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Journal of Neuroimmunology 181 (2006) 34–45

Journal of Neuroimmunology

www.elsevier.com/locate/jneuroim

The role of the proteasome–ubiquitin pathway in regulation of the IFN-γ mediated anti-VSV response in neurons

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Received 10 March 2006; received in revised form 20 June 2006; accepted 26 July 2006

Abstract

Pharmacologic inhibition of the proteasome resulted in increased NOS-1 protein levels and increased NO production by neuronal cells. This correlated with an increased antiviral effect of IFN-γ against the replication of vesicular stomatitis virus (VSV) replication *in vitro*. We also observed that a regulatory protein, Protein Inhibitor of NOS-1 (PIN) was down-regulated by IFN-γ treatment, and more ubiquitinated PIN accumulated in IFN-γ treated neurons. In cells of the reticuloendothelial system, IFN-γ treatment induces the expression of a set of *l*ow *m*olecular weight MHC-encoded *p*roteins (LMPs), which replace the β-subunit of the proteasome complex during the proteasome neosynthesis, resulting in a complex termed the immunoproteasome. LMP2, -7, and -10 were induced and the immunoproteasome was generated by IFN-γ treatment in neuronal cells. Importantly, we observed that IFN-γ induced inhibition of VSV protein synthesis was not dependent on ubiquitination.

Keywords: NOS-1; PIN; Ubiquitination; VSV

1. Introduction

IFN-γ is a critical antiviral mediator in eliminating viruses from the CNS (Komatsu et al., 1996; Chesler and Reiss, 2002). IFN-γ up-regulates NOS-1 expression post-transcriptionally and increases NO production, which is essential in the clearance of VSV infection by neurons (Bi and Reiss, 1995; Komatsu et al., 1996; Chesler et al., 2004). VSV is not unique; other neurotropic viruses whose replication is inhibited by NO include CMV (Kosugi et al., 2002), HIV (Hori et al., 1999), HSV-1 (Kodukula et al., 1999), MHV (Lane et al., 1997), Poliovirus (Komatsu et al., 1996), and Reovirus (Goody et al., 2005). But how this antiviral process is regulated largely remains unknown.

We propose that IFN-γ enhanced antiviral activity in neurons through alteration of the proteasome degradation of host proteins to induce NOS-1 expression and activity which can inhibit VSV protein synthesis (Bi and Reiss, 1995). Protein Inhibitor of NOS-1 (PIN) is a protein which complexes with NOS-1, preventing functional homodimer formation (Jaffrey and Snyder, 1996; Fan et al., 1998). Previous studies indicated that treatment of neurons with prostaglandin E_2 (PGE_2), an inhibitor of NOS-1 activity, induces PIN expression in neurons. Conversely, pharmacologic inhibition of cyclooxygenase-2 *in vitro* and in mice enhances NOS-1 activity, down-regulates PIN expression, and enhances clearance of virus and survival (Chen and Reiss, 2002).

The proteasome is an intracellular complex that degrades polyubiquitin-conjugated proteins (Ciechanover, 1994).

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doi:10.1016/j.jneuroim.2006.07.018

Previous studies showed that ubiquitination of NOS-1 occurs (Bender et al., 2000) and that irreversible inactivators of NOS enhance the proteolytic turnover of the enzyme by a mechanism involving the proteasome (Noguchi et al., 2000). IFN treatment may regulate the expression or activity of E3 Ub-ligases or de-Ubiquitinases, thus altering the functional half-life of the target proteins. The proteasome–ubiquitin pathway might be involved in IFN-γ mediated regulation of NOS-1 in neurons.

MHC-encoded IFN-γ-inducible *l*ow *m*olecular weight *p*roteins (LMP2, LMP7 and LMP10) replace the β-subunits of the proteasome complex in antigen presenting cells (Groettrup et al., 1996), altering its substrate specificity (Van den Eynde and Morel, 2001). Immunoproteasomes containing the IFN-γ-inducible β-subunits produce class I-binding peptides more efficiently than those containing only constitutive subunits. However, neurons do not participate in antigen processing and presentation, and are generally incapable of expressing MHC molecules (Gogate et al., 1996; Joly and Oldstone, 1992).

The immunoproteasome has been observed in neurons in three human neurodegenerative diseases: Huntington's Disease (Diaz-Hernandez et al., 2003), Amylotropic Lateral Sclerosis (Cheroni et al., 2005), and Alzheimer's Disease (Mishto et al., 2006). This induction of the LMPs in neurons may be secondary to local secretion of inflammatory cytokines including IFN-γ by T cells and NK cells during inflammatory diseases.

In this study, we examined ubiquitination of PIN, NOS-1 and VSV proteins and the generation of the immunoproteasome in neurons following IFN-γ treatment. The results demonstrate that IFN-γ treatment of neurons increases PIN ubiquitination and degradation. NOS-1 levels and production of NO increases. Ubiquitin-modification and protein degradation does not contribute to the suppression of VSV protein accumulation in IFN-γ-treated neuronal cells. The immunoproteasome is generated by treatment of neuronal cells with IFN-γ.

2. Results

2.1. Pharmacologic inhibition of the proteasome increases the antiviral effect of IFN-γ in vitro

In order to determine if the proteasome degradative pathway contributed to the inhibition of accumulation of VSV proteins in IFN-γ-treated neuronal cells (Komatsu et al., 1996; Chesler et al., 2004), we examined the effect of two proteasome inhibitors Lactacystin (LC) and Geldanamycin (GA) on VSV infection in neurons. LC inhibits proteasome directly by modifying the proteasome's catalytic β-subunits (Craiu et al., 1997). GA reduces proteasome-mediated antigen presentation through inhibition of Hsp90 (Imai et al., 2003). In order to determine the optimal concentration of inhibitors to be used in our studies, neuronal cells were treated with a range of doses of inhibitors. The MTS assay was used to measure mitochondrial activity as a surrogate for cell viability following LC or GA treatment (Fig. 1A). The suppression of proteasome activity by the two inhibitors was confirmed by an assay of proteasome cleavage of Z-GGL-pNA (Fig. 1B). Treatment of cells with LC or GA alone results in inhibition of VSV replication in NB41A3 cells (Fig. 2A). When we combined the two drugs, viral replication was almost totally eliminated. In IFN-γ-treated

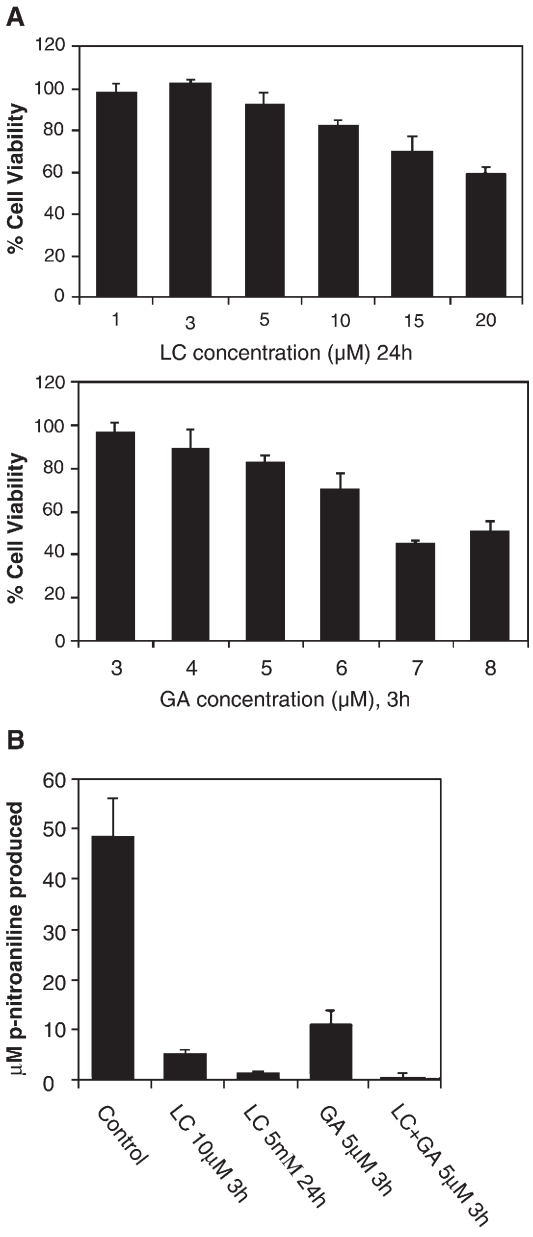

Fig. 1. Sensitivity of neurons to Proteasome Inhibition. (A) Cell viability (MTS) assay for NB41A3 cells treated with LC or GA. 2×10e6 NB41A3 cells were plated overnight before treatment of LC (24 h) or GA (3 h). Variable concentrations of LC or GA were used as indicated. Cell viability was measured using Promega MTS reagent. (B) LC and/or GA suppressed proteasome activity. NB41A3 cells were treated with LC and/or GA at indicated concentration and time. Cells were lysed and same amount of lysates from each sample were used to measure proteasome activity. Results are mean of triplicate samples.

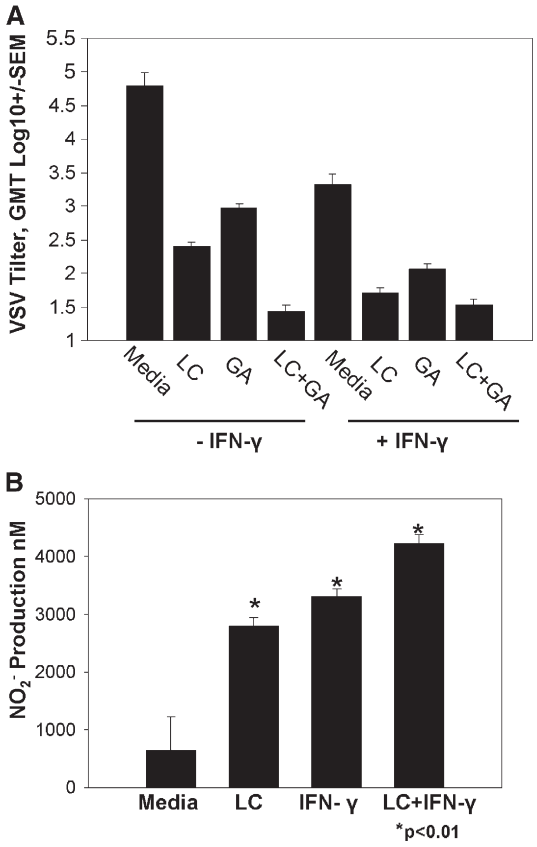

Fig. 2. (A) IFN-γ and proteasome inhibitors antagonize VSV replication in NB41A3 cells. NB41A3 cells were incubated with or without IFN-γ for 24 h; Lactacysin (10 μM) or Geldanamycin (5 μM) or both were added 3 h prior to infection with VSV at MOI = 0.1. At 7 h post infection, supernatants were harvested, and viral titers were determined by plaque assay. Viral titers presented are geometric mean titers from triplicate samples of each treatment. Data are representative of two experiments with similar results. (B) Lactacystin, IFN-γ, or both treatments induce NO production in NB41A3 cells. NB41A3 cells were treated with LC (3 μM, 24 h), IFN-γ (20 ng/ml, 48 h) or both. Supernatants were collected and a Griess assay was performed to measure the production of NO. Each sample was measured in triplicate. Results are representative of 3 independent experiments with similar trend (*$p < 0.01$).

NB41A3 cells, treated with LC or GA, the antiviral effect of IFN-γ was enhanced.

2.2. Inhibition of proteasome results in increased NO production and NOS-1 accumulation

NO is an important cellular inhibitor of viral replication (Reiss and Komatsu, 1998). The NOS-1 product, NO, can spontaneously combine with superoxide to form peroxynitrite (ONOO–), which readily degrades to NO_2^- (Love, 1999). To investigate the regulation of NOS-1 activity by the proteasome, we measured NO_2^- production by IFN-γ or LC treated neuronal cells. We treated NB41A3 neuroblastoma cells for 48 h with IFN-γ to allow accumulation of NO_2^- in the media. Fig. 2B shows NO_2^- production is increased by IFN-γ or LC treatment, consistent with the NOS-1 expression. This correlates well with the inhibition of VSV replication (Fig. 2A).

Ubiquitination and proteasome degradation of NOS-1 has been observed in transfected HEK cells (Bender et al., 2000). To determine if this were also true in mouse neuronal cells which constitutively express NOS-1, NB41A3 cells were treated for IFN-γ and/or LC for 24 h; the expression of NOS-1 mRNA was detected by RT-PCR and the protein by Western blot. In all related experiments, double treatment of IFN-γ and LC provide us the information of how IFN-γ effects the cell when proteasome activity was blocked. As expected, the mRNA level of NOS-1 is unaltered by IFN-γ or LC treatment (Fig. 3A). Similar results were observed at earlier time points (3 h, 12 h; data not shown). While NOS-1 protein levels increased four- to six-fold with IFN-γ or LC treatment (Fig. 3B; Komatsu et al., 1996; Chesler et al., 2004). The induction of NOS-1 protein in the presence of LC treatment was also observed in primary neuron cultures (Fig. 3C). This indicates both treatments of neuronal cells led to an accumulation of NOS-1 at the post-transcriptional level.

2.3. IFN-γ treatment inhibits NOS-1 degradation but ubiquitination is unchanged

The level of ubiquitination and degradation of NOS-1 following IFN-γ treatment of neurons was examined by immunoprecipitation (IP) and by pulse-chase experiments. Cells treated with LC served as a control for accumulation of NOS-1. Tubulin III was used as housekeeping and loading control for the IP experiment. As shown in Fig. 3D, unchanged levels of ubiquitinated NOS-1, molecular mass ~25 kDa higher than the NOS-1 monomer, were observed in both IFN-γ-treated neurons and the untreated cells. When the proteasome was inhibited, more Ub-NOS was observed in LC treated samples, but this was not affected by IFN-γ treatment.

Pulse-chase was performed to measure the degradation of NOS-1 in the presence or absence of IFN-γ or LC. As previously observed (Chesler et al., 2004), the half-life of NOS-1 increased 33%, from ~18 h to ~24 h in IFN-γ-treated neuronal cells (Fig. 3E). NOS-1 accumulation in IFN-γ-treated neuronal cells is secondary to slower degradation. We do not know at present whether activity of an E3 ligase is suppressed or whether a de-ubiquitinase specific for NOS-1 is up-regulated in IFN-γ-treated neurons. Work is in progress to examine gene expression in these cells under differing conditions to elucidate this question.

2.4. IFN-γ post-transcriptionally down-regulates PIN

To determine if loss of PIN were involved in the IFN-γ-mediated activation of NOS-1, we examined the expression of PIN following IFN-γ treatment in neuronal cells. RT-PCR indicated that the ratio of PIN mRNA to β-actin mRNA was unchanged by IFN-γ treatment (Fig. 4A),

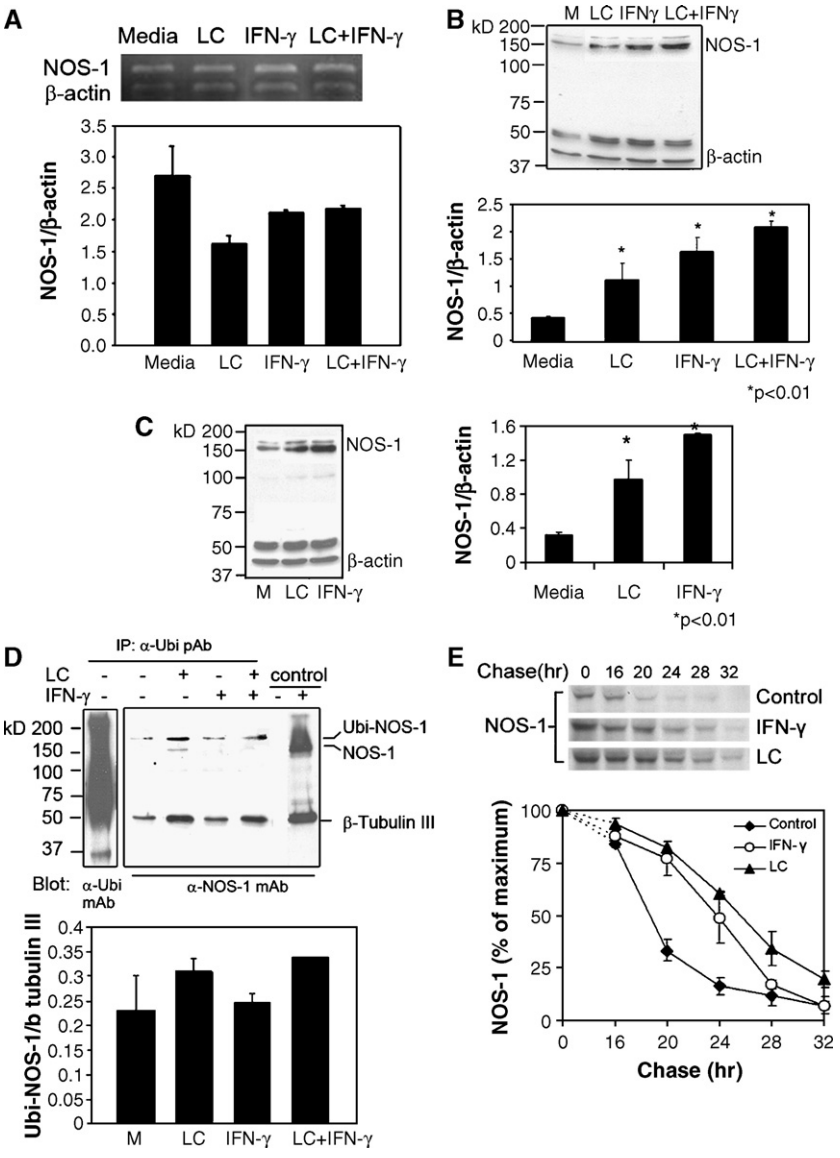

Fig. 3. Lactacystin or IFN-γ treatments induce NOS-1 expression post-transcriptionally with ubiquitination of NOS-1 unchanged. (A) Lactacystin or IFN-γ treatment does not alter NOS-1 mRNA levels in NB41A3 cells. NB41A3 cells were treated in triplicate for 24 h with medium, LC, IFN-γ or both. Total RNA was isolated and NOS-1 mRNA levels were determined by RT-PCR. The relative densities of bands were measured, and differences between reactions were normalized by β-actin levels. Data are represented as means ± S.E.M. (B) Lactacystin, IFN-γ, or both treatments induce NOS-1 expression in NB41A3 cells. NB41A3 cells were treated with LC, IFN-γ or both for 24 h. Total protein was collected and expression of NOS-1 and β-actin was measured by Western blot using anti-NOS-1 mAb and anti-β-actin mAb. (C) Lactacystin or IFN-γ treatment induces NOS-1 expression in primary neurons. Primary cultured neurons were treated with LC or IFN-γ for 24 h and expression of NOS-1 was detected by Western Blot. Results are representative of 3 independent experiments (*$p < 0.01$). (D) NB41A3 cells were treated with media (control), IFN-γ or LC, or both for 24 h. Ubiquitin conjugated proteins were precipitated using anti-ubiquitin pAb and blotted with anti-NOS-1 mAb. IP negative control (a sample with no antibody for IP step) was included. Each sample was loaded in duplicate. Results are representative of 3 independent experiments. (E) IFN-γ treatment results in prolonged half life of NOS-1. NB41A3 cells treated with IFN-γ (24 h) or LC (3 h) were metabolically labeled with [^{35}S]methionine/[^{35}S]cysteine mix for 2 h and chased for the indicated times. Same treatment (IFN-γ or LC) was present throughout the experiment. The cells were lysed and equal amount of lysates was used for each chase time. NOS-1 was analyzed by immunoprecipitation with anti-NOS-1 antibody and SDS-PAGE. The gel was exposed and the radioactivity was quantified. The values are the mean ± S.E. of three independent experiments.

while the protein level was significantly reduced in the IFN-γ-treated group (Fig. 4B). Earlier time points (3 h to 12 h, data not shown) were also examined with consistent results. Thus IFN-γ treatment of neuronal cells down-regulates PIN accumulation at the post-transcriptional level.

2.5. IFN-γ treatment enhances ubiquitination and degradation of PIN

To further investigate the mechanism of post-transcriptional down-regulation of PIN by IFN-γ, we performed an immunoprecipitation experiment to detect ubiquitination of

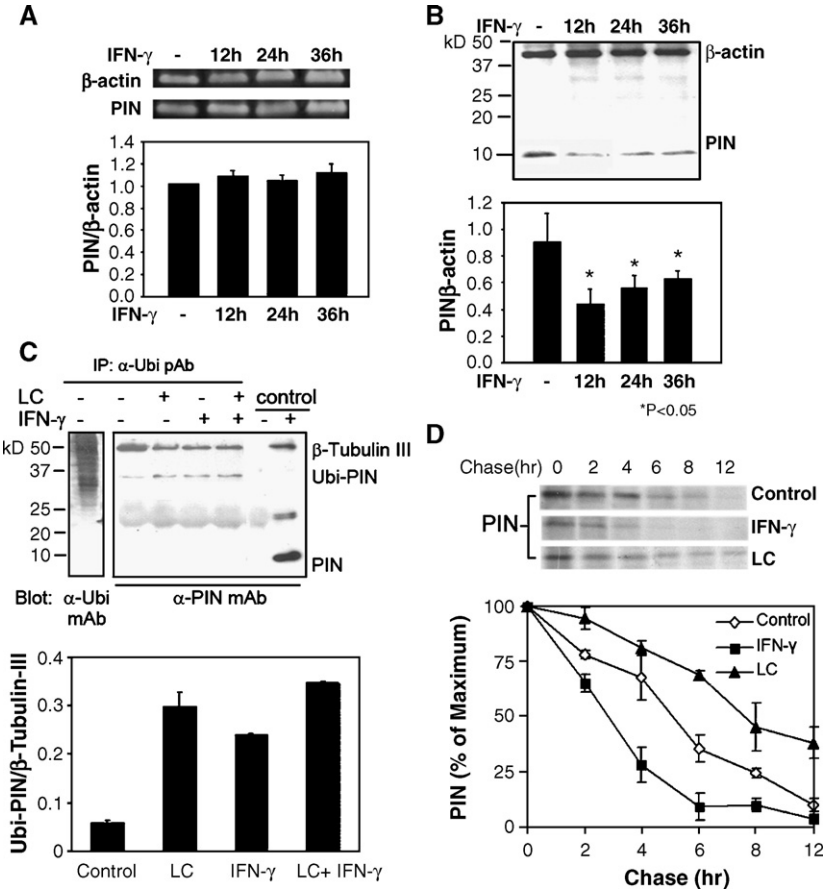

Fig. 4. PIN is post-transcriptionally down-regulated by IFN-γ and more ubiquitinated PIN is found in IFN-γ treated neuronal cells. (A) Treatment of neurons with IFN-γ does not alter PIN mRNA level. Total RNA was isolated from NB41A3 cells treated with IFN-γ for 0, 24, 48 or 72 h. RT-PCR was performed. 100 ng of total RNA was used for each reaction. Product sizes are as follows: PIN—390 bp; β-actin—540 bp. Each sample was amplified in duplicate. Results are representative of 3 independent experiments (*$p>0.05$). (B) Treatment of neurons with IFN-γ down-regulates PIN protein level. Whole cell lysate was isolated from NB41A3 cells treated with IFN-γ for 0 h, 24 h, 48 h or 72 h. The PIN protein level was determined by Western blot and normalized by β-actin. Results are representative of 3 independent experiments (*$p<0.05$). (C) More ubiquitinated PIN was found in IFN-γ treated neurons. Whole cell lysates were isolated from NB41A3 cells treated with LC or IFN-γ or both. Ubiquitin conjugated proteins were precipitated using anti-ubiquitin pAb and blotted with anti-PIN mAb. (D) IFN-γ treatment reduces half-life of PIN. NB41A3 cells treated with IFN-γ or LC were metabolically labeled with [^{35}S]methionine/[^{35}S]cysteine mix for 2 h and chased for the indicated times. Same treatment (IFN-γ or LC) was present throughout the experiment. The cells were lysed and PIN was analyzed by immunoprecipitation with anti-PIN antibody and SDS-PAGE. The gel was exposed and the radioactivity was quantified. The values are the mean±S.E. of three independent experiments.

PIN in both control and IFN-γ-treated neuronal cells. We precipitated ubiquitin-conjugated proteins using anti-ubiquitin pAb and blotted with anti-PIN mAb. As shown in Fig. 4C, PIN migrated at a molecular mass band of ~34 kDa; we interpret this to mean three ubiquitin units. Four-fold more ubiquitinated PIN was found in IFN-γ treated neuronal cells.

To measure the degradation of PIN, we performed a pulse-chase experiment. Based on preliminary results, the kinetics of PIN in cells was distinct from that of NOS-1 In comparison with control, PIN was degraded more rapidly in IFN-γ treated neurons (Fig. 4D). The half-life of PIN was reduced 40%, from ~5 h to ~3 h. As a control, LC treatment prolonged its half-life 50%, to ~7.5 h. We interpret these data to be consistent with IFN-γ induction of a Ub-ligase which modifies PIN and results in its degradation by the proteasome. The loss of PIN increases the active fraction of NOS-1.

2.6. Ubiquitin is not involved in IFN-γ-mediated inhibition of accumulation VSV proteins

IFN-γ treatment of neurons inhibits the production of VSV proteins ~75% and the release of infectious VSV by 30- to 1000-fold (Komatsu et al., 1996; Chesler et al., 2003). The inhibition of VSV protein synthesis is not associated with the dsRNA-activated protein kinase PKR which can suppress protein translation by phosphorylation of eIF2α (Chesler et al., 2003). Viral proteins can be degraded more rapidly when they are Ub-modified (Aviel et al., 2000).

NB41A3 cells were treated with IFN-γ and infected with VSV. Whole cell lysates were obtained at 5 h pi and a direct immunoblotting was performed to estimate total amount of VSV proteins in IFN-γ-treated as well as control cells (Fig. 5A). As previously observed (Komatsu et al., 1996; Chesler et al., 2003), IFN-γ treatment of neuronal cells resulted in

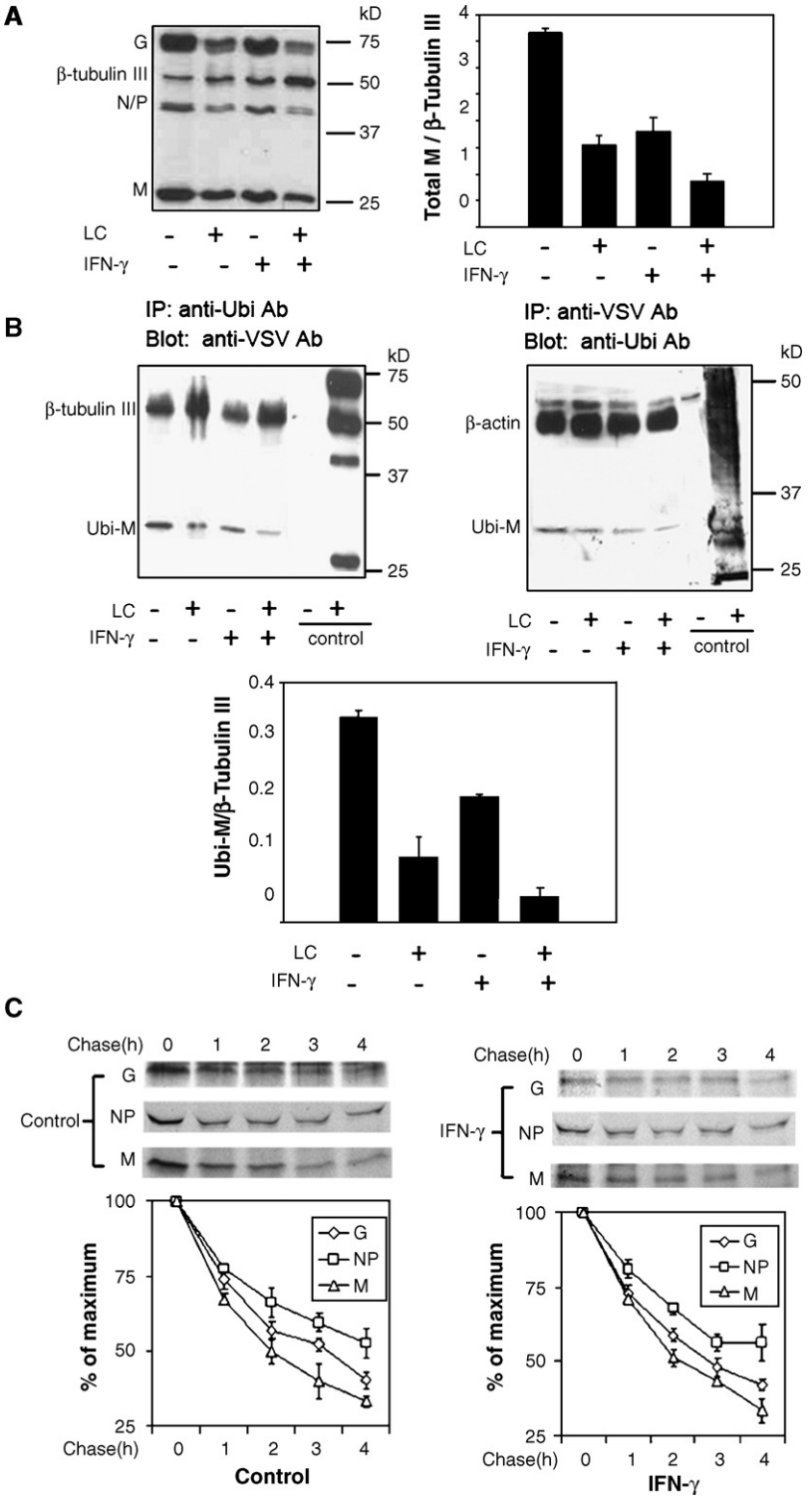

Fig. 5. Ubiquitin is not involved in IFN-γ mediated inhibition of viral synthesis. NB41A3 cells were pretreated with media (control), IFN-γ or LC, or both for 24 h prior to infection with VSV (MOI=0.1) and the 2 LC treated groups will be continuously treated with LC post infection. At 5 h pi, whole cell lysates were extracted. (A) The total amount of VSV proteins was determined by Western blot on duplicate samples with anti-VSV pAb, GAPDH was used as loading control. Results are representative of 3 independent experiments. (B) Ubiquitin pAb and β-tubulin III pAb were used for immunoprecipitation prior to SDS-PAGE; samples were blotted with anti-VSV pAb and anti-β-tubulin III mAb, and visualized with Pierce ECL kit. For the same samples, we also did IP with anti-VSV pAb, blotted with anti-Ubiquitin mAb and similar results were observed. The relative densities of the gel bands were quantitated and the amount of ubiquitinated M was normalized by total amount of β-tubulin. (C) Half-lives of VSV proteins are not changed by IFN-γ treatment. NB41A3 cells were pretreated with media (control) or IFN-γ for 24 h prior to VSV infection (MOI=1). After infection, cells were starved in Met/Cys free media for 30 min, metabolically labeled with [^{35}S]Met/[^{35}S]Cys mix for 1 h, and chased for indicated time. Equal amount of lysates were used and VSV proteins were immunoprecipitated with anti-VSV pAb and analyzed by SDS-PAGE. The gel was exposed and the radioactivity was quantified. The values are the mean±S.E. of three independent experiments.

inhibition of accumulation of VSV proteins (G, N, P, M), indicating that *either* viral protein synthesis is inhibited by IFN-γ treatment *or* viral proteins are rapidly degraded in IFN-γ treated neuronal cells. To determine if VSV proteins were targeted to the proteasomal pathway, we performed immunoprecipitations of ubiquitinated proteins and of viral proteins. Ubiquitinated VSV proteins were detected following immunoprecipitation with anti-ubiquitin by Western blot using anti-VSV pAb. As indicated in Fig. 5B, only M protein was found to be Ubiquitin modified in neuronal cells, which is consistent with the observation in BHK-21 cells of Harty et al. (2001). In IFN-γ-treated group, less Ub-M was detected, which correlated with decreased level of VSV proteins observed in NB41A3 cells following IFN-γ treatment. Ub-M was not over-represented.

Similar results were observed in a reciprocal IP in which anti-VSV pAb was used for precipitation and anti-ubiquitin mAb used for Western blotting (Fig. 5B). Thus IFN-γ treatment did not alter the ubiquitination of VSV M protein and proteasomal degradation cannot account for the IFN-γ-mediated inhibition in VSV protein accumulation.

A pulse-chase study was performed on VSV proteins in media and IFN-γ-treated cells to determine if the half-life of the viral proteins were shorter than in untreated neurons. If it were, this might indicate a proteolytic mechanism distinct from the ubiquitination-to-proteasome pathway, possibly autophagy, were active in the neurons. The result indicates that the degradation of VSV proteins is unchanged by IFN-γ treatment. As shown in Fig. 5C, the half-lives of VSV M, G, and NP proteins vary from 2 to 5 h, and there are no significant differences in between the control and IFN-γ-treated groups.

2.7. Immunoproteasome is induced by IFN-γ in murine neurons in vitro

The IFN-γ-induced immunoproteasome plays a major role in generating MHC I presenting peptides in antigen presenting cells. We investigated whether IFN-γ treatment resulted in the induction of the immunosubunits of the proteasome in murine neurons *in vitro*. Fig. 5A shows the induction of LMP2 mRNA expression and LMP7 protein by IFN-γ in NB41A3 cells. Similar results were observed in all three immunosubunits LMP2, LMP7 and LMP10. Untreated cells show no or extremely low levels of LMP expression while IFN-γ induced their expression at both mRNA and protein levels significantly. The mRNA induction was rapid and can be observed within 3 h of IFN-γ treatment (data not shown). All three components display maximum induction of expression at about 24 h treatment. To confirm that the immunosubunits are incorporated into the immunoproteasome, we isolated and immunoprecipitated the proteasome/immunoproteasome 20S complex from IFN-γ-treated and untreated NB41A3 cells and used Western blot to detect the incorporation of the three immunosubunits. Fig. 5B indicates that immunosubunits are found as components of the proteasomes isolated from IFN-γ-treated samples. The constitutive subunit α_5 was consistent in all samples. Similar observations were made using primary cultured neurons (not shown). Our results demonstrate that the immunoproteasome is generated in murine neurons by *in vitro* IFN-γ treatment.

3. Discussion

In this study, the contribution of proteasome–ubiquitin pathway in the IFN-γ-mediated anti-VSV response in neuronal cells was examined. We found that PIN is polyubiquitinated and rapidly degraded by proteasome in neurons (Fig. 4). IFN-γ treatment of neuronal cells leads to increased synthesis and slower degradation of NOS-1 (Fig. 3), resulting in production of NO and inhibition of viral replication (Fig. 2). Ub-modification and degradation of VSV proteins was unchanged in IFN-γ-treated neurons (Fig. 5). Immunoproteasome components LMP2, -7, and -10 are induced rapidly and incorporated into the proteasome in IFN-γ-treated neurons (Fig. 6).

Among VSV proteins, only M was polyubiquitinated, but its ubiquitination profile is unchanged by IFN-γ treatment (Fig. 5). Among the three isoforms of NOS, NOS-1 is the only type expressed in neurons. The mechanisms by which the viral replication is inhibited NO remain to be clarified. Peroxynitrite, ONOO(−), the product of superoxide and NO, may attack tyrosines, serines and cysteines of proteins and results in NO_2Y, NO_2S, and NO_2C (Gu et al., 2002; Radi, 2004). Such modifications may alter viral polymerase activity, protein interactions, or morphogenesis (Simon et al., 1996; Saura et al., 1999). NO_2-modified proteins may not be optimal substrates for Ub-modification, as well.

In eukaryotic cells, one of the primary pathways for the degradation of proteins in the cytosol and nucleus is via the proteasome–ubiquitin pathway (Zwickl et al., 1999). IFN-γ treatment altered the ubiquitination of PIN, likely through up-regulation of Ub-ligases and/or dampening of specific deUBases. It was reported 4 or more Ub units may be required for degradation of proteins by the proteasome (Hicke, 2001; Lederkremer and Glickman, 2005). We found that inhibition of proteasome prolonged half-life of PIN. However, co-IP experiment only identified triply-Ub-modified PIN, this could due to the rapid degradation of polyubiquitin chain and/or the affinity of the commercial antibodies used. Identifying the specific enzymes critical for this pathway will help elucidate some of the mechanisms of IFN-mediated antiviral responses.

Ubiquitination of viral proteins have been found in several virus infections (Hu et al., 1999; Kim et al., 2003; Eom et al., 2004). The ubiquitination of VSV M protein is involved in regulation of viral budding (Harty et al., 2001) and has been shown to be critical for morphogenesis of other viruses (Khor et al., 2003). Our examination of ubiquitinated VSV proteins in neurons showed only M protein is significantly ubiquitinated and the amount of Ub-M was unchanged by IFN-γ treatment. Thus ubiquitin does not

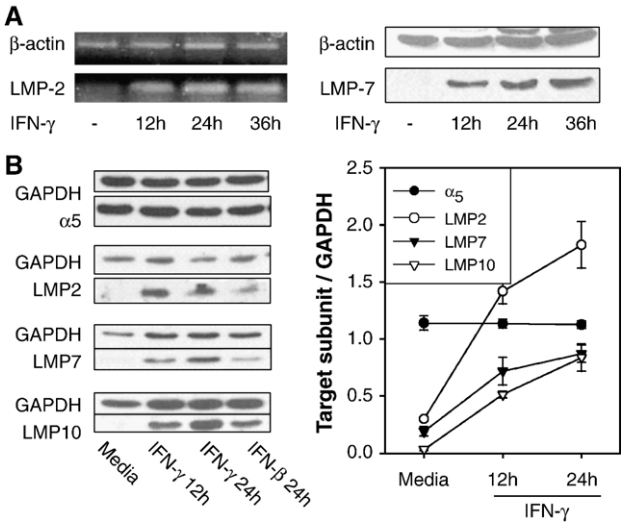

Fig. 6. (A) LMPs are induced in NB41A3 cells by IFN-γ. Treatment of neurons with IFN-γ induces LMP2 mRNA level. Total RNA was isolated from NB41A3 cells treated with IFN-γ for 0, 12, 24 or 36 h. RT-PCR was performed. Product sizes are as follows: LMP2—237 bp; β-actin—540 bp. Each sample was amplified in duplicate. Results are representative of 3 independent experiments (*$p<0.01$); IFN-γ induces LMP7 in NB41A3 cells. NB41A3 cells were treated with IFN-γ 20 ng/ml for 24 h, 48 h and 72 h. LMP7 levels were tested by Western blot. Raw 264.7 cells treated with IFN-γ for 24 h serve as positive control for anti-LMP7 antibody (not shown). Data are representative of three experiments with consistent trends. (B) Immunoproteasome is generated in neurons with IFN-γ treatment. NB41A3 cells were treated with media (control), IFN-γ (12 h and 24 h) and IFN-β (24 h). Whole lysates were extracted from each treatment. Proteasome 20S complex were purified from each lysate (200 μg) by IP using anti-proteasome 20S pAb. The purified proteasome complex was boiled with dissociation sample buffer and the presence of the constitutive subunit α_5 and the immunoproteasome subunits LMP2, 7, 10 were detected by Western blot. Anti-GAPDH pAb was used in both IP and immunoblotting as assay control. To eliminate the heavy and light IgG chains in the immunoblotting, Rabbit Trueblot™ beads and secondary antibody set was used whenever a rabbit anti-mouse primary antibody was used for blot. IFN-β treatment is used as positive control. Same results were observed in 3 independent experiments.

appear to contribute to the more rapid degradation of VSV proteins in IFN-γ-treated infected neurons. However, degradation of VSV by proteasome/immunoproteasome is still possible. Ubiquitin conjugation may not be required for the degradation of oxidized proteins by proteasome (Shringarpure et al., 2003). VSV infection induces oxidative stress in neurons (Komatsu et al., 1999).

A major function of the immunoproteasome is to produce antigenic peptides presented to $CD8^+$ T cells (Kloetzel and Ossendorp, 2004). However, little has been studied about the substrate specificity of the immunoproteasome. The immunoproteasome has been observed in three human neurodegenerative diseases: ALS, AD and HD (Diaz-Hernandez et al., 2003; Cheroni et al., 2005; Mishto et al., 2006). It is unclear what the effect of substitution of constitutive subunits with immunoproteasome LMPs has on the neuron's physiology in these diseases or in our studies. This alteration is likely to be secondary to infiltration of IFN-γ-secreting inflammatory cells from circulation in response to parenchymally-produced chemoattractants. Clearly, however, this is not necessary for processing and presentation of immunogenic short peptides to be presented on class I MHC molecules, as the vast majority of neurons are not able to express MHC and even when artificially induced to express MHC class I, incapable of presenting to T cells (Joly and Oldstone, 1992).

Although the impact of the immunoproteasome on inhibition of VSV replication remains unknown, it may be involved in degradation of VSV proteins through ubiquitin-independent pathways. A number of related ubiquitin-like proteins, including SUMO, Nedd8 and ISG15 have been identified that can modify to proteins and modulate proteins at the posttranslational level (Hochstrasser, 2000). ISG15 is the best defined Ub-like protein that is induced by IFN and microbial challenge, including virus infection. Upon induction, ISG15 conjugates with a variety of proteins, and may prevent the ligation of ubiquitin (Liu et al., 2003). The gain of function modulation of Jak-1, STAT-1 proteins by ISG15 has been observed (Malakhova et al., 2003) implying a role of ISG15 in cytokine induced cellular alterations. Work is in progress on the contribution of these homologues in IFN-γ-treated neurons on the inhibition of VSV replication.

Another degradative pathway is autophagy (Suhy et al., 2000; Schmid and Munz, 2005; Levine and Yuan, 2005), in which cytoplasmic domains are engulfed and fuse with the lysosome for destruction and recycling of proteins. Although we initially chose to focus on the proteasome, work is in progress to determine the contribution of autophagy in cytokine-treated neurons.

Our study indicated that, in addition to its key role in cellular protein degradation, the proteasome–ubiquitin pathway is involved in many aspects of IFN-γ-regulated antiviral responses in murine neurons *in vitro*. We also report generation of immunoproteasome in the murine neurons in culture. Ubiquitin and ubiquitin-like-proteins, with their roles in modulation of IFN-γ signaling components may be critical mediators in the IFN-γ-regulated antiviral responses. IFN-γ treatment results in increased translation and longer half-life of NOS-1. At the same time, there is depletion of a NOS-1 regulatory protein, PIN by the ubiquitin–proteasomal pathway. Viral proteins accumulate more slowly in IFN-γ-treated neurons, but this is apparently not due to Ub-modification and degradation.

4. Experimental procedures and materials

4.1. Cell cultures and reagents

NB41A3 cells, RAW 264.7 cells and L929 cells were purchased from ATCC. NB41A3 cells were maintained in F-12K medium containing 15% horse serum (Summit Biotech), 2.5% fetal bovine serum (FBS, Atlanta Biologicals), and 1% penicillin/streptomycin at 37 °C/5% CO_2. RAW 264.7 cells were grown in DMEM low glucose media

with 10% FBS and 1% penicillin/streptomycin at 37 °C/5% CO_2. L929 cells were maintained in DMEM low glucose media containing 10% FBS, 1 mM L-glutamine, HEPES buffer and 1% penicillin/streptomycin at 37 °C/5% CO_2. Unless otherwise stated, all tissue culture reagents were obtained from Mediatech.

VSV, Indiana serotype, San Juan strain, originally obtained from Alice S. Huang (The Children's' Hospital, Boston) was used in all viral studies. Recombinant murine IFN-γ and IFN-β were purchased from R&D Systems and used at a concentration of 20 ng/ml and 100 units/ml in all experiments. Lactacystin (LC, EMD Biosciences) and Geldanamycin (GA, Sigma) were used at concentration of 5 μM unless otherwise stated.

4.2. Primary neuron cultures

Newborn TgNSE-D^b mice (the gift of Michael B.A. Oldstone, The Scripps Research Institute; reference Joly and Oldstone, 1992) that constitutively express H-$2D^b$ driven by the neural specific enolase promoter in neurons were used as a source of neurons for primary culture. They were positively selected by panning using Mouse anti-H-$2D^b$ mAb (hybridoma cell lines HB36 and HB19 purchased from ATCC). Poly-D-lysine-coated 24 well plates (BD) were precoated with 100 μl goat–anti-mouse IgG (1:50, Vector) at 37 °C for 3 h. The plates were then coated with 200 μl anti-H-$2D^b$ monoclonal antibody (3 mg/ml) at 37 °C overnight and rinsed twice with HBSS prior to use. Otherwise primary cultures were prepared as previously described (Chesler et al., 2003). Olfactory bulbs were dissected from P0 (<24 h old) TgNSE-D^b mice, dissociated by enzymatic digestion (porcine trypsin 2.5%; Life Technologies, NY), and manual trituration using flame polished Pasteur pipettes. Cultures were plated in Dulbecco's minimal essential medium (DMEM) with 10% fetal bovine serum (FBS), 1% Penicillin/Streptomycin (Life Technologies, NY), 10 mM KCl, 10 μM L-glutamine, and 10 μM AraC (Sigma) for 1 h at 37 °C/5% CO_2. Remove the media with floating cells after 1 h and add same media with AraC. After 24 h, the medium was replaced with Neurobasal Medium (Life Technologies, NY) with 1% N2 supplement (Life Technologies), 0.5 mM L-glutamine, 25 μM L-glutamic acid, 10 mM KCl, 1% Penicillin/Streptomycin, and 10 μM AraC. All experiments were performed on day 6 of culture.

4.3. Cell viability (MTS) assay

Cell viability was determined by the MTS (Promega, Madison, WI) colorimetric assay following LC or GA treatment. The assay is based on soluble formazan production by dehydrogenase enzymes found in metabolically active cells. Cells were cultured with 100 μl media in 96 well plates and samples were seeded in three wells per time point at 2.5×10^3 cells per well. 100 μl of freshly mixed MTS reagent was added into each well and incubated for 1 h. Absorbance was determined at 490 nm using a micro-plate reader (Model 550, Bio-Rad) and the results expressed as the mean absorbance of triplicate experiments ± S.E.

4.4. Assay of proteasome activity

Cell lysates containing active 20S proteasome were prepared from NB41A3 cells by 20S lysis buffer containing 25 mM Tris × Cl, pH 7.4, 1 mM EDTA, 1 mM NaN_3. The assay is based on the proteasome-catalyzed cleavage of *p*-nitroaniline from *N*-benzyloxycarbonyl-Gly-Gly-Leu-*p*-nitroanilide (Z-GGL-pNA; Sigma) (Current protocols in protein science, *21.6*). 200 μg of cell lysate was diluted in 240 μl 0.05 M Tris × Cl (pH 7.5), mixed with 10 μl of 10 mM Z-GGL-pNA and incubated in 37 °C for 30 min. The reaction was stopped by adding 0.25 ml of 10% TCA. Samples were centrifuged at and 0.25 ml clear supernatant was mixed with 0.25 ml of 0.1% $NaNO_2$, incubating at room temperature for 3 min. 0.25 ml of 0.5% ammonium sulfamate was added and mixed for 2 min. Then 0.5 ml of 0.05% NNEDA was added to the sample following by vigorous vortex. Liberated *p*-nitroaniline was converted to a red-violet colored product and the absorbance of the chromogen was read in a microplate reader (model 550; Bio-Rad) at 540 nm against the blank. A standard curve (10 to 100 nmol *p*-nitroaniline) was established.

4.5. Plaque assay

Viral titers were determined in triplicates using serially diluted culture culernatants on monolayers of L929 cells as previously described (Komatsu et al., 1996). Data are presented as Geometric Mean Titer ± S.E.M., log (pfu).

4.6. RT-PCR

mRNA levels were determined by multiplex RT-PCR using the Qiagen One-Step RT-PCR kit. Total RNA (100 ng) was used in each reaction. The following program was used to carry out the reaction: 50 °C for 30 min, 95 °C for 15 min, 32 cycles of 94 °C for 1 min, 58 °C for 30 s, 72 °C for 1 min, then 72 °C for 10 min, and 4 °C hold. The primers were used in these reactions are listed in Table 1. Products were resolved by 1% agarose gel electrophoresis, stained using the SybrGold dye (Molecular Probes), and visualized using a UV transilluminator. Results were digitally photographed using a Kodak DC290 camera controlled by the Kodak 1D

Table 1
Primers used for RT-PCR and size of products

NOS-1	agcatgcctggcggaacgcctc	cagcaatgttaatctccacc	692 bp
β-Actin	gtgggccgctctaggcaccaa	ctctttgatgtcacgcacgatttc	540 bp
LMP2	aacgtggtgaagaacatctc	gatggcatttgtggtgaaac	237 bp
LMP7	cacactcgccttcaagttcc	ctttcacccaaccgtcttcc	551 bp
LMP10	cttgtgttccgagatggagt	agagctgttggtggaagcca	460 bp
PIN	tagcgaccggctgtcttctg	ggatcactgggtgtttggca	392 bp

analytic software (Eastman Kodak, Rochester, NY). DNA band densities were determined using the UN-SCAN-IT software.

4.7. Griess assay

The concentration of NO_2^- in culture supernatants was determined by assaying the stable end product of NO (Hewett, 1999). Briefly, equal volumes (100 μl) of experimental sample and Griess reagent (1 part 0.1% sulfanilamide in 60% acetic acid, 1 part 0.1% napthylenediamine dihydrochloride [Sigma] in distilled H_2O) were mixed and incubated at room temperature for 10 min. The reaction produces a pink color, which was quantified at 540 nm by microplate reader. Each sample was read in triplicate. A standard curve was made from sodium nitrite dissolved in blank medium.

4.8. Western blot

NB41A3 cells or primary neurons were cultured and treated with IFN-γ or LC for indicated period of time. Cells were lysed and protein concentrations were determined using the Bio-Rad DC protein assay, and 2 mg/ml solutions were prepared and boiled for 5 min in dissociation buffer. Samples were resolved by 7.5%, 10% or 12% SDS-PAGE electrophoresis, transferred to Transblot membranes (Bio-Rad), and probed for 1 h at ambient temperature or overnight at 4 °C simultaneously with primary antibodies, and then probed with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at ambient temperature. Membranes were visualized with Pierce PicoWest Enhanced™ Chemiluminescent Reagents. Film exposure was on Kodak BioMax film for 30 s to 2 min. Protein band densities were quantitated by UN-SCAN-IT software.

4.9. Antibodies

Mouse anti-mouse NOS-1 monoclonal antibody (mAb) (1:2000) and mouse anti-mouse PIN (1:100 for Western blot, 1:30 for IP)) mAb were purchased from BD Pharmingen. Rat anti-mouse PIN mAb (1:1000 for Western blot, 1:100 for IP) were purchased from Alexis. Mouse anti-mouse class III β-tubulin (1:10,000) mAb was obtained from RDI Diagnostics; rabbit anti-β-tubulin III pAb (1:600) was purchased from Covence. Rabbit anti-mouse LMP2 (1:300), anti-LMP7 (1:1000), anti-LMP10 (1:1000), anti-proteasome 20S (1:100) pAb and mouse anti-mouse α_5 mAb were purchased from Biomol. Anti-β-actin pAb (1:2000) was purchased from Abcam. Mouse anti-mouse β-actin (1:100,000) was obtained from Sigma. Mouse anti-mouse ubiquitin mAb (1:1000) and rabbit anti-mouse ubiquitin (1:300) pAb were purchased from AB Reagent and Dako, respectively. Sheep anti-VSV pAb was a generous gift from Dr. Alice S. Huang (when at The Children's Hospital, Boston). HRP conjugated anti-mouse secondary antibody (1:2000) was purchased from Vector, Donkey anti-sheep (1:4000) and Donkey anti-rabbit (1:40,000) secondary antibodies, both HRP conjugated, were purchased from Jackson. β-actin and/or class III β-tubulin were used as loading controls since both of them have been used in related approaches (Habelhah et al., 2004; Golde et al., 2003) and neither was reported to interact with the proteasome–ubiquitin pathway.

4.10. Co-immunoprecipitation

NB41A3 cells were plated and treated with IFN-γ and/or Lactacystin for 24 h. Whole cell lysates were extracted and 400 μg protein from each sample was used. Immunoprecipitations of NOS-1 and β-tubulin from whole cell lysates with rabbit anti-NOS-1 pAb (1:40) and rabbit anti-β-tubulin III pAb were performed at 4 °C overnight. Then, 20 μl protein A/G beads (Pierce) were added and rotated at 4 °C for minimum 3 h. Beads were collected and washed with lysis buffer for 3 times, prior to boiling with dissociation buffer for 5 min. The precipitate was detected by Western blot using mouse anti-Ubiquitin mAb and mouse β-tubulin mAb. Quantitation of the Ubi-NOS-1/β-tubulin ratio was performed using UN-SCAN-IT software. At the same time, IP of Ubiquitin and β-tubulin from whole cell lysate using anti-ubiquitin pAb and anti-β-tubulin pAb. The precipitate was detected in Western blots with anti-NOS-1 mAb, anti-Ubiquitin mAb and anti-β-tubulin mAb. The Ubi-NOS-1/β-tubulin ratio was determined. Three independent experiments, with each sample in duplicate, were performed to achieve statistical significance.

Co-IP of PIN and ubiquitin was performed in the same manner, excepted for using anti-PIN antibody instead of anti-NOS-1 antibody. For the VSV-ubiquitin co-immunoprecipitation, NB41A3 cells treated with IFN-γ and/or Lactacystin was infected with VSV at MOI = 0.1 and lysed at 5 h pi. 200 μg of whole cell lysate was used in each reaction. Anti-Ubi pAb and anti-Tubulin β III pAb were used for IP. Anti-VSV pAb and anti-Tubulin III mAb were used in the immunoblotting. Ubi-VSV/β-tubulin ratio was quantitated.

4.11. Metabolic labeling and immunoprecipitation

NB41A3 cells were cultured in 6-well plates (2.5×10^5 cells/well) and incubated with 1.0 ml of Met/Cys free DMEM medium containing 10% dialyzed FBS for 30 min, and then pulsed with Met/Cys-free DMEM medium containing 100 μCi of [^{35}S]Met[^{35}S]Cys mix. Following the 1 or 2 h labeling period, the medium was changed to chasing medium (starvation medium with 2 mM Met/Cys added back). At appropriate time intervals, cell pellets were collected, frozen and stored at −80 °C.

Cells were lysed and centrifuged at 14,000 rpm. Equal amounts of radioactive lysates were precleared with 50 μl protein A/G beads (Pierce) at 4 °C for 1 h. Antibodies against NOS-1, PIN or VSV were added and incubated under agitation 4 °C overnight. Subsequently, 25 μl of protein A/G

beads was added to the radioactive material and incubated for 1 h at 4 °C. Then beads were washed 3 times in lysis buffer, denatured in SDS-containing sample buffer and loaded on a 10% or 12% SDS-polyacrylamide gel. The gel was dried at 60 °C for 1.5 h and exposed at −80 °C for 1–7 days. The radioactivity was quantified by UN-SCAN-IT software.

4.12. Statistical methods

Student's *t*-test was used in all statistical analyses. Unless otherwise stated, all data were presented in mean ± S.E.M.

Acknowledgement

We are grateful to Dr. Alice S. Huang for the gift VSV and sheep anti-VSV antibody, Michael BA Oldstone for the mice constitutively expressing MHC I in neurons, to Dr. Michele Pagano (NYU School of Medicine) and Dr. Jane A. McCutcheon (NYU College of Dentistry) for helpful discussions.

This work was supported by a NYU Research Challenge Fund award N5385, and NIH grants DC003536 and NS039746 to C.S.R.

References

Aviel, S., Winberg, G., Massucci, M., Ciechanover, A., 2000. Degradation of the Epstein–Barr virus latent membrane protein 1 (LMP1) by the ubiquitin–proteasome pathway. Targeting via ubiquitination of the N-terminal residue. J. Biol. Chem. 275, 23491–23499.

Bender, A.T., Demady, D.R., Osawa, Y., 2000. Ubiquitination of neuronal nitric-oxide synthase in vitro and in vivo. J. Biol. Chem. 275, 17407–17411.

Bi, Z., Reiss, C.S., 1995. Inhibition of vesicular stomatitis virus infection by nitric oxide. J. Virol. 69, 2208–2213.

Chen, N., Reiss, C.S., 2002. Distinct roles of eicosanoids in the immune response to viral encephalitis: or why you should take NSAIDS. Viral Immunol. 15, 133–146.

Cheroni, C., Peviani, M., Cascio, P., Debiasi, S., Monti, C., Bendotti, C., 2005. Accumulation of human SOD1 and ubiquitinated deposits in the spinal cord of SOD1G93A mice during motor neuron disease progression correlates with a decrease of proteasome. Neurobiol. Dis. 18, 509–522.

Chesler, D.A., Reiss, C.S., 2002. The role of IFN-gamma in immune responses to viral infections of the central nervous system. Cytokine Growth Factor Rev. 13, 441–454.

Chesler, D.A., Munoz-Jordan, J.L., Donelan, N., Garcia-Sastre, A., Reiss, C. S., 2003. PKR is not required for interferon-gamma inhibition of VSV replication in neurons. Viral Immunol. 16, 87–96.

Chesler, D.A., McCutcheon, J.A., Reiss, C.S., 2004. Posttranscriptional regulation of neuronal nitric oxide synthase expression by IFN-gamma. J. Interferon Cytokine Res. 24, 141–149.

Ciechanover, A., 1994. The ubiquitin–proteasome proteolytic pathway. Cell 79, 13–21.

Craiu, A., Gaczynska, M., Akopian, T., Gramm, C.F., Fenteany, G., Goldberg, A.L., Rock, K.L., 1997. Lactacystin and clasto-lactacystin beta-lactone modify multiple proteasome beta-subunits and inhibit intracellular protein degradation and major histocompatibility complex class I antigen presentation. J. Biol. Chem. 272, 13437–13445.

Diaz-Hernandez, M., Hernandez, F., Martin-Aparicio, E., Gomez-Ramos, P., Moran, M.A., Casiano, J.G., Ferrer, I., Avila, J., Lucas, J.J., 2003. Neuronal induction of the immunoproteasome in Huntington's disease. J. Neurosci. 23, 11653–11661.

Eom, C.Y., Heo, W.D., Craske, M.L., Meyer, T., Lehman, I.R., 2004. The neural F-box protein NFB42 mediates the nuclear export of the herpes simplex virus type 1 replication initiator protein (UL9 protein) after viral infection. Proc. Natl. Acad. Sci. U. S. A. 101, 4036–4040.

Fan, J.S., Zhang, Q., Li, M., Tochio, H., Yamazaki, T., Shimizu, M., Zhang, M., 1998. Protein inhibitor of neuronal nitric-oxide synthase, PIN, binds to a 17-amino acid residue fragment of the enzyme. J. Biol. Chem. 273, 33472–33481.

Gogate, N., Swoveland, P., Yamabe, T., Verma, L., Woyciechowska, J., Tarnowska-Dziduszko, E., Dymecki, J., Dhib-Jalbut, S., 1996. Major histocompatibility complex class I expression on neurons in subacute sclerosing panencephalitis and experimental subacute measles encephalitis. J. Neuropathol. Exp. Neurol. 55, 435–443.

Golde, S., Coles, A., Lindquist, J.A., Compston, A., 2003. Decreased iNOS synthesis mediates dexamethasone-induced protection of neurons from inflammatory injury in vitro. Eur. J. Neurosci. 18, 2527–2537.

Goody, R.J., Hoyt, C.C., Tyler, K.L., 2005. Reovirus infection of the CNS enhances iNOS expression in areas of virus-induced injury. Exp. Neurol. 195, 379–390.

Groettrup, M., Kraft, R., Kostka, S., Standera, S., Stohwasser, R., Kloetzel, P.M., 1996. A third interferon-gamma-induced subunit exchange in the 20S proteasome. Eur. J. Immunol. 26, 863–869.

Gu, Z., Kaul, M., Yan, B., Kridel, S.J., Cui, J., Strongin, A., Smith, J.W., Liddington, R.C., Lipton, S.A., 2002. *S*-nitrosylation of matrix metalloproteinases: signaling pathway to neuronal cell death. Science 297, 1186–1190.

Habelhah, H., Takahashi, S., Cho, S.G., Kadoya, T., Watanabe, T., Ronai, Z., 2004. Ubiquitination and translocation of TRAF2 is required for activation of JNK but not of p38 or NF-kappaB. EMBO J. 23, 322–332.

Harty, R.N., Brown, M.E., McGettigan, J.P., Wang, G., Jayakar, H.R., Huibregtse, J.M., Whitt, M.A., Schnell, M.J., 2001. Rhabdoviruses and the cellular ubiquitin–proteasome system: a budding interaction. J. Virol. 75, 10623–10629.

Hewett, S.J., 1999. Interferon-gamma reduces cyclooxygenase-2-mediated prostaglandin E2 production from primary mouse astrocytes independent of nitric oxide formation. J. Neuroimmunol. 94, 134–143.

Hicke, L., 2001. Protein regulation by monoubiquitin. Nat. Rev., Mol. Cell Biol. 2, 195–201.

Hochstrasser, M., 2000. Evolution and function of ubiquitin-like protein-conjugation systems. Nat. Cell Biol. 2, E153–E157.

Hori, K., Burd, P.R., Furuke, K., Kutza, J., Weih, K.A., Clouse, K.A., 1999. Human immunodeficiency virus-1-infected macrophages induce inducible nitric oxide synthase and nitric oxide (NO) production in astrocytes: astrocytic NO as a possible mediator of neural damage in acquired immunodeficiency syndrome. Blood 93, 1843–1850.

Hu, Z., Zhang, Z., Doo, E., Coux, O., Goldberg, A.L., Liang, T.J., 1999. Hepatitis B virus X protein is both a substrate and a potential inhibitor of the proteasome complex. J. Virol. 73, 7231–7240.

Imai, J., Maruya, M., Yashiroda, H., Yahara, I., Tanaka, K., 2003. The molecular chaperone Hsp90 plays a role in the assembly and maintenance of the 26S proteasome. EMBO J. 22, 3557–3567.

Jaffrey, S.R., Snyder, S.H., 1996. PIN: an associated protein inhibitor of neuronal nitric oxide synthase. Science 274, 774–777.

Joly, E., Oldstone, M.B., 1992. Neuronal cells are deficient in loading peptides onto MHC class I molecules. Neuron 8, 1185–1190.

Khor, R., McElroy, L.J., Whittaker, G.R., 2003. The ubiquitin-vacuolar protein sorting system is selectively required during entry of influenza virus into host cells. Traffic 4, 857–868.

Kim, J.H., Kang, S., Kim, J., Ahn, B.Y., 2003. Hepatitis B virus core protein stimulates the proteasome-mediated degradation of viral X protein. J. Virol. 77, 7166–7173.

Kloetzel, P.M., Ossendorp, F., 2004. Proteasome and peptidase function in MHC-class-I-mediated antigen presentation. Curr. Opin. Immunol. 16, 76–81.

Kodukula, P., Liu, T., Rooijen, N.V., Jager, M.J., Hendricks, R.L., 1999. Macrophage control of herpes simplex virus type 1 replication in the peripheral nervous system. J. Immunol. 162, 2895–2905.

Komatsu, T., Bi, Z., Reiss, C.S., 1996. Interferon-gamma induced type I nitric oxide synthase activity inhibits viral replication in neurons. J. Neuroimmunol. 68, 101–108.

Komatsu, T., Srivastava, N., Revzin, M., Ireland, D.D., Chesler, D., Shoshkes Reiss, C., 1999. Mechanisms of cytokine-mediated inhibition of viral replication. Virology 259, 334–341.

Kosugi, I., Kawasaki, H., Arai, Y., Tsutsui, Y., 2002. Innate immune responses to cytomegalovirus infection in the developing mouse brain and their evasion by virus-infected neurons. Am. J. Pathol. 161, 919–928.

Lane, T.E., Paoletti, A.D., Buchmeier, M.J., 1997. Disassociation between the in vitro and in vivo effects of nitric oxide on a neurotropic murine coronavirus. J. Virol. 71, 2202–2210.

Lederkremer, G.Z., Glickman, M.H., 2005. A window of opportunity: timing protein degradation by trimming of sugars and ubiquitins. Trends Biochem. Sci. 30, 297–303.

Levine, B., Yuan, J., 2005. Autophagy in cell death: an innocent convict? J. Clin. Invest. 115, 2679–2688.

Liu, M., Li, X.L., Hassel, B.A., 2003. Proteasomes modulate conjugation to the ubiquitin-like protein, ISG15. J. Biol. Chem. 278, 1594–1602.

Love, S., 1999. Oxidative stress in brain ischemia. Brain Pathol. 9, 119–131.

Malakhova, O.A., Yan, M., Malakhov, M.P., Yuan, Y., Ritchie, K.J., Kim, K. I., Peterson, L.F., Shuai, K., Zhang, D.E., 2003. Protein ISGylation modulates the JAK–STAT signaling pathway. Genes Dev. 17, 455–460.

Mishto, M., Bellavista, E., Santoro, A., Stolzing, A., Ligorio, C., Nacmias, B., Spazzafumo, L., Chiappelli, M., Licastro, F., Sorbi, S., Pession, A., Ohm, T., Grune, T., Franceschi, C., 2006. Immunoproteasome and LMP2 polymorphism in aged and Alzheimer's disease brains. Neurobiol. Aging 27, 54–66.

Noguchi, S., Jianmongkol, S., Bender, A.T., Kamada, Y., Demady, D.R., Osawa, Y., 2000. Guanabenz-mediated inactivation and enhanced proteolytic degradation of neuronal nitric-oxide synthase. J. Biol. Chem. 275, 2376–2380.

Radi, R., 2004. Nitric oxide, oxidants, and protein tyrosine nitration. Proc. Natl. Acad. Sci. U. S. A. 101, 4003–4008.

Reiss, C.S., Komatsu, T., 1998. Does nitric oxide play a critical role in viral infections? J. Virol. 72, 4547–4551.

Saura, M., Zaragoza, C., McMillan, A., Quick, R.A., Hohenadl, C., Lowenstein, J.M., Lowenstein, C.J., 1999. An antiviral mechanism of nitric oxide: inhibition of a viral protease. Immunity 10, 21–28.

Schmid, D., Munz, C., 2005. Immune surveillance of intracellular pathogens via autophagy. Cell Death Differ. 12 (Suppl. 2), 1519–1527.

Shringarpure, R., Grune, T., Mehlhase, J., Davies, K.J., 2003. Ubiquitin conjugation is not required for the degradation of oxidized proteins by proteasome. J. Biol. Chem. 278, 311–318.

Simon, D.I., Mullins, M.E., Jia, L., Gaston, B., Singel, D.J., Stamler, J.S., 1996. Polynitrosylated proteins: characterization, bioactivity, and functional consequences. Proc. Natl. Acad. Sci. U. S. A. 93, 4736–4741.

Suhy, D.A., Giddings Jr., T.H., Kirkegaard, K., 2000. Remodeling the endoplasmic reticulum by poliovirus infection and by individual viral proteins: an autophagy-like origin for virus-induced vesicles. J. Virol. 74, 8953–8965.

Van den Eynde, B.J., Morel, S., 2001. Differential processing of class-I-restricted epitopes by the standard proteasome and the immunoproteasome. Curr. Opin. Immunol. 13, 147–153.

Zwickl, P., Voges, D., Baumeister, W., 1999. The proteasome: a macromolecular assembly designed for controlled proteolysis. Philos. Trans. R. Soc. Lond., B Biol. Sci. 354, 1501–1511.