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Involvement of the Ubiquitin-Proteasome System in the Early Stages of Wallerian Degeneration

Report

Qiwei Zhai,[1,4] Jing Wang,[1,4] Anna Kim,[1] Qing Liu,[2] Ryan Watts,[3] Eric Hoopfer,[3] Timothy Mitchison,[2] Liqun Luo,[3] and Zhigang He[1,*]
[1]Division of Neuroscience
Children's Hospital
[2]Department of Cell Biology
Harvard Medical School
Boston, Massachusetts 02115
[3]Department of Biological Sciences
Stanford University
Stanford, California 94305

Summary

Local axon degeneration is a common pathological feature of many neurodegenerative diseases and peripheral neuropathies. While it is believed to operate with an apoptosis-independent molecular program, the underlying molecular mechanisms are largely unknown. In this study, we used the degeneration of transected axons, termed "Wallerian degeneration," as a model to examine the possible involvement of the ubiquitin proteasome system (UPS). Inhibiting UPS activity by both pharmacological and genetic means profoundly delays axon degeneration both in vitro and in vivo. In addition, we found that the fragmentation of microtubules is the earliest detectable change in axons undergoing Wallerian degeneration, which among other degenerative events, can be delayed by proteasome inhibitors. Interestingly, similar to transected axons, degeneration of axons from nerve growth factor (NGF)-deprived sympathetic neurons could also be suppressed by proteasome inhibitors. Our findings suggest a possibility that inhibiting UPS activity may serve to retard axon degeneration in pathological conditions.

Introduction

Axon degeneration occurs frequently in many types of chronic neurodegenerative diseases and in injuries to axons caused by toxic, ischemic, or traumatic insults (Raff et al., 2002; Coleman and Perry, 2002). It may lead to separation of the neuron from its targets, resulting in loss of neuronal function. In the past, much effort has been focused on understanding the nature of neuronal cell death in these diseases (Yuan and Yankner, 2000). However, strategies designed to prevent neuronal cell death have resulted in only limited success in preventing or delaying neurodegeneration. One possibility is that neuronal cell death occurs too late in these diseases, so that it may not be an efficient target for therapy. Thus, interfering with the process of axon degeneration may represent an additional and complementary therapeutic avenue for these diseases.

The simplest model of axon degeneration known to date is the self-destructive process observed at the distal portion of a transected axon upon injury, termed "Wallerian degeneration' (Waller, 1850). In vertebrates, the distal part of an axon can remain viable and conduct action potentials in vivo for up to a few days, after which they undergo a rapid structural destruction where the axolemma and axonal cytoskeleton are dismantled (reviewed by Griffin et al., 1995). Interestingly, the axons undergoing Wallerian degeneration do not seem to possess detectable activation of the caspase family cysteine proteases (Finn et al., 2000), the molecular hallmarks of apoptosis activation, suggesting that Wallerian degeneration and apoptosis may represent two distinct self-destruct programs.

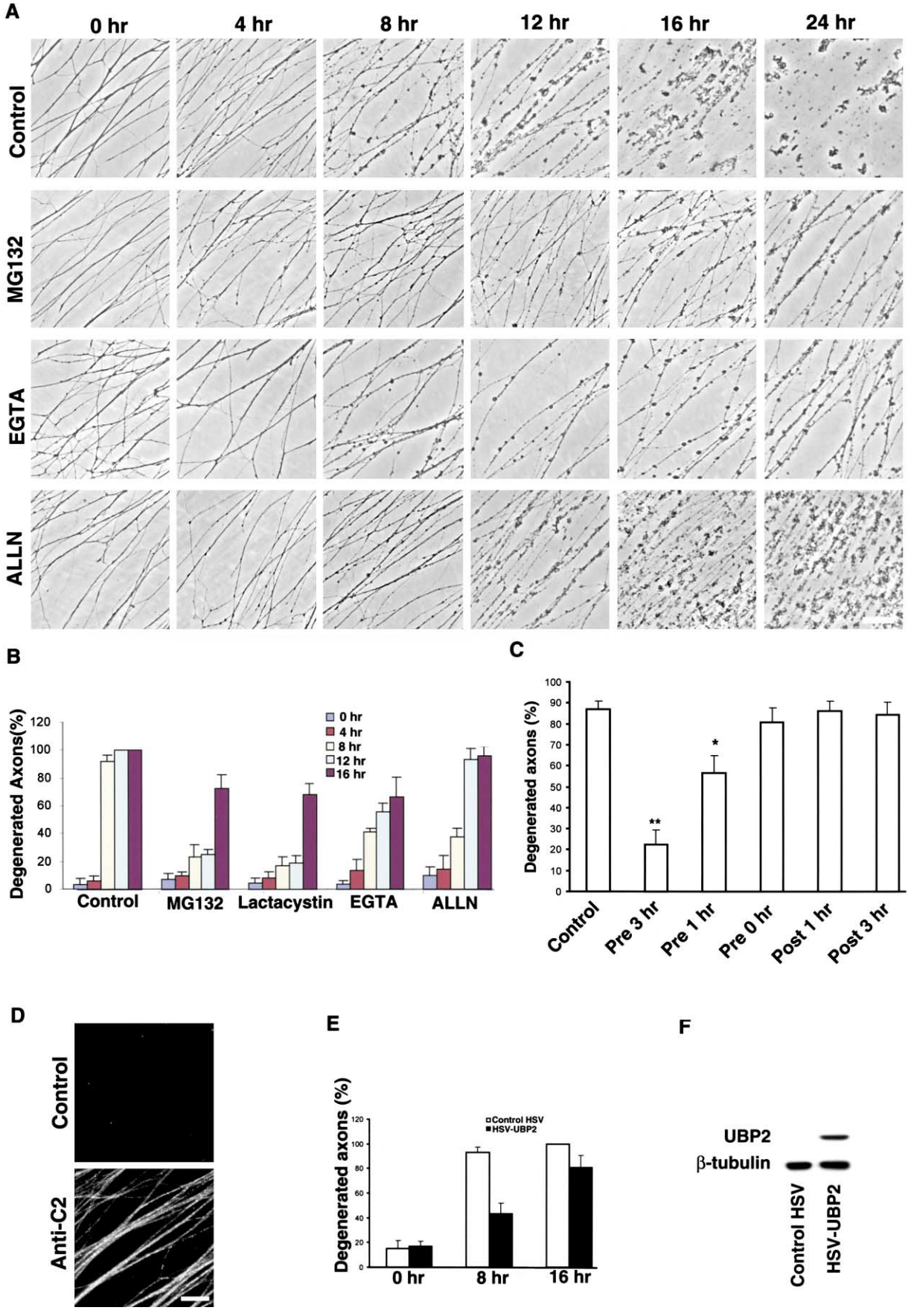
Because most neuronal proteins are synthesized in the soma and carried to the axon by specialized axonal transport systems, degeneration of the transected axons has long been thought to result from starvation for necessary proteins and other materials. However, the recent discovery of a spontaneously occurring mutant mouse strain, C57BL/*Wld*^{s}, whose axons survived for as long as weeks after transection (Lunn et al., 1989; Glass et al., 1993), suggests that Wallerian degeneration involves an active and regulated autodestruction program. The ubiquitin-proteasome system (UPS) has been implicated as a common mechanism for selective protein degradation in a variety of biological processes, such as axonal pathfinding (Campbell and Holt, 2001) and synapse formation (DiAntonio et al., 2001). In addition, an intragenic deletion in the gene encoding ubiquitin carboxy-terminal hydrolase has recently been found to account for the axon degeneration phenotype of the gracile axonal dystrophy (*gad*) mouse (Saigoh et al., 1999). Also, the slow Wallerian degeneration phenotype in the *Wld*^{s} mice has been attributed to the overexpression of a fusion protein that consists of the amino-terminal 70 amino acids of UFD2, an E4 protein involved in polyubiquitination, and the nicotinamide mononucleotide adenylyltransferase (Nmnat) (Mack et al., 2001), implying a potential involvement of UPS in Wallerian degeneration. However, the region of UFD2 in the fusion protein of *Wld*^{s} mice is absent in the functionally conserved homolog of UFD2 in yeast (Koegl et al., 1999), thus leaving it unclear whether there is contribution from UPS to the phenotype of *Wld*^{s} mice. In this study, we provide both pharmacological and genetic evidence that UPS inhibition can profoundly delay Wallerian degeneration, possibly by stabilizing microtubule skeletons at the early stages of Wallerian degeneration.

Results

Inhibiting the UPS Delays Wallerian Degeneration

Wallerian degeneration in most transected mammalian nerve fibers is a complex sequence that entails degradation of the axon, changes in the ensheathing and glial

*Correspondence: zhigang.he@tch.harvard.edu
[4]These authors contributed equally to this work.


A
0 hr
4 hr
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MG132
EGTA
ALLN
B
Degenerated Axons(%)
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12 hr
16 hr
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Lactacystin
EGTA
ALLN
C
Degenerated axons (%)
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Pre 0 hr
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Post 3 hr
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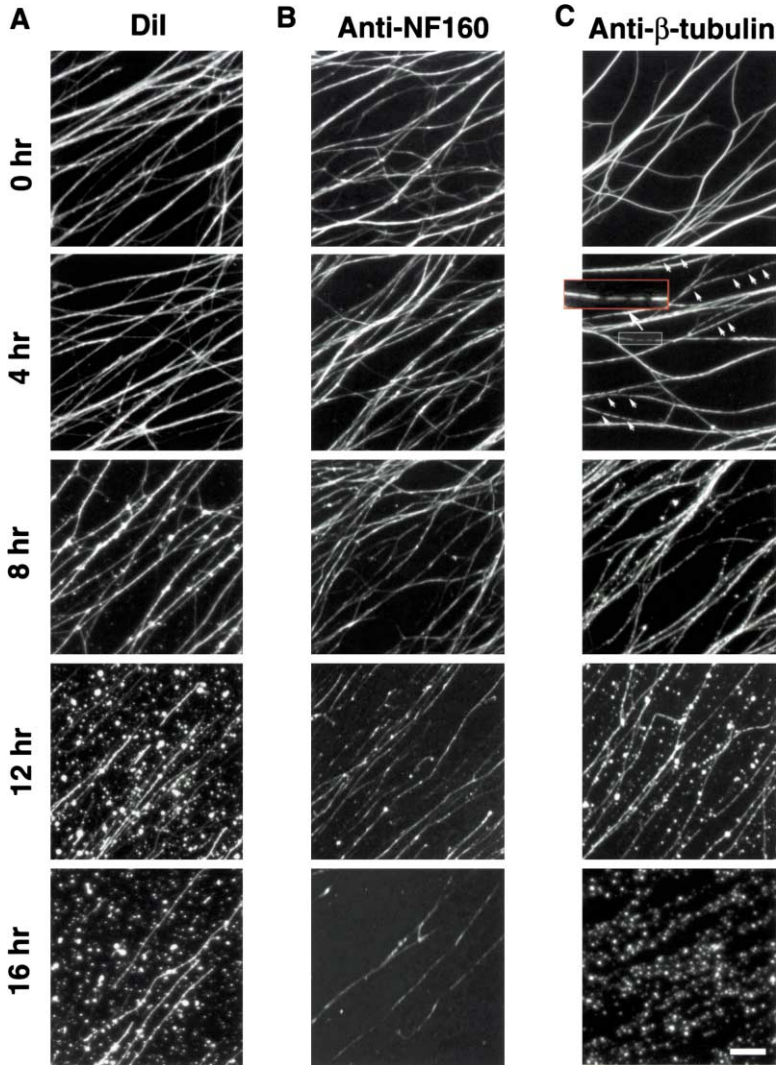

Figure 2. Characterization of Axonal Markers during Wallerian Degeneration

Cultured SCGs were fixed at indicated post-axotomy time points and either labeled with DiI to show the plasma membrane structures (A) or immunostained with antibodies against NF160 (B) or β-tubulin (C) to show the structures of microtubules and neurofilaments. Scale bar, 10 μm.

cells, alternations in the blood-tissue barriers, and responses of macrophages (reviewed by Griffin et al., 1995). To study the underlying mechanisms involved in the axon degeneration process, we have used the degeneration of transected axons from cultured primary neurons as models (George et al., 1995; Wang et al., 2001a). In cultured sympathetic superior ganglia (SCGs) derived from postnatal day 3 rats, soma-deprived axons undergo a typical degenerative process (Figure 1A). The severed axons first appear normal for 6–10 hr, depending upon the duration of culturing prior to axotomy. For SCGs that have been cultured for 5 days, the severed axons begin to develop such signs of early degeneration as focal swelling and beading approximately 8 hr after the axons have been separated from their cell bodies (Figures 1A and 1B). In most cases, the axons completely degenerated and detached after 12–16 hr post-axotomy (Figure 1A). Similar degenerative processes

Figure 1. Inhibiting UPS Delays Wallerian Degeneration

(A) Wallerian degeneration is delayed by MG132, EGTA, and ALLN. MG132 (20 μM), EGTA (2 mM), or ALLN (50 μM) was added to 5 day cultured SCGs prior to axotomy, and representative phase images were taken at the indicated time points postaxotomy. Scale bar, 10 μm.

(B) Quantification of axon degeneration results. Statistical analysis was done by two-way ANOVA using Graphpad Prism. $p < 0.0001$ for MG132 versus control, lactacystin (20 μM) versus control, EGTA versus control, and ALLN versus control.

(C) Quantification showing the protective effects of MG132 pretreatment but not posttreatment from axon degeneration at 8 hr postaxotomy. MG132 (20 μM) was added to the cultures 0, 1, or 3 hr prior to or after axotomy. Only the groups of pretreatment for 3 hr or 1 hr are significantly different (**$p < 0.001$; *$p < 0.05$, Student's t test) from control.

(D) Immunostaining of sympathetic axons with control IgG or rabbit antibodies against proteasome 20S subunit C2. Scale bar, 10 μm.

(E) Quantification of axon degeneration results. Statistical analysis was done by two-way ANOVA using Graphpad Prism. HSV-UBP2 versus control HSV, $p < 0.0001$.

(F) Western blotting showing the expression of UBP2 in infected SCGs. The same blot was reblotted with anti-β-tubulin to show an equal amount of cellular proteins loaded in both lanes. Scale bar, 8 μm.

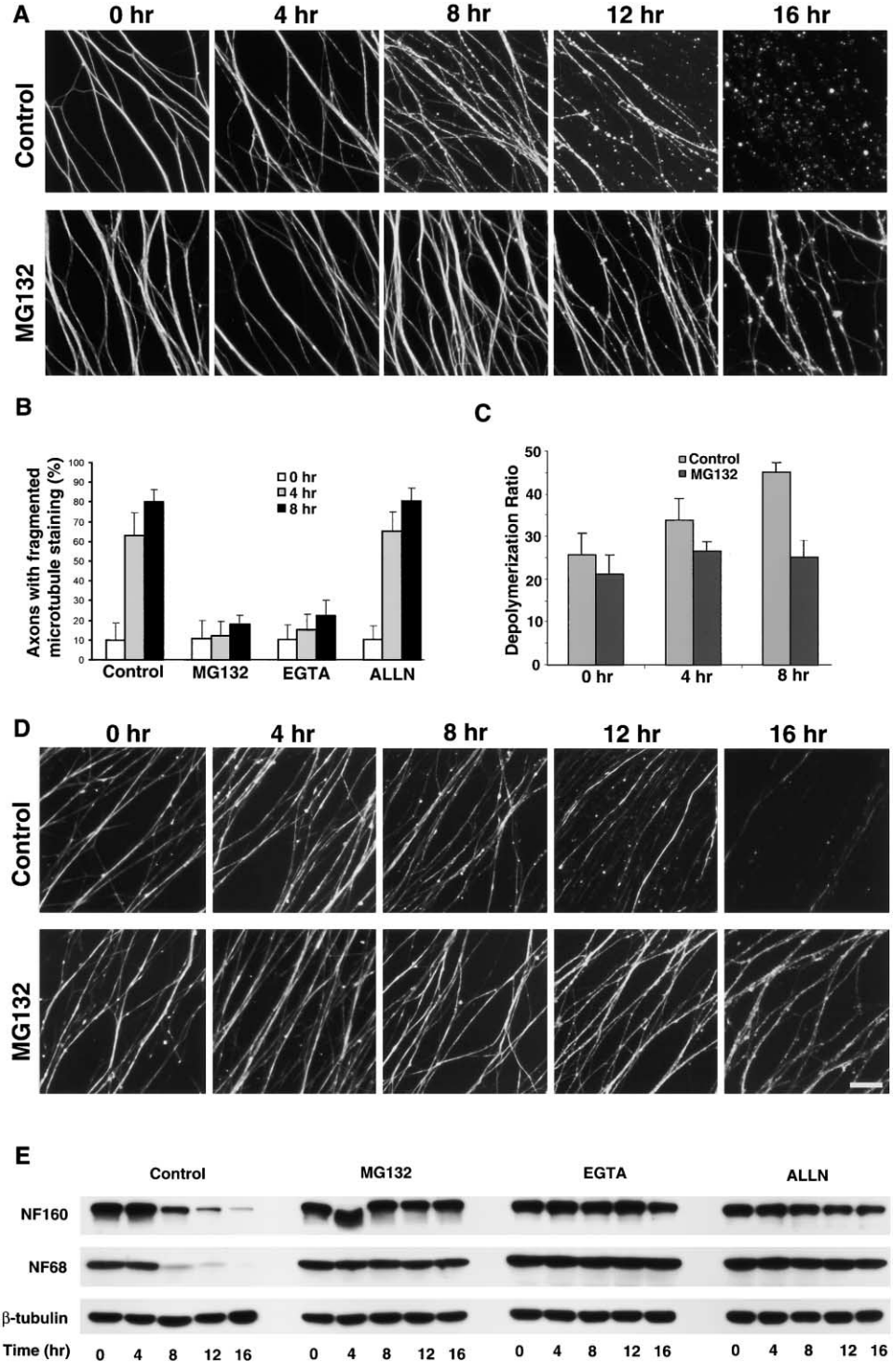

Figure 3. Proteasome Inhibitors Protect Microtubule Destabilization during Wallerian Degeneration

(A) Cultured SCGs were pretreated with or without MG132 (20 μM), axotomized, and fixed at indicated postaxotomy time points, and immunostained with an anti-β-tubulin antibody to show the structures of microtubules.

(B) Quantification of axons with microtubule fragmentation staining in different conditions (control, MG132 [20 μM], EGTA [2 mM], or ALLN [50 μM]). Statistical analysis was done by two-way ANOVA using Graphpad Prism. $p < 0.0001$ for MG132 versus control and EGTA versus control.

(C) MG132 prevents axotomy-elicited microtubule depolymerization. Statistical analysis from five experiments was done by two-way ANOVA using Graphpad Prism. In the absence of MG132, the groups of 4 and 8 hr posttransected axons were significantly different from the 0 hr group ($p < 0.0001$). MG132-treated groups (except for 0 hr) were significantly different from control ones ($p < 0.0001$).

(D) Neurofilament degeneration during Wallerian degeneration is protected by MG132 (20 μM). Scale bar, 10 μm.

(E) Western blotting showing the effects of MG132 (20 μM), EGTA (2 mM), or ALLN (50 μM) on protein degradation of neurofilament components and β-tubulin in transected axons.

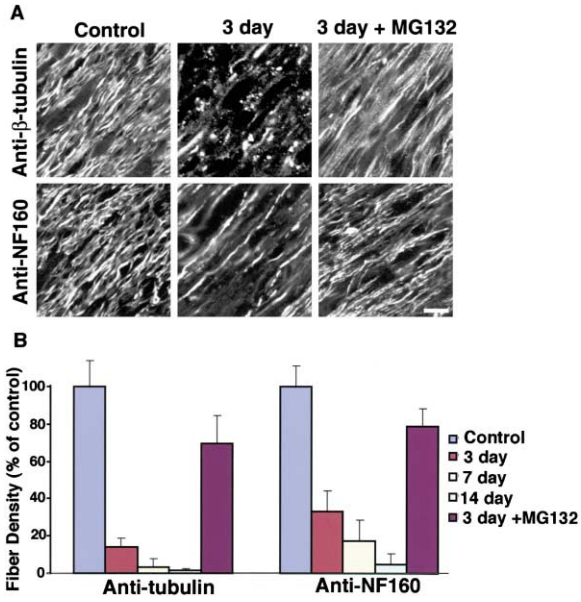

Figure 4. Inhibiting the UPS Delays Wallerian Degeneration of Crushed Optic Nerves In Vivo

(A) Immunostaining micrographs of the distal portion of transected optic nerves with neuron-specific anti-β-tubulin and anti-NF160 antibodies. Scale bar, 8 μm.

(B) Quantification of the fiber structures by immunostaining on optic nerve sections from control, 3, 7, or 14 days without MG132 and 3 days with MG132. Statistical analysis was done by two-way ANOVA using Graphpad Prism. For both β-tubulin and NF160 staining, the groups of 3, 7, and 14 days are significantly different from the control groups (all $p < 0.001$). For all of the time points except for control groups, the groups of β-tubulin staining are significantly different from those of NF160 staining (all $p < 0.001$). There are significant differences between 3 days with or without MG132 for both NF160 and β-tubulin staining (paired Student's t test, $p < 0.001$).

were also observed in other types of cultured neurons, such as chicken dorsal root ganglion neurons and retinal ganglion cells (data not shown).

We next attempted to examine whether UPS is involved in axotomy-triggered Wallerian degeneration by using specific pharmacological inhibitors. It is known that for UPS to degrade a specific substrate protein, the sequential action of three enzymes, including a ubiquitin activating enzyme (E1), a ubiquitin conjugating enzyme (E2), and a ubiquitin ligase (E3), are required to covalently link ubiquitin to the substrate. Polyubiquitinated proteins are then targeted for degradation by the 26S proteasome, which is composed of two 19S proteasome regulatory particles and a 20S core. Substrate ubiquitination may also be reversed by ubiquitin proteases (reviewed by Weissman, 2001). Consistent with previous evidence for the existence and functioning of the UPS in axons (Campbell and Holt, 2001), components of UPS machinery, such as subunit C2 of the proteasome 20S (Figure 1D), were found in both intact and transected axons from sympathetic neurons.

To perturb UPS activity, we used two mechanistically different proteasome inhibitors: MG132, a peptide-based reversible proteasome inhibitor; and lactacystin, a synthetic irreversible proteasome inhibitor. When included in SCG cultures, these proteasome inhibitors did not significantly alter the gross morphology of the uncut axon but profoundly delayed the Wallerian degeneration process (Figures 1A and 1B). Compared to untreated transected axons that began developing early signs of degeneration 8 hr postaxotomy, those treated with both MG132 and lactacystin did not show degeneration until 16 hr postaxotomy (Figures 1A and 1B). Twenty-four hours postaxotomy, when most of the axon debris has detached from the culture surface in control cultures, proteasome inhibitor-treated explants still retained many axons with some degeneration signs (Figure 1A). Because of the difficulty in quantifying axons with signs of later stages of degeneration, such as debris and detachment, we have quantified only the results of axons with early signs of degeneration in Figure 1B to illustrate the effects of proteasome inhibitors on delaying the onset of degeneration. This effect appeared to be specific for proteasome inhibitors, as the inclusion of a caspase inhibitor, Z-VAD-fmk, or a serine protease inhibitor, pepstatin A, did not result in any detectable protective effect (data not shown). Interestingly, such a protective effect only occurred when these proteasome inhibitors were added to the cultures prior to axotomy (Figure 1C). As it is known that these inhibitors are able to quickly execute their inhibitory effects upon addition to the cultures (Campbell and Holt, 2001), these results suggest a role of UPS in the early stages of Wallerian degeneration.

To provide additional evidence for the involvement of the UPS in Wallerian degeneration, we constructed recombinant herpes simplex viruses (HSV) to express in cultured sympathetic neurons a yeast ubiquitin protease (UBP2), which has been shown to reverse substrate ubiquitination across different species (Baker et al., 1992; DiAntonio et al., 2001; Watts et al., 2003). We found that sympathetic neurons infected with HSV expressing UBP2, but not control HSV, showed a significantly slower rate of degeneration (Figures 1E and 1F). However, the protective effects of UBP2 overexpression were less robust than those of the proteasome inhibitors, presumably due to the low expression levels of UBP2 (Figure 1F). Thus, the proteasome inhibitors were used for the rest of the study.

Consistent with previous studies (Finn et al., 2000), addition of ALLN (50 μM), an inhibitor of calcium-dependent proteases calpains, did not affect the development of the axonal beading appearance but slightly delayed the disintegration of transected axons into debris (Figures 1A and 1B). These results together suggest that UPS and calpains may act on different stages of Wallerian degeneration. Interestingly, application of the calcium chelator EGTA (2 mM) resulted in a much stronger protective effect on Wallerian degeneration than ALLN (Figure 1A) and other calpain inhibitors (data not shown). This observation suggests that the calpains might not be the only calcium-dependent event involved in Wallerian degeneration. The UPS is unlikely to be activated by elevated intracellular calcium levels, because the axon degeneration elicited by calcium ionophore A23187 or ionomycin were blocked by EGTA but not MG132 (data not shown). However, we cannot rule out the possibility that there is a calcium-dependent step in the participation of the UPS in Wallerian degeneration.

Microtubule Destabilization at the Initiation Stage of Wallerian Degeneration In Vitro

We next examined which cellular events might correlate with the involvement of the UPS and calpains in Wallerian degeneration. To this end, we monitored the integrity of different subcellular organelles in the transected axons by both immunocytochemistry and membrane labeling (Figure 2). Plasma membrane staining with both a lipophilic dye DiI (Figure 2A) and antibodies against an axonal surface molecule p75 (data not shown), as well as cytoplasmic staining with an antibody against PGP9.5, an abundant axonal cytoplasmic protein (George et al., 1995; Finn et al., 2000, data not shown), revealed characteristic morphological changes of Wallerian degeneration as depicted in Figure 1A, with the onset of swelling and beading followed by fragmentation and detachment of axonal debris. Likewise, immunostaining with an anti-neurofilament NF160 (2H3) antibody showed a similar time course of degeneration (Figure 2B), consistent with previous observations that neurofilaments are broken down during Wallerian degeneration (Schlaepfer et al., 1984; Finn et al., 2000). At the protein level, we observed a corresponding cleavage of the neurofilament subunits neurofilament 160 (NF-160) and neurofilament 68 (NF68) as detected by Western blotting (Figure 3E). Apart from these axonal markers, however, the earliest detectable change appears to occur in the microtubules of transected axons. Immunostaining with antibodies against both α-tubulin (data not shown) and β-tubulin (Figures 2C, 3A, and 3B) revealed fragmented microtubule structures in approximately 60% of the transected axons 4 hr after axotomy (Figures 2C and 3B), when other subcellular structures were still largely intact, as suggested by both phase contrast image analysis (Figure 1A) and immunostaining (Figures 2A and 2B). After 12 hr postaxotomy, the stained signals of fragmented microtubule structures weakened gradually and were replaced by many spheroid bodies in severed axons. By 16 hr after axotomy, almost all of the detected signals were spheroid bodies along the tracts of original axons. Surprisingly, even 16 hr after axotomy, when the majority of the axonal NF160 and NF68 had been degraded, most of the tubulin proteins still remained intact, and the protein level remained stable according to Western blotting (Figure 3E).

To provide additional biochemical evidence for such an axotomy-elicited microtubule destabilization in transected axons at early stages of Wallerian degeneration, we separated monomeric/dimeric (soluble, S) and polymerized (pellet, P) tubulins from transected axons and measured the depolymerization ratio (S/[S + P]) at different time points. As shown in Figure 3C, microtubule depolymerization in transected axons was significantly increased as early as 4–8 hr postaxotomy. These results together suggest that the breakdown of neurofilaments and microtubules might be mediated by different mechanisms, with neurofilament proteins being cleaved and microtubule polymers simply being disassembled.

The UPS and Calpains Mediate the Breakdown of Axonal Cytoskeleton Components

To explore the possible link between these cellular and molecular changes during Wallerian degeneration, we next examined whether proteasome inhibitors, calpain inhibitors, and EGTA affect neurofilaments and microtubules in transected axons. Both MG132 and EGTA profoundly retarded the degeneration of all examined subcellular markers, including both the destabilization of microtubules (Figures 3A–3C) and the cleavage of neurofilaments (Figures 3D and 3E, data not shown). However, ALLN only delayed neurofilament degradation (Figure 3E), not the fragmentation of microtubules (Figure 3B). While confirming a critical involvement of calpains in the cleavage of neurofilaments (Schlaepfer et al., 1984; Finn et al., 2000), these results also suggest that both calcium-dependent events and the UPS are critical for the disassembly of microtubules and perhaps other early events of Wallerian degeneration.

MG132 Slows Down Wallerian Degeneration of Transected Optic Nerve In Vivo

To assess whether these observations from in vitro culture experiments hold true in vivo, we first performed immunostaining experiments to examine the alterations of microtubules and neurofilaments in transected optic nerves (Figure 4). In uncut optic nerves, antibodies against both neurofilament and tubulin components revealed numerous fiber structures of the axon cytoskeleton (Figures 4A and 4B). In optic nerves 3 days after axotomy, when an anti-NF160 antibody still detected many linear neurofilaments fibers, an anti-β-tubulin antibody revealed largely spheroid bodies, reminiscent of the microtubule changes observed in vitro 16 hr postaxotomy (Figure 2). Similar staining patterns were observed in transected optic nerves 7 or 14 days postaxotomy (Figure 4B). As previous studies implicated neurofilament degradation as an early marker of Wallerian degeneration (Glass et al., 2002), our results suggest that destabilization of microtubules might occur prior to that of neurofilaments following axotomy.

To examine whether proteasome inhibitors would retard Wallerian degeneration in vivo, we applied gelfoams presoaked with vehicle (PBS) or MG132 to the lesion sites and examined the transected optic nerves 3 days postaxotomy by immunostaining. As expected, MG132 significantly slowed down the breakdown of microtubules and neurofilaments (Figures 4A and 4B) as well as axolemma as detected by anti-PGP9.5 antibody staining (data not shown) in transected optic nerves. However, since the effect of drugs presoaked in gelfoams is likely to decrease over time, we could not determine the maximum extent to which these proteasome inhibitors could slow down Wallerian degeneration in vivo. Nevertheless, these results together suggest that inhibiting UPS activity is able to delay Wallerian degeneration both in vitro and in vivo.

Proteasome Inhibitors also Delay Neurite Degeneration in NGF-Deprived Sympathetic Neurons

In addition to transected axons, axons from neurons that have been deprived of their physiological trophic factors may also undergo a Wallerian-like degeneration process (reviewed by Raff et al., 2002). If the distal part of an axon of a cultured sympathetic neuron is locally deprived of NGF, that part of the axon degenerates while the rest of the cell and axon survives (Campenot, 1982). Moreover, it has been shown that such NGF deprivation-triggered axon degeneration is delayed in the sympa-

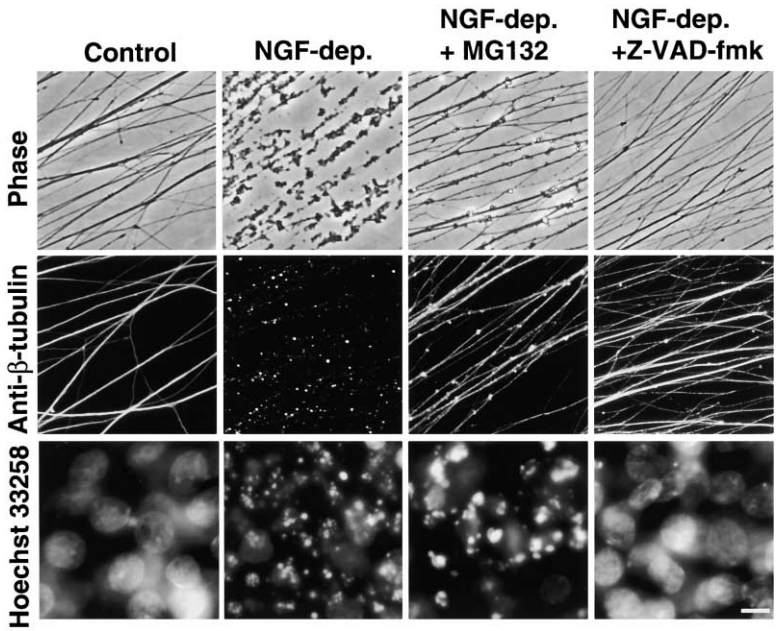

Figure 5. Inhibiting the UPS Prevented Axon Degeneration but Not Soma Death of NGF-Deprived Sympathetic Neurons

Alter changing to the medium with NGF (control) or without NGF (NGF-dep.) in the presence or absence of MG132 (20 mM) or Z-VAD-fmk (20 mM), SCGs were kept in culture for an additional 36 hr prior to acquisition of phase contrast images (phase) and fixation. The fixed cultures were then stained with anti-β-tubulin antibodies to show the microtubule structures and with Hoechst 33258 to show fragmented nuclei. Scale bar, 8 μm for phase and anti-β-tubulin and 4 μm for Hoechst 33258 micrographs.

thetic neurons from *Wld*^{s} mice (Deckwerth and Johnson, 1994). We thus decided to investigate whether UPS is also involved in NGF deprivation-triggered axon degeneration. Consistent with previous studies (for example, Deckwerth and Johnson, 1994), 36 hr after NGF deprivation, all of the cultured sympathetic neurons underwent a typical apoptosis program, with death of cell bodies as detected by nuclei staining with Hoechst 33258 (a few normally stained nuclei are glial cells) and degeneration of axons as indicated by both morphological criteria and immunostaining (Figure 5). The caspase inhibitor Z-VAD-fmk efficiently prevented both cell body death and neurite degeneration (Figure 5). However, in the presence of MG132, while these neuronal cell bodies still showed clear apoptotic staining, the majority of the axons were still intact (Figure 5), suggesting that MG132 specifically delays axon degeneration but not the death of cell bodies. In addition, overexpression of the yeast UBP2 protein by a recombinant HSV also showed a qualitatively similar but less profound protective effect on the degeneration of axons and not the death of cell bodies (data not shown). Thus, it is possible that the molecular events underlying Wallerian degeneration may also participate in other pathological conditions that have been thought to depend only on the apoptosis machinery.

Discussion

In this study, we have used both pharmacological and genetic means to show that the UPS plays a critical role in the early stages of Wallerian degeneration, possibly by eliciting the destabilization of axon microtubules that in turn triggers a late phase of axon degeneration. We also demonstrate that proteasome inhibitors are able to delay neurite degeneration but not cell body apoptosis in NGF-deprived sympathetic neurons, providing additional support that Wallerian degeneration operates via an apoptosis-independent molecular mechanism.

Involvement of the UPS in the Early Stages of Wallerian Degeneration

How is the UPS involved in Wallerian degeneration? Axon degeneration is protected only by pretreatment with the proteasome inhibitors, suggesting an involvement of UPS at the early stage(s) of Wallerian degeneration. This notion is further substantiated by the results that UPS inhibitors are able to retard the development of early signs of Wallerian degeneration. There are two possible mechanisms that may account for the early involvement of UPS in Wallerian degeneration. For one, it can activate many E3 proteins to trigger a generic protein degradation program. Alternatively, it may activate a single or a subset of E3s that mediate the degradation of key protein(s) that can then trigger a secondary phase of the degenerative process. Our results favor the latter possibility, as microtubule fragmentation, a specific and earliest detectable change during Wallerian degeneration, is dependent on UPS. Although proteasome inhibitors can also retard the degeneration of other axonal structures, such as axolemma and neurofilaments, it is possible that these alterations are secondary to the disruption of microtubules in the transected axons. Consistent with this notion, we found that proteasome inhibitors cannot protect the Wallerian-like neurite degeneration elicited by microtubule destabilizing agents, such as colchicine and vincristine (data not shown). It remains unclear whether microtubule destabilization is the only immediate consequence upon the activation of UPS in the transected axons during Wallerian degeneration.

Microtubule Destabilization in Wallerian Degeneration

While microtubule destabilization is sufficient to induce axon degeneration (Luduena et al., 1986; Wang et al., 2001b), disruption of neurofilaments by the introduction of anti-NF160 into *Xenopus* embryos did not affect the distribution of microtubules and other axon cytoskele-

ton structures (Walker et al., 2001). Moreover, we found that latrunculin B, an actin depolymerizing agent, failed to induce axon degeneration in our assays (data not shown). Thus, among the three major polymer components of the axon cytoskeleton, microtubules may play a particularly important role in maintaining the integrity of axons.

How are the axonal microtubules disrupted during Wallerian degeneration? Our results indicate that although an increased portion of microtubules appear fragmented and depolymerized, tubulin proteins per se are not cleaved at least at the early stages of Wallerian degeneration. Thus, the axonal tubulin proteins are not likely to be direct UPS substrates. It is known that the stability of microtubules in the neurons is controlled by a balance between stabilizing factors, notably microtubule associated proteins (MAPs; Garcia and Cleveland, 2001), and destabilizing factors, such as the Kin kinesin KIF2 (Desai et al., 1999). It is conceivable that, upon axotomy, engagement of UPS may lead to microtubule destabilization by degrading the stabilizing factors or the inhibitory molecules of destabilizing factors. Although our preliminary results suggest that the protein levels of two abundant MAPs (MAP1 and Tau) are stable at early stages of Wallerian degeneration, future studies are needed to examine whether other less abundant MAPs or microtubule destabilization factors may be the targets of UPS-mediated proteolysis. In addition, the fact that microtubule fragmentation is also protected by EGTA but not by calpain inhibitors suggests that an unknown calcium-dependent event might cooperate with UPS to induce the destabilization of axon microtubules during Wallerian degeneration.

The Physiological and Pathological Relevance of the Mechanisms of Wallerian Degeneration

The realization that Wallerian degeneration is brought about by an active program rather than a passive "wasting-away" process suggests that such a molecular mechanism may also function in normal physiological contexts. It is known that selective axon removal is an important means of fine-tuning or remodeling neuronal connectivity during development and in the adult (O'Leary and Koester, 1993; Sanes and Lichtman, 1999). Interestingly, a recent study examining the stereotyped pruning of γ neurons of the *Drosophila* mushroom bodies during metamorphosis demonstrated that such pruning is mediated by local degeneration, which also requires the action of UPS (Watts et al., 2003). Strikingly, in this pruning process, disruption of the microtubule cytoskeleton is also the earliest detectable change. These observations strongly suggest that some types of developmental axon pruning and Wallerian degeneration may share at least some common molecular mechanisms.

In addition to normal physiological contexts, axonal degeneration has frequently been observed in many types of neurodegenerative diseases and after various neuronal insults (Raff et al., 2002; Coleman and Perry, 2002). Such axon degeneration could occur either together with or independent of cell body apoptosis. For apoptosis-independent local axon degeneration, a typical example is the "dying back" process in aging Purkinje neurons (Chen and Hillman, 1999) and in peripheral nerves damaged by a wide variety of toxic, metabolic, and infectious insults (Raff et al., 2002). In these cases, axon degeneration begins distally and spreads toward the cell body. Although the underlying mechanisms for these local axon degenerations remain largely unclear, existing evidence supports a potential mechanistic link between such a dying-back process and Wallerian degeneration. For instance, the dying-back degeneration elicited by vincristine, a clinical anti-tumor drug with a common side effect of inducing axonal degeneration-associated peripheral neuropathy (Schaumburg et al., 1992), is protected in neurons from *Wld*^{s} mice (Wang et al., 2001b). Interestingly, a mutation in the Uch-l1 gene accounts for dying-back axon degeneration and the formation of spheroid bodies in degenerating nerve terminals in *gad* mice (Saigoh et al., 1999), pointing to the importance of UPS in maintaining the integrity of axons. It is known that the specificity of UPS-mediated proteolysis is determined by specific E2/E3 and perhaps hydrolases. Thus, a future challenge will be to identify specific E2/E3 molecules involved in maintaining the integrity of axons and mediating the degeneration of axons.

Experimental Procedures

Explant Cultures, Axotomy, and NGF Deprivation

SCGs dissected from P3 rats were cultured for 5 days in 6-well plates precoated with poly-D-lysine and laminin in DMEM/F12 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 ng/ml NGF, 10 μM cytosine arabinoside, and antibiotics. For axotomy, axons were cut through the axonal halo around each ganglion with a microscalpel and the ganglion was removed, leaving the transected axons in the culture dish. To deprive the neuronal culture of NGF, the medium was removed on day 5 in culture and substituted with the same medium without NGF. To observe the effects of different pharmacological perturbations on axon degeneration, MG132 and lactacystin were added to the cultures 3 hr prior to axotomy or NGF deprivation. Other inhibitors, including ALLN, pepstatin A, V-ZAD-fmk, and EGTA, were added 1 hr prior to axotomy or NGF deprivation. At indicated time points postaxotomy or after NGF deprivation, two representative phase micrographs were taken from each explant. To quantify percentages of axons with degeneration (with beading and/or fragmented appearance) from phase images, at least 200 singly distinguishable axons were blindly scored from several randomly taken images of entire explants per condition from duplicate explants per experiment and from three independent experiments.

Immunocytochemistry was performed with the following antibodies: monoclonal anti-β-tubulin (TUJ1) and anti-NF160 (2H3), polyclonal antibodies against p75 neurotrophin receptor (Covance).

To determine how much tubulin protein is in soluble (or depolymerized) form, we followed a standard procedure (Jacob and McQuarrie, 1996; Lund et al., 2002) to treat transected axons with polymer stabilizing buffer (100 mM Pipes [pH 6.9], 5 mM EGTA, 5 mM $MgCl_2$, 20% glycerol, 5% DMSO, 0.2% Triton X-100, protease inhibitor cocktail, 12 μM Taxol). After centrifugation (16,000 $\times$ *g* (90 min, 4°C), the supernatant (S) and pellet (P) fractions were mixed with SDS-containing sample buffer, resolved in SDS-PAGE, and detected by Western blotting with an anti-β-tubulin antibody. The amount of tubulin protein in each sample was determined by comparing the intensity of the bands with standard samples by NIH imager program. Depolymerization ratio was calculated as [S/(S + P)].

Optic Crushing, Fixation, and Immunohistochemistry

For optic nerve crushing, we followed the protocol described by Meyer et al. (1994). To study the protective effect of MG132, a

gelfoam presoaked with PBS or 50 μM MG132 was attached to the lesion site of the optic nerve for 60 min prior to crushing (mimicking the pretreatment) and was kept in the lesion site after crushing. To assess the protective effects of MG132, fiber structures as detected by immunostaining were counted from sections at similar distances to lesion sites and were presented as fiber density (fiber number/0.01 mm × 0.1 mm, vertical to the axon fibers).

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