Original Article
Calcium homeostasis disruption and endoplasmic reticulum stress mediates ischemia/reperfusion-induced PC12 cells apoptosis

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Abstract: Background: Ischemic stroke is a major cause of morbidity and mortality in the world today. Endoplasmic reticulum stress (ERS) is an essential signaling event for neuronal injury resulting from IR. Calcium signals play important roles in physiological activities which regulate many fundamental cellular processes. However, the role of intracellular calcium signals and ERS at the early stage of IR-induced apoptosis remains largely elusive. Objective: To investigate whether intracellular Ca2+ participate in IR-induced apoptosis as an upstream messenger in an in vitro model of ischemia-reperfusion (IR). Methods: An in vitro model of IR was established according to literature. The expression of SERCA2, GRP78, caspase-12 and CHOP were evaluated by western blot during different reperfusion time intervals. Results: The apoptotic rates and [Ca 2+]i in PC12 cells increased in a reperfusion time-dependent manner, the vitality of SERCA decreased with reperfusion time gradually. A stable SERCA protein decrease was observed throughout the entire time course. The expression of GRP78, caspase-12 and CHOP were up-regulated after IR. Conclusion: IR induced [Ca2+]i elevation at the early stage of apoptosis, which might involve Ca2+ efflux from the endoplasmic reticulum and Ca2+ influx from extracellular medium. Calcium signals and ERS were the important upstream messengers in IR-induced apoptosis in PC12 cells.

Keywords: Calcium signals, endoplasmic reticulum stress, SERCA, apoptosis, ischemia/reperfusion

Introduction

Ischemic stroke is a major cause of morbidity and mortality in the world today [1]. It results from a temporary or permanent reduction of cerebral blood flow which leads to functional and structural damage in different brain regions. Patients once found ischemic stroke require intensive care unit admission for constant monitoring. Furthermore, it is the leading cause of adult disability which brings a heavy burden to their family and society as well. However, there has been little progress toward the development of treatments to improve its prognosis in over 20 years since the introduction of the thrombolytic drug, recombinant tissue plasminogen activator [2, 3]. Therefore, comprehensive researches in the field of stroke are needed to further understand stroke pathophysiology and to explore novel therapeutic strategies.

The ischemic cascade and reperfusion injury are characterized by the following biochemical events: bioenergetic failure, ionic imbalance, acidosis, excitotoxicity, oxidative stress and inflammation, culminating in cell death via necrosis or apoptosis [4]. Studies have revealed that endoplasmic reticulum stress (ERS) is an essential signaling event for neuronal injury resulting from ischemia/reperfusion (I/R) [5-8]. The endoplasmic reticulum (ER) is an important organelle in eukaryotic cells which serves as the quality control center for proteins, ensuring their proper folding, assembly, and post-translational modifications [9, 10]. When the ER cannot process proteins effectively, ERS ensues. ER function is sensitive to environmental conditions and cellular changes. Certain stimuli such as ischemia, hypoxia, and hypertension might trigger the accumulation of unfolded proteins in the ER lumen, leading to the unfolded protein response (UPR) which involves expansion of ER
membranes, accelerated degradation of unfolded proteins, increased translation of folding chaperones, and inhibition of other protein synthesis [11, 12]. UPR is a protective reaction to restore endoplasmic reticulum function impaired by stress. However, when this stress is excessive or prolonged, cell death via apoptotic pathways is triggered [13].

It is well known that intracellular Ca\(^{2+}\) plays multiple roles in the induction or regulation of processes related to cell survival and cell death. The ER is the main Ca\(^{2+}\)-storage organelle and therefore plays a central role in intracellular Ca\(^{2+}\) signaling [14]. The steady state of ER Ca\(^{2+}\)-store content level is controlled by the balance between ER Ca\(^{2+}\)-ATPases (SERCA) and the ER basal Ca\(^{2+}\)-leak mechanisms predominantly via the inositol 1,4,5-trisphosphate (IP3) receptor (IP3R) [15-17]. Disruption of Ca\(^{2+}\) homeostasis in the ER is well documented to trigger ER stress [18, 19] and apoptosis [20]. However, to date, little has known about whether intracellular calcium signals operate at the early stage of IR-induced apoptosis and whether the ER-associated pathway is involved in this mechanism.

In this study, an in vitro model of Ischemia-reperfusion established by Yidong Cao et al. [21] was utilized to investigate whether intracellular Ca\(^{2+}\) participated in IR-induced apoptosis in PC12 cells as an upstream messenger. The apoptotic rate, the influence of reperfusion on the changes of intracellular calcium concentration ([Ca\(^{2+}\)]\(_i\)) and the vitality of SERCA were measured at different reperfusion time. In addition, the expression of SERCA2, GRP78, caspase-12 and CHOP, the key elements in the ER-associated pathway [9], were evaluated during different reperfusion time intervals.

**Materials and methods**

**Main reagents and instrument**

Uncomplete Dulbecco’s modified Eagle’s medium (DMEM) with high glucose was purchased from Keygen Biotech (Nanjing, Jiangsu, China). Fetal bovine serum (FBS) was obtained from Si-Ji-Qing Biotechnology Co. (Hangzhou, Zhejiang, China). Annexin V-fluorescein isothiocyanate (V-FITC) was purchased from BD Pharmingen (SanDiego, CA, USA), and Fluo-3/AM was purchased from Beyotime Institute of Biotechnology (Haimen, Jiangsu, China). Ethylene glycol tetraacetic acid (EGTA), TritonX-100 and adenosine triphosphate (ATP) were purchased from VWR Life Science AMRESCO (Solon, Ohio, USA), ATPase/GTPase activity assay kit was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Anti-GRP78 rabbit monoclonal antibody, anti-caspase-12 rabbit monoclonal antibody and anti-CHOP mouse monoclonal antibody were obtained from Abcam Co. (Cambridge, MA, UK). Anti-GAPDH mouse monoclonal anti-body was purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). The goat anti-rabbit IRDy®680CW secondary antibody and goat-mouse IRDye®680RD secondary antibody were purchased from LI-COR Biosciences (Lincoln, Nebraska, USA). All the other reagents are analytical grade, made in China.

**Cell culture**

Murine PC12 pheochromocytoma cells were purchased from the Cell Bank of Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). PC12 cells were cultured in uncomplete DMEM medium of high glucose supplemented with 10% (v/v) heat-inactivated FBS, 100 U/ml penicillin, 100 ìg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO\(_2\) and 95% air. Cells were routinely passaged every other day. Prior to treatment, cells were harvested and seeded on plastic six-well culture plates at 4×10\(^5\) cells/ml and allowed to grow adherently for 24 h. After that, cells at logarithmic phase were ready for treatment.

**Apoptotic rate assay by flow cytometry method**

Ischemia-reperfusion was induced in PC12 cells by exposed to Earle’s solution with sugar-free (130 mmol/L NaCl, 5.7 mmol/L KCl, 1.8 mmol/L CaCl\(_2\), 26 mmol/L NaHCO\(_3\), 0.8 mmol/L MgSO\(_4\)•7H\(_2\)O, 1.2 mmol/L NaH\(_2\)PO\(_4\)•2H\(_2\)O, pH 7.2-7.4) in hypoxia box (37°C, 5% CO\(_2\)/95% N\(_2\)) for 2 h. Subsequently, cells were exposed to original medium, keeping at 37°C in a humidified atmosphere of 5% CO\(_2\)/95% air. PC12 cells were divided into six groups. Five of them were ischemia-reperfusion groups with Ischemia/Reperfusion (Is/Re) injury of 2 h/0 h, 2 h/6 h, 2 h/12 h, 2 h/24 h, 2 h/48 h, respectively. The control group was treated with Earle’s solution (130 mmol/L NaCl, 5.7 mmol/L KCl, 1.8...
mmol/L CaCl₂, 26 mmol/L NaHCO₃, 0.8 mmol/L MgSO₄•7H₂O, 1.2 mmol/L NaH₂PO₄•2H₂O, 36.3 mmol/L glucose, pH 7.2-7.4). Cells were harvested and washed twice with ice-cold phosphate-buffered saline (PBS) after treatment. The apoptotic and viable cells were quantified by annexin V-FITC apoptosis detection kit according to the manufacturer’s instructions. Apoptotic analysis was performed using a flow cytometer (FACS can; BectonDickinson Immunocytometry Systems, Franklin Lakes, NJ, USA).

**[Ca²⁺]i measurement**

The changes of intracellular calcium concentration ([Ca²⁺]) during the application of EDTA (5

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**Figure 1.** Quantitative analysis result and representative images of apoptosis in PC12 cells as illustrated by FACS: A. Control; B. Is/Re injury of 2 h/0 h; C. Is/Re injury of 2 h/6 h; D. Is/Re injury of 2 h/12 h; E. Is/Re injury of 2 h/24 h; F. Is/Re injury of 2 h/48 h; G. The apoptosis rate of PC12 cells induced by ischemia-reperfusion at different times. Values represent means ± SD of three determinations. Significance: *P<0.05, **P<0.01, compared with the control.
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mM) and Tritonx100 (0.1%) were monitored by LSCM using fluo-3/AM as a calcium fluorescent indicator that could monitor real-time alterations of \([Ca^{2+}]_i\). Fluo-3/AM was dissolved in DMSO to make 1 mM stock solution, and stored at -80°C. Cells were loaded with fluo-3/AM (3 μM) for 45 min in a humidified incubator which provided an atmosphere of 5% CO2 and 95% air at a constant temperature of 37°C. \([Ca^{2+}]_i\) was measured using an inverted laser scanning confocal microscope (Olympus, Japan). The excitation wavelength was 488 nm and fluorescence images were captured at 525 nm. All fluorescence measurements were made at room temperature (25°C). \([Ca^{2+}]_i\) was calculated by the following equation:

\[ [Ca^{2+}]_i \text{ (nmol/L)} = Kd \left( \frac{F - F_{\text{min}}}{F_{\text{max}} - F} \right) \]

where Kd is 400 nmol/L at 37°C, F is the control level before treatment, \(F_{\text{min}}\) is the minimum exposure level during the application of EGTA and \(F_{\text{max}}\) is the maximum exposure level during the application of Tritonx100. Each data point was an average value calculated from 15 to 54 samples. Data were expressed as mean ± SD.

Measurement of the vitality of SERCA

The vitality of SERCA in PC12 cells was determined by ATPase/GTPase Activity Assay Kit (sigma, USA). After establishing models, cells were harvested, homogenized with ice cold assay buffer and centrifuged at 14000 rpm for 10 minutes. The supernatant was collected into duplicate wells of a 96 well plate. According to the manufacturer’s protocol, start the reaction and incubate it for 30 min at room temperature. The absorbance of each well was recorded by a microplate reader at 620 nm.

Western blot analysis

The cells were harvested and then treated with ice-cold lysis buffer (1 mL lysis buffer, 10 μL phosphatase inhibitors, 1 μL protease inhibitor, 5 μL 100 mM phenylmethyl sulfonylefluoride). The soluble protein samples were electrophoresed on 8% or 15% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked in 5% skim milk and incubated with each primary antibody overnight at 4°C. The membranes were then incubated with their respective secondary antibodies (1:10000) for 2 h at RT. The blots were developed by IRASER bicolor image system (Oddsey, USA). The relative intensity of the protein bands was measured by spot densitometry analysis using GAPDH as loading control by Oddsey software.

Statistical analysis

All data was presented as mean ± SD. Statistical analysis was performed by SPSS 17.0 software. Statistical significance of differences between groups was analyzed by Student’s test or by one-way ANOVA analysis of variance when more than two groups were compared. A P-value of <0.05 was considered statistically significant.

Results

Apoptotic rate assay by flow cytometry method

According to the results of flow cytometry detection, exposure to Earle’s solution with sugar-free induced apoptosis in PC12 cells. As shown in Figure 1, the apoptotic rates of PC12 cells increased significantly after reperfusion of 6 h, 12 h, 24 h and 48 h. And it presented a time-dependent tendency.
The impact of reperfusion on intracellular calcium ([Ca²⁺]i) in individual cells was observed by real-time LSCM. As shown in Figure 2, reperfusion rapidly enhanced intracellular [Ca²⁺]i in individual cells with nearly a time-dependent manner. But a decrease was observed at 48 h of reperfusion which might be induced by plasmatorrhexis. Compared with control group, [Ca²⁺]i in PC12 cells had significant difference at 12 h (P<0.05), 24 h (P<0.01) and 48 h (P<0.05) (Table 1).

The morphological changes of PC12 cells at different times after reperfusion were shown in Figure 3. It can be observed that cells in control group had normal morphological features which nuclei membrane was distinct (Figure 2A). With the increase of reperfusion time, the nuclei membrane became fading (Figure 2B-E), which indicated the intracellular calcium ions influx to the cytoplasm. Cells became shrinkage, turned round and arranged loosely. Most of the PC12 cells floated in the nutrient medium at 48 h (Figure 2F).

Measurement of the vitality of SERCA

The influence of reperfusion on the vitality of SERCA in PC12 cells was measured by using ATPase/GTPase Activity Assay Kit. As shown in Figure 4, the vitality of SERCA decreased with reperfusion time gradually. A sudden drop occurred at 6 h to 12 h reperfusion time, which was in consistent with the above studies of [Ca²⁺]i. Compared with control group, the vitality of SERCA in PC12 cells displayed significant difference at 12 h, 24 h and 48 h with P values less than 0.01 (Table 2).

Effect of reperfusion on the expression of SERCA, GRP78, caspase-12 and CHOP

As shown in Figure 5, Ischemia/Reperfusion altered the expression level of SERCA, GRP78, Caspase-12 and CHOP in PC12 cells. A stable SERCA decrease was observed throughout the entire time course. At 12 h of reperfusion, SERCA was found to be significantly down-regu-
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**Table 2. The change of vitality for SERCA at different times after reperfusion (n = 8)**

<table>
<thead>
<tr>
<th>Groups</th>
<th>The vitality of SERCA (units/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.72 ± 0.32</td>
</tr>
<tr>
<td>0 h</td>
<td>2.52 ± 0.40</td>
</tr>
<tr>
<td>6 h</td>
<td>2.51 ± 0.28</td>
</tr>
<tr>
<td>12 h</td>
<td>2.05 ± 0.34**</td>
</tr>
<tr>
<td>24 h</td>
<td>1.66 ± 0.44**</td>
</tr>
<tr>
<td>48 h</td>
<td>0.87 ± 0.31**</td>
</tr>
</tbody>
</table>

Data are shown as mean ± SD. *P<0.05, **P<0.01, compared with the control.

lated compared to the control (P<0.05). At 24 h and 48 h, SERCA levels were further decreased (P<0.01). The results were consistent with the reduction of the vitality of SERCA, which indicated that the calcium homeostasis dysfunction often used as potentially useful marker of endoplasmic reticulum stress.

The expression of GRP78 serves as a good marker of ER stress because this protein is specifically activated under the conditions of ER dysfunction. In the present study, GRP78 levels kept rising from 0 h to 24 h post reperfusion (P<0.05 at 6 h and 12 h, P<0.01 at 24 h). However, the expression of GRP78 has a tendency to decrease at 48 h post reperfusion. The results of GRP78 expression indicated that Ischemia/Reperfusion injury had a severe effect on PC12 cells.

Similar to GRP78, Caspase-12 was also increased throughout the course of reperfusion. As shown in Figure 5, Caspase-12 was found to be significantly up-regulated at 0 h following reperfusion (P<0.05), and the expression further increased from 6 h to 24 h (P<0.01). The maximum expression level was about 4-fold increase compared with the untreated group.

The expression of CHOP has been shown to be specifically activated under conditions of disturbing the function of ER. Significant increase in the expression levels were observed at 6, 12, 24 and 48 h after reperfusion (P<0.05).

**Discussion**

Apoptosis, which was first identified by Wyllie et al. in 1972, is a highly regulated, energy-dependent, sequential form of cell death with certain features such as chromatin condensation, membrane blebbing, and typical DNA fragmentation [22]. Intracellular Ca^{2+} concentration is regulated very tightly as a signaling messenger. Disruption of Ca^{2+} homeostasis can induce apoptosis [23]. Ischemic injury is thought to initiate apoptosis, but for substantial apoptotic cell death to occur, reperfusion is necessary [24]. However, little has known about the ER-associated pathway and [Ca^{2+}]_{i} involved in apoptosis after ischemia reperfusion.

In this study, by establishing an in vitro model of ischemia-reperfusion, we investigated whether intracellular calcium signals operate at the early stage of IR-induced apoptosis. The major findings are that IR induced [Ca^{2+}]_{i} elevation in PC12 cells at the early stage of apoptosis, and that Ca^{2+} efflux from the endoplasmic reticulum and Ca^{2+} influx from extracellular medium may be involved in this process. In addition, under ERS condition, GRP78 was up-regulated and the pro-apoptotic pathways mediated by CHOP and caspase-12 were promoted eventually.

Recent researches have demonstrated that ERS induce apoptosis both in vitro and in vivo, which play an essential role in IR-induced impairment. Signaling pathways involved in this apoptosis were investigated as well [8, 21]. Intracellular Ca^{2+} signaling has been proved to be an important messenger in apoptosis. The formation of these Ca^{2+} signals is dependent on numerous cellular Ca^{2+}-binding and Ca^{2+}-transporting proteins presenting in various cell compartments. ER forms the main intracellular Ca^{2+} store. Because of the active extrusion of Ca^{2+} by pumps in the plasma membrane or in intracellular organelles, such as SERCA in the ER, the resting cytosolic [Ca^{2+}] remains very low (~100 nM). Due to SERCA activity and intraluminal Ca^{2+} binding proteins, the ER can accumulate Ca^{2+} in more than thousandfold excess compared to the cytosol [25, 26]. Excessive Ca^{2+}-release events from the ER will lead to cell death via mitochondrial pathways [14].

In our experiment, we found that reperfusion significantly increased the apoptotic rates in a time-dependent manner, and the rapid enhanced intracellular [Ca^{2+}]_{i} in individual cells was observed in nearly the same manner at the early stage of apoptosis. The results suggested that the increase of intracellular [Ca^{2+}]_{i} is closely related to cell apoptosis after I/R. Intracellular Ca^{2+} was a potent upstream pro-
apoptotic messenger acting at the early stage of IR-induced apoptosis in PC12 cells. Furthermore, the vitality of SERCA significantly decreased with reperfusion time gradually. A sudden drop occurred at 6 h to 12 h reperfusion time, which was in correlation with the variation tendency of $[Ca^{2+}]$. Inhibiting the SERCA pump could induce $[Ca^{2+}]$ elevation, and then significantly enhance apoptosis. Thus it revealed that calcium movement in and out of the ER is tightly regulated by the SERCA at the early stage of apoptosis. SERCA pump may serve as a potential target to ameliorating cerebral ischemia-reperfusion injury.

Folding and processing newly synthesized proteins are vital functions of the ER that are sensitive to a variety of stress conditions. The UPR is activated to restore ER function impaired by stress [27]. However, if ER stress persists and the protein folding demand/capacity balance cannot be restored, UPR triggers apoptosis [28]. Upon UPR activation, GRP78, a sentinel marker for ER stress under pathologic condi-
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GRP78 levels kept rising from 0 h to 24 h post reperfusion due to the severe effect of I/R injury on PC12 cells, thus demonstrated that GRP78 functions an anti-apoptotic molecule under ER stress at the early stage of IR-induced apoptosis. A previous study pointed out that the increased expression of GRP78 attenuates the induction of CHOP during ER stress and reduces ER stress-induced apoptosis [30]. And caspase-12 is significantly activated following the aggravation of reperfusion injury, which can promote ER stress cascade reactions and aggravate cell apoptosis [31]. In our experiment, significant increase in the expression levels of CHOP were observed at 6, 12, 24 and 48 h after reperfusion. Caspase-12 was also increased throughout the course of reperfusion. The results were consistent with literatures.

Currently, the only validated therapy for ischemic stroke is thrombolysis, which must be administered within 4-5 h of symptom onset [32]. Due to its narrow therapeutic time window and concerns about hemorrhagic complications, thrombolysis is still not used regularly [33]. Thus, a better understanding of the multiple mechanisms involved in ERS may help uncover useful therapeutic approaches for ischemic stroke. Researchers have been focusing on ERS and calcium dyshomeostasis after I/R injury, for regulating calcium homeostasis and inhibiting cell apoptosis mediated by ERS has become a new way of drug resistance to cerebral ischemia-reperfusion injury.

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Disclosure of conflict of interest

None.

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