($n = 2$, mean age 60 ± 14 years) skeletal muscle biopsies. To test antibody specificity, blocking experiments were performed with the immunogenic peptide used to raise the antiserum. Antibody solutions were preincubated for 1 h with either the immunogenic or an irrelevant control peptide at 100 μg/ml. The solution was centrifuged to remove any precipitate and the supernatant applied to the sections being analyzed. All other procedures were identical to those described above.

Viral Vectors for Heterologous Gene Expression—Herpes simplex virus (HSV)-based vectors harboring full-length βAPP-751, the APP C-terminal fragment C100, and β-amyloid 1–42 (Aβ42) were prepared as described previously (20). In this study, C100 and C99 are used interchangeably. C100 specifically refers to an engineered cDNA with the open reading frame encoding the C99 fragment of βAPP that retains the methionine from position −1 relative to the sequence of Aβ. The C100 and Aβ constructs were engineered to contain an N-terminal signal peptide to direct their synthesis and processing through the secretory pathway, as would normally occur for βAPP. A doxycycline-inducible adenoviral expression construct containing Aβ42 (Ad-TRE-Aβ42) and adenovirus harboring the TetON positive regulator (Ad-TetON), which encodes the activator rtTA, were as described by Magrane *et al.* (29). For the HSV-based constructs, cells were infected at a multiplicity of infection of 0.5 to 1 based on myoblast counts prior to differentiation. The adenoviruses were added at a 4:1 ratio of Ad-TRE-Aβ42 to Ad-TetON, to a total multiplicity of infection of 100–200, and expression was induced 24–48 h later by the addition of doxycycline (Sigma) to 1 μg/ml final concentration. Human wild-type Parkin containing an N-terminal Myc epitope tag was cloned alongside an internal cytomegalovirus promoter into the FUGW vector backbone.[4] The lentiviral constructs were packaged in human embryonic kidney 293FT cells with vesicular stomatitis virus as an envelope and the delta 8.9 plasmid, which contains genes for core proteins and enzymes, to reverse transcribe the lentiviral RNA genome after infection (30). Lentiviral infection was performed (multiplicity of infection 5–10) 18 h prior to infection with Ad-TRE-Aβ42.

Lactate Dehydrogenase Release Assay—Conditioned culture medium was clarified by centrifugation and lactate dehydrogenase enzymatic activity measured using the BioVision lactate dehydrogenase cytotoxicity assay kit (BioVision, Inc.; Mountain View, CA). Activity is determined by the conversion of lactate to pyruvate in the presence of the tetrazolium compound INT to produce a colored formazan. The reaction products were read using a Molecular Devices multiwell plate reader at a wavelength of 490 nm. 100-μl aliquots of test medium were assayed in triplicate for each treatment condition.

Protein Extractions, Electrophoresis, and Western Blotting—For routine whole cell lysates, cultures were extracted with radioimmune precipitation buffer containing 1% Nonidet P-40, 0.5% deoxycholate, and 0.1% sodium dodecyl sulfate (SDS) plus protease inhibitors (Complete Protease Inhibitor; Roche Applied Science). To assess the amount of soluble *versus* insoluble Aβ in the muscle cultures, the cells were washed with phosphate-buffered saline, scraped from the dish, and collected by centrifugation. Pelleted cells were resuspended in a solution of 1% SDS containing protease inhibitors. After disruption by repeated pipetting, the mixture was centrifuged at 13,000 × *g* for 20 min. The resulting supernatant was removed and identified as "soluble." The remaining

[4] C. E. H. Moussa, V. Veereshwarayya, K. M. Rosen, G. Qin, and H. W. Querfurth, manuscript in preparation.

pellet was triturated and then vortexed in a solution of 70% formic acid (Sigma) for 5 min at room temperature. The "insoluble" formic acid fraction was then neutralized by the addition of 1 M Tris-hydroxymethyl-aminomethane. Protein determinations were performed on diluted samples using the microscale version of the Bio-Rad protein assay kit. For Western blotting, proteins were electrophoretically separated and subsequently transferred to polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA). After incubation with specific primary antibodies, blots were incubated with secondary antibody linked to horseradish peroxidase (DAKO/Cytomation, Carpinteria, CA) and blots developed using enhanced chemiluminescence reagents and film from Amersham Biosciences. For analysis of β-amyloid and C-terminal fragments of βAPP, samples were fractionated on 4–12% acrylamide Bis-Tris gels using MES running buffer. For other proteins, standard SDS-polyacrylamide gels were utilized.

RESULTS

Histologic and immunochemical analyses of skeletal muscle biopsies from patients affected with IBM identify the presence of atrophic, vacuolated, and angulated myofibers that often contain intracytoplasmic inclusions consisting of the β-amyloid peptide, other proteolytic fragments of βAPP, and hyperphosphorylated neurofilament proteins and the microtubule-associated protein tau (31, 32). The cytoplasmic inclusions also contain high levels of aggregated ubiquitin (24). These characteristics together with the findings of high endogenous levels of expression of the *Parkin* gene in skeletal muscle and the colocalization of Parkin with Lewy bodies in Parkinson disease (11), led us to examine human IBM biopsy material for the presence of Parkin reactivity. In muscle derived from IBM samples, but not from normal, age-matched specimens or those from polymyositis cases, affected myofibers were found to contain elevated levels of intramyofibrillar Parkin, as detected by immunoreactivity with antibody HP2A. This finding suggests that intracellular accumulation or aggregation of Parkin may also be a feature of this muscle disorder (Fig. 1). Examination of muscle biopsies from all samples indicated that Parkin expression is ubiquitous in skeletal muscle fibers, but only in the affected regions of IBM muscle biopsies was there a dramatic increase in the staining of some fibers. To establish antibody specificity, blocking experiments were performed where the antibody solution was preincubated with the immunizing peptide prior to their application. Preincubation with the immunizing peptide, but not the control peptide, led to a total elimination of parkin staining. Of note, the specificity of the HP2A antibody staining has also been recently confirmed in human brain specimens of a Parkin-mutant Parkinson disease case (33). Co-accumulation of both Parkin and ubiquitin, and of β-amyloid together with ubiquitin, occurred in a significantly increased proportion of myofibers staining for either ($\chi 2 = 47.4$ and 90.8, respectively, $p < 0.05$). Only IBM specimens showed intramyofiber Aβ deposition (data not shown, see Ref. 19).

Previous reports in mice and flies have indicated that the absence of parkin increases susceptibility to mitochondrial toxins and results in dysfunction of both muscle cells and spermatocytes (14, 15, 34). After finding increased Parkin immunoreactivity in affected IBM skeletal myofibers, we examined the sensitivity of primary skeletal muscle cultures derived from either wild-type or parkin-null mice to the effects of both mitochondria-specific and other toxins. Primary myotube cultures were treated 3–5 days after the switch to differentiation medium, when mature, multinucleated, electrically active, contracting myotubes are numerous. To establish the sensitivity of these cells to non-mitochondrial toxins, we tested the effects of calcium ionophore (A23187) and hydrogen peroxide (H_2O_2). Cultures were incubated with increasing doses of these reagents for 24 h and the medium assayed for the release of lactate dehydrogenase. A comparison of muscle cultures derived from wild-type *versus* parkin knock-out mice showed no differential sensitivity to these toxic compounds over a range of several doses (Fig. 2). The response, however, was significantly different when cultures were tested for sensitivity to the mitochondrial toxins rotenone and CCCP, affecting complex 1 activity and electron transport coupling, respectively. In both cases, the absence of parkin led to a specific increase in muscle cell death to these inhibitors of mitochondrial-based oxidative phosphorylation (Fig. 2).

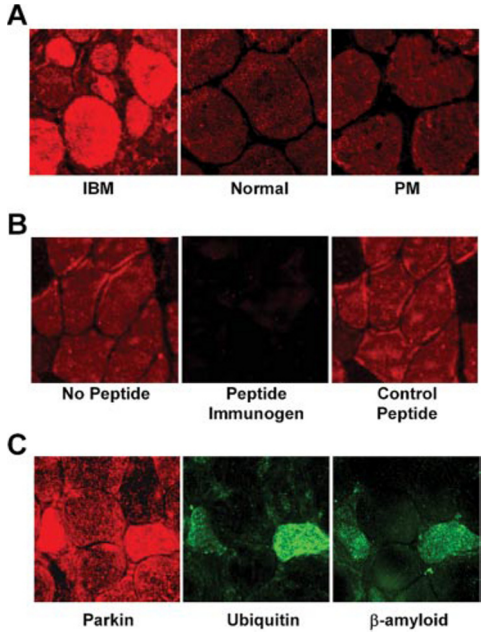

FIGURE 1. **Parkin accumulation in affected IBM myofibers.** *A*, representative fields from an IBM (*left*), a normal (*center*), and a polymyositis (*right*) muscle biopsy are shown. All samples were processed at the same time with the same stock of rabbit anti-Parkin antibody HP2A and subsequently visualized by epifluorescence microscopy. Photographic exposure indices were held constant for all of the images. *B*, adjacent sections from a normal skeletal muscle biopsy were incubated with antibody HP2A alone (*left*) or antibody solution that was previously incubated with an excess of the immunizing peptide HPK2 (*center*) or an irrelevant control peptide (*right*). *C*, co-localization of Parkin, ubiquitin, and β-amyloid. A single section from a representative IBM case was double stained for Parkin (*left*) and ubiquitin (*center*), while an adjacent section was stained with antibody 6E10 against β-amyloid. Rare fibers that were immunopositive for Aβ or ubiquitin were present only in IBM cases but not in normal or polymyositis specimens (results not shown).

Affected IBM muscle fibers contain substantial accumulations of β-amyloid, and we previously have shown that directed β-amyloid overexpression induces cell death in cultured skeletal muscle fibers (20). The accumulation of β-amyloid, as well as Parkin, in IBM myofibers (Fig. 1) suggested that Parkin might act to directly or indirectly influence the capacity of the muscle to degrade fragments derived from βAPP processing (35). To examine this possibility, we infected primary muscle cultures derived from both parkin knock-out and wild-type mice with HSV-based constructs that target the expression of human holo-βAPP, of C100, and of Aβ42. Whole cell extracts were prepared 24 h after viral infection and examined by Western blot for expression of the transduced proteins (Fig. 3). Muscle cells lacking parkin (Fig. 3*A*) accumulate higher levels of both Aβ and the APP C-terminal fragment, C99, generated by β-secretase, compared with wild-type cells infected with the same constructs (Fig. 3*B*). The absence of murine parkin also leads to an increased accumulation of higher order oligomeric assemblies of β-amyloid in cells expressing Aβ42, *i.e.* dimers and trimers, forms that are regarded to be more toxic *in vivo* than the amyloid monomer alone (36–38). Further evidence for the function of parkin and proteasomal

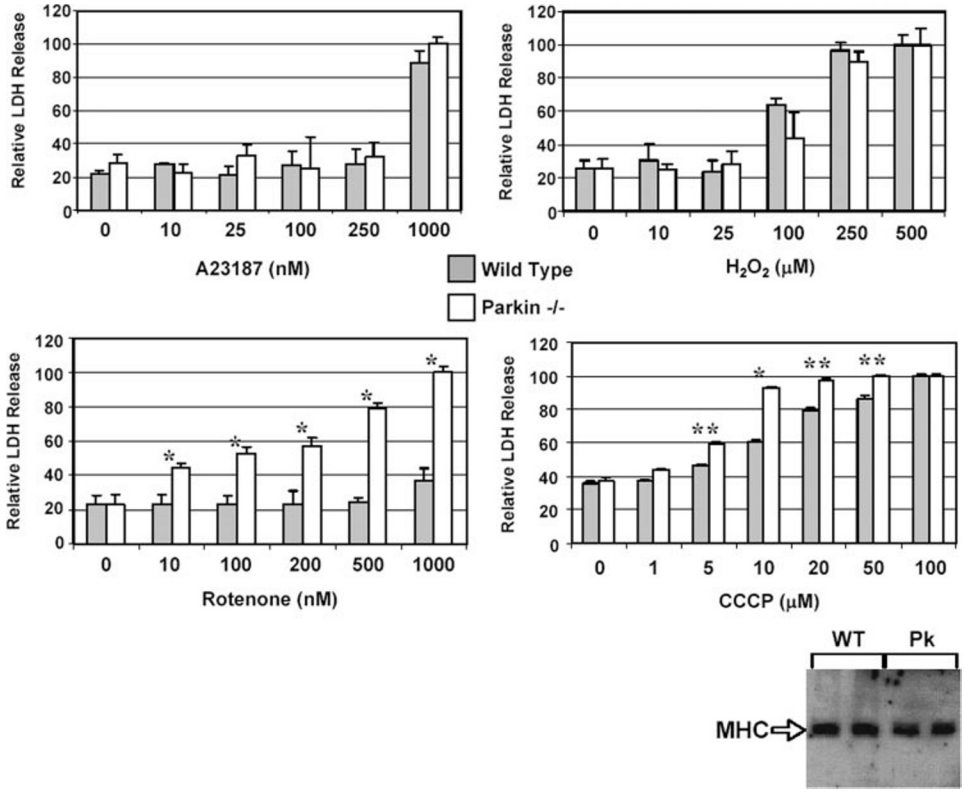

FIGURE 2. **parkin-null murine myotubes are more sensitive to mitochondrial toxins.** Comparison of the effects of various agents for inducing cell death in skeletal muscle cultures derived from either wild-type (*shaded*) or parkin knock-out (*open*) mice. Lactate dehydrogenase activity was measured in conditioned medium after 24-h treatments with A23187, H_2O_2, and CCCP. Rotenone treatments were for 1 h. *Bars* show the means ± S.E. All samples were measured in at least triplicate. (*, $p < 0.001$; **, $p < 0.05$). *Inset*, Western blot for myosin heavy chain protein in extracts prepared from duplicate cultures of both wild-type (*WT*) and parkin −/− (*Pk*) primary muscle cultures as a measure of differentiation of the cultures.

activity in the regulation of Aβ is given as an increase in Aβ levels as well in the presence of proteasome inhibition (Fig. 3*B*, *far right panel*). In contrast, parkin-null muscle cultures infected with virus expressing holo-βAPP failed to show any increase in levels of the full-length holoprotein compared with wild-type cultures infected with the same vector (Fig. 3*C*), suggesting that pathways in which parkin is involved do not regulate steady-state levels of βAPP itself. This experiment also provided an internal control showing that the absence of parkin did not lead to any alteration in susceptibility to either viral infection or expression of the transduced gene. We next examined the pattern of accumulation of C-terminal-derived fragments (CTFs) of βAPP using an antibody directed against the last 20 amino acids of the precursor (39). The proteolytically generated C99 CTF is the immediate precursor to β-amyloid, whereas the C83 CTF is produced through the action of α-secretase and is cleaved within the β-amyloid sequence (40). We infected muscle cultures for 24 h with HSV-βAPP or -C100 constructs and examined them for the presence of these CTFs (Fig. 3*D*). In the absence of any transducing vector, muscle cultures lacking parkin accumulate the endogenous α-secretase product C83 to higher levels than do wild-type muscle cultures. Overexpression of the C99 fragment in parkin knock-out muscle shows the same elevated levels of endogenous C83 as in the parkin knock-out control lane and the expected boost in levels of C99. In addition, expression of holo-βAPP promoted higher C83 levels without any change in β-secretase-cleaved products in either wild-type or parkin-deficient mice. Importantly, generalized inhibition of the proteasome using MG-132 also causes a substantial accumulation of βAPP CTF C83. These results suggest that the biological activity of Parkin in skeletal muscle may affect pathways that degrade both βAPP CTFs and β-amyloid.

The accumulation of proteolytic byproducts of βAPP metabolism, some of which exert toxic properties *in vivo*, would be expected to have survival implications for myotubes that lack parkin. In previous studies from our laboratory we have determined the required duration of expression and associated dose of HSV-Aβ42 to promote muscle cell death (20). In the present studies, we purposely limited the time after viral infection of the myotubes before harvest or morphologic examination of the myotube cultures in order to allow us to ascertain any enhancement in muscle cell death in the absence of parkin. Wild-type and knock-out cultures were infected with HSV-Aβ42 for 16 h and then examined by microscopy for myotube attrition. Tabulating the data from counting 20 microscopic fields for each condition, we found that levels of β-amyloid that are typically sublethal for wild-type cells induced significantly higher myotube death in parkin knock-out myotubes (Fig. 4). The lowered threshold for cell death is supported by the finding that the levels of the apoptosis-dependent cleaved form of caspase 9 are increased in the same β-amyloid-expressing parkin knock-out cultures (Fig. 4*C*). Additional support for this alteration is provided by the enhanced release of cytochrome *c* to the cytosolic fractions in parkin knock-out cultures (Fig. 4*D*).

In AD brain and cell culture models thereof, cellular deposition of Aβ proceeds by coalescing into inclusion-like aggregates (41, 42). A fraction of intracellular Aβ may accumulate in an SDS-stable, insoluble pool (43, 44). The overexpression of β-amyloid in skeletal myofibers leads to the formation of intracellular filaments within inclusions prior to cell death (20). Therefore, we speculated that the relative solubility of β-amyloid might be altered in muscle when parkin is absent. Parallel cultures of parkin-deficient and wild-type skeletal muscle were infected with the adenoviral construct harboring the doxycycline-inducible β-amyloid (Ad-TRE-Aβ) (as in Fig. 4, *C* and *D*) (29). The advantage of this system is to define the exact timing and extent of Aβ expression by titration of both viral and doxycycline doses. This approach offers an alternative to the continuous, high level Aβ expression generated when using herpes virus-derived vectors. After infection of the myotubes for 18 h, new doxycycline-containing muscle differentiation medium was added to the cultures for 24 h to activate gene expression. Cultures were first extracted in 1% SDS and then cleared by centrifugation to produce a

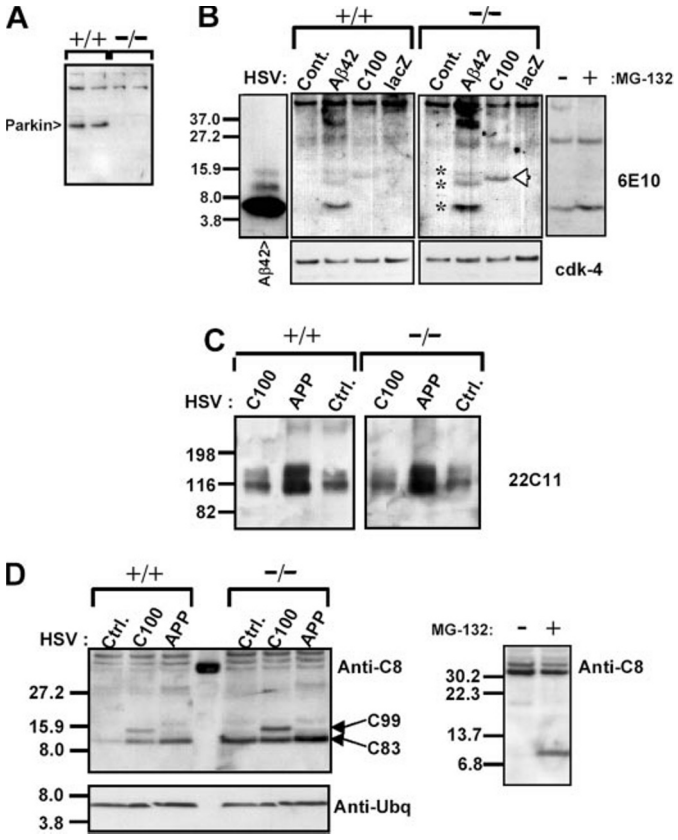

FIGURE 3. **parkin-null murine myotubes show enhanced accumulation of Aβ42 and βAPP CTFs.** *A*, Western blot analysis for parkin on duplicate samples of skeletal muscle cultures derived from either wild-type (+/+) or parkin knock-out (−/−) mice separated on 10% polyacrylamide-SDS gels. parkin was detected using antibody Prk8. *B*, comparisons of Aβ sequence-containing peptide products of βAPP after infection with the virus vectors indicated. 25 μg of total protein was electrophoresed for each sample on a 4–12% acrylamide Bis-Tris gel and probed using antibody 6E10 against human β-amyloid 1–17. (*) identifies monomeric and oligomeric Aβ species. The *arrowhead* indicates the CTF C99. cdk-4 was used as a control for equal loading. In the *right panel*, identical primary muscle cultures were infected with Aβ42 virus followed by treatment either with (+) or without (−) proteasome inhibitor MG-132 for 18 h. *C*, characterization of holo-βAPP levels in wild-type (+/+) and parkin-deficient (−/−) cultures after infection with HSV-C100 or HSV-βAPP. 20 μg of protein for each sample were fractionated on 8% polyacrylamide SDS gels. βAPP was detected using antibody 22C11. *D*, analysis of C-terminal fragment expression by Western blot using antibody C8 directed against the C terminus of βAPP. Equal amounts of protein were fractionated on 4–12% acrylamide Bis-Tris gels. The β-secretase product, C99, and the α-secretase product, C83, are indicated. The blot was stripped and reprobed for monoubiquitin. The additional two lanes to the right show that blockade of proteasome activity with MG-132 leads to enhanced accumulation of CTFs.

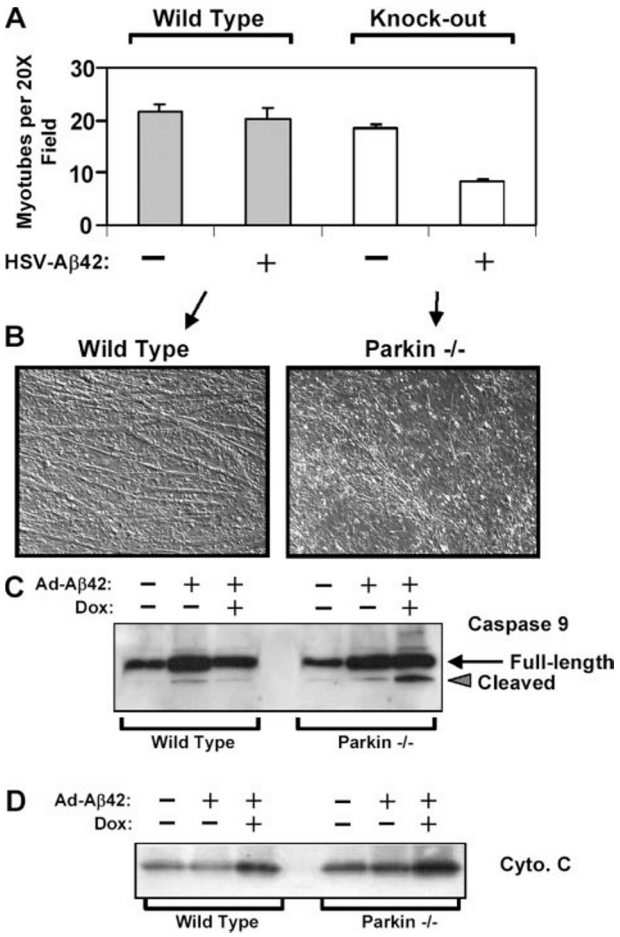

FIGURE 4. **Enhanced cell death by β-amyloid in parkin −/− cultures.** *A*, primary myotube cultures in 8-chamber slides were infected for 16 h using HSV-Aβ42. 20 random fields were photographed (at ×20), and myotubes were counted in each. Only those myotubes that were long enough to cross at least the midpoint of two adjacent boundaries were included. *Bars* represent the means ± S.E. *B*, representative microscopic fields photographed under Hoffman modulation contrast for each type of culture in the presence of amyloid overexpression. *C*, cultures as indicated were infected with the inducible Ad-Aβ42, and expression was induced with doxycycline for 24 h as shown. Extracts were assayed for the presence of cleaved (activated) caspase 9. Induction of amyloid expression in the parkin knock-out culture results in higher levels of cleaved caspase 9. *D*, amyloid expression leads to increased release of cytochrome *c* from the mitochondria. Cytosolic fractions were prepared from cultures treated as indicated and examined for the presence of cytochrome *c*.

soluble extract. Because β-amyloid has a propensity to form detergent-insoluble aggregates, the post-SDS pellet was extracted with 70% formic acid to yield an insoluble extract. Samples from both parkin knock-out and wild-type muscle cultures were examined by Western blot for differential partitioning of the overexpressed Aβ. A standardized amount of synthetic β-amyloid 1–42 was run on all gels for comparison. As expected, in the absence of parkin we observed an increase in the relative proportion of β-amyloid (including oligomers) that was distributed into the detergent-insoluble, formic acid-extractable compartment (Fig. 5).

The increased sensitivity of muscle cells to both β-amyloid accumulation (Fig. 4, *A* and *B*) and mitochondrial toxins in the absence of parkin suggests a function for Parkin in protecting muscle from mitochondrial damage. To more fully develop this notion, we sought to determine whether higher levels of Parkin would afford protection against these insults in normal, wild-type skeletal muscle. For these experiments we utilized a lentiviral vector that was engineered to express wild-type human Parkin (Lv-Park).[4] Primary muscle cultures from wild-type mice were infected with 5–10 multiplicity of infection of Lv-Park 2 days prior to the switch to differentiation medium. Parkin-overexpressing and control muscle cultures were subjected to treatment with 10 μM CCCP for 24 h and then assayed for release of lactate dehydrogenase. This dose was chosen because our previous results, as shown in Fig. 2, defined this concentration to differentiate the sensitivities between wild-type and parkin knock-out muscle. In Fig. 6*A*, overexpression of wild-type human Parkin is shown to confer a significant rescue effect (~50%) against cell death induced by CCCP treatment when compared with control cultures. Importantly, when wild-type muscle cultures were made to overexpress Parkin for at least 2 days prior to the induction of Aβ42 expression, there was a similarly significant decrease (~66%) in β-amyloid-induced toxicity (Fig. 6*B*). To better understand the mechanism by which lentiviral-directed Parkin overexpression decreased sensitivity to β-amyloid toxicity, extracts were prepared from both control and Parkin-overexpressing wild-type muscle cultures that were subsequently infected with adenoviral β-amyloid constructs and induced by addition of doxycycline. Western blot analysis revealed an ~2-fold

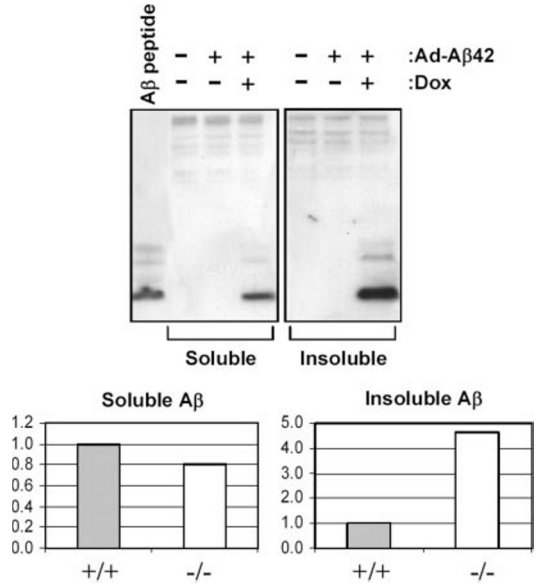

FIGURE 5. **The absence of parkin alters the partitioning of amyloid between detergent-soluble and -insoluble compartments.** Wild-type and knock-out cultures were infected with Ad-TRE-Aβ42 and induced with doxycycline for 24 h. Samples of soluble and insoluble fractions from representative knock-out cultures are shown fractionated on 4–12% acrylamide Bis-Tris gel. Western blots were analyzed using monoclonal antibody 6E10 to detect β-amyloid. For all experiments, 50 ng of synthetic Aβ peptide was electrophoresed on the same gel. *Bar graphs* provide the densitometric results for Aβ signals in all fractions after correction to the synthetic peptide signal and normalization to total protein in the original extract. Wild-type samples were set equal to a value of 1. In the parkin-deficient muscle, a greater proportion of intracellular β-amyloid is found in the insoluble fraction.

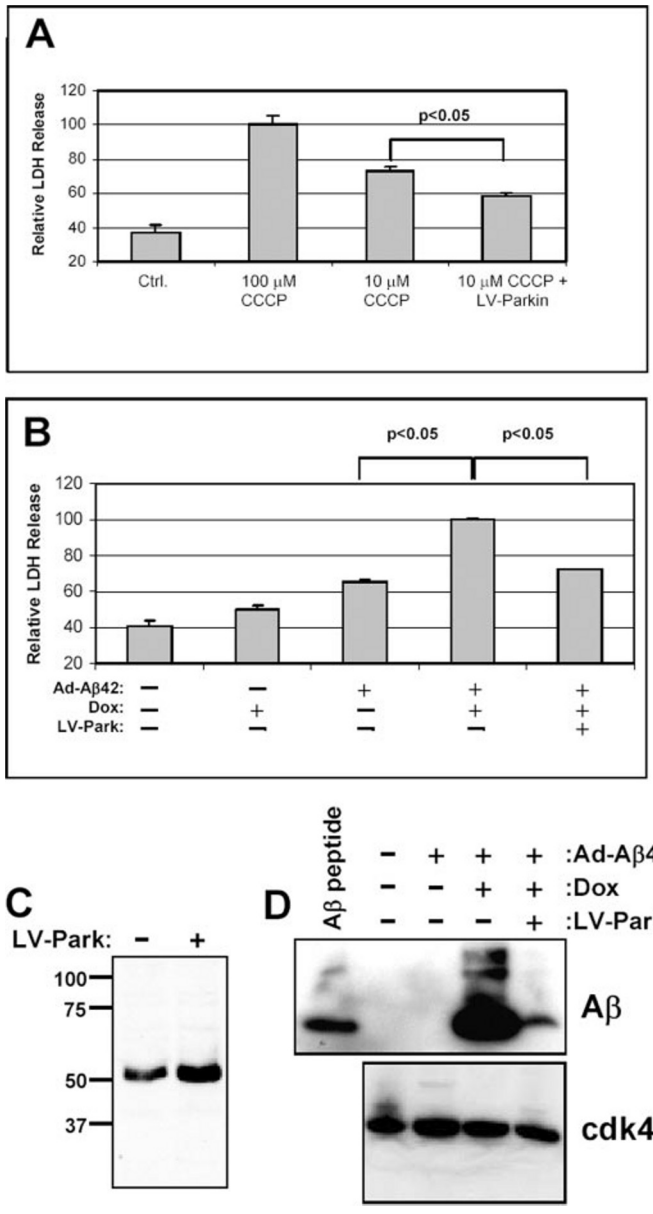

FIGURE 6. **Parkin is protective against mitochondrial and Aβ42-derived insults.** *A*, test of cell death measurements using lactate dehydrogenase release assay. Wild-type muscle cultures were treated with the doses of CCCP indicated corresponding to control (no drug) and maximal (100 μM) cell death. Prior to incubation with 10 μM CCCP one set of cultures was infected with lentiviral parkin as indicated under "Results." All samples were tested in triplicate. *B*, an analogous experiment was performed to measure the ability to resist β-amyloid-induced cell toxicity. Selected wild-type muscle cultures were infected with Ad-TRE-Aβ and induced with doxycycline after a preceding infection with lentiviral-parkin (*Lv-Park*). All samples were tested in triplicate. *Bars* indicate the mean ± S.E. *C*, Western blot using Prk8 on muscle cultures that either were or were not infected with Lv-Park. Viral expression increases parkin levels ~2-fold. *D*, Parkin expression reduces steady-state Aβ levels in skeletal muscle cultures. Extracts were prepared from parallel cultures treated with inducible adenovirus and lentivirus as indicated. After fractionation of 25 μg of protein/lane on 4–12% acrylamide Bis-Tris gels, samples were transferred to Immobilon-P and probed with antibody 6E10.

increase in Parkin levels after Lv-Park infection (Fig. 6*C*), which was sufficient to reduce Aβ42 levels by ~80% (Fig. 6*D*). We concluded that the biological effect of Parkin promoted protection of mitochondria against select toxins in skeletal muscle and reduced the steady-state levels of intracellular β-amyloid, thereby reducing its toxicity.

DISCUSSION

The confluence of recently published data highlights the role of the E3 ubiquitin ligase Parkin in the etiology of autosomal recessive Parkinson disease (reviewed in Ref. 45). The studies point to several possible pathways by which Parkin's function, and its loss through mutation, leads to cell loss in catecholaminergic nuclei. In this study we have analyzed the impact of murine parkin deficiency on metabolism and viability in skeletal muscle. Skeletal muscle is a major site of normal Parkin expression (46). These experiments were undertaken to jointly address the role of Parkin and the biology of β-amyloid in muscle, a leading candidate in the etiology of human IBM. Our data show that primary cultures derived from skeletal muscle lacking parkin display increased sensitivity to the effects of the mitochondrial toxins rotenone and CCCP as well as to Aβ-42.

The role of Parkin in mitochondrial function has continued to grow as several groups have pointed to prominent alterations in mitochondrial function and cellular susceptibility to various toxic insults in the absence of this protein. Palacino *et al.* (15) used a proteomic approach to show that the ventral midbrain in parkin knock-out mice has altered expression of proteins related to oxidative stress and mitochondrial function. They concluded that oxidative damage and reduced cellular respiratory capacity, as opposed to strictly toxic substrate accumulation, have the greater pathological significance when parkin function is absent. Using a *Drosophila* model, Greene *et al.* (13) used a genomics approach to identify loss-of-function mutations in genes that respond to oxidative stress and critically enhance the parkin-null phenotype and identified reduced glutathione levels as an important modifier. Studies by both Greene *et al.* (34) and Pesah *et al.* (14) in parkin ortholog-null flies described substantial mitochondrial pathology and cell loss involving the indirect flight muscles, a site of elevated metabolic activity. The fact that murine parkin knock-out muscle appears to be substantially more sensitive to mitochondrial-specific toxins, but not to *non*-mitochondrial toxins, targeted to calcium dysregulation and peroxide-induced cell stress again implicates parkin in mitochondrial homeostasis.

Of note, to date no electrophysiological or histopathological data have been provided in human Parkin-null cases.

The relationship between Aβ accumulation and mitochondrial dysfunction in Alzheimer disease, as shown by others in neurons (reviewed in Refs. 47, 48), is supported by our data in myocytes and also reveals a novel protective role for Parkin outside the central nervous system. Although no increase in Aβ plaques has been observed in carefully analyzed Parkin-deficient human brain (33), we have extended the investigation of Parkin biology to show a protective role in a non-α-synuclein-linked inclusion disorder with degenerative features, IBM. Whether the enhanced accumulation of β-amyloid we observe in the absence of Parkin is related more to oxidative dysfunction from mitochondrial toxicity or to the loss of Parkin's ubiquitin ligase activity and role in proteasome-mediated degradation remains to be shown. The co-accumulation of both Parkin and ubiquitin in affected IBM myofibers suggests an involvement of the proteasome in this disorder, similar to the accumulation of Parkin in the Lewy bodies of sporadic Parkinson disease (11). On the other hand, mitochondrial uncoupling leads to a similar enhancement in the accumulation of intracellular β-amyloid in mixed neuron-glia cultures from Down syndrome brain (49). Other studies have shown that increases in free radical stress leads to an increase in levels of cellular Aβ (50). Another possible mechanism for the protective role of Parkin follows from the observation that oxidative stress-induced generation of reactive oxygen species leads to an increase in insoluble parkin and an associated transfer into a detergent-insoluble compartment (51, 52). Thus, Parkin by virtue of a neutralizing role for reactive oxygen species could confer protection from mitochondrial toxins and Aβ42 accumulation (53).

The activity of the proteasome complex has previously been implicated in the degradation of the C-terminal fragments of βAPP (54–56). However, the significance of proteasome function in the pathobiology of IBM remains unclear, although both the unfolded protein response (23) and proteasomal inhibition (57) have been implicated in the abnormal handling of proteins and the cellular dysfunction in this disorder. In addition to the accumulation of myotoxic fragments of the β-amyloid precursor protein (19, 20, 26), inflammatory responses are also activated (reviewed in Ref. 58). Our finding of a general decline in the ability of muscle lacking parkin to degrade βAPP CTFs, both C83 and C99, also worsened by the addition of MG132, supports an involvement of the ubiquitin-dependent proteasomal system in the normal degradation of these fragments. Higher levels of C99 and/or widespread impairment of intracellular peptide degradation would be expected to further increase Aβ levels in these cells. Our study represents the first report on the role of mammalian parkin protein in muscle cells and demonstrates that its absence leads to the accumulation of β-amyloid, a suspected myotoxin (18, 20, 59). Further investigations into the coordinate relationship between proteasomal dysfunction and mitochondrial impairment may lead to a better understanding of the causal steps and the array of derangements that are central to the development of IBM. The identification of protective molecules, such as parkin, with dual properties likely points to important areas for future therapeutic development. Likewise, future genotyping may reveal a contributing role for the parkin gene in IBM susceptibility, and future neurophysiological and histopathological studies may identify changes in muscle cell metabolism in Parkin-deficient human Parkinson disease cases.

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