Original Article

Modification of HSP proteins and Ca$^{2+}$ are responsible for the NO-derived peroxynitrite mediated neurological damage in PC12 cell

Jun Wen¹*, Hua Li²*, Yudan Zhang², Xia Li², Fang Liu³

¹Department of Emergency, Xi’an Children’s Hospital, Xi’an 710003, China; ²Department of Neurology, Xi’an Children’s Hospital, Xi’an 710003, China; ³Department of Ultrasound, Xi’an Children’s Hospital, Xi’an 710003, China. *Equal contributors.

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Abstract: Peroxynitrite as one crucial metabolite of NO-derived agents has been well multi-investigated to inspect its potential role and sought to define its concrete mechanism underlying the memory loss and impaired cognition involved in pathological processes. In this investigation, the cell viability was assessed by the MTT assay. The neurotoxicity of peroxynitrite was analyzed by using immunohistochemical measurements in cultured PC12 cells to explore the underlying mechanisms. The generation of ROS was evaluated by a fluorometry assay by a fluorometry assay. Apoptosis was assayed by annexin V-FITC and PI staining with flow cytometry. [Ca$^{2+}$]i was examined by using the microspectrofluorometer. Hsp70 was detected by western blot assay. The results revealed that PC12 cells were inhibited by peroxynitrite both in a dose-dependent and time-dependent manner. The level of ROS in PC12 cells exposed to SIN-1 was increased in a dose-dependent manner. The result indicated that the SIN-1 induced apoptosis of PC12 cells in a dose-dependent manner. Quercetin inhibited the viability of PC12 cells in a concentration-dependent manner. [Ca$^{2+}$]i was increased gradually when cells treated with quercetin alone and also increased with treatment of dantrolene-containing. Hsp70 was detected by western blot assay. The results revealed that PC12 cells were inhibited by peroxynitrite both in a dose-dependent and time-dependent manner. The level of ROS in PC12 cells exposed to SIN-1 was increased in a dose-dependent manner. The result indicated that the SIN-1 induced apoptosis of PC12 cells in a dose-dependent manner. Quercetin inhibited the viability of PC12 cells in a concentration-dependent manner. [Ca$^{2+}$]i was increased gradually when cells treated with quercetin alone and also increased with treatment of dantrolene-containing. Hsp70 was significantly decreased in SIN-1-treated group compared with that of control group ($P<0.01$). In conclusion, Ca$^{2+}$ homeostasis and chaperone Hsp70 were critically involved in peroxynitrite induced nitrosative stress as protective. Peroxynitrite acts as the pathological agent in learning and memory defects in CNS disorders associated with challenge.

Keywords: Peroxynitrite, neurotoxicity, Ca$^{2+}$, Hsp70, cognition

Introduction

Peroxynitrite, the product of the diffusion-controlled bi-radical reaction between nitric oxide and superoxide radical, is a short-lived oxidizing and nitrating specie and commonly considered to be involved in NO-induced modification of cellular functions including cell apoptosis and death [1-3]. Accumulating evidences suggest that the pharmacological products and inhibition of peroxynitrite formation or decomposition may possibly shed light on mechanisms of peroxynitrite-mediated injuries include massive injuries including vascular diseases, ischemia-reperfusion injury, circulatory shock, inflammation and neurodegeneration implicated with impaired cognitive function in the manners of lipid peroxidation, thioloxidation, receptor/protein nitration, and tyrosine phosphatase activation as proposed [2, 4, 5].

Prior studies have revealed that mild oxidative and nitrosative stress in NO-related conditions mediates neuronal function and signaling transduction while excessive accumulation of free radicals contribute to neuronal injury or death [3]. Under neurodegenerative circumstances, overactivation of both synaptic and extra synaptic NMDARs trigger excessive influx of Ca$^{2+}$ ions, generating neurotoxic levels of free radical associated with protein misfolding, mitochondrial dysfunction, synaptic injury and eventual neuronal loss, instead of the physiological role of mediating neuronal function and survival in basal conditions when the NMDARs were mildly activated [5-7]. Collaboration between Stuart...
Lipton and Jonathan Stamler groups initially discovered and characterized that NO inhibits excessive NMDAR activity through S-nitrosylation, the covalent reaction of an NO group with a reactive cysteine thiol on target proteins, emerged as the principal mechanism exerting NO bioactivity. The survey implicate that the NMDAR/NO pathway might potentially represent a therapeutic target in CNS diseases associated cognitive dysfunction of different levels [4, 8, 9].

Additionally, it is worth noting that many neurodegenerative disorders implicated with misfolding, aggregation and accumulation of amyloid fibrils both inside and outside the neurons are characterized by conformational changes in proteins regulated by a network of interactive molecules, known as the chaperone system, which is composed of molecular chaperones and co-chaperones [2, 10, 11]. The heat shock protein70 (Hsp70)-based chaperone machinery controlled the polyglutamine androgen receptor (polyQAR) involved in neurodegenerative protein aggregation. 3-morpholinosydnonimine chlorhydrate leads to a dose-dependent increase in Hsp70 expression and cell apoptosis and death as well in monocyte tests derived from human donors. What is more, overexpression of Hsp70 interacting protein (Hip), a co-chaperone enhancing binding of Hsp70 to its substrates, promotes client protein ubiquitination and polyQAR clearance [12, 13]. These findings highlight the therapeutic potential of Hsp70 and provide new insights into the role of the chaperone machinery in protein quality control.

Although these phenomena suggested peroxynitrite was responsible for cytotoxicity and cellular injury, we propose a hypothesis that peroxynitrite-mediated biological effects. Under physiological pH, SIN-1 undergoes an autooxidation chemical process to produce equal nitric oxide and superoxide, which will in turn lead to the formation of peroxynitrite.

In the present study, we measured the acute exposure of peroxynitrite on PC12 cells and tried to address the issue with more intuitive evidence that whether cognitive impairments induced by peroxynitrite was combined with NMDARs-mediated glutamatergic transmission and could Hsp70 play certain neuroprotective effect.

Materials and methods

Reagents

RPMI 1640 cell culture medium was purchased from GIBCO Invitrogen. Trypsinase was purchased from Ameresco Corporation. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyldetrazolium bromide (MTT), fetal bovine serum (FBS) and N-(2-mercaptopropionyl)-glycine (N-MPG) were all purchased from Sigma Chemical Co., St. Louis, MO, USA. The ROS testing kit was purchased from Genmed Sciences Inc., USA. The Annexin V-fluorescein isothiocyanate (FITC) propidium iodide (PI) apoptosis detection kit was from Biopharma Corporation, USA. Plastic culture microplates and flasks used in the experiment were supplied by Corning Incorporated (Costar, Corning, NY, USA). Parafilm membrane was purchased from Parafilm Corporation. PVDF membrane was purchased from Millipore Corporation. Primary and secondary antibodies were purchased from Santa Cruz Corporation. Protein marker was purchased from Fermentas Corporation.

Cell culture

PC12 rat pheochromocytoma cells were obtained from Institute of Basic Medical Sciences Chinese Academy of Medical Sciences. The cells were maintained on plastic culture microplates with RPMI 1640 (pH 7.4) supplemented with 5% fetal bovine serum and 10% horse serum at 37°C in the humidified atmosphere of 5% CO₂. All medium included 100 U/mL of penicillin and 100 U/mL of streptomycin.

Acute peroxynitrite exposure and drug application

As a peroxynitrite donor, SIN-1 (3-morpholinosydnonimine) is widely applied for examining peroxynitrite-mediated biological effects. Under physiological pH, SIN-1 undergoes an auto-oxidation chemical process to produce equal nitric oxide and superoxide, which will in turn lead to the formation of peroxynitrite.

SIN-1 was purchased from Alexis (USA). It was diluted directly in ultra-pure grade water and pre-incubated for 2 h at room temperature to yield the appropriate concentration of peroxynitrite before being applied. 1 mM SIN-1 generates ONOO⁻ at a speed of 1 μM/min and 500 μM SIN-1 forms ONO2⁻ at a speed of 0.5 μM/ min. The preservation of SIN-1 stock solution should avoid light using the tin foil package strictly and diluted to certain concentrations just before drug application.

Quercetin, known as an inhibitor of HSP synthesis, was dissolved in dry DMSO to make 0.2 M
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stock solution and diluted to different concentrations before experiments. EGTA was dissolved in water to make 20 mM stock solution and diluted to 500 μM before use. Dantrolene sodium was dissolved in dry DMSO to make a 20 mM stock solution and diluted to 30 μM just before use. CdCl\textsubscript{2} was dissolved in water to make 0.1 M stock solutions and then added to standard external recording solution for a final trivalent cation concentration of 100 μM just before use.

Cell viability assay

The cell viability was assessed by the MTT assay, which was based on the reduction of the dye MTT to formazan crystals, an insoluble intracellular blue product, by cellular dehydrogenases. Cells were seeded on 96-wells plates with 1×10\textsuperscript{4} cells in 200 μL medium per well and cultured 24 h for stabilization. After 6, 12, 24 and 36 h incubation, 20 μL MTT was added to each well with a final concentration of 2 mg/mL, and afterwards the cells were cultured at 37°C for 4 h. The medium was then removed carefully and 150 μL DMSO was added in and mixed with the cells thoroughly until formazan crystals were dissolved completely. This mixture was measured in an ELISA reader (EIX 800, Bio-TEK, USA) with a wave length of 570 nm. The cell viability was expressed as a percentage of the viability of the control culture. Meanwhile, the dose of peroxynitrite and quercetin used in assays of apoptosis and [Ca\textsuperscript{2+}] was based on the results of the MTT test as well as the western blot analysis.

Detection of ROS and the effect of N-(2-mercaptopropionyl)-glycine (N-MPG)

The generation of ROS was evaluated by a fluorometry assay by a fluorometry assay using intracellular oxidation of dichlorodihydrofluorescein diacetate (DCFH-DA). The cells in logarithmetic growth phase were incubated in 6-well plate for 24 h for stabilization, then the medium was replaced with medium containing different concentrations (100, 250, 500 μM and 1 mM) of SIN-1 for 24 h. After exposure, cells were washed with phosphate-buffered saline (PBS) and resuspended at a concentration of 1×10\textsuperscript{6} cells/ml and stained by the staining solution for 20 min. The cells were detected and analyzed by flow cytometry.

The effect of N-(mercaptopropionyl)-glycine (N-MPG), known as a kind of ROS scavenger, was detected by MTT assay. After stabilized on a 96-well plate, cells were pre-treated with N-MPG (300 μmol/L) for 30 min. Following the procedures of standard method described above the suspension was diluted to the final concentration of 50 μg/ml and followed incubation for 24 h. Then the cells were exposed to different concentrations of SIN-1 (100, 250, 500 μM and 1 mM), while cultured cells without the peroxynitrite exposure served as the vehicle control.

Detection of apoptotic cells with flow cytometry

Apoptosis was assayed by annexin V-FITC and PI staining followed by analysis with flow cytometry (BECKMAN-COULTER, USA). The methodology followed the procedures as described in the annexin V-FITC/PI detection kit. The cells were exposed to SIN-1 with the concentration of 100, 250, 500 μM and 1 mM for 24 h. Eventually, the cells were resuspended in 400 μl 1× binding buffer at a concentration of 1×10\textsuperscript{6} cells/ml. The cells were then stained with 5 μl annexin V-FITC and 10 μl PI for 15 min at the room temperature in the dark. Then the cell suspension was ready for the analysis by the flow cytometry.

[Ca\textsuperscript{2+}] measurements

PC12 cells grown on coverslips were plated and cultured at 37°C in 1640 medium for 30 min. Then cells were loaded with 3 μM Fura-2/AM at room temperature allowed to de-esterify for a minimum of 15 min and washed 3 times with HBSS buffer (NaCl 110 mM; KCl 5 mM, CaCl\textsubscript{2} 1.25 mM; Na\textsubscript{2}HPO\textsubscript{4} 0.3 mM; KH\textsubscript{2}PO\textsubscript{4} 0.3 mM; MgSO\textsubscript{4} 0.8 mM; NaHCO\textsubscript{3} 4 mM; Glucose 5.6 mM; HEPES 15 mM). After that, the coverslip was transferred in a perfusion chamber with 1 mL HBSS containing dantrolene sodium (an inhibitor of calcium release from the ryanodine-sensitive ER stores, 30 μM) or CdCl\textsubscript{2} (a non-specific calcium channel blocker, 100 μM) or none. After cell identification, fluorescence emission at 510 nm wavelength was monitored with excitation at 340 and 380 nm using the microspectrofluorometer. The F340/F380 was directly related to [Ca\textsuperscript{2+}].

Western blot analysis

PC12 cells were applied with different concentrations of quercetin (0, 50, 100, 150, 200 μM) for 6 h while the acute treatment of peroxynitrite as described above. Then protein samples...
were abstracted in each treatment group, and the concentration was quantified by bicinchoninic acid assay according to manufacturers’ instructions. Protein samples were solubilized by boiling for 5 min, fractioned and separated on 8% SDS-polyacrylamide gel electrophoresis and electrotransferred to polyvinylidene difluoride (nitrocellulose) membranes (PVDF). Each sample of 15 μL contains 20 μg total protein. Then membranes were incubated at room temperature for 2 h in a blocking buffer which contained 5% skimmed fat-free milk. According to the protein marker, the PVDF membranes were then incubated with primary antibody described above for overnight at 4°C in a blocking buffer containing primary antibody separately at a 1:5000 dilution (Hsp70) and a 1:2500 dilution (β-actin). Subsequently, the membranes were washed three times for 10 min with Tris-buffered saline/Tween 20 (TBST) buffer and incubated for 2 h at room temperature with a secondary antibody at a 1:4000 dilution in a blocking buffer. Blots were washed another three times with TBST and detected with chemiluminescent HRP substrate (Immobilon western). Negative control samples were treated with species-appropriate IgG instead of primary antibody. The figures showed representative results from experiments repeated at least three times. For the analysis, quantitation was performed by scanning and determination of the intensity of the hybridization signals. Image J was used to evaluate differences between the sample of interest and its respective β-actin.

**Statistical analysis**

All data were expressed as mean ± standard deviation (S.D.) and analyzed by Origin 8.0 and SPSS 17.0. Statistical analysis were performed by A one-way ANOVA followed by the post-hoc test of Dunnett’s multiple comparison to determine whether there were significant differences between individual groups. Statistical significance was established when P<0.05. There was a minimum of six animals per age group.

**Results**

**Cell viability depletion by peroxynitrite**

As shown in Figure 1A, the cell viability was decreased with the increasing concentration and incubation time. After 6 h incubation, cell viability was decreased, the viability of the cells incubated with the concentration of 100 μM peroxynitrite was not significantly inhibited, but there were significant changes in concentrations of 250 μM, 500 μM and 1 mM. With time increasing, significant inhibition of the SIN-1 was observed at the concentrations of 500 μM and 1 mM compared with that of control group (59.1 ± 5.19, 52.3 ± 4.62)% after 24 h exposure of SIN-1. ANOVA analysis and Dunnnett’s test revealed that PC12 cells were inhibited by peroxynitrite both in a dose-dependent and time-dependent manner.

As shown in Figure 1B, the viability of PC12 cells cultured with the SIN-1 (500 μM) for 24 h
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was 56.94%. When pre-treated with ROS scavenger N-MPG, the viability of PC12 cells was significantly increased to 88.14%. Then the treatment for 24 h was chosen for subsequent experiments on SIN-1-induced cell death.

Measurement of ROS generation

After the PC12 cells were exposed to the SIN-1 of different concentrations of 100, 250, 500 μM and 1 mM for 24 h, the generation of the ROS was elevated. As shown in Figure 2, the ratio of DCF-positive cells was 24.15%, 29.49%, 48.37% and 71.46% at the concentration of 100, 250, 500 μM and 1 mM, respectively compared with the control group (3.21%). Data revealed the level of ROS in PC12 cells exposed to SIN-1 was increased in a dose-dependent manner.

The apoptosis of PC12 cells induced by SIN-1

The apoptosis rate of PC12 cells was increased from 4.87% in control group to 16.24%, 28.57% and 38.38% after the cell exposure to the SIN-1 (100, 250, 500 μM) for 24 h, respectively (Fi-
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Figure 3. The peroxynitrite induced apoptotic cell death in PC12 cells. The flow cytometry assay was carried out for detection of apoptotic cells. (A-C) The cells were treated with 0 μmol/L (A), 250 μmol/L (B), 500 μmol/L (C) of SIN-1 or 24 h. (D) The cells incubated with the peroxynitrite (500 μmol/L) were pre-treated with N-MPG. (E) The corresponding linear diagram of flow cytometry was shown. n = 3, mean ± SEM, *P<0.05.

Effects of quercetin treatment on cell viability

When pre-treated with Hsp inhibitor Quercetin in different concentrations (50, 100, 200 μM), the viability of PC12 cells was significantly decreased compared to the control group as the MTT assay revealed. Except the 50 μM group, the viability of PC12 cells was decreased...
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![Graph](image1)

**Figure 4.** The quercetin application in PC12 cells. The MTT assay was carried out for detection of cell viability. Different concentration of quercetin was applied in PC12 cells. Cells cultured without quercetin served as the control. Results represent the means of three separate experiments, and error bars represent the standard error of the mean, \( ^* P<0.05 \).

Significantly (\( P<0.05, P<0.01 \)). ANOVA analysis revealed that quercetin inhibited the viability of PC12 cells in a concentration-dependent manner (Figure 4).

**Time tracing of \([\text{Ca}^{2+}]_i\) after quercetin treatment in PC12 cells**

SIN-1-treated PC12 cells were exposed to Quercetin (200 μM) as well as different treatments as indicated in **Figure 5** which showed that either quercetin alone (I), dantrolene-containing (30 μM) (II) or Cd\(^{2+}\)-containing (100 μM) (III) were applied and observed. \([\text{Ca}^{2+}]_i\) was increased gradually when cells treated with quercetin alone and also increased with treatment of dantrolene-containing. However, with the treatment of Cd\(^{2+}\)-containing, \([\text{Ca}^{2+}]_i\) was remarkably decreased, and kept at a lower level (\( F_{340}/F_{380} = 0.236 \)).

**Hsp70 and β-actin protein expression assayed by western blot**

PC12 cells in well cultured condition were treated with different concentrations of quercetin. As indicated in **Figure 6**, expression of Hsp70 was significantly decreased in SIN-1-treated group compared with that of control group (\( P<0.01 \)) except the 100 μM concentration group. Besides, this decrease was in a concentration-dependent way in higher concentration applied. There are significant differences of Hsp70 protein among these SIN-1 groups as demonstrated in the **Figure 6**.

**Discussion**

Neurodegenerative diseases are a heterogeneous group of illnesses with distinct clinical phenotypes and genetic etiologies although most neurodegenerative diseases are sporadic with no known genetic linkage. The increase in human lifespan especially in industrialized countries has been accompanied by a marked prevalence of neurodegenerative diseases characterized by progressive dysfunction and loss of neurons [2, 14]. Over the past several years, a pivotal role for oxidative stress shared as a common pathogenic pathway in neurodegenerative diseases is gaining increasingly more acceptance, suggesting that excessive activation of glutamate receptors by excitatory amino acids leads to a number of deleterious consequences, including impairment of calcium buffering, generation of free radicals, activation of the mitochondrial permeability transition and even secondary cytotoxicity [14]. But the underlying mechanisms through which the free radicals participate in neurodegeneration still need further investigation. The aim of the present study was to explore further into the free radicals and cellular damage in PC12 cells to investigate the underlying mechanism involved.

Thus in the present study, the cytotoxicity of the peroxynitrite was assessed in PC12 cells cultured with different concentrations in multiple methods which revealed that peroxynitrite impaired the PC12 cell in a concentration-and time-dependent manner probably by inducing
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intercellular ROS stress and led to cellular apoptosis. What’s more, Hsp70 protein might act to be protective in PC12 cells in damaged conditions and calcium played a very important role for cell living. Firstly, after exposure to varied dose of peroxynitrite, the cell viability and ROS formation were assayed as well as the cell apoptosis. Results revealed that followed the peroxynitrite exposure, massive oxidative stress was tested and ROS level were evaluated and positively correlated with concentrations applied. These were consistent with previous reports. Prior studies in both the morphology and DNA fragmentation of several cell types including cortical neurons, HL-60 cells and rat thymocytes in culture had first revealed a role for peroxynitrite in cell apoptosis [3, 15, 16]. It’s reported in recent studies as well that microglial activation implicated in neurodegenerative disorders are capable of releasing neurotoxic agents, such as proinflammatory cytokines and toxic oxygen/nitrogen species (ROS/RNS) and accumulating evidence shows that activated microglia can damage or kill neurons in vitro by generating nitric oxide (NO) in nanogram grade [2, 17]. NO and superoxide in pathology and stress conditions react to form the neurotoxic peroxynitrite, which has been suggested to be involved in AD. Depending on the dose applied, NO-generating agents exert a dual facilitatory and inhibitory modulation on glutamatergic transmission. It was reported that SIN-1 inhibited excitatory synaptic responses in rat RVLM by decreasing glutamate release mainly through peroxynitrite acting to release adenosine to activate A1 adenosine receptors on presynaptic site. Systematic disorders in diseases enhance oxidative stress and ONOO- mediated oxidization is considered 1000-fold greater than hydrogen peroxide-mediated, and subsequent ONOO formation may enhance development of systemic inflammatory response leading to vicious circle causing severe damages. All studies proposed that peroxynitrite is in possession of cytotoxicity either in vivo or in vitro study in massive cells. What’s more, so far in this aspect, several approaches and signaling pathway to mediate damage has been detected and verified. All these evidences are supportive and consistent with my results proposed in this paper. By generation of massive ROS and massive S-nitrosylation in cellular proteins as detected in the present studies, the exposure of peroxynitrite caused cellular death and apoptosis, possibly by trigger of inflammation response, excitotoxicity of glutamate and oxidant injury of free radicals in various cell types to exert toxic effects.

Hsp70 has been recognized as a potential cell-protective protein almost since its original description in 1974 [18, 19]. Hsp70 is referred to be a protein chaperone that occurs at many stages in the life of a cell by regulating its level of expression during development and through the cell cycle [19-21]. In the present study, we found that expression of Hsp70 was down-regulated significantly when quercetin, a bioflavonoid which was widely used in the researches associated with Hsp70, was applied to the PC12 cells as analyzed [22, 23]. However, the up-expression of Hsp70 was determined by Western Blotting when SIN-1 was applied alone to the cultured PC12 cell. The role of Hsp70 exerted in this study was interesting and seemed to conflicting. Many neurodegenerative diseases manifest conformational changes in proteins that result in misfolding and aggregation [24]. For example, degenerating brain contains aberrant accumulations of misfolded, aggregated proteins, such as a-synuclein and...
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synphilin-1 in PD, and amyloid-β (Aβ) and Tau in AD. Other diseases with aggregates or inclusion bodies include Huntington’s (polyQ), ALS and prion diseases [24-26]. On the other hand, it’s been implied that peroxynitrite causes substantial S-nitrosylation to modify protein function to be responsible for neurodestructive consequences. S-nitrosylation is widely considered as an indicator of ONOO$^-$ toxicity as well which modulates the function of a broad spectrum of extracellular, cytoplasmic membrane and nuclear proteins, in some ways akin to phosphorylation [16, 27]. Recent studies have linked nitrosative stress to protein misfolding and neuronal cell death [28, 29]. The theoretical connection suspected could probably account for the death and apoptosis in SIN-1-exposed PC12 cells as well. Decrease in tyrosine nitration had showed to be capable of attenuating post-bypass inflammation in a rat-CPB model, as evidenced by smaller plasma level of IL-6 and IL-8. Furthermore, increased free Ca$^{2+}$ levels and consequent nitrosative stress are associated with chaperone and proteasomal dysfunction, resulting in accumulation of misfolded aggregates. Molecular chaperones, such as glucose-regulated protein 78 and heat-shock protein, had been showed to provide neuroprotection by facilitating proper protein folding. And as demonstrated in Western blotting, we further made the identification of the inducible chaperone Hsp70 after the treatment with SIN-1. The expression of Hsp70 increased over the concentration of SIN-1 adopted. Our results suggested that Hsp70 expression was induced in response to peroxynitrite exposure to exert to afford protection. This verdict we proposed in this manuscript is consistent with related studies before. A boost in Hsp70 protein was observed in the SIN-1 exposed PC12 cells and manifested that Hsp70 protein was inducible under nitrosative stress situation which might participate in elimination and transportation of misfolded proteins to afford neuroprotective function. To adapt the substantial S-nitrosylation modification of proteins and even aggresomes formation in the cell, Hsp70 was abundantly induced and expressed sought to protect the cell from disregulation and death.

Apoptosis is essential for cell development and homeostasis in eukaryotic organisms, and apoptosis is involved in many diseases including cancers and neurodegenerative disorders such as Alzheimer’s and Parkinson’s diseases. Alteration of intracellular calcium homeostasis plays a great role in the process of apoptosis. The [Ca$^{2+}$] can be changed by the influx of extracellular Ca$^{2+}$ and the release of internal Ca$^{2+}$ stores through channels in the endoplasmic or sarcoplasmic reticulum. In consequence, we sought to explore whether the molecular chaperone Hsp70 played a role during this pathological process since a common sign shared in many neurodegenerative diseases is the accumulation of misfolded proteins that adversely affect neuronal function, connectivity and plasticity and trigger cell death signaling pathways. Since Hsp70 inhibit the cell apoptosis, we examined the connection between Hsp70 and [Ca$^{2+}$], Dantrolene sodium, an inhibitor of Ca$^{2+}$ release from internal stores, and Cd$^{2+}$, a non-specific calcium channel blocker were used respectively to investigate the interaction of Hsp70 and extracellular/intracellular Ca$^{2+}$. Results of microspectrofluorometry showed that Hsp70 was down regulated while [Ca$^{2+}$] was increased gradually, which demonstrated that Hsp70 could inhibit the increase of [Ca$^{2+}$]. However, [Ca$^{2+}$] was decreased when the SIN-1-treated PC12 cells were exposed to quercetin and Cd$^{2+}$. This results indicated that calcium channels could play an important role in the process of [Ca$^{2+}$] increase and Hsp70 probably took part in the inhibition of influx of extracellular Ca$^{2+}$ but had no effect on the release of Ca$^{2+}$ from the intracellular stores. Multiple reports and researches had provided with electrophysiological evidence that SIN-1at micromolar concentrations altered GABAA-receptor-mediated synaptic transmission in developing rat hippocampal slices via the formation of ONOO-. And this manifested that ion channels could be shared as the common targeting spot in both ONOO- associated injuries and Hsp70 protective function in cellular regulation. These results also indicated that the process of the cell apoptosis induced by SIN-1-treated might be triggered by the influx of extracellular Ca$^{2+}$, which was inhibited by the expression of Hsp70 afterwards.

Concerning several lines of evidence demonstrated the antiapoptotic effects of Hsp70 induced by various stress and agents, including NO, oxidative stress, tumor necrosis factor and anticancer drugs. However, the concrete mechanism of antiapoptotic effects of Hsp70 is not fully understood. Hsp70/dj1 or dj2 chaperone pair prevents apoptosis by interacting with Bax
through ATPase domain of Hsp70 and preventing translocation to the mitochondria in CHOP-induced apoptosis. Two groups reported a direct interaction between Apaf-1 and Hsp70 that prevents apoptosome formation. Also, Hsp70 directly binds to apoptosis-inducing factor (AIF), inhibiting AIF-dependent apoptosis mediated by the peptide domain of Hsp70 which is not chaperone-activity dependent. Hsp70 overexpression can also inhibit caspase-dependent events that occur much later in apoptosis, such as activation of cytosolic phospholipase A2 and changes in nuclear morphology. Hsp70 could also protect cells from forced expression of caspase-3. Thus, heat-shock proteins also inhibit events occurring downstream of caspase activation. Stress signals are well-known targets for Hsp70. Insults such as heat shock, UV irradiation, and oxidant stress activate the c-Jun N-terminal kinase (JNK) pathway and induce phosphorylation of c-Jun following by transcription through AP-1. Hsp70 inhibits stress signal induced cell death by suppressing JNK activation. Wild type Hsp70 is suggested to be necessary to suppress caspase-9 and-3 activities in stress signal-induced apoptosis. Hsp70 appears to affect the Bid-dependent apoptotic pathway negatively by inhibiting JNK activation by unclear mechanisms. In hematopoietic cells, Hsp70 is able to protect against TNF-induced apoptosis. Using Drosophila as a model system, Hsp70 was identified as having a major role in preventing cell death and protein aggregation in polyglutamine and Parkinson’s model systems.

In the present study, we showed that SIN-1 impaired cellular function and regulation in PC12 cell to cause apoptosis and death via ROS generation and Ca$^{2+}$ ion homeostasis. Hsp70 might exert neuroprotective role in the peroxynitrite exposure as inducible expression, and can be considered as a strategy for neonatal action control in pathological and neurodegenerative disease.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Xia Li and Dr. Fang Liu, Departments of Neurology and Ultrasound, Xi’an Children’s Hospital, 17 Xi’jun Yuan Xiang Road, Xi’an 710003, China. E-mail: liixiaxan@yeah.net (XL); liufffang@yeah.net (FL)

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