行政院國家科學委員會補助專題研究計畫成果報告

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Proteomic analysis of lipopolysaccharide-induced apoptosis in PC12 cells

We employed rat pheochromocytoma PC12 cells as our model system to identify cellular proteins that accompany Escherichia coli lipopolysaccharide (LPS)-induced apoptosis, based on a proteomic approach. Cell viability tests revealed that naïve PC12 cells underwent cell death in a dose-dependent manner after treatment with LPS. Flow cytometric analysis confirmed that apoptosis was primarily responsible for the observed cell death. Two-dimensional electrophoresis in conjunction with N-terminal sequencing, immunoblot, matrix-assisted laser desorption/ionization-time of flight analysis or computer matching with protein databases further revealed that the LPS-induced apoptosis is accompanied by an augmented level of calreticulin, calcium binding protein 50, endoplasmic reticulum protein 60 (ERP60), heat shock protein 60 (HSP60) or HSP90, and a reduced level of amphoterin, c oxidase polypeptide Va-liver or ERP29. These proteins are associated with endoplasmic reticulum, mitochondria or cell membrane, and are with known or potential roles in apoptosis. Their identification therefore provides an impetus for further delineation of the cellular and molecular basis of apoptotic cell death and sepsis based on proteomic profiling of PC12 cells.

Keywords: Apoptosis / Lipopolysaccharide / PC12 cells / Proteome map / Two-dimensional gel electrophoresis

1 Introduction

PC12 cell is an established cell line that is derived from rat pheochromocytoma [1]. It has been used extensively as an in vitro model system to study neuronal cell fate, including survival, proliferation, differentiation, and apoptosis [1–3]. Many important signaling pathways or molecular regulators involved in PC12 cell fate have been established in recent years [4–9], based on techniques in molecular biology, immunology or cell biology. However, a sizable number of crucial molecules that may be engaged in the regulation of physiological or pathological responses of PC12 cells, which in turn control their cell fate, remain to be identified.

Sepsis is a detrimental clinical phenomenon that is associated with profound cardiovascular abnormalities and high mortality rate [10]. It is a severe systemic inflammatory response triggered by an interaction between lipopolysaccharide (LPS), the major structural component of the outer leaflet of Gram-negative bacteria, and macrophages or other host cells [11,12]. The most often mentioned underlying mechanism stipulates the progressive release of a variety of mediators, including proinflammatory cytokines such as interleukin 8 (IL8), IL1-β and IL6 [13–16], nitric oxide [NO; 10, 17–19] and tumor necrosis factors [TNF; 10, 17, 18].

Activation of NO [5, 20–22] or TNF-α [22] by LPS also induces apoptosis. Considering that sepsis is an important clinical phenomenon that inevitably engages cell death and the biological responses to LPS are remarkably complex [15], it is reasonable to propose that many more mediators, particularly cellular proteins, should accompany LPS-induced apoptosis. This proposal was evaluated in the present study based on a proteomic analysis of protein profiles during LPS-induced apoptosis in PC12 cells. Our initial results indicated that apoptotic cell death of PC12 cells was accompanied by both augmented and reduced levels of proteins that are present in the cell membrane, mitochondria or endoplasmic reticulum, and are with known or potential roles in apoptosis.

2 Materials and methods

2.1 Materials

PC12 cells were obtained from the Culture Collection and Research Center, Food Industry Research and Development Institute (Hsinchu, Taiwan, Republic of China). DMEM, horse serum, and bovine serum were purchased...
from Gibco (Grand Island, NY, USA), Escherichia coli LPS (serotype 0111:B4), DTT, CHAPS, propidium iodide staining solution, Triton X-100 and 3-[4,5-dimethylthiazol-2,5]-diphenyl tetrazolium bromide (MTT) were obtained from Sigma (St. Louis, MO, USA), Coomassie Brilliant Blue R-250 was supplied by Bio-Rad (Richmond, CA, USA). Sodium citrate was obtained from Serva (San Diego, CA, USA), annexin V-FITC apoptosis detection kit II from Pharmingen (San Diego, CA, USA), and antisera from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other chemicals were purchased from Merck (Rahway, NJ, USA).

2.2 Cell culture

PC12 cells were cultured in DMEM containing 10% horse serum and 5% fetal bovine serum and were incubated in an atmosphere containing 10% CO2 at 37°C. The medium was changed every 2–3 days. To avoid the effect of cell density on protein synthesis [23], cells were seeded at a density of 2 x 10^6 cells per poly-L-lysine-coated 150 mm diameter polystyrene dish. Protein concentration was determined as described by Bradford [24].

2.3 Cell viability test

The viability of PC12 cells was measured using the MTT assay [25]. Cells were incubated for 4 h at 37°C in 100 μL of fresh culture medium and 10 μL of MTT (0.5 mg/mL). After removal of the MTT solution, the formazan crystals were dissolved by adding 100 μL of 10% SDS in 0.01 M HCl. The concentration of the formazan product was determined spectrophotometrically at an absorbance wavelength between 570 and 630 nm.

2.4 Flow cytometric analysis

PC12 cells (10^6) were washed in Dulbecco’s PBS and resuspended in binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 0.25 mM CaCl2) with annexin V-FITC and 2.5 μg/mL propidium iodide. After incubation at room temperature for 15 min in the dark, the fluorescence emitted by cells was analyzed with a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). Cells with phosphatidylserine residues on the outer leaflet of the cell membrane and without loss of cell integrity (PS-/PI+) were considered to be apoptotic cells, and PS+/PI- and PS+/PI+ were taken respectively as necrotic and viable cells [26].

2.5 Sample preparation for proteomic analysis

The protein samples were concentrated by precipitation in 10% trichloroacetic acid (TCA) and 0.1% DTT overnight at -20°C, and subsequently centrifuged at 15,000 rpm for 30 min at 4°C [27]. The pellet was air-dried for a few minutes after being washed twice with ice-cold acetone. The dry precipitated sample (500 μg) was reconstituted with 250 μL of rehydration solution (8 M urea, 2% CHAPS, 0.5% IPG buffer, 15 mM DTT and trace bromophenol blue).

2.6 Two-dimensional gel electrophoresis

In the first dimension, IEF was carried out with commercially available immobilized pH gradients (linear pH gradient 3–10, 13 cm in length). The gels were rehydrated overnight by placing the strips gel side down in an IPGphor strip holder (Amersham Pharmacia Biotech, Uppsala, Sweden), with the rehydration solution covered by mineral oil. IEF was carried out using an IPGphor™ isolectric focusing system (Amersham Pharmacia Biotech) at 20°C. A three-phase program was used for both analytical and preparative gels. The first phase was set at 500 V for 1 h, and the second at 1000 V for 1 h. The final phase was a linear gradient spanning from 8000 V to 16,000 V for 2 h. After the IEF run, the IPG gel strips were kept at ~80°C or prepared directly for second-dimensional electrophoresis.

Prior to second-dimensional electrophoresis, the IPG gel strips were incubated at room temperature for 15 min in an equilibration solution (50 mM Tris–HCl, pH 8.8, 6 M urea, 2% SDS, 30% glycerol) containing 1% DTT. This was followed by incubation for 5 min in the same equilibration solution containing 2.5% iodoacetamide. The gels were subsequently subject to a second dimensional run [28], using a Hoefer™ SE 600 (Amersham Pharmacia Biotech) at 4°C on running SDS-PAGE gels (15 x 15 cm) without stacking. The IPG gel strips were embedded on top of the gels with 1% agarose. Electrophoresis was carried out at 30 mA/gel for 5 h until the bromophenol blue reached the bottom of the gel. The 2-DE gels were stained either with silver nitrate [29] or overnight with Coomassie blue.

The silver stained 2-DE gels were scanned in a Molecular Dynamics (Sunnyvale, CA, USA) densitometer and the images were processed using Adobe Photoshop and PowerPoint software. Protein spots were quantified and numbered using ImageMaster 2D Elite software (Amersham Pharmacia Biotech) and checked manually to eliminate artifacts due to gel distortion, abnormal silver staining or poorly detectable spots. The protein level of each
spot is expressed as a percentage of total spot volume in the 2-DE gel. Protein levels with an increase or decrease > 40% over untreated controls after LPS treatment were considered as substantial changes.

2.7 N-terminal amino acid sequencing

Proteins were electroblotted onto PVDF membranes using a Multiphor II semidry blotter (Amersham Pharmacia Biotec) in conjunction with transfer buffer (10 mM CHAPS, 10% methanol) for 1.5 h at 24 V. The PVDF membrane-bound proteins were visualized by staining with 0.1% Coomassie Brilliant Blue R-250 in 50% aqueous methanol for 5 min, and destained in 40% methanol and 10% acetic acid. Membranes were dried at 37°C and stored at -20°C until further analysis. Selected protein spots were excised using pipette tips of various sizes and were subjected to amino acid sequencing by Edman degradation, using an automatic protein/peptide sequencer (Applied Biosystems 492, Foster City, CA, USA).

2.8 Immunoblotting

Immunoblotting was used to either confirm the results from N-terminal amino acid analysis, or to identify new proteins. PVDF membrane-bound proteins were detected by Western blot analysis using specific antisera. Proteins were visualized by alkaline phosphatase-linked reaction. The antisera used in this study included goat anti-HSP60 (SC-1052) and rabbit anti-HSP90 (SC-7947) antisera from Santa Cruz Biotechnology.

2.9 In-gel digestion

To further identify protein spots that were otherwise undetectable by either N-terminal amino acid analysis or immunoblotting, in-gel digestion was performed by modifying the procedures described by Kaji et al. [30]. Protein spots excised from the Coomassie blue stained gel were destained in microtubes with 0.2 mL acetonitrile for 15 min and dried completely in a centrifugal evaporator. The dried gels were rehydrated on ice for 45 min with a digestion buffer composed of 0.04 mg/mL of modified trypsin (Promega, Madison, WI, USA) in 0.005% zwittergent 3–16 and 50 mM NH4HCO3. After removing excess solution, proteins were further digested at 37°C for 15 h. The resultant peptides were extracted with 5% acetonitrile in 5% formic acid, desalted and concentrated using in-tip reversed-phase resin (ZipTip C18: Millipore, Bedford, MA, USA).

2.10 MALDI-TOF MS

Peptide mixtures were eluted from the ZipTip with 75% acetonitrile in 0.1% TFA, applied to the sample target and air-dried. After mixing with the matrix (a-cyano-4-hydroxycinnamic acid dissolved in 50% acetonitrile, 0.1% TFA), the sample was analyzed in a biflex III MALDI-TOF mass spectrometer system (Bruker-Franzen Analytik, Bremen, Germany). The peptide masses were measured as monoisotopic masses. To identify proteins, the measured monoisotopic masses of peptide were analyzed using WWW search programs MS-Fit provided by UCSF (http://prospector.ucsf.edu/ucsfhtml3.2/msfit.htm) with NCBI database.

2.11 Computer search for protein identity or similarity

Search for similarity of amino acid sequence was performed using FASTA, BLAST, and TFASTA computer programs of the Genetics Computer Group (Madison, WI, USA) to screen protein databases [31]. The analysis software TagIdent, available from the ExPASy internet server at http://expasy.hcuge.ch/ch2d/pi_tool.html was also used to identify sequenced proteins and to calculate theoretical pI and Mr [32]. The observed pI and Mr were determined by carbamyllyte pI calibration markers and molecular weight markers available from ImageMaster 2D Elite software, after aligning the 2-DE gel with known protein spots.

3 Results

3.1 LPS decreases viability of PC12 cells

Incubation for 24 h with LPS at concentrations ranging from 10-2-10-6 ng/mL resulted in a dose-related reduction in cell viability of naïve PC12 cells cultured in DMEM supplemented with 10% horse serum and 5% fetal bovine serum (Fig. 1A). PC12 cells became highly aggregated and detached (Fig. 1B) after treatment with LPS at 10-6 ng/mL for 24 h.

3.2 LPS induces apoptosis in PC12 cells

We next analyzed the nature of LPS-induced cell death by subjecting the detached PC12 cells to flow cytometric analysis (Fig. 2). That early stage of apoptosis has taken place was indicated when 46% of the cells became positively stained with annexin V-FITC 24 h after LPS treatment at 10-6 ng/mL. The minute number (< 1%) of cells that were double-labeled with annexin V and propidium iodide suggested minimal engagement of necrosis.
### 3.3 Changes in protein profile of PC12 cells during LPS-induced apoptosis

Figure 3 shows that a total of 532 protein spots were visualized in the silver stained 2-DE reference map of cellular proteins in PC12 cells at the pH range of 3–10. Intriguingly, many of these protein spots underwent an increase or decrease in intensity by over 20% after the induction of apoptosis by LPS. We have thus far identified 13 of these protein spots. N-terminal sequencing first identified six of them to be amphoterin, calcium binding protein 50 (CBP-50), cytochrome c oxidase polypeptide Vla-liver, endoplasmic reticulum protein 29 (ERP29), ERP60 and heat shock protein 60 (HSP60). Immunoblotting further identified an additional five protein spots as HSP60, and another as a group of HSP90 isoforms with observed pI between 5.21 and 5.76. The last spot was identified by MALDI-TOF analysis, which revealed that the tryptic peptide mass fingerprint with 16 peptide masses (Fig. 4A) matched with that of calreticulin (Fig. 4B, C). The relevant characteristics of these 13 protein spots are given in Table 1. Comparison of the proteome maps for control samples and PC12 cells treated with LPS at $10^6$ ng/mL for 24 h (Fig. 5, Table 2) revealed that whereas calreticulin, CBP-50, ERP60, HSP60 and HSP90 manifested an augmented intensity, amphoterin, cytochrome c oxidase polypeptide Vla-liver and ERP29 underwent a reduction.

### 4 Discussion

Heneka et al. [5] reported recently that exposure of nerve growth factor differentiated PC12 cells to TNF-α and LPS resulted in apoptosis that is mediated by NO. The present study further demonstrated that documented apoptotic cell death can also be induced in naïve PC12 cells, and LPS alone is efficacious. Our proteomic analysis additionally revealed that this LPS-induced apoptosis is accompanied by an augmented level of calreticulin, CBP-50, ERP60, HSP60 or HSP90 and a reduced level of amphoterin, cytochrome c oxidase polypeptide Vla-liver or ERP29. These proteins are associated with endoplasmic reticulum, mitochondria or cell membrane. Intriguingly, amphoterin, CBP-50, ERP29 and ERP60 have not hitherto been reported to be expressed in PC12 cells, despite their relative abundance in the 2-DE map.
4.1 Proteins with augmented intensity during LPS-induced apoptosis

The endoplasmic reticulum is the major intracellular Ca^{2+} storage site [33, 34], and Ca^{2+} binding proteins are responsible for the regulation of Ca^{2+} accumulation and release [35–37]. Both calreticulin (also known as calregulin) and CBP-50 are Ca^{2+} binding proteins and are mainly located at the periphery of the endoplasmic membrane [38]. They belong to the CREC family of proteins [39, 40], which derived its acronym from cab-45, reticulocalbin, ERC-45 and calumenin. Although the functional proper-
ties of these proteins are largely unknown, they consist of a number of EF-hand proteins that exert low affinity Ca\(^{2+}\) binding activities [41]. Prior stress of the endoplasmic reticulum induces expression of calreticulin that renders cells resistant to cell death caused by oxidants [42]. It follows that the increased level of calreticulin seen during LPS-induced apoptosis may reflect a compensatory mechanism that confers protection on PC12 cells by rendering better Ca\(^{2+}\) buffering or decreased Ca\(^{2+}\) release. As another endoplasmic Ca\(^{2+}\) binding protein, CBP-50 was previously purified from the synaptic membrane fraction of guinea pig brain [43]. It is likely that CBP-50 shares the same protective mechanism as calreticulin.

ERP60 is distributed within the lumen of endoplasmic reticulum, and is a member of the stress-response machinery of the endoplasmic reticulum [44, 45]. It is an isofrom of protein disulfite isomerase that has two thiodixin-like domains [46]. Originally thought to be a phosphoinositide-specific phospholipase c isozyme [47], it was later reported to be a cysteine protease [48]. Functionally, it plays a role in the degradation of misfolded proteins in endoplasmatic reticulum [48]. The up-regulation of ERP60 may therefore represent a regulatory mechanism for the elimination of abnormal or misfolded proteins in the endoplasmic reticulum of LPS-treated PC12 cells.

HSP60 is located in the mitochondrial matrix. It can interact with p21 ras [49] and may prevent misfolding and promote refolding or proper assembly of unfolded polypeptides generated under stress conditions [50]. Samali et al. [51] demonstrated in Jurkat cells that induction of apoptosis activates mitochondrial procaspase-3 and its dissociation from HSP60. This chaperon protein may in turn accelerate activation of caspase-3 by cytochrome c, the hallmark event of apoptosis. HSP60 also stimulates macrophages to express IL-12 and IL-15, and rapidly releases TNF-\(\alpha\) [52]. Overexpression of HSP90 increases the rate of apoptosis in the monoblastoid cell line U937 after treatment with TNF-\(\alpha\) and cycloheximide [53]. These events are therefore consistent with our observed increase in HSP60 or HSP90 during LPS-induced apoptosis in PC12 cells.

Figure 4. Identification of calreticulin by MALDI-TOF analysis. Tryptic peptide spectrum (A) of calreticulin contains sixteen matched peptides with observed \(m/z\) values and tryptic cutting sites. The amino acid sequence of all matched peptides in calreticulin are listed in (B), or underlined in the internal sequence (C).
## Table 1. Proteins that changed their expression levels in LPS induced apoptotic PC12 cells

<table>
<thead>
<tr>
<th>Spot no.</th>
<th>kDa</th>
<th>pI</th>
<th>N-terminal sequence</th>
<th>Gene name</th>
<th>SWISS-PROT accession no.</th>
<th>Protein description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>24.72</td>
<td>28.73</td>
<td>5.62 6.58</td>
<td>GKGDPKR</td>
<td>HMG1 P07155</td>
<td>Amphoterin*</td>
</tr>
<tr>
<td>2</td>
<td>48.00</td>
<td>56.26</td>
<td>4.33 4.12</td>
<td>CALR</td>
<td>P18418</td>
<td>Calcitulin</td>
</tr>
<tr>
<td>3</td>
<td>37.00</td>
<td>55.00</td>
<td>4.40 4.12</td>
<td>KPTKKDRV</td>
<td>None O35783</td>
<td>CBP-50*</td>
</tr>
<tr>
<td>4</td>
<td>12.30</td>
<td>10.69</td>
<td>9.30 7.30</td>
<td>SGAGHGEES</td>
<td>COX8A1 P10818</td>
<td>Cytochrome C oxidase polyepptide Via-liver</td>
</tr>
<tr>
<td>5</td>
<td>28.57</td>
<td>31.03</td>
<td>6.23 6.58</td>
<td>LHTKCALPD</td>
<td>ERP29 P52555</td>
<td>ERP29*</td>
</tr>
<tr>
<td>6</td>
<td>56.60</td>
<td>59.90</td>
<td>5.88 6.29</td>
<td>SDVLETDEN</td>
<td>None P11598</td>
<td>ERP60*</td>
</tr>
<tr>
<td>7</td>
<td>60.96</td>
<td>61.86</td>
<td>5.30 5.30</td>
<td>-</td>
<td>HSP60 P19227</td>
<td>HSP60</td>
</tr>
<tr>
<td>8</td>
<td>60.96</td>
<td>62.06</td>
<td>5.30 5.38</td>
<td>-</td>
<td>HSP60 P19227</td>
<td>HSP60</td>
</tr>
<tr>
<td>9</td>
<td>60.96</td>
<td>62.06</td>
<td>5.30 5.47</td>
<td>-</td>
<td>HSP60 P19227</td>
<td>HSP60</td>
</tr>
<tr>
<td>10</td>
<td>60.96</td>
<td>61.66</td>
<td>5.30 5.57</td>
<td>-</td>
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<td>HSP60</td>
</tr>
<tr>
<td>11</td>
<td>60.96</td>
<td>61.46</td>
<td>5.30 5.67</td>
<td>AKDVKGADARMLQ</td>
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<td>HSP60</td>
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<tr>
<td>12</td>
<td>60.96</td>
<td>61.46</td>
<td>5.30 5.76</td>
<td>-</td>
<td>HSP60 P19227</td>
<td>HSP60</td>
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<tr>
<td>13</td>
<td>83.19</td>
<td>90.21</td>
<td>5.06 5.21-5.76</td>
<td>-</td>
<td>HSP90 P34058</td>
<td>HSP90</td>
</tr>
</tbody>
</table>

N-terminal sequences are shown using the single letter for amino acid residues. Theoretical (Theor.) pI and M values were calculated using the "ExPASy Compute pI/Mw tool" at http://expasy.hcuge.ch/ch2d/pi_tool.html. Observed values (Observ.) for pI and M, were calculated using the ImageMaster 2D Elite software. + indicates novel proteins found expressed in PC12 cells.

## 4.2 Proteins with reduced intensity during LPS-induced apoptosis

Amphoterin is a membrane-bound receptor protein and is a developmentally regulated heparin-binding protein that is abundant in embryonic brain. It is localized in a diffused manner in stationary cells but becomes clearly localized to growth cones in neuronal cells [54, 55]. Amphoterin is also found to be the endogenously occurring ligand that binds to the extracellular moiety of RAGE, the receptor for advanced glycation end products [56]. The neurite outgrowth and cell survival are coregulated by amphoterin via RAGE-amphoterin interaction [57]. These findings imply that amphoterin may play a role in regulating neurite outgrowth and in increasing cell survival of PC12 cells. Down-regulation of amphoterin in LPS-treated PC12 cells, on the other hand, may contribute to our observed highly detached apoptotic cells following LPS treatment.

Cytochrome c oxidase polyepptide Via-liver is a cytochrome c oxidase isoform that is involved in energy metabolism [58]. A deficiency in cytochrome c oxidase also correlates with apoptosis in muscle [59]. The reduction of this oxidase in PC12 cells during LPS-induced apoptosis implies a deficiency in the generation of ATP at the mitochondria, leading to apoptosis. ERP is also a member of the stress-response machinery of the endoplasmic reticulum [44, 45] and is the first eukaryotic protein disulfide isomerase (PDI)-related protein for which the structures of all domains have been determined [60]. PDI expression is induced by LPS in splenic B cells and BCL1 cells [61], and its up-regulation renders protection against apoptotic cell death [62]. However, unlike ERP60, ERP29 does not seem to exhibit PDI activity [42, 60]. This may account for the differential expression of ERP60 and ERP29 during LPS-induced PC12 cell apoptosis.

## Table 2. Changes in protein levels in LPS-induced apoptotic PC12 cells

<table>
<thead>
<tr>
<th>Protein</th>
<th>Protein levels</th>
<th>% control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>LPS</td>
</tr>
<tr>
<td>Detection on silver stained 2-DE gels</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amphoterin</td>
<td>0.20 ± 0.02</td>
<td>0.11 ± 0.02</td>
</tr>
<tr>
<td>Calcitulin</td>
<td>0.65 ± 0.06</td>
<td>0.92 ± 0.04</td>
</tr>
<tr>
<td>CBP-50</td>
<td>0.47 ± 0.02</td>
<td>0.86 ± 0.08</td>
</tr>
<tr>
<td>Cytochrome C oxidase polyepptide Via-liver</td>
<td>0.35 ± 0.06</td>
<td>0.21 ± 0.04</td>
</tr>
<tr>
<td>ERP29</td>
<td>0.41 ± 0.02</td>
<td>0.21 ± 0.04</td>
</tr>
<tr>
<td>ERP60</td>
<td>0.54 ± 0.02</td>
<td>0.99 ± 0.15</td>
</tr>
<tr>
<td>Detection on Western blot</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSP60</td>
<td>1.49 ± 0.28</td>
<td>2.56 ± 0.06</td>
</tr>
<tr>
<td>HSP90</td>
<td>0.46 ± 0.08</td>
<td>0.81 ± 0.02</td>
</tr>
</tbody>
</table>

The protein level of each spot is expressed as a percentage of total spot volume in the 2-DE gel. Results represent mean ± SD values from three independent experiments.
5 Conclusions

The present study provided our initial analysis of the changes in protein profiles of PC12 cells during LPS-induced apoptosis. We recognize that an augmented protein level in the proteome map may imply an increase in de novo synthesis or a decrease in degradation; and vice versa for reduced protein expression. Likewise, the detected alterations in protein levels in PC12 cells during LPS-induced apoptosis should be regarded as correlative rather than causative. However, the identification of LPS-induced alterations in 13 proteins from PC12 cells with known or potential association with apoptosis should provide a new impetus for further delineation of the cellular and molecular basis of apoptotic cell death and sepsis.

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6 References
