Cytoskeleton/Endoplasmic Reticulum Collapse Induced by Prostaglandin J2 Parallels Centrosomal Deposition of Ubiquitinated Protein Aggregates*

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Many neurodegenerative disorders, such as Parkinson disease, exhibit inclusion bodies containing ubiquitinated proteins. The mechanisms implicated in this aberrant protein deposition remain elusive. In these disorders signs of inflammation are also apparent in the affected central nervous system areas. We show that prostaglandin J2 (PGJ2), an endogenous product of inflammation, disrupts the cytoskeleton in neuronal cells. Furthermore, PGJ2 perturbed microtubule polymerization in vitro and decreased the number of free sulfhydryl groups on tubulin cysteines. A direct effect of PGJ2 on actin was not apparent, although actin filaments were altered in cells treated with PGJ2. This cyclopentenone prostaglandin triggered endoplasmic reticulum (ER) collapse and the redistribution of ER proteins, such as calnexin and catechol-O-methyltransferase, into a large centrosomal aggregate containing ubiquitinated proteins and α-synuclein. The PGJ2-dependent cytoskeletal rearrangement paralleled the development of the large centrosomal aggregate. Both of these events were replicated by treating cells with brefeldin A, which impairs ER/Golgi transport. PGJ2 also perturbed 26 S proteasome assembly and activity, which preceded the accumulation of ubiquitinated proteins as detergent/salt-insoluble aggregates. Our data support a mechanism by which, upon PGJ2 treatment, cytoskeleton/ER collapse coincides with the relocation of ER proteins, other potentially neighboring proteins, and ubiquitinated proteins into centrosomal aggregates. Development of these large perinuclear aggregates is associated with disruption of the microtubule/ER network. This aberrant protein deposition, triggered by a product of inflammation, may be common to other compounds that disrupt microtubules and induce protein aggregation, such as MPP+ and rotenone, found to be associated with neurodegeneration.

Protein aggregates containing ubiquitinated proteins are detectable in a variety of degenerative diseases. These diseases range from neurological disorders, such as Alzheimer disease, Parkinson disease, amyotrophic lateral sclerosis, and Huntington disease, to liver diseases, such as Wilson disease and alcoholic hepatitis, to name a few (1). Whether these protein aggregates are pathogenic or represent a coping mechanism to prolong survival of the affected cells, including neurons and hepatocytes, is a hotly debated issue (2). The protein aggregates, however, are indicative of a malfunction of the normal process of protein turnover because they are not prevalent in healthy cells.

Most of these protein aggregates are detectable with antibodies that react with ubiquitinated proteins but their major structural components vary from cell type to cell type. For example, tau proteins are found in cortical neurofibrillary tangles, α-synuclein in dopaminergic Lewy bodies, and huntingtin in intranuclear inclusions of striatal projection neurons (reviewed in Ref. 3). Full characterization of the components and mechanisms leading to the formation of these protein aggregates is lacking.

Notably, most of the diseases associated with the accumulation of ubiquitinated proteins in intracellular inclusions also exhibit signs of inflammation in the respective damaged areas (reviewed in Ref. 4). Our studies focus on one of the products of inflammation, prostaglandin J2 (PGJ2),3 because recent reports indicate that these cyclopentenone prostaglandins may play an important role in neurodegeneration. For example, the levels of 15d-PGJ2, a PGJ2 metabolite, were found to be elevated in spinal cord motor neurons of patients with amyotrophic lateral sclerosis (5). Furthermore, J2 prostaglandins were shown to be neurotoxic and pro-oxidant agents (6, 7), to up-regulate the expression of cyclooxygenase-2 (8), to inhibit ubiquitin isopeptidase activity (9) as well as UCH-L1 and UCH-L3 (10), and to induce the accumulation of ubiquitinated proteins (6) and neuronal apoptosis (5). All of these events are associated with neurodegeneration.

We recently demonstrated that J2 prostaglandins induce the accumulation of ubiquitinated proteins into distinct aggregates in neuronal cells (10). To understand the mechanisms leading to the formation of these protein aggregates and to identify some of their components, we investigated the effects of PGJ2 on human neuroblastoma SK-N-SH cells. Herein, we show that PGJ2 damages the cytoskeletal structure. The ensuing microtubule disruption and ER collapse coincides with the formation of

References

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3. The abbreviations used are: PGJ2, prostaglandin J2; COMT, catechol-O-methyltransferase; DAPI, 4',6-diamidino-2-phenylindole; DTNB, 5,5′-di-thiobis(2-nitrobenzoic acid); 15d-PGJ2, 15-deoxy-A15,14-prostaglandin J2; ER, endoplasmic reticulum; MTOC, microtubule organizing center.
protein aggregates co-localized with the centrosome/microtubule organizing center (MTOC). In addition to ubiquitinated proteins, the PGJ2-induced aggregates contain calnexin, catechol-O-methyltransferase (COMT), and α-synuclein. Thus, products of inflammation, such as PGJ2, destabilize the microtubule network resulting in collapse of the ER and trigger the formation of protein aggregates containing ubiquitinated proteins. Most of the ubiquitinated proteins accumulate as insoluble aggregates. In conclusion, disruption of the microtubule and ER networks in conjunction with the accumulation of ubiquitinated proteins, both caused by PGJ2, may represent mechanisms shared by other inducers of protein aggregation associated with neurodegeneration.

**EXPERIMENTAL PROCEDURES**

**Materials**—PGJ2 was from Cayman Chemical (Ann Arbor, MI). Colchicine and brefeldin A were from Sigma. Kits to assess tubulin and actin polymerization were from Cytoskeleton, Inc. (Denver, CO). The following primary antibodies were used: rabbit polyclonal anti-COMT (1:500, from Chemicon Int., Temecula, CA), goat polyclonal anti-calnexin (C-20, 1:40), and anti-COX-1 (C-20, 1:1,000) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), mouse monoclonal anti-actin (1:200, clone AC-20), and mouse monoclonal anti-γ-tubulin (1:200, clone GTU-88) from Sigma, rabbit polyclonal anti-α-tubulin (1:200) from Abcam Inc. (Cambridge, MA), mouse monoclonal anti-α-synuclein (1:200) from Zymed Laboratories Inc. (San Francisco, CA), mouse monoclonal and rabbit polyclonal anti-ubiquitinated proteins (1:250) from Chemicon Int. (Temecula, CA) and Dako Cytomation (Carpinteria, CA), respectively, mouse monoclonal anti-αB-crystallin (1:500) and anti-S8 (1:1,000), both proteasome subunits, were obtained from BIOMOL (Plymouth Meeting, PA). The respective secondary antibodies with fluorophores (1:50) were from Jackson ImmunoResearch Laboratories (West Grove, PA).

**Cell Cultures**—SK-N-SH cells are a human neuroblastoma cell line derived from peripheral tissue (11). The cells were maintained as described in Ref. 8 and were obtained from ATCC.

**Cell Treatments**—SK-N-SH cell cultures were treated for the indicated times with vehicle (Me2SO) or with different concentrations of PGJ2 in Me2SO added directly to serum-containing medium. The final Me2SO concentration in the medium was 0.5%. At the end of the incubation, all cultures were washed twice with phosphate-buffered saline and processed for the different assays as described below. Cell washes removed unattached cells, therefore subsequent assays were performed on adherent cells only.

**Immunofluorescence**—After treatment SK-N-SH cells were rinsed with phosphate-buffered saline, fixed in ice-cold methanol:acetone (1:1) for 10 min at −20 °C, and co-incubated with the antibodies listed in each figure. Slides were mounted with Vectashield medium containing DAPI (Vector Laboratories, Inc., Burlingame, CA). Cell staining was visualized with an OPTIPHOT-2 fluorescence microscope (NIKON, Melville, NY).

**In Vitro Tubulin and Actin Polymerization**—Standard tubulin and actin polymerization assays were performed with kits from Cytoskeleton Inc. following the manufacturer’s recommendations. Tubulin polymerization was assessed at 340 nm every 60-s with a temperature-regulated Power Wave 200™ microplate scanning spectrophotometer, Bio-Tek Instruments, Inc. (Winooski, VT). Actin polymerization was assessed at 365/407 nm every 60-s with a 650-105 Fluorescence Spectrophotometer equipped with a 150 Xenon Power Supply, PerkinElmer Life Sciences. Data were analyzed with KC4 Kinetics for Windows, from Bio-Tek Instruments, Inc.

**DTNB Assay for Free Tubulin Sulphydryl Groups**—Tubulin (3 μg/μl, 50 μl) in general tubulin buffer with 1 mM GTP was incubated under basal conditions with 5 μl of general tubulin buffer alone or containing Me2SO (1.5%), PGJ2 (273 or 545 μM), or PGE2 (545 μM) for 90 min, with gentle rocking at 37 °C. Free sulphydryl groups were assessed with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) essentially as described in Ref. 12. A standard curve with l-cysteine (10–20 nmol) was used to calculate the free sulphydryls in the tubulin samples.

**Western Blotting**—Western blot analysis was carried out by SDS-PAGE on 8% polyacrylamide gels. Samples were boiled for 5 min in Laemmli buffer and loaded onto the gel (30 μg of protein/lane for total cell lysates, 5 μg of protein/lane for fractionation). Following electrophoresis, proteins were transferred to an Immobilon-P membrane (Millipore, Bedford, MA). The membrane was probed with an antibody that reacts with poly-ubiquitinated proteins (1:1,500, from Dako Cytomation). When specified, the membrane was stripped and reprobed with the anti-COX-1 antibody. Antigens were visualized by a chemiluminescent horseradish peroxidase method with the ECL reagent. Semi-quantitative analysis of protein detection was done by image analysis with the Image PC program from NIH as described (13).

**In-gel Proteasome Activity and Detection**—Upon treatment with vehicle (Me2SO) or 15 μM PGJ2 cells were washed twice with phosphate-buffered saline to remove extracellular prostaglandins and were then harvested with the following buffer A: 50 mM Tris-HCl, pH 7.4, 5 mM MgCl2, 5 mM ATP (grade 1; Sigma), 1 mM dithiothreitol, and 10% glycerol. Following homogenization the protein content of the cleared supernatants (determined with the Bradford assay) was adjusted to equal concentrations with buffer A. The cleared supernatants (15 μg of protein/lane for proteasome activity and 5 μg of protein/lane for Western blotting) were resolved by non-denaturing PAGE using a modification of the method described in Ref. 14. We used a four-gel layer consisting of equal amounts, from the bottom up, of 6, 5, 4, and 3% polyacrylamide with Rhinohide™ polyacrylamide strengthener (Molecular Probes). Bromphenol blue was added to the protein samples prior to loading. Non-denaturing minigels were run at 125 volts for 3 h. The gels were then incubated on a rocker for 30 min at 37 °C with 15 ml of 0.4 mM Suc-LLVY-AMC in buffer B (buffer A modified to contain 1 mM ATP only). Proteasome bands were visualized upon exposure to UV light (360 nm) and were photographed with a NIKON Cool Pix 8700 camera with a 3-4219 fluorescent green filter (Peca Products, Inc.). Proteins on the native gels were transferred (110 mA) for 2 h onto polyvinylidene difluoride membranes. Western blot analyses were then carried out sequentially for detection of the 26 S and 20 S pro-
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tesomes with anti-α4 and anti-S8 subunit antibodies. The anti-α4 antibody reacts with a core particle subunit, and therefore detects both the 26 S and 20 S proteasomes. The anti-S8 antibody reacts with a regulatory particle subunit thus only detecting 26 S proteasomes. Antigens were visualized by a chemiluminescent horseradish peroxidase method with the ECL reagent.

Subcellular Fractionation—After treatment for 24 h, SK-N-SH cells in 10-cm dishes (4.5 × 10^6 cells/ml) were rinsed twice with phosphate-buffered saline. Cells were harvested by gently scraping into ice-cold homogenization buffer (15 mM Tris-HCl, pH 7.6, 1 mM dithiothreitol, 0.25 mM sucrose, 1 mM MgCl2, 2.5 mM EDTA, 2.5 mM EGTA and protease inhibitor mixture (Sigma) supplemented with 1 mM Na3VO4, 25 mM NaF, and 2 mM sodium pyrophosphate). All subsequent steps were carried out at 4 °C. Homogenization and subcellular fractionation were performed as described in Ref. 15. Briefly, samples were homogenized with a Dounce homogenizer (30 strokes) followed by a 10-min centrifugation at 1,000 g to obtain the crude nuclear pellet (P1) and supernatant fraction (S1). S1 was centrifuged at 10,000 g to collect the pellet (P2). The S2 supernatant was further centrifuged at 162,000 g for 1 h in a Beckman TL-100 (Palo Alto, CA) to collect the cytosolic fraction (S3) and the microsomal pellet (P3). The P2 pellet was resuspended in homogenization buffer containing 1% Triton X-100 and 400 mM KCl, sonicated five times for 5 s each, washed on a shaker for 1 h followed by a 10-min centrifugation at 20,000 g to obtain the Triton X-100-insoluble and Triton X-100-soluble fractions. The P1 and Triton X-100-insoluble pellets were re-suspended in denaturing buffer (10 mM Tris-HCl, pH 7.3, 1% SDS, and 1 mM Na3VO4).

Protein Concentration—Concentration was determined with the bicinchoninic acid assay kit (Pierce) or the Bradford assay (Bio-Rad).

RESULTS

PGJ2 Disrupts the Cytoskeleton Structure—To determine the effect of PGJ2 on the cytoskeleton we visualized two of its components, namely microtubules and actin filaments, by immunofluorescence analysis with anti-α-tubulin and anti-actin antibodies, respectively. We chose to treat cells with 15 μM PGJ2 for 24 h, because in our previous studies, these conditions clearly induced both accumulation and aggregation of ubiquitinated proteins as well as neuronal cell death (10).

In the absence of PGJ2, microtubules and actin filaments were intact, straight, and oriented parallel to the longest axis of the SK-N-SH cells (Fig. 1, A, B, and E). Upon treatment for 24 h with 15 μM PGJ2, the appearance of the microtubule network and actin filaments was altered. The microtubules were fragmented and displayed a circular arrangement surrounding the nucleus (Fig. 1, C and F) and the actin filaments exhibited an amorphous pattern (Fig. 1D). The shape of the cell complemented the change observed for the microtubule network. Control cells were elongated (Fig. 1, A, B, and E), whereas PGJ2-treated cells displayed a round to square shape (Fig. 1, C, D, and F).

The alterations in microtubule appearance elicited by PGJ2 suggest that this product of inflammation affects microtubule polymerization. We thus investigated the effect of PGJ2 on the in vitro polymerization of highly purified (>99%) bovine brain tubulin at pH 6.9 and 37 °C, as described under “Experimental Procedures.” The effect of PGJ2 on microtubule assembly was determined as a function of time with a turbidimetric assay. The in vitro microtubule polymerization assay requires high tubulin concentrations (3 mg/ml or 54.5 μM) for turbidity assessment, thus we increased the PGJ2 concentrations in the in vitro assay, accordingly. Other compounds found to oxidize tubulin sulfhydryls, such as peroxynitrite, were tested at similar concentrations (12).

Standard polymerization reactions in the absence of drug or solvent (crosses) or in the presence of 1.5% Me2SO (prostaglandin solvent, open triangles), or 545 μM PGE2 (solid triangles), or 273 and 545 μM PGJ2 (open and solid circles, respectively) are shown in Fig. 2A. The curves represent the average of two experiments. The Vmax for each curve is listed in Fig. 2B. Tubulin polymerizes with a Vmax of ~5.5 milli-OD units per minute (mOD/min) in the general tubulin buffer plus GTP. Me2SO alone (1.5% final concentration) eliminates the nucleation phase of polymerization and enhances the Vmax of the growth (elongation) phase by ~4-fold. Others have reported that Me2SO induces self-assembly of tubulin (16). Although the

FIGURE 1. PGJ2 perturbs the structure of the cytoskeleton. Immunofluorescence staining of SK-N-SH cells treated with vehicle only (Me2SO, A, B, and E) or 15 μM PGJ2 (C, D, and F) for 24 h. Microtubules were visualized with an anti-α-tubulin antibody (A, C, E, and F, Texas Red) and actin filaments with an anti-actin antibody (B and D, fluorescein isothiocyanate). n, nucleus. Scale bar = 10 μm.
mechanism is not well characterized, it was suggested that solvents such as Me2SO alter the H2O structure around the tubulin dimers lowering the free energy of polymerization.

In the presence of PGJ2 (1.5% Me2SO) tubulin polymerization was hindered as depicted by a significant decrease in $V_{\text{max}}$ and in levels of assembled microtubules when compared with Me2SO alone (Fig. 2, A and B). The $V_{\text{max}}$ was lowered by 28 and 43% by 273 and 545 μM PGJ2, respectively (Fig. 2B). PGJ2, a prostaglandin that does not cause aggregation of ubiquitin dimers lowering the free energy of polymerization. Fig. 2C depicts the decrease in the levels of DTNB-reactive sulfhydryl groups in tubulin elicited by PGJ2 treatment. A 22 and 35% drop in free sulfhydryls was observed when tubulin was treated with 273 and 545 μM PGJ2, respectively. The decrease in free tubulin sulfhydryl groups induced by PGJ2 correlated well with its inhibition of microtubule polymerization (Fig. 2, A and B).

Prostaglandins of the J2 series are unique among the prostaglandin family as they contain α,β-unsaturated carbonyl groups in their cyclopentenone ring (17). One of these prostaglandins, 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2), was shown to induce cysteine-targeted protein oxidation in SH-SY5Y cells (18). Furthermore, tubulin was one of the proteins that was identified as being S-oxidized in these cells. Microtubules are composed of the heterodimeric protein tubulin that contains 20 cysteines: 12 in the α- and 8 in the β-subunit (19, 20). We thus reasoned that PGJ2 may hinder microtubule polymerization by inducing the S-oxidation of free sulfhydryl groups in tubulin. To test our hypothesis, we assessed the levels of free sulfhydryl groups in purified tubulin incubated in the absence and presence of PGJ2, using DTNB. This reagent forms a colored product measurable at 412 nm when it reacts with free sulfhydryl groups. Fig. 3C depicts the decrease in the levels of DTNB-reactive sulfhydryls in tubulin elicited by PGJ2 treatment. A 22 and 35% drop in free sulfhydryls was observed when tubulin was treated with 273 and 545 μM PGJ2, respectively. The decrease in free tubulin sulfhydryl groups induced by PGJ2 correlated well with its inhibition of microtubule polymerization (Fig. 3, A–C). No decline in free tubulin sulfhydryl groups was detected in the presence of 545 μM PGE2, a prostaglandin that does not contain α,β-unsaturated carbonyl groups.

PGJ2 also disrupted the structure of actin filaments in SK-N-SH cells. Thus we assessed the effect of PGJ2 on in vitro actin polymerization by pyrene fluorescent enhancement as described under “Experimental Procedures.” Me2SO alone (1.5% final concentration) slightly enhanced the $V_{\text{max}}$ of actin polymerization by ~1.4-fold, when compared with general actin buffer (Fig. 3B). However, the levels of polymerized actin were not altered by Me2SO (Fig. 3A). In the presence of PGJ2 or...
PGE2 the $V_{\text{max}}$ and polymerized actin levels were equivalent to the ones observed for Me$_2$SO and general actin buffer (Fig. 3, A and B). We thus conclude that PGJ2 failed to directly affect actin polymerization in this in vitro assay.

**PGJ2 Induces the Collapse of the ER and Formation of Centrosomal Protein Aggregates**—It is well established that the morphology and stability of the ER are maintained by its attachment to microtubules (21, 22). Furthermore, microtubule disruption causes the ER to retract toward the nucleus (21, 22). Because we observed that PGJ2 disrupts microtubule integrity, we investigated the effect of PGJ2 on ER morphology assessed by immunofluorescence analysis with an anti-calnexin antibody. Calnexin is a membrane-bound ER resident protein and they exhibited a diffuse distribution throughout the cytoplasm and nucleus (Fig. 6, A and B). Low levels of ubiquitinated proteins were detected in PGJ2-treated cells retracted toward the nucleus (21, 22). Nuclei (n) were detected with DAPI (A, E, and I) and for COMT/calnexin (D, H, and L). Arrows point to large calnexin/COMT aggregates. Scale bar = 10 μm.

**The Protein Aggregates Induced by PGJ2 Contain Ubiquitinated Proteins, α-Synuclein, COMT, and Calnexin**—Under control conditions, only low levels of ubiquitinated proteins were detected and they exhibited a diffuse distribution throughout the cytoplasm and nucleus (Fig. 6, B and J). Most of the ubiquitinated proteins in PGJ2-treated cells were consistently concentrated in a single large, perinuclear aggregate (Fig. 6, F and N, arrows). Low levels of ubiquitinated protein immunoreactivity were also detected in small punctuated aggregates scattered throughout the cells.

We established that in PGJ2-treated cells, the large perinuclear aggregate contained high levels of α-synuclein (Fig. 6G, arrow). The latter is one of the major components of dopaminergic Lewy bodies detected in the substantia nigra of Parkinson disease patients. Under control conditions, α-synuclein immunoreactivity exhibited a punctated appearance more or less evenly distributed throughout the cell with very low levels in the nucleus, if any (Fig. 6C).

In PGJ2-treated cells, most of the ubiquitinated proteins are co-localized in the large perinuclear aggregate with α-synuclein (Fig. 6H) and COMT (Fig. 6P). As shown in Fig. 4, the distribution of COMT matched that of calnexin in the protein aggregates. Therefore, it appears that the microtubule and ER collapse triggered by PGJ2 treatment coincides with the redistribution of these three proteins, i.e. calnexin, COMT, and α-synuclein, as well as ubiquitinated proteins into the large protein aggregates.
Formation of Protein Aggregates in PGJ2-treated Cells Is Time-dependent and Coincides with Microtubule Collapse—Because calnexin and COMT have similar subcellular distributions, we focused our studies on COMT, because the activity of this enzyme may be relevant to neurodegeneration (26). No microtubule alterations or protein aggregates were detected by immunofluorescence analysis after 4 h of treatment with 15 μM PGJ2 (Fig. 7, A and B). Eight hours after PGJ2 treatment a slight indication of microtubule disruption as well as COMT concentration around the nuclei were noticeable (Fig. 7, C and D). At 16 h post-treatment (Fig. 7, E and F) the microtubules appeared already fragmented and formed a circular network around the nuclei; COMT immunoreactivity emerged as a large aggregate co-localized with the centrosome depicted by γ-tubulin immunostaining. These changes observed after 16 h of treatment with 15 μM PGJ2 were similar to the ones detected upon 24 h of treatment (Fig. 7, G and H).

Biochemical Analysis of PGJ2-induced Protein Aggregation—The protein aggregation and microtubule disruption patterns induced by PGJ2 were mimicked by treating cells with colchicine, a drug that disrupts the microtubule/ER network. Large perinuclear aggregates containing ubiquitinated proteins (Fig. 8A, arrow) and calnexin (Fig. 8B, arrow) as well as microtubule collapse (Fig. 8C) were observed in colchicine-treated cells. Perturbation of Golgi function by treating cells with brefeldin A did not duplicate this effect of PGJ2 treatment on protein aggregation. Ubiquitinated proteins were detected mostly in the nuclei and the perinuclear/ER area (Fig. 8, D and G). As expected, calnexin staining was very low in the nuclei of brefeldin A-treated cells, because calnexin is an ER resident protein (Fig. 8E). Microtubule structure was also disrupted by treatment with brefeldin A but not to the extent observed with colchicine or PGJ2 (compare Fig. 8C (colchicine) and H (brefeldin A) with I (control)). As assessed by Western blot analysis (Fig. 8, bottom panels), colchicine and brefeldin A failed to increase the levels of ubiquitinated proteins in SK-N-SH cells. Actually, when compared with control conditions, we observed a ∼70 and 80% decrease in ubiquitinated protein levels in cells treated for 16 h or longer with brefeldin A or colchicine, respectively (Fig. 8, bottom panels). PGJ2 Inhibits the Activity and Perturbs the Assembly of the 26 S Proteasome—To address the time-dependent effects of PGJ2 on the proteasome, we compared proteasome activity and levels by non-denaturing gel electrophoresis of crude extracts from control and PGJ2-treated SK-N-SH cells. In control cells, most of the proteasome activity assessed with the short substrate Suc-LLVY-AMC coincided with the 26 S holoenzyme (not the 20 S) form of the proteasome (Fig. 9, panels labeled with Chy). The activity of the 26 S proteasome was clearly decreased upon 4 h of treatment with 15 μM PGJ2. Semi-quantification by image analysis, as described under “Experimental Procedures,” detected a decrease in 26 S proteasome activity by at least 10 and 35% after 4 and 24 h of treatment with 15 μM PGJ2, respectively.

Western blot analysis of the native gels probed with an antibody that reacts with the α4 subunit of the core particle revealed that the levels of the 26 S holoenzyme are greater than the 20 S proteasome in control cells (Fig. 9, panels labeled with α4). However, an obvious decrease in the 26 S holoenzyme (by more than 45% as semi-quantified by image analysis) was observed upon treatment with PGJ2, even at the 4-h time point. This was further confirmed by stripping and reprobing the immunoblot with an antibody that reacts with the S8 subunit (an ATPase) of the 19 S regulatory particle (Fig. 9, panels labeled with S8). These findings establish that PGJ2 treatment...
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![Image of DAPI, Ub-conj., α-synuclein, Merge](image_url)

**FIGURE 6.** Distribution of ubiquitinated proteins, α-synuclein, and COMT in PGJ2-treated cells. Double immunofluorescence staining of SK-N-SH cells treated with vehicle only (Me2SO, A-D and I–L) or 15 μM PGJ2 (E–H and M–P) for 24 h. The distribution of ubiquitinated proteins was visualized with anti-ubiquitinated protein antibodies (B and F, Texas Red; J and N, fluorescein isothiocyanate), α-synuclein with the anti-α-synuclein antibody (C and G, fluorescein isothiocyanate), and COMT with the anti-COMT antibody (K and O, Texas Red). Nuclei (n) were detected with DAPI (A, E, I, and M, blue). Merged images are shown in D, H, L, and P. Arrows point to large perinuclear aggregates. Scale bar = 10 μm.

decreases 26 S proteasome activity and promotes its disassembly. Free 19 S particles were not detected, in accordance with a report showing that the 19 S and its subcomplexes are undetectable as free particles in yeast (27).

Although inhibition of the 26 S proteasome was detected upon 4 h of treatment with 15 μM PGJ2 a rise in the levels of ubiquitinated proteins, which are degraded by the 26 S proteasome, was only apparent after at least 8 h of incubation (Fig. 9, bottom panel). The levels of ubiquitinated proteins increased by as much as ~2-fold after 16 h of treatment with 15 μM PGJ2, whereas COX-1 levels did not.

To characterize the aggregates of ubiquitinated proteins that accumulate upon PGJ2 treatment we performed subcellular fractionation followed by Western blot analysis for ubiquitinated proteins. As shown in Fig. 10, upon PGJ2 treatment most ubiquitinated proteins were detected in the Triton X-100/salt-insoluble fraction, suggesting that they occur as insoluble aggregates. However, some ubiquitinated proteins were also detected in the crude nuclear pellet as well as microsomal fraction.

**DISCUSSION**

The mechanisms implicated in the formation of aggregates containing ubiquitinated proteins detected in neurodegenerative disorders remain elusive. We recently demonstrated that J2 prostaglandins, which are endogenous products of inflammation, trigger the aggregation of ubiquitinated proteins (10). However, the mechanisms leading to this aberrant protein aggregation and the components of the distinct protein deposits were not defined.

To elucidate the mechanism of protein aggregation induced by PGJ2, we investigated its effect on the cytoskeletal structure of human neuroblastoma SK-N-SH cells. Herein, we show that PGJ2 disrupts the structural integrity of microtubules and actin filaments in the SK-N-SH cells. Furthermore, we established that PGJ2 perturbs the in vitro polymerization of highly purified tubulin from bovine brain. Notably, the reactive oxygen species peroxynitrite was shown to oxidize tubulin sulphhydrils and impair microtubule polymerization (12). Furthermore, a product of lipid peroxidation, 4-hydroxynonenal, covalently modifies proteins by forming Michael adducts. Interestingly, 4-hydroxynonenal also disrupts neuronal microtubules and modifies cellular tubulin (28). We established that PGJ2 has similar effects. It disrupts microtubule integrity, impairs microtubule polymerization, and decreases the number of free sulphhydrils on tubulin cysteines. The unsaturated carboxyls of J2 prostaglandins are known to covalently modify free cysteines in proteins by the Michael addition reaction (17). Moreover, J2 prostaglandins increase the intracellular levels of products of lipid peroxidation, such as 4-hydroxynonenal and acrolein (6). Thus, disruption of microtubule integrity detected in PGJ2-treated cells may be caused by the formation of complexes between PGJ2 (or its metabolites) and tubulin and/or by a more indirect mechanism mediated by, for example, formation of 4-hydroxynonenal.

We observed that actin filaments were disrupted in cells treated with PGJ2. However, we could not detect a direct effect of this prostanooid on actin, as assessed by an in vitro actin polymerization assay involving pyrene fluorescent enhancement. Unlike the heterodimeric protein tubulin that contains 20 cysteines (12 in the α- and 8 in the β-subunit), actin contains only five cysteines. From these, only one (Cys-374) seems to be susceptible to Michael addition (29). It is possible that the in vitro assay is not sensitive enough to detect polymerization changes caused by only one cysteine per actin molecule. Alternatively, PGJ2 may have no direct effect on actin. Instead, the effect of PGJ2 on microtubules observed in SK-N-SH cells may...
Aggresomes induced by overexpression of several mutant proteins, such as the cystic fibrosis transmembrane conductance regulator, or by proteasome inhibitors, were previously reported to be surrounded by but not co-localized with the ER (24, 30). The PGJ2-induced large aggregates that we observed share some of the aggresome properties, such as being co-localized with the centrosome/MTOC and containing detergent/salt-insoluble proteins. However, aggresomes are cytoplasmic and their formation requires an intact cytoskeleton. In contrast, PGJ2-induced aggregates occur in parallel with the collapse of the microtubule/ER network. In addition, their formation can be mimicked by treating cells with colchicine, a drug that like PGJ2, disrupts microtubules. Brefeldin A, which interferes with Golgi function, did not mimic the effect of PGJ2 on protein aggregation. In fact, ubiquitinated proteins were trapped in the nucleus and perinuclear area of brefeldin A-treated cells. Taken together, these results demonstrate that PGJ2-induced aggregates are subcellular structures that are different from aggresomes.

The accumulation of ubiquitinated proteins induced by PGJ2 could be the result of its inhibition of deubiquitinating enzymes (9, 10) and of the 26 S proteasome. When the peptidase activities of the proteasome were assessed in total cell lysates, one study showed that J2 prostaglandins decrease proteasome activity (31), whereas others report the opposite, i.e. that these agents do not inhibit proteasome activity (9, 10). These conflicting results may be due to different harvesting and assay buffers and to the lack of specificity of the substrates used to assess proteasome activity in total cell lysates. For instance, it is well established that the substrate Suc-LLVY-AMC is cleaved not only by the proteasome (32) but also by other chymotrypsin-like proteases as well as by calpains (33), and thus does not specifically reflect proteasome activity in total cell lysates. To circumvent this difficulty, we assessed proteasome activity by an in-gel assay in cells harvested with a buffer that preserves the assembly of the 26 S proteasome. Our studies demonstrate that in control cells, the majority of the proteasome chymotrypsin-like activity is associated with the 26 S proteasome, suggesting that the free 20 S core particle is nearly inactive. A similar phenomenon was observed in yeast (34). In addition, our studies revealed a PGJ2-dependent decline in proteasome activity after 4 h of treatment. This decline in activity paralleled a decrease in the levels of the

trigger a cascade of cellular responses that will then perturb microfilament assembly.

It is well established that disruption of the microtubule network leads to ER collapse, thus forming an aggregate of membranes around the nucleus (reviewed in Ref. 22). Our studies show that disruption of the microtubule structure induced by PGJ2 treatment coincides with ER collapse into a large perinuclear aggregate that is co-localized with a centrosome/MTOC marker. As far as we know, ours is the first study showing that microtubule/ER collapse coincides with the formation of protein aggregates in cells treated with PGJ2. We observed a similar effect in rat primary cortical neurons treated with PGJ2 (23).

The protein aggregates induced by treatment with PGJ2 contained not only ubiquitinated proteins, but also α-synuclein, calnexin, and COMT. The two latter proteins, i.e. calnexin and COMT, have not yet been identified as components of protein aggregates. Based on these data, we propose a model in which cytoskeleton/ER collapse induced by PGJ2 is likely to displace many intracellular proteins causing them to co-localize in protein aggregates. These protein aggregates are likely to include an unspecific collection of proteins depending on their proximity to the collapsing microtubule and ER networks, levels of expression, aggregation susceptibility, and cell type.

**FIGURE 7. Disruption of the cytoskeleton by PGJ2 parallels the formation of protein aggregates.** Double immunofluorescence staining of SK-N-SH cells treated with 15 μM PGJ2 for 4 (A and B), 8 (C and D), 16 (E and F), and 24 h (G and H). Microtubules were visualized with the anti-α-tubulin antibody (A, C, E, and G, Texas Red), the centrosome/MTOC with the anti-γ-tubulin antibody (B, D, F, and H, fluorescein isothiocyanate), and COMT with the anti-COMT antibody (B, D, F, and H, Texas Red). Merged images are shown in B, D, F, and H. Scale bar = 10 μm.
FIGURE 8. Induction of protein aggregation following treatment with colchicine and brefeldin A. Double immunofluorescence staining of SK-N-SH cells treated for 24 h with 15 μM colchicine (A–C), 15 μM brefeldin A (D–H), and vehicle (Me2SO, I). The distribution of ubiquitinated proteins was visualized with the anti-ubiquitinated protein antibody (A and D, Texas Red), calnexin with the anti-calnexin antibody (B and E, fluorescein isothiocyanate), and microtubules with the anti-α-tubulin antibody (C, H, and I, Texas Red). Nuclei (n) were detected with DAPI (G, blue). Merged images are shown in F and G. Arrows point to large perinuclear aggregates. Scale bar = 10 μm. Lower panels, cells were harvested for Western blot analysis to detect ubiquitinated proteins (30 μg of protein/lane) after treatment with 15 μM brefeldin A (left panel) or 15 μM colchicine (right panel) for 4, 8, 16, and 24 h, or Me2SO for 4 and 24 h. Equal protein loading was demonstrated by reprobing the immunoblots for COX-1.
26 S holoenzyme indicating that PGJ2 disrupts the assembly state of 26 S proteasomes. These findings support the notion that, like in yeast, the 20 S proteasome is more or less inactive in SK-N-SH cells. Moreover, reduction in 26 S proteasome activity induced by PGJ2 reflects its disassembly. Notably, recent studies demonstrated that treatment of SH-SY5Y cells with 15d-PGJ2, a PGJ2 metabolite, induced formation of 15d-PGJ2/proteasome conjugates (31) and oxidation of the S6 ATPase subunit of the 26 S proteasome (35). These post-translational modifications could contribute to 26 S proteasome disassembly and to the observed reduction in its activity.

Overall, our data support the notion that cytoskeleton/ER collapse induced by an endogenous product of inflammation, PGJ2, may be closely associated with the neurodegenerative process of aberrant protein deposition. Interestingly, recent studies have demonstrated that parkin binds strongly to and stabilizes microtubules (36, 37). It was suggested that because parkin is anchored to and stabilizes microtubules and that many of its substrates are transmembrane or membrane-associated proteins, parkin may be optimally suited to meet the challenge of perturbed protein degradation in the ER (37). Furthermore, α-synuclein appears to be a microtubule-associated protein, in this aspect resembling tau protein. Wild type α-synuclein was shown to induce the in vitro polymerization of tubulin into microtubules, whereas the two mutant forms of α-synuclein associated with Parkinson disease do not (38). Moreover, toxins such as MPP+ and rotenone, which induce Parkinson disease symptoms in animal models, strongly hinder microtubule polymerization (39–41). The findings from all of these studies seem to converge on the premise that preserving microtubule integrity may be critical to neuronal homeostasis. Disruption of the microtubule network will most likely cause defects in axonal transport, a mechanism that seems to be common to many neurodegenerative disorders (reviewed in Ref. 42).

Physiological concentrations of prostaglandins in body fluids are found to be in the pico-nanomolar range (43). However, their levels rise considerably under pathological conditions such as hyperthermia, infection, and inflammation, reaching the micromolar range at the site of damage (44). The PGJ2 concentrations tested in our studies reflect, most likely, endogenous PGJ2 concentrations produced under pathological conditions. The rise in neurotoxic prostaglandins, such as J2 prostanoids, in the central nervous system might be an integral component of the cellular response to an insult evoked by physical, chemical, or microbial stimuli. However, the resulting neuronal cell death may have devastating effects as, in the vast majority of cases, neurons lost to disease cannot be replaced. It is thus critical that we learn more about the effects of neuroinflammation and its products, to be able to design therapies that...
will prevent endangered neurons from dying. Among the many products of arachidonic acid signaling, cyclopentenone prostaglandins such as PGJ2, seem to contribute to pathogenic mechanisms that lead to neurodegeneration (45, 46).

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