Prostaglandin J2 Alters Pro-survival and Pro-death Gene Expression Patterns and 26 S Proteasome Assembly in Human Neuroblastoma Cells*§

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Many neurodegenerative disorders are characterized by two pathological hallmarks: progressive loss of neurons and occurrence of inclusion bodies containing ubiquitinated proteins. Inflammation may be critical to neurodegeneration associated with ubiquitin-protein aggregates. We previously showed that prostaglandin J2 (PGJ2), one of the endogenous products of inflammation, induces neuronal death and the accumulation of ubiquitinated proteins into distinct aggregates. We now report that temporal microarray analysis of human neuroblasto sma SK-N-SH revealed that PGJ2 triggered a “repair” response including increased expression of heat shock, protein folding, stress response, detoxification and cysteine metabolism genes. PGJ2 also decreased expression of cell growth/maintenance genes and increased expression of apoptotic genes. Over time pro-death responses prevailed over pro-survival responses, leading to cellular demise. Furthermore, PGJ2 increased the expression of proteasome and other ubiquitin-proteasome pathway genes. This increase failed to overcome PGJ2 inhibition of 26 S proteasome activity. Ubiquitinated proteins are degraded by the 26 S proteasome, shown here to be the most active proteasomal form in SK-N SH cells. We demonstrate that PGJ2 impairs 26 S proteasome assembly, which is an ATP-dependent process. PGJ2 perturbs mitochondrial function, which could be critical to the observed 26 S proteasome disassembly, suggesting a cross-talk between mitochondrial and proteasomal impairment. In conclusion neurotoxic products of inflammation, such as PGJ2, may play a role in neurodegenerative disorders associated with the aggregation of ubiquitinated proteins by impairing 26 S proteasome activity and inducing a chain of events that culminates in neuronal cell death. Temporal characterization of these events is relevant to understanding the underlying mechanisms and to identifying potential early biomarkers.

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‡ The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables S1–S3.

§ Data were deposited in the Gene Expression Omnibus (GEO) repository under the accession number GSE4329.

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Neuroinflammation is a central nervous system (CNS) response to injury (1) and is characteristic of many neurodegenerative disorders (reviewed in Ref. 2). Neuroinflammation is induced by a variety of insults, such as neurotoxins including tri-methylin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), rotenone, lipopolysaccharide (LPS), and HIV glycoprotein gp-120 (3). In the initial stage of the reaction, astrocytes, and/or microglia are activated to release pro-inflammatory cytokines (TNF-α, IL-1β), free radicals (nitric oxide, superoxide, perhydroxyl), and/or anti-inflammatory mediators (TNF-β1, cyclopentenone prostaglandins) (4). As a result, several intracellular pathways are affected in neurons as well as glia. For example, TNF-α leads to caspase-8-mediated apoptosis and an intracellular increase in calcium (5), both TNF-α and IL-1β activate NF-κB and MAPK signaling pathways (3) and free radicals induce inflammatory reactions and oxidative stress (6). In addition, anti-inflammatory mediators, such as TNF-β1 and cyclopentenone prostaglandins, induce apoptosis, and inhibit NF-κB (7). Neuroinflammation may thus be a major contributor to neuronal damage observed in neurodegenerative disorders.

Cyclooxygenase-2 (COX-2) has emerged as a major player in inflammation (reviewed in Ref. 8). Cyclooxygenases are bifunctional enzymes that catalyze cyclooxygenation of arachidonic acid to prostaglandin G2 (PGG2) followed by the hydroperoxida- tion of PGG2 to prostaglandin H2 (PGH2) with the generation of free radicals as by-products (9). Specific reductases, isomerases, and synthases then convert PGH2 to other prostaglandins and thromboxane A2 (10). The brain expresses cyclooxygenase-1 (COX-1) and COX-2 under normal physiological conditions, but COX-2 levels are dynamically regulated by pro-inflammatory signals and by physiological neuronal plasticity (11). The cellular levels of COX-2 are dynamically regulated by pro-inflammatory mediators via transcriptional activation (12). There seems to be a close correlation between COX-2 up-regulation and neurodegeneration. For instance, COX-2 is up-regulated in neurofibrillary tangle-containing and

2 The abbreviations used are: CNS, central nervous system; AD, Alzheimer disease; ALS, amyotrophic lateral sclerosis; AMC, 7-amido-4-methylcou-marin; COX-1 and COX-2, cyclooxygenase 1 and 2; Me2SO, dimethyl sulfoxide; EASE, Expression Analysis Systematic Explorer; PD, Parkinson disease; PG, prostaglandins; ROS, reactive oxygen species; Seq., sequesitosome 1/p62; Suc, succinyl; UPP, ubiquitin-proteasome pathway; Ub, ubiquitin; ER, endoplasmic reticulum; TNF, tumor necrosis factor; IL, interleukin; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MAPK, mitogen-acti-vated protein kinase; JNK, c-Jun N-terminal kinase; RT, reverse transcription.
damaged neurons in the frontal cortex (13) and hippocampal pyramidal layer of AD patients (14) and the spinal cord of ALS patients (15). In addition, increased cyclooxygenase activity indicated by a higher level of PGE2, was detected in the substantia nigra of PD patients (16, 17).

Prostaglandins (PGJ2) is derived from PGD2, the principle cyclooxygenase product synthesized in the mammalian CNS. PGD2 is produced by PGD2 synthases, which are enzymes that catalyze the isomerization of PGH2 to PGD2 (18). PGD2 readily undergoes in vivo and in vitro non-enzymatic dehydration to generate PGJ2, which is further converted to 15-deoxy-D12,14-PGJ2 (15d-PGJ2) (19). J2 prostaglandins regulate gene expression through different transcriptional pathways. For example, 15d-PGJ2 seems to be an endogenous agonist for the transcription factor peroxisome proliferator-activated receptor (PPARγ) (reviewed in Ref. 20). J2 prostaglandins also regulate gene expression through PPARγ-independent transcription, including activation of p38MAPK and JNK pathways (21, 22) and inhibition of the NF-κB pathway (23, 24). Unlike most other classes of eicosanoids, J2 prostaglandins are characterized by the presence of a cyclopentenone ring with α,β-unsaturated carbonyl groups that are able to form Michael adducts with nucleophiles such as free sulfhydryls in cysteine residues of glutathione and cellular proteins (19). That J2 prostaglandins may play a role in neurodegeneration is supported by the finding that 15d-PGJ2 is elevated in spinal cord motor neurons of ALS patients (25). In addition, these prostaglandins induce apoptosis (26–28) and the accumulation and aggregation of polyubiquitinated proteins in neuronal cells (29). Their chemical properties in conjunction with their pro-oxidant and ubiquitin-proteasome pathway disrupting effects render these prostaglandins extremely neurotoxic and capable of inducing neuronal cell death (reviewed in Ref. 30).

In this study, we investigated by temporal microarray analysis the gene expression profile of human neuroblastoma SK-N-SH cells treated with PGJ2. We identified possible mechanisms by which PGJ2 leads to the accumulation and aggregation of ubiquitinated proteins and to neuronal cell death.

**EXPERIMENTAL PROCEDURES**

**Cells**—Human neuroblastoma SK-N-SH cells were maintained at 37 °C and 5% CO2 in minimal essential media (MEM) with Eagle’s salts containing 2 mM L-glutamine, 1 mM sodium pyruvate, 0.4% MEM vitamins, 0.4% MEM nonessential amino acids, 100 units/ml penicillin, 100 μg/ml streptomycin, and 5% normal fetal bovine serum. These cells are derived from peripheral tissue (31).

**Cell Treatments**—Cells were treated at 37 °C for different times with vehicle (0.5% Me2SO) or different concentrations of PGJ2 (Cayman Chemical Co., Ann Arbor, MI) in Me2SO. The drug was added dropwise directly into serum-containing medium with a gentle swirl of the culture plate. At the end of the indicated incubation times, the cultures were washed twice with phosphate-buffered saline, and the cells were harvested as previously described (32). Cell washes removed unattached cells; hence subsequent assays were performed on adherent cells only.

**Temporat Microarray Analysis of Gene Expression**—The CAG Human 19K arrays were printed at the Center for Applied Genomics (Newark, NJ). The arrays consist of 65-mer oligonucleotides (Human OligoLibrary, Compugen, San Jose, CA) representing 18,664 human genes and 197 controls spotted onto poly-L-lysine coated glass microscope slides.

SK-N-SH cells were treated with 20 μM PGJ2 for 4, 8, 16, and 24 h. Each time point was repeated twice. For each time point, total RNA from control and PGJ2-treated cells was isolated with the Qiagen RNeasy mini kit (Valencia, CA) according to the manufacturer’s protocol. RNA was evaluated for quantity and integrity by taking OD measurements at 260/280 nm and agarose gel electrophoresis.

cDNAs were generated from 5 μg of total RNA per sample by reverse transcription (RT) for 2 h at 42 °C using the 3DNA Submicro™ Oligo Expression Array detection kit (Genisphere, Hatfield, PA) and Superscript II (Invitrogen, Carlsbad, CA). For each microarray two consecutive hybridizations were performed with cDNAs and the fluorescent probes following the manufacturer’s specifications. Briefly, in the first hybridization reaction the solution containing the mixture of cDNAs obtained from control and treated cells was loaded onto the CAG Human 19K array and incubated overnight at 55 °C in a hybridization chamber (GeneMachines, San Carlos, CA). In the second hybridization reaction Cy3 (green) and Cy5 (red) fluorescent probes were used to label the cDNAs from control and PGJ2-treated cells, respectively. The second hybridization was carried out for 3 h at 65 °C in the hybridization chambers. This cDNA co-hybridization provides a measure of the relative gene expression levels between the two cell populations. The arrays were scanned using an Axon GenePix 4000B Scanner (Molecular Devices, Sunnyvale, CA) with laser intensities adjusted to bring the brightest spot just below saturation and the data extracted with the Axon GenePix Pro software.

**Analysis of Microarray Data**—Analysis of the microarray data were performed using TIGR MultiExperiment Viewer (MEV 3.1). The data were normalized globally so the median log ratios for each experiment were equal to zero. The data were filtered so that only non-flagged spots with a diameter above 70 and with a signal intensity of 2-fold above background in at least one channel were given a relative expression value: PGJ2-treated versus control. In addition, we filtered out the genes with more than 20% of their values missing across the experiments. A one class SAM analysis was performed with the MEV software comparing all the time points to the untreated cells to find genes with changes that occurred consistently in all time points. A median FDR of zero (delta = 2.11, Pi0Hat = 0.44) and an average change of at least 1.8-fold were considered our cut off for significance. A hierarchical clustering was then performed on samples and genes (Fig. 1). To identify biological themes within the gene list we used the Expression Analysis Systematic Explorer (EASE, Ref. 33) algorithm from the MEV software (34) with the annotation of the Human OligoLibrary, Fisher exact test, p < 0.01. Data were deposited in the Gene Expression Omnibus (GEO) repository under the accession number GSE4329.

**Real-time PCR Analysis**—Total RNA was extracted from control and PGJ2-treated cells as described above for the
TABLE 1
Real time RT-PCR analysis of the expression of genes of the ubiquitin-proteasome pathway
SK-N-SH cells were treated with Me$_2$SO (0.5%) or 15 or 20 µM PGJ2 for 24 h and analyzed for expression of genes of the ubiquitin-proteasome pathway by real time RT-PCR. The values are given as relative fold change of mRNA expression in PGJ2-treated versus Me$_2$SO-treated control cells. GAPDH is included as a control. Positive values imply an increased expression and negative values a decreased expression in PGJ2-treated versus the Me$_2$SO-treated control cells.

| Genes                      | Real-time PCR (15 µM PGJ2) | Real-time PCR (20 µM PGJ2) | Microarray (20 µM PGJ2) | fold-change |
|----------------------------|-----------------------------|----------------------------|-------------------------|-------------|
| 20 S Proteasome            |                             |                            |                         |             |
| α2 (PSMA2)                 | 2.22                        | 2.23                       | ND*                     |             |
| α3 (PSMA3)                 | ND                          | ND                         | 2.67                    |             |
| α5 (PSMA5)                 | 2.99                        | 2.62                       | 5.29                    |             |
| α6 (PSMA6)                 | ND                          | ND                         | 2.49                    |             |
| β2 (PSMB2)                 | 2.62                        | 2.34                       | ND                      |             |
| β3 (PSMB3)                 | 1.43                        | 1.71                       | ND                      |             |
| β4 (PSMB4)                 | 1.99                        | 1.78                       | ND                      |             |
| β7 (PSMB7)                 | 2.23                        | 2.41                       | 5.73                    |             |
| 26 S Proteasome            |                             |                            |                         |             |
| ATPase 5 (PSMCS)           | 3.32                        | 3.73                       | ND                      |             |
| Non-ATPase 1 (PSMD1)       | 3.53                        | 4.11                       | 4.07                    |             |
| Non-ATPase 3 (PSMD3)       | 3.01                        | 2.9                        | ND                      |             |
| Non-ATPase 6 (PSMD6)       | ND                          | ND                         | 3.34                    |             |
| Non-ATPase 8 (PSMD8)       | 1.66                        | 1.83                       | ND                      |             |
| Non-ATPase 12 (PSM8D)      | ND                          | ND                         | 3.96                    |             |
| Ubiquitin-conjugating enzyme|                            |                            |                         |             |
| UBE2H                      | 2.42                        | 8.78                       | ND                      |             |
| Control                    |                             |                            |                         |             |
| GAPDH                      | 1.1                         | −1.05                      | 1.28                    |             |

* ND, not determined.

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microarray analysis. 4 µg of RNA were reverse-transcribed with the ReactionReady™ First Strand cDNA Synthesis kit from SuperArray (Frederick, MD). The PCR primers to amplify the human cDNAs listed in Table 1 were purchased from SuperArray (Frederick, MD, proprietary sequence). Quantitative amplification of the human cDNAs was monitored with SYBR Green using real-time PCR. PCR amplification was carried out in 25 µl of ROX PCR Master Mix (SuperArray) containing 0.2 µM of each primer and 1 µl of the reverse transcription reaction in a 7500 Real-time PCR system (Applied Biosystems). Thermal cycling conditions comprised of an initial step at 95 °C for 15 min for denaturation and for activation of the HotStart TaqDNA polymerase, followed by 40 cycles of: 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s. The reaction ended with a 5-min incubation at 72 °C. Expression of the target genes in cells treated with PGJ2 was normalized to expression under control (0.5% Me$_2$SO-treated) conditions by means of the comparative C$_t$ (threshold cycle) method. The C$_t$ is defined as the number of cycles required for the fluorescence signal to exceed the detection threshold. The values are given as the means of two independent determinations.

Western Blotting—Following the indicated treatments, cell extracts were prepared as described previously (32), and 20 µg of protein/lane were boiled for 5 min in Laemmli buffer and analyzed by SDS-PAGE on 10% polyacrylamide gels followed by Western blotting for detection of the proteasome subunits with the respective antibodies (1:1,000) obtained from BIOMOL (Plymouth Meeting, PA). The mouse monoclonal anti-sequestosome antibody (1:500) was obtained from BD Transduction Lab (San Diego, CA). Antigens were visualized by a standard chemiluminescent horseradish peroxidase method with the ECL reagent. Protein concentration was determined with the bicinchoninic acid protein assay kit from Pierce.

In-gel Proteasome Activity and Detection—Cells treated for 24 h with vehicle (Me$_2$SO) or PGJ2 (15 µM and 20 µM) 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 MgCl$_2$, 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, Bio-Rad) was adjusted to equal concentrations with buffer A. The cleared supernatants were resolved by non-denaturing PAGE using a modification of the method described in Ref. 35. 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 V for 2.5 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 proteasomes with anti-α4- and anti-S8-subunit antibodies obtained from BIOMOL. The anti-α4 antibody reacts with a core particle subunit, 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.

RESULTS

Effect of PGJ2 on Differential Gene Expression in Human Neuroblastoma SK-N-SH Cells—A total of 18,664 genes were evaluated over four time points, 4, 8, 16, and 24 h after treatment with 20 µM PGJ2 (Fig. 1). Of these, 5,298 genes remained after filtering for missing data and/or low expression levels. We found that 389 genes expressed in the SK-N-SH cells showed statistical significance (SAM median FDR = 0) and at least a 1.8-fold increase or a 1.8-fold decrease in expression for four time points after acute treatment with 20 µM PGJ2. Up-regulation was dominant over down-regulation at all time points with 334 genes found to be up-regulated and 55 down-regulated.

Biological Themes Enriched in Genes Differentially Expressed in Human Neuroblastoma SK-N-SH Cells after Treatment with PGJ2—We performed an EASE analysis on the 334 up- and 55 down-regulated genes differentially expressed after PGJ2-treatment. We found enrichment in 11 functional groups based on the oligonucleotide annotation from the Compgen Source data base (supplemental Table S1). Genes involved in heat shock response, protein folding, stress response, ubiquitin cycle, ubiquitin-dependent protein degradation, polyubiquitination, apoptosis, cysteine metabolism, and heavy metal response, were up-regulated more than would be expected using a random sampling. Categories corresponding to down-
FIGURE 1. Cluster analysis of microarray data. Overall analysis of eight experiments carried-out for microarray analysis of the gene expression profiles in SK-N-SH cells after treatment with 20 μM PGJ2 for 4, 8, 16, and 24 h. Down-regulated genes in PGJ2-treated cells are represented in green (Cy3) and up-regulated genes in red (Cy5). The horizontal bar above provides a visual indication of the level of change in PGJ2-treated cells versus control.
regulated genes included cell growth/maintenance and transcription regulation. The percentage and number of genes significantly up- or down-regulated within each of the functional groups is shown in Fig. 2. It is clear that from all the biological themes analyzed, ubiquitin-dependent protein degradation, heat shock, protein folding, and apoptosis display the largest number of up-regulated genes. Transcription regulation exhibits the largest number of down-regulated genes.

A longitudinal study provided a general idea of the levels of expression of the known genes with significantly altered expression over all time points against untreated controls (Fig. 3). PGJ2 treatment significantly induced changes in gene expression that lasted for at least 24 h. Expression of heat shock genes increased sharply by 8 h and then leveled off reaching a maximum of ~10-fold by 24 h (Fig. 3A). Protein folding gene expression exhibited a modest flat increase throughout the 24-h period (Fig. 3A). Expression of genes involved in polyubiquitination, ubiquitin-dependent degradation, and ubiquitin cycle showed a steady increase from 4 to 24 h, never reaching a plateau (Fig. 3B). Polyubiquitination gene expression was the highest of the three latter functional groups reaching a maximum of ~5.5-fold by 24 h. Changes in mRNA levels of apoptosis and stress response genes lagged behind, showing a modest and flat increase up to 16 h then steeply rising to a maximum of ~4-fold by 24 h (Fig. 3C). Down-regulation of transcription regulation genes showed a steady decline throughout the 24-h period (Fig. 3E). The expression of cell growth/maintenance genes decreased steeply by 8 h, then leveling off up to 24 h. Based on these results, it seems that initially the cells try to overcome the deleterious effects of PGJ2 by up-regulating heat shock, protein folding, polyubiquitination, Ub-dependent degradation, and ubiquitin cycle genes. Pro-death responses, such as induction of apoptosis are triggered at a later post-treatment time point.

Heat Shock, Protein Folding, and Stress Response—The expression of 14 heat shock genes was up-regulated for all time points (supplemental Table S1 and Fig. 3). Notably, HSP70, HSP90, and HSP105 were among the 21 top genes that exhibited the greatest level of up-regulation in SK-N-SH cells treated for 24 h with 20 μM PGJ2. In particular, HSP 70 (HSP70B/HSPA6) was the most highly expressed gene of all reaching a 69-fold increase in mRNA level upon treatment with 20 μM PGJ2 for 24 h. Expression of genes involved in protein folding (12 genes), including two genes with DnaJ domains, were consistently found to be up-regulated. Stress response genes (four) were steadily up-regulated up to 16 h and then exhibited a sharp increase by 24 h. These data strongly indicate the active involvement of a “repair” response in an attempt to rescue the cells from stress induced by PGJ2, which is a neurotoxic product of inflammation. Moreover, it is consistent with the established role of repair gene products which are sensitive to a large variety of stress-inducing agents.

Ubiquitin-Proteasome Pathway (UPP)—A large number of genes (23 genes) involved in the UPP were affected by PGJ2 treatment (supplemental Table S1 and Fig. 3). At least eight genes encoding for 26 S proteasome subunits, including subunits from the 20 S and 19 S particles, were significantly up-regulated over control conditions. Additional microarray data support a requirement for increased levels of ubiquitination and protein degradation by the UPP upon PGJ2 treatment. Accordingly, mRNA levels of the ubiquitin hydrolase UCH-L1 and of at least four ubiquitin ligases, including culin 7 and SMURF2, were significantly up-regulated in cells treated with PGJ2. Moreover, the mRNA levels of the Ub C gene were up-regulated by as much as 13-fold 24 h post PGJ2-treatment. Ub C, which encodes nine tandemly repeated ubiquitins, is one of the members of the human ubiquitin multigene family that is stress-inducible (36).

Mechanisms Involved in PGJ2 Cytotoxicity—J2 prostaglandins were shown to induce apoptosis in SH-SY5Y neuronal cells (25). Our studies established that at least nine genes associated with the induction of apoptosis were up-regulated by PGJ2, strongly supporting that J2 prostaglandins trigger a pro-apoptotic response in neuronal cells.

Increased mRNA levels were observed in eight genes related to oxidative stress (supplemental Tables S1 and S2). One of these genes, heme oxygenase 1, was up-regulated 30-fold upon 4-h treatment with 20 μM PGJ2 (supplemental Table S2). Heme oxygenase 1 is thought to be a novel protective factor against inflammation and oxidative stress (reviewed in Ref. 37). Other genes in this group are associated with protein thiolation: (i) glutamate-cysteine ligase is the first rate-limiting enzyme in glutathione synthesis (supplemental Table S1), (ii) glutathione peroxidase detoxifies hydrogen peroxide (supplemental Table S2), (iii) cystathionine is involved in the conversion of methionine to cysteine, which is important for glutathione synthesis (supplemental Table S1), and (iv) metal-regulatory transcription factor 1, which is an activator of the metallothionein promoter (supplemental Table S1). In addition, NAD(P)H menadione oxireductase 1, which is a quinone reductase, and

| Term                              | # Up | # Down | Total on Chip | Fisher Exact Test |
|-----------------------------------|------|--------|---------------|------------------|
| heat shock response               | 14   | 26     | 0.0000        |                  |
| ubiquitin-dependent protein       | 15   | 83     | 0.0000        |                  |
| degradation                       |      |        |               |                  |
| protein folding                   | 12   | 81     | 0.0000        |                  |
| polyubiquitination                | 5    | 18     | 0.0000        |                  |
| apoptosis                         | 9    | 114    | 0.0002        |                  |
| cysteine metabolism              | 2    | 3      | 0.0009        |                  |
| cell growth and maintenance       | 4    | 5      | 256           | 0.0012           |
| stress response                   | 4    | 34     | 0.0031        |                  |
| heavy metal response              | 2    | 5      | 0.0031        |                  |
| transcription regulation          | 6    | 8      | 831           | 0.0040           |
| ubiquitin cycle                   | 3    | 22     | 0.0068        |                  |

FIGURE 2. Comparison of increased and decreased gene expression within each gene ontology category found to be significantly enriched using EASE. The histogram compares the percentage of genes whose expression was increased (gray bars, UP) or decreased (black bars, DOWN) in expression for four time points after acute treatment with 20 μM PGJ2, within each gene ontology category. EASE analysis was performed separately on the 55 genes found to be down-regulated and the 334 genes found to be up-regulated following treatment.
superoxide dismutase 1, which metabolizes superoxide radicals, were also up-regulated by PGJ2-treatment (supplemental Table S2). These findings support the notion that prostaglandins of the J2 series are potential inducers of oxidative stress (38).

At least four genes encoding detoxifying enzymes were found to be up-regulated (supplemental Table S2). These include two UDP glucuronosyltransferases, arylacetamide deacetylase and a glycine-N-acetyltransferase.

Other Mechanisms Affected by PGJ2—We found that PGJ2 increased the mRNA levels of genes encoding for calcium-dependent, cytoskeleton-related and lysosomal proteins (supplemental Table S2).

From the calcium-dependent group of genes: one is similar to the complement decay accelerating factor (C-type lectin domain family 14), one participates in the transport of secretory vesicles (synaptotagmin), one is a calcium channel subunit and the prion protein, (ii) a huntingtin-interacting protein, (iii) an aminopeptidase, (iv) a potassium channel modulator, and (v) a cholinergic receptor subunit (supplementary Table S2). Changes in the expression levels of all of these genes may be relevant to neurodegeneration.

PGJ2 increased the mRNA levels of eight genes associated with the cytoskeleton and/or intracellular vesicular transport. Interestingly, 15d-PGJ2 was recently shown to disrupt the microtubule cytoskeletal network in breast cancer cells (41) and to induce cytoskeletal reorganization in mesangial cells (42). We have found a similar phenomenon in neuronal cells.3

The mRNA levels of three genes encoding lysosomal enzymes were up-regulated by more than 4-fold. Interestingly, the increase in mRNA levels for two of these genes that are lysosomal proton pumps was most apparent after 16 h of treatment with PGJ2 (Fig. 3D), thus later than the observed increase in components of the UPP (Fig. 3B) and overlapping with apoptosis (Fig. 3C).

We also observed a rise in mRNA levels of a group of genes of dissimilar functions: one involved in inflammation (TGF-β), one associated with glucose metabolism (pyruvate carboxylase), and five encoding for (i) the prion protein, (ii) a huntingtin-interacting protein, (iii) an aminopeptidase, (iv) a potassium channel modulator, and (v) a cholinergic receptor subunit (supplementary Table S2). Changes in the expression levels of all of these genes may be relevant to neurodegeneration.

Potential Early Biomarkers—We attempted to single out genes that, because of their extreme increase in mRNA levels upon 4 h of treatment with PGJ2 (greater than 10-fold), may be considered to be potential early biomarkers of inflammation related to neurodegeneration. There were eight genes that fell under this category (supplemental Table S3). Interestingly, the gene that exhibited the highest increase in mRNA levels (48-fold) upon 4 h of treatment with PGJ2 potentially encodes for a C-type lectin domain family 14 protein. Although not yet fully

3 Ogburn, K. D., and Figueiredo-Pereira, M. E. (2006) J. Biol. Chem. doi:10.1074/jbc.M600635200.
characterized it resembles DAF (decay-accelerating factor), which in neutrophils is up-regulated within minutes of activation in a calcium-dependent manner (43). Neuronal up-regulation of DAF, also known as CD55, was recently suggested to be a key protective signaling mechanism in brain cells to withstand complement activation and survive within an inflammatory site (44). If the prediction is correct, this protein may be an early indication of neuronal stress related to inflammation. Overall, the changes in gene expression revealed by our temporal microarray studies provide potential clues to the etiology of neurodegeneration associated with pro-inflammatory conditions.

Increases in Proteasome Subunit mRNA Levels Induced by PGJ2 Do Not Correspond to Elevated Protein Levels—A more detailed analysis of increased expression of some UPP genes by PGJ2 was carried out by real-time RT-PCR. Alterations in mRNA expression are shown as the relative mRNA levels in treated (15 or 20 μM PGJ2) versus control (MeSO, 0.5%) cells. Treatment of SK-N-SH cells for 24 h with 15 or 20 μM PGJ2 resulted in a 1.4–4.1-fold increase in mRNA levels of the tested proteasome genes encoding subunits of 20 S core and 19 S regulatory particles of the 26 S proteasome (Table 1).

To investigate whether the increased expression of proteasome genes corresponded to an elevation in protein levels, we performed Western blot analysis of SK-N-SH cells treated for 24 h with 10, 15, and 20 μM PGJ2. As shown in Fig. 4, it is clear that after 24 h of treatment with PGJ2 the protein levels of the proteasome subunits were not considerably altered. Failure to increase proteasome subunit levels was not because of an overall decrease in protein synthesis. Accordingly, sequestosome/p62 (Seq.) levels increased in a PGJ2-dependent manner reaching a peak in cells treated with 15 μM PGJ2 (Fig. 4). As previously shown anti-Seq. immunoreactivity is barely detected in control cells (45).

Inhibition of the Activity of the 26 S Proteasome by PGJ2 Involves Proteasome Disassembly—To further address the 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. 5, left panel). A concentration-dependent decrease in the chymotrypsin-like activity of 26 S as well as 20 S proteasomes was observed in cells treated with 15 and 20 μM PGJ2.

Western blot analysis of the native gels probed with an antibody that reacts with the α4-subunit of the core particle revealed that the 26 S and 20 S proteasome levels are similar in control cells (Fig. 5, middle panel). However, an increase in 20 S proteasome with a concomitant decrease in the 26 S holoenzyme was observed upon treatment with PGJ2 (Fig. 5, middle panel). PGJ2 thus perturbs the assembly of the 26 S proteasome. This view was supported by the finding that stripping and reprobing the immunoblot with an antibody that reacts with the S8 (ATPase) subunit of the 19 S regulatory particle (right panel). Proteasomal 26 S and 20 S forms are indicated on the right.
PGJ2 Alters Gene Expression Patterns and Proteasome Assembly

**DISCUSSION**

These studies identify by temporal microarray and conventional biochemical analyses, potential mechanisms leading to the development of two hallmarks of neurodegeneration: cell death and the formation of inclusion bodies containing ubiquitinated proteins. We focused on the effects of PGJ2, a neurotoxic product of inflammation. In these studies SK-N-SH cells were treated with concentrations of PGJ2 in the micromolar range (10–20 μM). For the temporal microarray analysis, cells were treated with 20 μM PGJ2 to permit detection of changes in gene expression at all time points investigated (4, 8, 16, and 24 h). Although physiological concentrations of prostaglandins in body fluids are in the piconanomolar range (47), their levels rise considerably under pathological conditions such as hyperthermia, infection and inflammation, reaching micromolar range at the site of damage (48–50). J2 prostaglandins cannot be accurately measured in biological fluids because they have a short half-life and bind avidly to free sulfhydryl groups in intracellular proteins and GSH. Consequently, the in vivo concentrations of J2 prostaglandins may be underestimated. Attempts to measure the endogenous levels of these highly reactive cyclopentenone eicosanoids should focus on assessing intracellular PGJ2/protein complexes (51). Moreover, J2 prostaglandins were reported to covalently modify their protein targets, thus their effects could be cumulative (23). Although the in vivo concentrations of PGJ2 and its metabolites (12- PGJ2 and 15d-PGJ2) are currently unknown their in vivo occurrence was clearly demonstrated (Refs. 50 and 52 and reviewed in Ref. 53). Interestingly, the levels of 15d-PGJ2 were found to be elevated in spinal cord motor neurons of ALS patients (25). PGJ2 and its metabolites are thus produced in the CNS and their concentrations are likely to be increased in response to pro-inflammatory stimuli, particularly during the resolution phase of the inflammatory response (50).

The results from our temporal microarray analysis of gene expression in cells treated with PGJ2 revealed that the cells initiate a putative “repair” response that includes increased expression of genes of the following functional groups: heat shock, protein folding, stress response, ubiquitin-dependent protein degradation, ubiquitin cycle, and polyubiquitination. Increased expression of all of these genes indicates a cellular attempt to rescue and/or remove abnormal proteins generated by the proteotoxic effects of PGJ2. If the damaging effects of PGJ2 cannot be reversed by these and other repair mechanisms, then pro-death pathways, including apoptosis, are activated most likely to remove damaged cells. Studies with SH-SY5Y cells, a subline of the human neuroblastoma SK-N-SH cells, confirmed by DNA fragmentation that J2 prostaglandins induce apoptosis (25). The resulting neuronal cell death may have devastating effects as, in the vast majority of cases, neurons lost to disease processes cannot be replaced.

Prostaglandins of the J2 series exert some of their damaging effects through oxidative stress. They were shown to decrease glutathione levels, glutathione peroxidase activity, mitochondrial membrane potential and to increase the production of protein-bound lipid peroxidation products, such as acrolein and 4-hydroxy-2-nonenal (38). These effects suggest that prostaglandins of the J2 series are either a source of markedly increased ROS generation or modulators of ROS sensitivity (54). That PGJ2 induces oxidative stress is further supported by our findings showing increased expression of genes linked to the antioxidant response. In addition, our results demonstrate that PGJ2 disrupts the intracellular sulfhydryl homeostasis as it induces up-regulation of genes associated with cysteine metabolism. Furthermore, PGJ2-treatment increased the mRNA levels of genes encoding for calcium-dependent and cytoskeletal-related proteins, supporting the notion that these eicosanoids exert a complex array of effects that are relevant to neurodegeneration. As conveyed in an excellent recent review (30), the CNS production of highly reactive cyclopentenone prostaglandins, such as the J2 series, may represent a novel pathogenic mechanism common to many neurodegenerative diseases. The temporal and spatial characterization of the effects of these agents may lead to the identification of early biomarkers of these neurodegenerative conditions that are associated with inflammation. In our studies we detected one potential candidate, i.e. a putative member of the C-type lectin domain family, the expression of which is highly increased at an early time point following PGJ2 treatment.

Besides being associated with oxidative stress, PGJ2 also induces the accumulation and aggregation of polyubiquitinated proteins, suggesting that it impairs the UPP (29). Although expression of at least eight proteasome genes was increased by PGJ2, we did not observe a parallel rise in their protein levels. Failure to increase proteasomal subunit levels was not caused by an overall translation shut-off by PGJ2 treatment. As we previously observed, Seq. protein levels were increased by PGJ2 treatment (45). It is possible that free proteasomal subunits are unstable under these conditions. Interestingly, our temporal microarray analysis revealed that PGJ2 increased the mRNA levels of genes encoding for lysosomal components later than those encoding for UPP components. This could indicate an effort to bypass a defect in protein degradation caused by UPP impairment.

Up-regulation of proteasome subunit gene expression failed to overcome or prevent the decrease in proteasome activity observed in PGJ2-treated cells. Recent studies demonstrated that treatment of SH-SY5Y cells with 15d-PGJ2 induced formation of 15d-PGJ2/proteasome conjugates (55) and oxidation of the S6 ATPase subunit of the 26 S proteasome (56). These modifications could contribute to decreased proteasome activity. The effect of PGJ2 on proteasome activity is controversial. Whereas some studies show that J2 prostaglandins fail to inhibit proteasome activity in cells (29, 57) others report the opposite, i.e. that J2 prostaglandins decrease proteasome activity (55). To address this issue 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 coincides with the 26 S proteasome, suggesting that the free 20 S core particle is nearly inactive. A similar phenomenon was observed in yeast (58). Furthermore, our studies revealed a PGJ2-dependent decline in proteasome activity. This decline paralleled a shift of the 26 S proteasome to the 20 S core particle, indicating that PGJ2 disrupted the assembly state of 26
disorders share a common mechanism, whether is whether different forms of neurodegenerative disorders associated with the accumulation and aggregation of ubiquitinated proteins. The CNS production of such as J2 prostaglandins, may play a crucial role in neurodegenerative disorders associated with the accumulation and inability to degrade ubiquitinated proteins. This in turn would lead to the accumulation of ubiquitinated proteins in the cells.

Overall, we conclude that products of neuroinflammation, such as J2 prostaglandins, may play a crucial role in neurodegenerative disorders associated with the accumulation and aggregation of ubiquitinated proteins. The CNS production of these cyclopentenone eicosanoids may represent a novel pathogenic mechanism leading to many neurodegenerative conditions (reviewed in Ref. 30). A key question that remains unanswered is whether different forms of neurodegenerative disorders share a common mechanism, i.e. neuroinflammation. If so, how can the enormously varied etiology, presentation and time course of these devastating disorders be explained? For example, whereas head injury is a rapid, accidental event, PD is characterized by damage to specific brain regions resulting in motor disturbances and chronic degeneration (60). This variety of disease manifestations could be correlated with the primary brain region affected by the injurious event, its severity and duration.

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