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Prolyl Endopeptidase is Revealed Following SILAC Analysis to be a Novel Mediator of Human Microglial and THP-1 Cell Neurotoxicity

ANDIS KLEGERIS,[1] JANE LI,[2] THEO K. BAMMLER,[2] JINGHUA JIN,[2] DAVID ZHU,[2] DANIEL T. KASHIMA,[2] SHENG PAN,[2] SADAYUKI HASHIOKA,[3] JOHN MAGUIRE,[4] PATRICK L. McGEER,[3*] AND JING ZHANG[2]

[1]*Unit of Biology and Physical Geography, Barber School of Arts and Sciences, University of British Columbia Okanagan, Kelowna, British Columbia, Canada*

[2]*Division of Neuropathology, Harborview Medical Center, University of Washington School of Medicine, Seattle, Washington*

[3]*Kinsmen Laboratory of Neurological Research, University of British Columbia, Vancouver, British Columbia, Canada*

[4]*Department of Pathology and Laboratory Medicine, University of British Columbia, Vancouver, British Columbia, Canada*

KEY WORDS

Alzheimer disease; mass spectrometry; neuroinflammation; Parkinson disease; proteomics

ABSTRACT

Reactive microglial cells may exacerbate the pathology in some neurodegenerative disorders. Supernatants of stimulated human microglial cells, or their surrogate THP-1 cells, are lethal to cultured human neuroblastoma SH-SY5Y cells. To explore this neurotoxicity, we examined the spectrum of proteins generated by THP-1 cells using the technique of stable isotope labeling by amino acids in cell culture (SILAC). Unstimulated cells were grown in medium with light L-[$^{12}C_6$] arginine while cells stimulated by lipopolysaccharide (LPS) plus interferon-γ (IFN-γ) were grown in medium with heavy L-[$^{13}C_6$] arginine. Proteins isolated from the media were digested with trypsin, and relative concentrations of generated peptides determined by mass spectrometry. More than 1,500 proteins or putative proteins were identified. Of these, 174 were increased and 189 decreased by more than twofold in the stimulated cell supernatant. We selected one upregulated protein, prolyl endopeptidase (PEP), for further investigation of its potential contribution to neurotoxicity. We first confirmed its upregulation by comparing its enzymatic activity in stimulated and unstimulated cell supernatants. We then evaluated two specific PEP inhibitors, Boc-Asn-Phe-Pro-aldehyde and *Z*-Pro-Pro-aldehyde-dimethyl acetal, for their potential to reduce toxicity of stimulated THP-1 cell and human microglia supernatants towards SH-SY5Y cells. We found both to be partially protective in a concentration-dependent manner. Inhibition of PEP may be a therapeutic approach to neurodegenerative disorders including Alzheimer and Parkinson diseases.

INTRODUCTION

The neuroinflammatory hypothesis of neurodegenerative diseases proposes that inflammation contributes to the pathogenesis in a number of neurological disorders. The key finding is the presence of reactive microglial cells in pathologically affected areas. This phenomenon was originally described for Alzheimer, Parkinson, Pick and Huntington diseases, amyotrophic lateral sclerosis (ALS), ALS/Parkinsonism-dementia complex of Guam, Shy-Drager syndrome, multiple sclerosis, and AIDS encephalopathy (McGeer et al., 1988). It has since been found to apply to many other degenerative conditions. Microglia, which are believed to orchestrate the inflammatory reactions, have been shown to be neuroprotective or neurotoxic depending on the experimental model. The strength and nature of the stimuli as well as the functional state of surrounding cells may determine the outcome (Kadiu et al., 2005; Klegeris and McGeer, 2005b; Simard and Rivest, 2006).

The damaging effect of microglia to host tissue has been illustrated by a number of *in vitro* and *in vivo* studies (reviewed in Block et al., 2007). Activated microglia secrete an array of potentially neurotoxic materials, including glutamate, quinolinic acid, tumor necrosis factor-α (TNF-α), soluble FasL, and reactive oxygen intermediates. The toxicity may also be mediated by unknown soluble proteins (Flavin et al., 1997; Moriguchi et al., 2003), and several groups have identified proteolytic enzymes as candidate neurotoxins. These include tissue plasminogen activator (Flavin et al., 2000), cathepsin B (Gan et al., 2004; Kingham and Pocock, 2001), matrix metalloproteinases (Kim et al., 2006; Nuttall et al., 2007; Zhang et al., 2003), and chymotrypsin-like proteases (Klegeris and McGeer, 2005a). It is probable that microglial neurotoxicity is mediated by a combination of different toxins, which may vary depending on the nature of the stimulating agents and the surrounding medium.

To explore this possibility further, we undertook a proteomics study of supernatants from stimulated and

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*Correspondence to: Patrick L. McGeer, Kinsmen Laboratory of Neurological Research, University of British Columbia, Vancouver, BC V6T 1Z3, Canada. E-mail: mcgeerpl@interchange.ubc.ca

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unstimulated microglia and their surrogate THP-1 cells. Our goal was to confirm the presence of known neurotoxins and to identify novel proteins with a potential to contribute to microglial toxicity. The screening technique we employed is termed stable isotope labeling by amino acids in cell culture (SILAC) which is combined with analysis by mass spectrometry (MS). Since the required amount of protein cannot be harvested from the usual sources of human primary microglia, we used human THP-1 cells as a surrogate model. They are transformed human mononuclear cells, which have a range of properties similar to microglia and other mononuclear phagocytes. They can release such products as interleukin (IL)-1β, prostaglandin E_2, superoxide anion, TNF-α, and various other neurotoxins (Combs et al., 1999; Giulian et al., 1990; Klegeris and McGeer, 2005a; Lorton et al., 1996; Schwende et al., 1996; Yates et al., 2000).

We compared the protein composition of cell supernatants harvested from unstimulated THP-1 cells with cells stimulated by lipopolysaccharide (LPS) and interferon (IFN)-γ. We chose this combination because it has consistently caused the most significant neurotoxic response of THP-1 cells as well as human microglia. SILAC methodology allowed us to identify more than 1,500 proteins or putative proteins based on known DNA sequences. In addition, we found 174 proteins or putative proteins upregulated by more than twofold and 189 downregulated by more than twofold.

On the list of significantly upregulated proteins was prolyl endopeptidase (PEP, EC 3.4.21.26), a potential new candidate for neurotoxicity. We confirmed this by measuring upregulated enzyme activity in stimulated THP-1 cell supernatants. We further found that specific inhibitors of this enzyme partially reduced toxicity of both stimulated THP-1 cells and human microglia towards SH-SY5Y neuroblastoma cells.

MATERIALS AND METHODS

Materials

PEP inhibitors Boc-Asn-Phe-Pro-aldehyde and *Z*-Pro-Pro-aldehyde-dimethyl acetal were purchased from Bachem Bioscience (King of Prussia, PA). Human recombinant IFN-γ, IL-6 and TNF-α were from PeproTech Canada (Ottawa, ON, Canada). Human recombinant α-synuclein was a kind gift from Dr. B.I. Giasson, University of Pennsylvania, Philadelphia, PA. Antibodies used in ELISA assays were as follows: for TNF-α capture, mouse monoclonal (1:2,000, Chemicon, Temecula, CA); for TNF-α detection, biotinylated rabbit polyclonal (1:200, PeproTech Canada). Other reagents, including the PEP substrate *Z*-glycyl-L-proline-4-nitroanilide were purchased from Sigma-Aldrich (St. Louis, MO).

Cell Culture

The human monocytic THP-1 cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA). The human neuroblastoma SH-SY5Y cell line was a gift from Dr. R. Ross, Fordham University, NY. They were grown in Dulbecco's modified Eagle medium-nutrient mixture F12 Ham (DMEM-F12, Invitrogen Canada, Burlington, ON) supplemented with 10% fetal bovine serum (FBS, Invitrogen) containing gentamicin (50 μg mL^{-1}). They were used without initial differentiation. Adult human microglial cells were isolated from surgically resected temporal lobe tissue according to a previously published protocol and grown in the same medium (Klegeris and McGeer, 2005a). Immunostaining with antibodies against CD68, which stains microglia as well as macrophages, and against glial fibrillary acidic protein (GFAP), which is a marker of astrocytes, showed that the isolated cultures contained more than 95% microglial cells.

SILAC Analysis of Proteins

For SILAC analysis of proteins, our previously published procedures were used with minor modifications (McLaughlin et al., 2006; Zhou et al., 2005). Two groups of THP-1 cells were grown in DMEM media without standard L-arginine (L-Arg) (JRH Biosciences, Lenexa, KS). Unstimulatd THP-1 cells were grown in medium supplemented with a light L-[$^{12}C_6$]Arg isotope while cells to be stimulated were grown in the same medium supplemented with the heavy L-[$^{13}C_6$]Arg isotope (Cambridge Isotope Laboratories, Andover, MA). Both had 10% dialysed FBS (Invitrogen) added. They were grown for five generations over a 5-week period in order to achieve near complete incorporation of the L-Arg isotopes into proteins (Ong et al., 2002). Subsequently, cells were washed twice with phosphate-buffered saline (PBS), plated at a concentration of 5×10^5 cells mL^{-1}, and treated in corresponding media in the absence of FBS. L-[$^{13}C_6$]Arg-labeled THP-1 cells were treated for 48 h with 0.5 μg mL^{-1} of LPS and 150 U mL^{-1} of IFN-γ while L-[$^{12}C_6$]Arg-labeled cells were treated with PBS only. The cell-free supernatants were collected, concentrated by SpeedVac centrifugation and protein concentrations measured by the Bradford assay. Equal amounts of total protein from control and LPS+IFN-γ-treated groups were pooled together for proteomic analysis. Supernatants from 25 million THP-1 cells were needed to harvest a sufficient amount of protein for the SILAC analysis. Since a typical human surgical sample yields less than 1 million recoverable microglial cells SILAC analysis of the secretome of mature microglial cells was not possible.

In-Gel Digestion of Proteins in Culture Supernatants Followed by Protein Identification Using LCQ Tandem Mass Spectrometry (MS/MS)

The collected supernatants were desalted by Zeta Desult Spin Column (Pierce Biotechnology, Cat no. 89892, Rockford, IL), and concentrated by Amicon Ultra-15 Centrifugal Filter Device (no. UFC 900524, Millipore,

Billerica, MA) with MWCO 5000. Samples were subjected to 10–20% gradient SDS-PAGE, and the gel was cut into nine sections after staining with Coomassie Blue. The sections were reduced, alkylated, and then digested with trypsin (Promega, Madison, WI) overnight at 37°C following the conventional in-gel digestion procedure (Kinter and Sherman, 2002). The efficiency of digestion was confirmed by silver staining, demonstrating a loss of most, if not all, protein bands after digestion, with a corresponding appearance of a smear of low molecular proteins/peptides (data not shown). The digested peptides were desalted with Sep-Pak Vac reverse-phase (RP) C18 cartridge (Waters, Milford, MA), concentrated in SpeedVac (Thermo, Waltham, MA), and resuspended in 0.4% formic acid. Each resulting peptide mixture was separated by strong cation exchange (SCX) and reverse phase (RP) liquid chromatography (LC) before it was analyzed with an electron spray ionization (ESI) mass spectrometer (LCQ DECA PLUS^{XP}; ThermoElectron, San Jose, CA). Settings for the LC-MS/MS were the following: six fractions were eluted from SCX using a binary gradient of 2–90% solvent D (1.0 M ammonium chloride and 0.1% formic acid in 5% acetonitrile) versus solvent C (0.1% formic acid in 5% acetonitrile). Each fraction was then injected onto an RP column automatically with the peptides being resolved using a 300-min binary gradient of 5–80% solvent B (acetonitrile and 0.1% formic acid) versus solvent A (0.1% formic acid in water). A flow rate of 160 μL min^{-1} with a split ratio of 1/80 was used. Peptides were eluted directly into the MS capable of data-dependent acquisition. Each full MS scan was followed by two MS/MS scans of the two most intense peaks in the full MS spectrum with dynamic exclusion enabled to allow detection of less-abundant peptide ions. MS scan events and HPLC solvent gradients were controlled by the Xcalibur software (ThermoElectron).

MS/MS Data Analysis

The proteins in the sample were later identified automatically using the computer program Sequest^{TM}, which searched the MS/MS spectra against the Human International Protein Index (IPI, v3.01) database (*http://www.ebi.ac.uk/IPI/IPIhuman.html*). Search parameters for the SILAC-labeled samples used in this study were the following: +6 Da for ^{13}C isotopic-labeled arginine, +16 Da for oxidized methionine, +57 Da for carbamidomethyl; mass tolerance ±3 Da. Potential peptides and proteins were further analyzed with two computer software programs, PeptideProphet^{TM} and ProteinProphet^{TM} based on statistical models. PeptideProphet uses various SEQUEST scores and a number of other parameters to calculate a probability score for each identified peptide. The peptides were then assigned a protein identification using the ProteinProphet software. ProteinProphet allows filtering of large-scale data sets with assessment of predictable sensitivity and false-positive identification error rates. Typically, when an error rate of 5% is accepted, less than 1% of proteins filtered by ProteinProphet can be found by decoy database search (Jin et al., 2007). In this study, only those proteins or putative proteins that were identified by two or more unique peptides are reported. The ratio between isotopically light (control) and heavy (LPS+IFN-γ treatment) forms of each protein was calculated using the automated statistical analysis of protein abundance (ASAP) ratio program. All of these methods are freely accessible at the website of the Institute for Systems Biology (*http://www.systemsbiology.org/Default.aspx?pagename_proteomicssoftware*).

Bioinformatics Analysis of Proteins Identified

All proteins identified were analyzed by gene ontology (GO) for protein categorization (Camon et al., 2004). Briefly, queries using the International Protein Index (IPI) values were submitted to the EMBL-EBI IPI database using the Bioconductor (Gentleman et al., 2004) software package. Bioconductor commands were used to construct and send dbfetch queries to the IPI database via a URL, for example, http://www.ebi.ac.uk/cgi-bin/dbfetch?db=IPI$id=IPI00298853. These IPI query results were further processed in Bioconductor to construct a master table containing IPIs, Entrez Gene IDs, Gene Symbols, and Gene Descriptions. The Entrez Gene IDs were used in conjunction with the Bioconductor biomaRt package (Durinck et al., 2005) to query the Ensembl BioMart database for the gene ontology IDs (GOIDs) as well as GO categories of the three gene ontologies (Biological Process, Molecular Function, Cellular Component). Because any Entrez Gene ID can occur in more than one GO category, the calculation of the numbers of proteins in selected GO categories, whether a cellular component or a biological process, was done on a priority basis. This means that a gene/protein was removed from the master table after it was classified so that multiple counting of the gene/proteins was avoided.

Secretion of PEP and the Toxic Action of THP-1 Cells

For experiments not involving SILAC, human THP-1 cells were seeded into 24-well plates at a concentration of 4.5×10^5 cells per well in 0.9 mL of DMEM-F12 medium containing 5% FBS without phenol red (Invitrogen). The cells were incubated in the presence or absence of various stimuli for 48 h. The following stimuli were used: IFN-γ alone; LPS alone or in combination with IFN-γ; α-synuclein alone or in combination with IFN-γ; and IL-6 plus TNF-α. Subsequently, 0.1 mL of the cell-free supernatants was transferred to 96-well plates for PEP activity measurements, while 0.4 mL was transferred to each well of 24-well plates containing SH-SY5Y cells. The neuronal cells had been plated 24 h earlier at a concentration of 2×10^5 mL^{-1} in 0.4 mL of DMEM-F12 medium containing 5% FBS. After 72 h of incubation, the neuronal

cell survival was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as described earlier (Klegeris and McGeer, 2005a). PEP enzymatic activity was measured colorimetrically by previously described methods (Hauzer et al., 1981; Makinen et al., 1994) with minor modifications; 100 μL of cell-free supernatants were mixed with an equal volume of PBS (pH 7.5) containing 200 μM PEP substrate *Z*-glycyl-L-proline-4-nitroanilide. Following 20-min incubation at 37°C, optical densities (OD) were measured by using a microplate reader with a 405 nm filter.

Inhibitory Effects of PEP Inhibitors on Toxicity of Supernatants from THP-1 and Human Microglial Cells

For protease inhibitor evaluation, experiments were performed using previously described methods (Klegeris and McGeer, 2005a). THP-1 cells were seeded into 10-cm tissue culture plates at a concentration of 5×10^5 mL^{-1} in 15 mL of DMEM-F12 medium containing 5% FBS, while human microglial cells were seeded into 6-cm plates at a concentration of 6×10^4 mL^{-1} in 4 mL of the same medium. After 1-h incubation, THP-1 cells were stimulated by a combination of LPS (0.5 μg mL^{-1}) plus IFN-γ (150 U mL^{-1}) for 24 h and their supernatants diluted 1.5 times with DMEM-F12 plus 5% FBS. High concentrations of stimuli were used in order to achieve maximal activation of THP-1 cells and to reduce day-to-day variability of supernatant toxicity.

For experiments involving human microglia, cells were first allowed to adhere through incubation at 37°C for 24 h. Subsequently, fresh medium was added to the cells and they were stimulated by a combination of α-synuclein (10 μg mL^{-1}) plus IFN-γ (150 U mL^{-1}). Following 48-h incubation, cell-free supernatants were harvested by centrifugation (275*g* for 10 min). Aliquots of the cytotoxic microglial and THP-1 cell supernatants were stored at −70°C for up to a month.

Human SH-SY5Y neuroblastoma cells were plated 24 h before experimental use at a concentration of 2×10^5 mL^{-1} in 0.4 mL of DMEM-F12 medium containing 5% FBS. Microglial and THP-1 cell supernatants were thawed, heated to 37°C, and 0.4 mL aliquots transferred to each well containing SH-SY5Y cells. PEP inhibitors, or their vehicle solvent dimethyl sulfoxide (DMSO), were added immediately after the transfer of supernatants to the SH-SY5Y cells. Neuronal cell survival was assessed after 72 h of incubation by two independent assays, the MTT and lactate dehydrogenase (LDH) assays as described earlier (Klegeris and McGeer, 2005a). The final concentration of the vehicle solvent in tissue culture medium was 0.5%.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA from THP-1 cells and human microglia was isolated by utilization of the Trizol reagent (Invitrogen), according to the manufacturer's instructions. Reverse transcription and PCR analyses were performed essentially as described before (Klegeris and McGeer, 2000). The PCR primers used to detect PEP (Genbank accession BC030636) were: forward, 5′ GAGCCAAGAGTTT TCCGAGAGGTGA 3′; and reverse, 5′ CCAGGATACC ACCCATGTGTCTCAC 3′. They were designed to produce a specific fragment of 247 bp and were synthesized by Invitrogen. PCR amplification was carried out using AmpliTaq Gold DNA polymerase (Perkin Elmer, Foster City, CA). The amplification program consisted of an initial denaturation step at 94°C, which was extended to 9 min in order to activate AmpliTaq Gold enzyme. This was followed by an annealing step at 55°C for 30 s, and an initial synthesis step at 72°C for 3 min. The remaining cycles were 1 min at 94°C, 30 s at 55°C, and 1 min at 72°C. After amplification, PCR products were separated on a 6% polyacrylamide gel and visualized by ethidium bromide staining. Polaroid photographs of the gels were taken.

Statistical Analysis

Data are presented as means ± SEM. The effects of various inhibitors were evaluated statistically by the randomized blocks ANOVA, followed by Fisher's least significant differences (LSD) test for multiple comparisons. The statistical significance of correlations between supernatant toxicity and PEP enzymatic activity was estimated by calculating Pearson correlation coefficient.

RESULTS

A total of 1,522 proteins were identified by two or more unique peptides through the IPI database. They are listed in alphabetical order as Supplementary Material Appendix I. Interpretation of the results at this early stage is difficult. While it is presumed that the majority were secreted, the abnormal release of intracellular proteins from dead or damaged cells cannot be excluded. Nevertheless, those proteins that are highly upregulated or highly downregulated are candidates for further investigation.

GO analysis revealed their diversity. In unstimulated cells, extracellular and membrane proteins accounted for 7.7% and 17.0%, respectively, of the total (Fig. 1A). Although most proteins can have several GO localizations, it is obvious that there were proteins with annotated intracellular localizations, e.g. mitochondria, endoplasmic reticulum and lysosomes (Figs. 1B,D).

Comparative concentrations in stimulated and unstimulated cell media of 1,294 proteins were possible. Of these, 174 were increased more than twofold while 189 were decreased more than twofold. The full list of these 363 candidates can be found in the Supplementary Material Appendix II.

GO analysis of cellular processes in unstimulated cells indicated that 2.7% and 7.7% of proteins belonged,

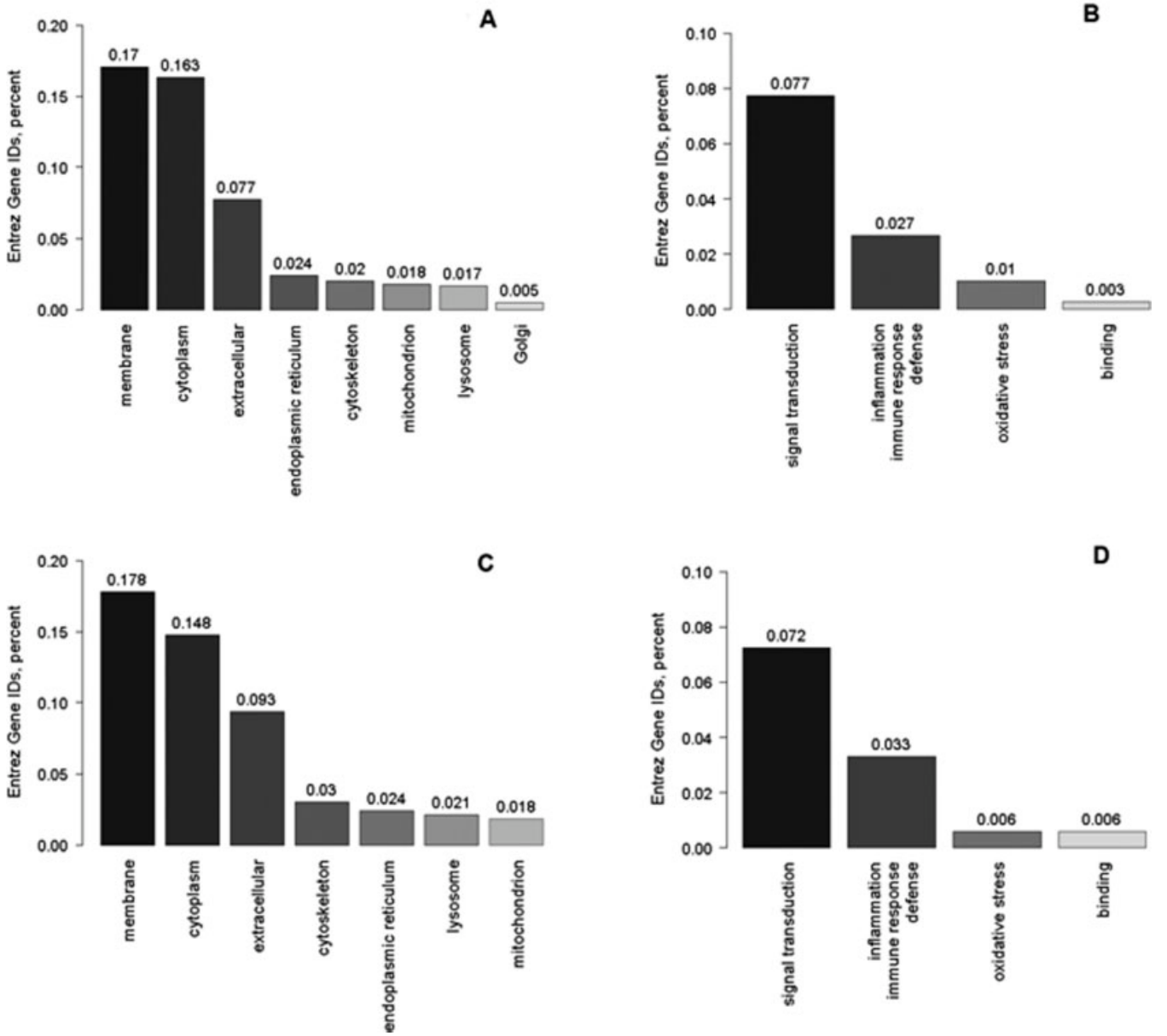

Fig. 1. Gene ontology (GO) analysis of the proteins identified in human THP-1 monocytic cell culture media. Panels (**A**) and (**C**) demonstrate proteins classified as cellular components with (C) being those with relative alterations after LPS + IFN-γ stimulation. Panels (**B**) and (**D**) show proteins classified on the basis of biological processes/cellular functions with (D) being those with relative alterations after LPS + IFN-γ stimulation. The distribution of proteins was identified according to GO analysis and annotations.

respectively, to inflammation and signal transduction categories (Fig. 1B). Other significant processes such as oxidative stress, and protein–protein or protein-small molecule binding, are also shown in Figs. 1B,D. GO analysis of cellular processes after LPS+IFN-γ treatment indicated that proteins corresponding to the extracellular and membrane compartments were 9.3% and 17.8%, respectively (Fig. 1C). Those falling in the categories of inflammation and signal transduction were 3.3% and 7.2%, respectively (Fig. 1D).

Table 1 gives a partial list of proteins, their precursors or fractions that were upregulated at least threefold in stimulated cells. Most were identified by more than four unique peptides. These included PEP, aldolase B, antithrombin III variant, protein kinase C gamma type, vascular cell adhesion protein 1, cathepsin B, collagenase type IV, cathepsin S, heme oxygenase 1, TNF, and cathepsin L.

Table 2 gives a partial list of proteins that were downregulated by twofold or more. These may be of equal interest since suppression of multiple pathways may be necessary for an inflammatory response to be successful. Furthermore, the healing process may require these suppressed pathways to be restored. Some of the prominent proteins included lysozyme C, huntingtin, xanthine dehydrogenase, α-1 antitrypsin, plasminogen, cystatin

TABLE 1. Partial List of Proteins, their Precursors or their Fractions that were Upregulated at Least Threefold in Stimulated Cells (see Supplementary Material Appendix II for the full list)

IPI number	Protein description	ASAP ratio[a]	Number of unique peptides
IPI00218407	Aldolase B	0	4
IPI00032179	Antithrombin III variant	0	5
IPI00009794	Calcium binding protein Cab45 precursor	0	3
IPI00007793	Interleukin-6 precursor	0	2
IPI00007207	Lysosomal acid lipase/cholesteryl ester hydrolase precursor	0	9
IPI00179415	Serine/threonine protein phosphatase 2B catalytic subunit, alpha isoform	0	3
IPI00007694	Splice isoform 1 of protein phosphatase methylesterase 1	0	4
IPI00018136	Splice Isoform 1 of vascular cell adhesion protein 1 precursor	0	4
IPI00007128	Protein kinase C, gamma type	0.05	4
IPI00022314	Superoxide dismutase (Mn), mitochondrial precursor	0.06	10
IPI00295741	Cathepsin B precursor	0.11	45
IPI00295400	Tryptophanyl-tRNA synthetase	0.11	60
IPI00185146	Importin 9	0.12	6
IPI00029260	Monocyte differentiation antigen CD14 precursor	0.12	17
IPI00419237	LAP3 protein	0.14	41
IPI00030781	Splice isoform 1 of Signal transducer and activator of transcription 1-alpha/beta	0.15	30
IPI00291866	Plasma protease C1 inhibitor precursor	0.16	21
IPI00000045	Splice isoform 1 of Interleukin-1 receptor antagonist protein precursor	0.19	7
IPI00292858	Thymidine phosphorylase precursor	0.19	17
IPI00027509	92 kDa type IV collagenase precursor	0.2	10
IPI00023234	Ubiquitin-like 2 activating enzyme E1B	0.21	5
IPI00299150	Cathepsin S precursor	0.24	23
IPI00375631	Interferon alpha-Inducible protein (clone IFI-15K)	0.24	8
IPI00001671	Tumor necrosis factor precursor	0.25	7
IPI00012887	Cathepsin L precursor	0.26	23
IPI00219757	Glutathione S-transferase P	0.26	20
IPI00215893	Heme oxygenase 1	0.26	5
IPI00470455	Splice Isoform 1 of Amyloid beta A4 precursor protein-binding family B member 2	0.26	2
IPI00007853	Gamma-interferon inducible lysosomal thiol reductase precursor	0.29	5
IPI00008164	Prolyl endopeptidase	0.31	14
IPI00004480	ADAM DEC1 precursor	0.31	17

[a]The ratio between isotopically light (unstimulated) and heavy (LPS+IFN-γ stimulated) forms of each protein was calculated using the automated statistical analysis of protein abundance (ASAP) ratio program, which normalizes the dataset internally. Zero indicates that the peptides were not detected in the unstimulated cells.

TABLE 2. Partial List of Proteins, their Precursors or their Fractions that were Downregulated at Least Twofold in Stimulated Cells (see Supplementary Material Appendix II for the full list)

IPI number	Protein description	ASAP ratio[a]	Number of unique peptides
IPI00064162	Deubiquitinating protein VCIP135	999	2
IPI00298853	Vitamin D-binding protein precursor	999	2
IPI00020586	Cytochrome P450 24A1, mitochondrial precursor	68.59	2
IPI00019038	Lysozyme C precursor	8.26	9
IPI00002335	Huntingtin	7.43	5
IPI00244391	Xanthine dehydrogenase	5.83	4
IPI00305457	Alpha-1-antitrypsin precursor	5.27	10
IPI00032220	Angiotensinogen precursor	4.11	32
IPI00022432	Transthyretin precursor	3.93	12
IPI00292657	NADP-dependent leukotriene B4 12-hydroxydehydrogenase	3.72	2
IPI00011285	Calpain 1, large (catalytic) subunit	3.65	9
IPI00019580	Plasminogen precursor	3.54	3
IPI00032293	Cystatin C precursor	3.39	27
IPI00019579	Complement factor D precursor	3.29	29
IPI00216319	Tyrosine 3/Tryptophan 5-monooxygenase activation protein, eta polypeptide	3.09	8
IPI00027230	Endoplasmin precursor	3.03	18
IPI00028064	Cathepsin G precursor	2.95	19
IPI00022462	Transferrin receptor protein 1	2.86	4
IPI00020599	Calreticulin precursor	2.7	6
IPI00023728	Gamma-glutamyl hydrolase precursor	2.27	18
IPI00017603	Coagulation factor VIII precursor	2.13	2
IPI00297284	Insulin-like growth factor binding protein 2 precursor	2.04	6
IPI00032063	Low-density lipoprotein receptor-related protein 1B precursor	2.04	5
IPI00032292	Metalloproteinase inhibitor 1 precursor	2.04	19

[a]See Table 1, "999" indicates that the peptides were not detected in the supernatants from stimulated cells.

C, complement factor D, cathepsin G, calretinin, coagulation factor VIII, insulin growth factor binding protein 2, low density lipoprotein receptor related protein 1b, and metalloproteinase inhibitor 1. The full list of identified proteins can be found in the Supplementary Material Appendix II.

As a first step in evaluating the significance of candidate proteins for neurotoxicity, we focused on PEP, which has already been implicated in the pathogenesis of Alzheimer disease (AD) due to its ability to process neuropeptides (Cunningham and O'Connor, 1997; Rossner et al., 2005). Initially, we investigated whether PEP

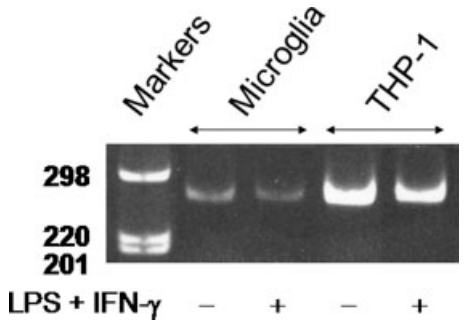

Fig. 2. Human microglia and monocytic THP-1 cells both express PEP mRNA. Polaroid photographs of typical ethidium bromide-stained gels demonstrating PCR products for PEP after 32 amplification cycles. The left lane contains the following size markers (from the top): 298, 220, and 201 base pairs. Three pairs of unstimulated and stimulated THP-1 cells and human microglia were analyzed. Stimulation was with LPS (0.5 μg mL^{-1}) plus IFN-γ (150 U mL^{-1}) for 24 h.

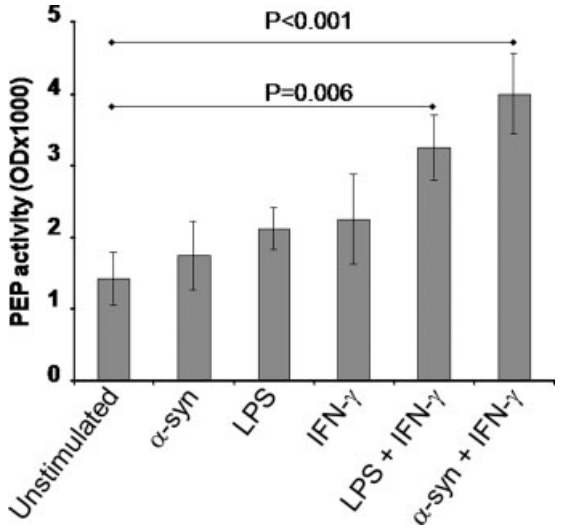

Fig. 3. Different stimuli cause increased PEP enzymatic activity in the supernatants of THP-1 cell cultures. Cells were seeded into 24-well plates at a concentration of 4.5×10^5 cells per well in 0.9 mL of DMEM-F12 medium without phenol red containing 5% FBS. PEP enzymatic activity was measured in the cell-free supernatants after 48-h incubation in the presence or absence of different stimuli (shown on the abscissa) by using a PEP substrate *Z*-glycyl-l-proline-4-nitroanilide. The following stimuli were used (from left to right): no stimulus (PBS only); α-synuclein (10 μg mL^{-1}); LPS (500 ng mL^{-1}); IFN-γ (150 U mL^{-1}); LPS (500 ng mL^{-1}) plus IFN-γ (150 U mL^{-1}); α-synuclein (10 μg mL^{-1}) plus IFN-γ (150 U mL^{-1}). Data (means ± SEM) from four to eight independent experiments are expressed as percent live cells. The *P* values were obtained by Fisher's LSD test for multiple comparisons.

mRNA was expressed by THP-1 cells as well as by primary human microglial cells, and if so, whether the expression levels were modulated by stimulation with a combination of LPS and IFN-γ. The results indicated that the mRNA is significantly expressed in both THP-1 cells and microglia, but the levels, if anything, appear to be mildly depleted after 24 h of stimulation (see Fig. 2).

Next, we confirmed that there was an increased extracellular concentration of PEP in stimulated cells by measuring its enzymatic activity. Figure 3 shows upregulation of PEP under a variety of stimulation conditions. LPS, IFN-γ or α-synuclein alone did not upregulate PEP enzymatic activity. However, combinations of LPS plus IFN-γ or α-synuclein plus IFN-γ increased PEP activity. When the SILAC activation paradigm of 0.5 μg mL^{-1} of LPS and 150 U mL^{-1} of IFN-γ was used, the activity was increased 2.3-fold (1.43 in unstimulated vs. 3.25 in stimulated cells). This enzymatic increase corresponded reasonably well with the 3.2-fold PEP protein increase estimated by SILAC (see Table 1).

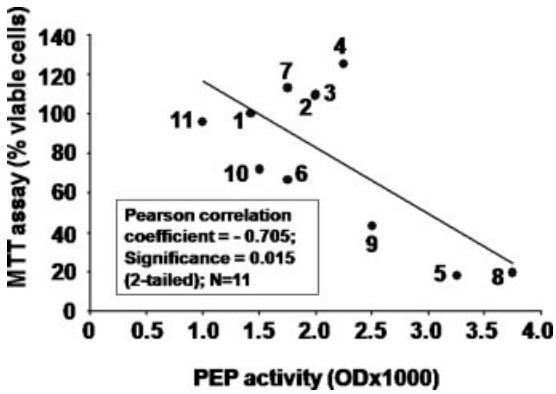

Fig. 4. A statistically significant correlation was observed between THP-1 cell supernatant toxicity towards SH-SY5Y cells and PEP activity in the same supernatants. Cells were seeded into 24-well plates at a concentration of 4.5×10^5 cells per well in 0.9 mL of DMEM-F12 medium without phenol red containing 5% FBS. PEP enzymatic activity was measured in the cell-free supernatants after 48-h incubation in the presence or absence of different stimuli (shown on the abscissa). The same supernatants were analyzed for their toxicity by transferring them into wells containing SH-SY5Y neuroblastoma cells and measuring their viability 72 h later by the MTT assay (shown on the ordinate). The following stimuli were used: 1, control (PBS only); 2, LPS (500 ng mL^{-1}); 3, LPS (50 ng mL^{-1}); 4, IFN-γ (150 U mL^{-1}); 5, LPS (500 ng mL^{-1}) plus IFN-γ (150 U mL^{-1}); 6, LPS (50 ng mL^{-1}) plus IFN-γ (15 U mL^{-1}); 7, α-synuclein (10 μg mL^{-1}); 8, α-synuclein (10 μg mL^{-1}) plus IFN-γ (150 U mL^{-1}); 9, α-synuclein (1 μg mL^{-1}) plus IFN-γ (15 U mL^{-1}); 10, IL-6 (10 ng mL^{-1}) plus TNF-α (10 ng mL^{-1}); 11, IL-6 (1 ng mL^{-1}) plus TNF-α (1 ng mL^{-1}). Data from four independent experiments are shown. The statistical significance was estimated by calculating Pearson correlation coefficient.

In the next series of experiments, we selected several different stimuli for THP-1 cells and showed that there was a statistically significant correlation between supernatant toxicity towards SH-SY5Y cells and PEP activity in those supernatants (see Fig. 4). LPS, IFN-γ or α-synuclein alone, or the combination of IL-6 plus TNF-α, had little effect on PEP enzyme activity or cell toxicity by the MTT assay. However, the combinations of LPS plus IFN-γ or α-synuclein plus IFN-γ increased PEP activity and decreased cell survival in a concentration-dependent manner. The selection of different stimuli and the concentrations used (see Fig. 4 legend) were based on our previous studies of neurotoxicity associated with such stimuli (Klegeris et al., 2005, 2006).

Finally, we performed pharmacological studies showing that two specific PEP inhibitors partially reduced the toxicity of supernatants from LPS plus IFN-γ-stimulated THP-1 cells (see Fig. 5) and from α-synuclein plus IFN-γ-stimulated human microglial cells (see Fig. 6). The data were obtained by mixing toxic supernatants with various concentrations of the inhibitors at the time the supernatants were transferred to the wells contain-

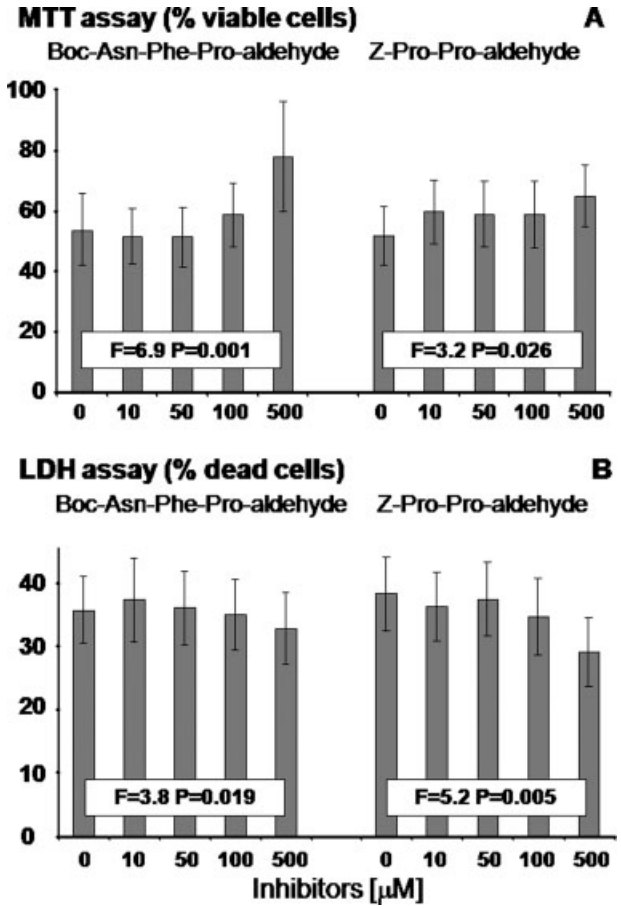

Fig. 5. Two selective inhibitors of PEP significantly reduce toxicity of THP-1 cell supernatants. SH-SY5Y cells were incubated in cell-free supernatants from human monocytic THP-1 cells that had been stimulated with LPS (500 ng mL^{-1}) plus IFN-γ (150 U mL^{-1}). Various concentrations of PEP inhibitors (shown on the abscissa) were added to the supernatants at the time of their transfer to SH-SY5Y cells. Viability of SH-SY5Y cells was assessed after 72 h by the MTT assay (**A**) or the LDH assay (**B**). Data from six to eight experiments are expressed as means ± SEM. The concentration-dependent effects of the inhibitors were evaluated by the randomized blocks design ANOVA. *F* and *P* values are shown in boxes.

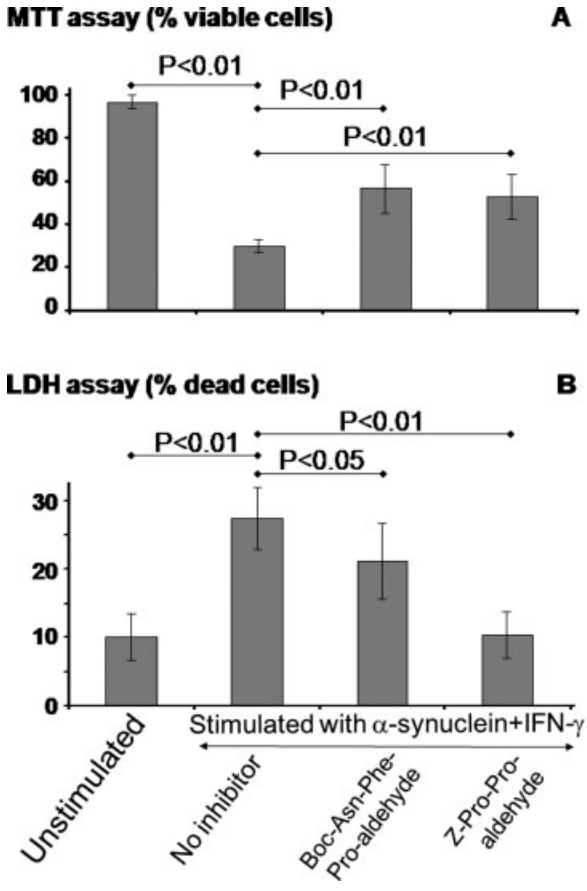

Fig. 6. The selective inhibitors of PEP significantly reduce toxicity of human microglial supernatants. SH-SY5Y cells were incubated in cell-free supernatants from unstimulated human microglia or microglia that had been stimulated with α-synuclein (10 μg mL^{-1}) plus IFN-γ (150 U mL^{-1}) for 48 h. PEP inhibitors (500 μM) or their solvent DMSO (No inhibitor) were added to the supernatants at the time of their transfer to SH-SY5Y cells. Viability of SH-SY5Y cells was assessed after 72 h by the MTT assay (**A**) or the LDH assay (**B**). Data (means ± SEM, $N = 3$) were collected by using microglia from two independent cases. The effects of the inhibitors were evaluated by the randomized blocks design ANOVA, followed by Fisher's LSD test for multiple comparisons.

ing SH-SY5Y cells. Furthermore, these inhibitors showed similar blocking activity when added to THP-1 cells before their stimulation, which was followed by transfer of supernatants 24 h later (data not shown). They also failed to inhibit TNF-α secretion by LPS plus IFN-γ-stimulated THP-1 cells, which was measured according to the previously published protocol (Klegeris et al., in press) (data not shown). This established that, at the concentrations used, neither of the inhibitors was toxic to THP-1 cells.

DISCUSSION

The primary goal of this study was to identify proteins that could contribute to human microglial neurotoxicity, including both upregulated and downregulated products. It was necessary to use THP-1 cells as a surrogate model for microglia in order to harvest sufficient material for SILAC analysis. This approach permitted us to quantify changes in the protein composition of monocytic THP-1 cell supernatants in response to stimulation with a combination of two powerful inflammatory mediators, LPS and IFN-γ. Supernatants of human THP-1 cells as well as microglia stimulated in this manner are known to be toxic to neuronal cells (Klegeris et al., 2005). Such neurotoxicity was confirmed in this study under the cell culture conditions used for the SILAC experiments (data not shown).

There have been several studies of the proteome of macrophages or other cell types. Gronborg et al. (2006) demonstrated the usefulness of the SILAC technique by distinguishing secreted proteins of pancreatic cancer-derived cells from those of non-neoplastic pancreatic ductal cells. They identified 145 differentially secreted proteins, several of which had not previously been associated with pancreatic cancer. Less sensitive techniques have been used to explore the monocyte-macrophage

secretome. Dupont et al. (2004) and Rakkola et al. (2007) studied by two dimensional gel electrophoresis the secreted and intracellular proteins from cultured monocyte-macrophages. Cibrowski et al. (2007) studied by one-dimensional electrophoresis and liquid chromatography tandem MS the secretome of HIV-1 infected human monocyte-derived macrophages. Many proteins of potential interest have been identified in these previous studies although the numbers were considerably fewer than have been identified by the SILAC technique used in this study.

A significant number of identified proteins belong to intracellular organelles. While such proteins may have been derived from dying cells, it is possible that either they, or their breakdown products, may be actively secreted. One good example is α-synuclein, a protein long believed to be an intracellular protein has now been demonstrated to be actively secreted into the extracellular space (Lee et al., 2005).

It cannot be overemphasized that proteomics analysis provides only a potential list of proteins that might be important in a process or in a cellular location. Moreover, some of the proteins could be misidentified due to deficiencies in the available databases. Consequently, all candidate proteins need to be validated before their biology is pursued.

Ratios for more than 1,200 of the identified proteins were determined; there were several whose upregulation would be expected after inflammatory stimulation. These included IL-6, complement proteins C5 and C3, cathepsin B, and TNF-α. Cathepsins B, S, and L were increased more than threefold, cathepsins H, Z, and D showed smaller increases, while cathepsin G was downregulated approximately threefold (see Tables 1 and 2 and Supplementary Material Appendix II). Genetic studies indicate that cathepsins B, D, and G could be involved in AD pathogenesis (Serretti et al., 2005). MMP-14 was elevated fivefold. Several matrix metalloproteinases including MMP-2, 3, and 9 have been shown to contribute to microglial toxicity (Jourquin et al., 2003; Kim et al., 2006; Wang and Tsirka, 2005; Zhang et al., 2003). There was downregulation of several protease inhibitors including metalloproteinase inhibitor 2 and α-1-antitrypsin precursor which were downregulated approximately fivefold. Because of the possible misidentification of proteins, the above observations need to be confirmed using alternate techniques and on samples collected from several experiments.

We selected PEP to demonstrate that approach. Measurements of PEP enzymatic activity in more than four independent experiments showed that stimulation of THP-1 cells with a combination of LPS and IFN-γ caused a 2.27-fold increase in PEP activity, which was in reasonable agreement with the 3.2-fold increase in protein concentration by SILAC evaluation.

PEP (EC 3.4.21.26), also known as prolyl oligopeptidase and post-proline cleaving enzyme, is a serine protease, which cleaves peptide bonds at the C-terminal side of proline residues (Cunningham and O'Connor, 1997; Gass and Khosla, 2007). It is found in blood, brain, and CSF (Momeni et al., 2003). It is speculated to be involved in activation of cell-mediated immunity, autoimmune, and inflammatory responses (Maes et al., 1994) with proposed connections to neurodegenerative processes occurring in Alzheimer's, Huntington's, and Parkinson's diseases (Mannisto et al., 2007). PEP is distributed widely in mammalian tissues and expressed at high levels in the brain (Bellemere et al., 2004; Irazusta et al., 2002). It is believed to act both extracellularly, where it is involved in the maturation and degradation of peptide hormones and neuropeptides, including substance P and neurotensin (Bellemere et al., 2004; Cunningham and O'Connor, 1997), and intracellularly where it may be part of signaling pathways or contribute to transport and secretion of proteins and peptides including Alzheimer amyloid beta (Aβ) protein (Rossner et al., 2005; Schulz et al., 2002, 2005). It has been speculated that PEP may play a role in the pathogenesis of AD based on studies showing abnormal expression of this enzyme in brain samples from AD patients and in transgenic mice overexpressing Alzheimer amyloid precursor protein (APP) (Cunningham and O'Connor, 1997; Laitinen et al., 2001; Rossner et al., 2005). PEP inhibitors have also been shown to have neuroprotective and memory-enhancing effects that have been attributed to their ability to modify neuropeptide metabolism (Cunningham and O'Connor, 1997; Makinen et al., 1994). PEP may be a key enzyme in the production of Aβ by neurons since a specific PEP inhibitor is reported to reduce Aβ secretion from the NG108-15 neuronal cell line (Shinoda et al., 1997). Taken together with our findings, these overall results strongly suggest that PEP activity be further investigated in a spectrum of degenerative neurological disorders.

We observed that there was a strong correlation between the PEP enzymatic activity in supernatants harvested under various stimulatory conditions and their toxicity towards SH-SY5Y neuroblastoma cells (see Fig. 4). This indirect evidence of possible PEP involvement in THP-1 cell toxicity was supported in a series of experiments, which showed that two specific PEP inhibitors in a concentration-dependent manner reduced toxicity of supernatants harvested from LPS plus IFN-γ-stimulated THP-1 cells (see Fig. 5) and from α-synuclein plus IFN-γ-stimulated human microglial cells (see Fig. 6). Because these inhibitors were only partially protective, it is evident that PEP represents only one component of a mixture of toxins responsible for the observed THP-1 cell toxicity.

PEP activity has been believed to act on oligopeptides of less than 3 kDa. However, recent data indicate that certain PEPs are capable of digesting 33-mer peptides and even cleaving intact proteins (see Gass and Khosla, 2007). Therefore, the substrate specificity and chain length restrictions for human monocytic cell PEP need to be investigated further. It is also possible that PEP is acting downstream to other proteases that make initial cuts in larger proteins.

In summary, our study has shown that SILAC methodology can be used to explore changes in the protein

composition of THP-1 monocytic cell cultures, thus adding to previous studies on the proteome of monocyte-derived cells (McLaughlin et al., 2006; Zhou et al., 2005). Stimulation of THP-1 cells caused significant upregulation and downregulation of several well-known proteolytic enzymes. All of these are candidates for further study. We validated data for only one such enzyme, PEP, which has already been implicated in regulation of cellular death in several *in vitro* and *in vivo* paradigms. Use of specific inhibitors indicated that PEP released from stimulated monocytic cells or microglia may contribute to the neurotoxic actions of these cells. PEP has been implicated in many physiological processes including psychiatric disorders, and while evidence of the precise physiological role is lacking, intense research is taking place on its pharmacology (reviewed by Garcia-Horsman et al., 2007). Although the mechanism of action is so far unknown, our data indicate that further *in vivo* testing of PEP inhibitors in animal models relevant to microglial activation may yield fruitful results.

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