Protective effect of ginsenoside Rg1 on dopamine-induced apoptosis in PC12 cells

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KEY WORDS ginseng; saponins; dopamine; apoptosis; PC12 cells; bcl-2 genes

ABSTRACT

AIM: To explore the possible molecular mechanism of exogenous dopamine-induced apoptosis in PC12 cells and the protective effect of ginsenoside Rg1. METHODS: Flow cytometric assay was used to quantify the apoptotic cells and measure the percentage of cells with positive Bcl-2 and Bax proteins. The morphology of apoptotic cells was evaluated by transmission electron microscopy. DNA fragmentation was observed by gel electrophoresis. Caspase-3 activity was determined by fluorescent spectrophotometer and the expression bcl-2 and bax mRNA by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). RESULTS: Dopamine 0.15, 0.30, 0.45, and 0.60 mmol/L induced PC12 cell apoptosis from 1.1 % ± 0.4 % (control) to 41 % ± 3 %, 46.4 % ± 2.7 %, 53 % ± 3 %, and 64.5 % ± 2.7 %, respectively. After treatment with dopamine 0.45 mmol/L following pretreatment with Rg1 10 μmol/L for 24 h, the percentage of apoptotic cells and caspase-3 activity decreased from 53 % ± 3 % and 683 ± 8 (mean fluorescence intensity, MFI) to 1.9 % ± 0.6 % and 325 ± 5, and the percentage of cells with positive Bcl-2 protein increased from 14.3 % ± 1.1 % to 25.9 % ± 1.6 %, however, the percentage of cells with positive Bax protein decreased from 48 % ± 3 % to 35 % ± 3 %, compared with group treated with DA 0.45 mmol/L alone. CONCLUSION: Ginsenoside Rg1 protected PC12 cells against apoptosis by inhibiting the activation of caspase-3 and regulating the ratio of Bcl-2 to Bax protein.

INTRODUCTION

Apoptosis is an evolutionary conserved phenomenon which regulates normal cellular turnover. Two families of proteins, Bcl-2 and aspartate-specific cysteine proteases (caspases), have shown to play an important role in implementing and regulating apoptosis. It has been shown that 1-methyl-4-phenylpyridinium (MPP+) exerted its proapoptotic action by activation of caspase-3-like proteinase in neuronal study[1]. Bcl-2, as the most important member of antiapoptotic proteins in Bcl-2 family, inhibited apoptosis by inhibiting the caspase-3-activating protein, ie, apoptosis protease-activating factor-1 (APAF-1)[2]. Bax has been proposed to exert its proapoptotic function as a homodimer, possibly by forming a mitochondrial channel that allows leakage of caspase-activating factors from the mitochondrial[3].

Dopamine (DA) was one of the major sources of reactive oxygen species (ROS) in central nervous system, which ultimately contributed to inhibitions of mitochondrial respiration, lipid peroxidation, and neuronal death[4]. Furthermore, DA-induced apoptosis was protected by some antioxidants such as glutathione and N-acetylcysteine[5], and by overexpressing Bcl-2 in PC12 cells[6]. Ginsenosides represented the major active ingredients of ginseng, which showed a variety of biomedical efficacies, such as immune modulation, anti-aging, anti-inflammatory, and anti-oxidation[7,8]. Several studies have shown that ginsenoside Rg1 prevented rat cortical neurons from apoptosis and panaxadiol saponins protected brain from the anoxic injury[9,10].

To explore the possibility of using ginsenoside Rg1 to treat some neurodegenerative diseases, such as
Parkinson’s disease (PD) in vitro, PC12 cells were used as a model system of PD in this study to investigate the apoptotic effect of DA and the protective role of ginsenoside Rgl1.

MATERIALS AND METHODS

Materials Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum, and horse serum were from Gibco BRL (Grand Island, NY, USA); DA and propidium iodide (PI) from Sigma (St Louis, MO, USA); Ginsenoside Rgl1 (purity > 98 %) from Department of Organic Chemistry of Bethun Medical University (Changchun, China); Reverse transcription polymerase chain reaction (RT-PCR) kit from Promega (Madison, WI, USA); Bcl-2 and Bax monoclonal antibody, caspase-3 assay kit, and caspase-3 inhibitor Ac-DEVD-CHO (N-acetyl-Asp-Glu-Val-Asp-aldehyde) from PharMingen (San Diego, CA, USA).

Cell cultures and treatments Differentiated PC12 cells were grown in the DMEM supplemented with 5 % heat-inactivated fetal bovine serum and 5 % horse serum, benzylpenicillin 100 kU/L, streptomycin 100 mg/L, and 1 % glutamine at 37 °C, in a humidified atmosphere of 5 % CO₂. Cells were seeded on a 6-well plate precoated with collagen and were passed every other day. To evaluate cell morphology after treatment with DA and with or without Rgl1, PC12 cells were seeded on a 6-well-plate with collagen-coated coverslips (1.8 cm × 1.8 cm). In all studies, the cell density was 5 × 10⁴/L and medium was changed the day before experiments. PC12 cells were incubated with various concentrations of DA (0.15 – 0.60 mmol/L), following pretreatment with or without Rgl1 10 μmol/L for 24 h according to the previous study [9].

Detection of apoptotic cells by flow cytometry The cultured cells were washed twice with cold phosphate buffer saline (in mmol/L: NaCl 13.7, KCl 0.27, Na₂HPO₄ 0.43, and KH₂PO₄ 0.14, pH 7.3) and then exposed to PI solution (PI 500 mg/mL and RNase A 50 mg/L) for 30 min at 37 °C. After being washed twice with phosphate buffer saline, the cells were measured by flow cytometer (FACScan, Becton Dickinson, USA). Data were analysed with CellQuest™ software (Becton Dickinson, USA).

DNA fragmentation Approximately 5 × 10⁶ cells from each experimental condition were harvested, then the extraction of DNA was followed as described [11]. In brief, the cultured cells were treated with lysis buffer (1 % NP-40 in edetic acid 20 mmol/L, Tris-HCl 50 mmol/L, pH 7.5, 10 μL per 1 × 10⁶ cells) for 10 s. Then the cells were centrifugated at 1600 × g for 5 min, and the supernatants were treated with 1 % SDS and RNase A 5 g/L at 56 °C for 2 h, followed with proteinase K (2.5 g/L) for at least 2 h at 37 °C. After addition of 0.5 volume of ammonium acetate 10 mol/L, DNA was precipitated with 2.5 volume of ethanol, and separated by electrophoresis in 1.0 % agarose gel containing ethidium bromide 0.1 mg/L. DNA was visualized under UV light.

Assay for caspase-3 activity After the above treatment, the cells were washed rapidly with cold phosphate buffer saline, then lysis buffer was added (Tris-HCl 10 mmol/L, Na₂HPO₄/NaH₂PO₄ 10 mmol/L, pH 7.5; NaCl 130 mmol/L, 10 % Triton-X 100, sodium pyrophosphate 10 mmol/L, cell density 1 × 10⁶/L). Caspase-3 activity was measured using fluorescent substrate peptide Ac-DEVD-AMC (N-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin). Cell lysates 100 μL were incubated with Ac-DEVD-AMC 100 μmol/L in 100 μL of incubation buffer (HEPES 20 mmol/L, pH 7.5; 10 % glycerol, DTT 2 mmol/L) at 37 °C for 60 min. The release of AMC was measured by fluorometer (RF-5301 PC, Japan, excitation at 380 nm and emission at 460 nm). Caspase-3 activity was expressed as relative mean fluorescence intensity (MFI).

Measurements of Bcl-2 and Bax proteins by flow cytometry The levels of Bcl-2 and Bax protein were measured as described by Liu and Zhu [12]. Briefly, cells were collected by centrifugation and washed with phosphate buffer saline. After fixed with 2 % paraformaldehyde for 20 min and permeabilization with 0.5 % Triton-X 100, cells were incubated with primary antibodies to Bcl-2 and Bax for 30 min, respectively, then incubated with corresponding FITC-conjugated goat anti-rabbit antibody for 30 min at 37 °C in the dark. After washing twice with phosphate buffer saline, cells were measured by flow cytometer and the percentage of positive cells were determined.

Identification of bcl-2 and bax mRNA by RT-PCR Total RNA was isolated from PC12 cells with guanidium isothiocyanate. First-strand cDNA was reverse transcribed from 2 μg of RNA in a total volume of 20 μL with AMV reverse transcriptase 12 U at 42 °C for 30 min. PCR primers were designed as described [13, 14]. The bcl-2 sense primer was 5’ GTGCCAGGACGTGC-CTCTA 3’, and bcl-2 antisense primer was 5’ GTTGTC-AGATGC CGTTCGAG 3’. The bax sense primer was
RESULTS

Protective effect of Rgl on dopamine-induced PC12 cell apoptosis After treatment with various concentrations of DA (0.15, 0.30, 0.45, and 0.60 mmol/L) for 24 h, apoptosis in PC12 cells was induced. Meanwhile, some nuclei of PC12 cells exhibited typical apoptotic characteristics, such as nuclear condensation and DNA fragmentation. In PC12 cells pretreated with Rgl 10 μmol/L for 24 h, cell apoptosis and DNA fragmentation were completely blocked (Fig 1, 2, 3, and Tab 1).

DA induced PC12 cell apoptosis by activating caspase-3 After treatment with different concentrations of DA for 24 h, the percentage of apoptotic cells and caspase-3 activity increased markedly. The apoptosis and caspase-3 activation were inhibited completely by pretreatment with Ac-DEVD-CHO (a specific inhibitor of caspase-3) or Rgl, indicating that apoptosis was mediated by caspase-3 activation (Tab 1).

Effects of DA and Rgl on the expression of Bcl-2/Bax protein and mRNA After treatment with DA 0.45 mmol/L, Bcl-2 protein was decreased as well as the ratio of Bcl-2/Bax, while Bax protein was increased. Meanwhile, the expression of bcl-2 mRNA was decreased and bax mRNA was increased. In PC12 cells pretreated with Rgl 10 μmol/L for 24 h, the levels of Bcl-2 protein and mRNA were increased, while Bax protein and mRNA were decreased, indicating Bcl-2 and Bax protein might be important regulators of DA-induced apoptosis in PC12 cells (Tab 2, Fig 4).

DISCUSSION

In the present study, we provided evidence that the DA-induced apoptosis in PC12 cells was regulated by caspase-3. First, exposure of the cells to DA activated the caspase-3. Second, the DA-stimulated caspase-3 activation preceded apoptotic processes (data not shown). The caspase-3 activation correlated with the subsequent...
Fig 3. DNA fragmentation after treatment with DA and Rgl in PC12 cells. Lane M: DNA marker; Lane 1: treatment with DA 0.45 mmol/L and Rgl 10 μmol/L; Lane 2: treatment with DA 0.45 mmol/L.

apoptosis. Third, and most importantly, a specific caspase-3 inhibitor Ac-DEVD-CHO blocked caspase-3 activation as well as apoptosis. Finally, ginsenoside Rg1, as an anti-oxidant, mostly inhibited caspase-3 activation and consequent apoptosis.

DA treatment also resulted in the decreased ratio of Bcl-2 to Bax proteins. It has been reported that bcl-2 antisense oligonucleotide increased the sensitivity to DA-induced cell death, and Bcl-2 protein overexpressing cells showed a marked resistance to DA toxicity.[15,16]

Meanwhile, it was proposed that the proapoptotic proteins promoted cell death by dimerizing with antiapoptotic proteins and that the susceptibility of a cell to apoptotic signals was regulated by the ratio of anti- to pro-apoptotic proteins.[16] Therefore, in the present study, when Bcl-2 protein was overexpressed, it heterodimerized with Bax protein and death was repressed.

DA could be metabolized to form ROS, which could trigger apoptosis in a variety of cell systems.[17] In

Tab 1. Percentage of apoptotic cells and activity of caspase-3 after treatment with different doses of DA following pretreatment with Rgl 10 μmol/L or caspase-3 inhibitor Ac-DEVD-CHO 0.1 mmol/L for 24 h. n = 3. *P < 0.01 vs DA alone-treated group.

<table>
<thead>
<tr>
<th>Group</th>
<th>0</th>
<th>0.15</th>
<th>0.30</th>
<th>0.45</th>
<th>0.60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apoptotic cells/%</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>DA</td>
<td>1.1±0.4</td>
<td>41±3</td>
<td>46.4±2.7</td>
<td>53±3</td>
<td>64.3±2.7</td>
</tr>
<tr>
<td>DA + Ac-DEVD-CHO</td>
<td>0.7±0.3</td>
<td>0.8±0.4</td>
<td>1.0±0.3</td>
<td>1.1±0.4</td>
<td>1.3±0.4*</td>
</tr>
<tr>
<td>DA + Rgl</td>
<td>1.0±0.4</td>
<td>1.5±0.7</td>
<td>1.9±0.6</td>
<td>2.0±0.7</td>
<td>1.9±0.5*</td>
</tr>
<tr>
<td>Activity of caspase-3/MFI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DA</td>
<td>216±6</td>
<td>434±5</td>
<td>354±9</td>
<td>603±8</td>
<td>455±9</td>
</tr>
<tr>
<td>DA + Ac-DEVD-CHO</td>
<td>214±4</td>
<td>316±6</td>
<td>325±5</td>
<td>346±9</td>
<td>331±18</td>
</tr>
<tr>
<td>DA + Rgl</td>
<td>209±6</td>
<td>306±4</td>
<td>312±6</td>
<td>325±5</td>
<td>303±6*</td>
</tr>
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</table>

Tab 2. Percentage of positive Bcl-2, positive Bax protein, and ratio of Bcl-2/Bax in PC12 cells after treatment with different concentrations of DA following pretreatment with Rgl 10 μmol/L for 24 h. n = 3. *P < 0.05, *P < 0.01 vs DA alone-treated group.

<table>
<thead>
<tr>
<th>Group</th>
<th>0</th>
<th>0.15</th>
<th>0.30</th>
<th>0.45</th>
<th>0.60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive Bcl-2 protein/%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DA</td>
<td>44.8±2.3</td>
<td>35.8±2.1</td>
<td>26.7±1.6</td>
<td>14.3±1.1</td>
<td>11.9±0.6</td>
</tr>
<tr>
<td>DA + Rgl</td>
<td>46.7±2.5</td>
<td>43±3</td>
<td>36.8±2.4</td>
<td>25.9±1.6*</td>
<td>22.4±1.0*</td>
</tr>
<tr>
<td>Positive Bax protein/%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DA</td>
<td>33±4</td>
<td>36.8±2.1</td>
<td>42.3±2.6</td>
<td>48±3</td>
<td>50±3</td>
</tr>
<tr>
<td>DA + Rgl</td>
<td>32.5±1.3*</td>
<td>34±4</td>
<td>35±3</td>
<td>37±3*</td>
<td></td>
</tr>
<tr>
<td>Ratio of Bcl-2/Bax</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DA</td>
<td>1.27±0.12</td>
<td>0.97±0.08</td>
<td>0.63±0.09</td>
<td>0.29±0.06</td>
<td>0.34±0.03</td>
</tr>
<tr>
<td>DA + Rgl</td>
<td>1.37±0.11</td>
<td>1.10±0.10*</td>
<td>1.09±0.05*</td>
<td>0.73±0.06*</td>
<td>0.66±0.03*</td>
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</tbody>
</table>
agreement with this idea, our data showed that ginsenoside Rgl could mostly block DNA fragmentation and PC12 cell apoptosis. Similarly, Rgl exerted protective effect on DA-induced apoptosis in PC12 cells by inhibiting the activation of caspase-3 and regulating the ratio of Bcl-2 to Bax protein.

In the present study, DA-induced apoptosis in a concentration range of 0.15–0.60 mmol/L. This concentration was much higher than that needed for physiological function in the synapse(16). However, we believed that our observation was of potential relevance to pathological situation. Furthermore, we just only investigated the roles of caspase-3 and Bcl-2 family during the DA-induced PC12 cell apoptosis. They were not likely to be the only regulatory factors. In short, one of the chief findings was that DA induced apoptosis through regulation of apoptotic proteins or genes. The second find-

Fig 4. Expression of bcl-2 and bax mRNA after treatment with DA and Rgl. A: bcl-2; B: bax. Lane M: DNA marker; Lane 1: control; Lane 2: DA 0.45 mmol/L; Lane 3: DA 0.45 mmol/L and caspase-3 inhibitor Ac-DEVD-CHO 0.1 mmol/L; Lane 4: DA 0.45 mmol/L and Rgl 10 μmol/L. The ratio of bcl-2/GAPDH mRNA from Lane 1 to Lane 4 were 0.427, 0.314, 0.400, and 0.325. The ratio of bax/GAPDH mRNA from Lane 1 to Lane 4 were 0.642, 0.745, 0.548, and 0.581.

REFERENCES


14. Tilly JL, Tilly KL, Kentor ML. Expression of members of the bcl-2 gene family in the immature rat ovary: equine chori-


人参皂甙 Rgl 对抗多巴胺诱导的 PC12 细胞凋亡

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关键词 人参；皂苷类；多巴胺；细胞凋亡；PC12 细胞；bcl-2 基因

目的：探讨外源性多巴胺诱导 PC12 细胞凋亡以及人
参皂甙 Rgl 保护作用的分子机制。方法：流式细
胞仪定量测定 PC12 细胞的凋亡和 Bcl-2、Bax 蛋白的表达；电子显微镜观察 PC12 细胞的形态；凝胶电泳
评价 DNA 的断裂；荧光分光光度计法测定 caspase-3 的活力；半定量 RT-PCR 分析 bcl-2 和 bax mRNA 的表达。结果：多巴胺（浓度为 0.15、0.30、0.45 和 0.60 mmol/L）诱导 PC12 细胞凋亡，各剂量组细胞凋亡率分别从对照组 1.1 % ± 0.4 % 增加到 41 % ± 3 %；46.4 % ± 2.7 %；53 % ± 3 % 和 64.5 % ± 2.7 %；人参皂甙 Rgl 10 μmol/L 预处理 24 h 后，多
巴胺 0.45 mmol/L 单独处理时，PC12 细胞的凋亡率和 Caspase-3 的活力分别从 53 % ± 3 % 和 683 ± 8 (平均荧光强度) 下降到 1.9 % ± 0.6 % 和 325 ± 5，Bcl-2 蛋白阳性率从 14.3 % ± 1.1 % 增加到 25.9 % ± 1.6 %，Bax 蛋白阳性率从 48 % ± 3 % 下降到
35 % ± 3 %。结论：人参皂甙 Rgl 通过抑制 Caspase-3 的激活并调节 Bcl-2 和 Bax 两者间蛋白的比值，对抗多巴胺对 PC12 细胞凋亡的诱导作用。

（责任编辑 吴民淑）