Plumbagin protects against hydrogen peroxide-induced neurotoxicity by modulating NF-κB and Nrf-2

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Abstract

Introduction: Redox signaling initiates pathogenesis of neuronal degeneration. Plumbagin is a potential antioxidant with anti-inflammatory, anti-cancer and radio sensitizing properties. In the present study, we aimed to determine the protective role of plumbagin against H₂O₂-induced neurotoxicity in PC12 cells by determining nuclear factor κB (NF-κB) and nuclear factor E2-related factor 2 (Nrf-2) pathways.

Material and methods: We analyzed oxidative stress by determining reactive oxygen species (ROS) and nitrite levels, and antioxidant enzyme activities. Nrf-2 and NF-κB p65 nuclear localization was determined through immunofluorescence. Further, nuclear levels of p-Nrf-2 and downstream expression of NAD(P)H quinone dehydrogenase 1 (NQO1), heme oxygenase-1 (HO-1) and glutathione-s-transferase (GST) were determined by western blot. Anti-inflammatory activity was analyzed by evaluating NF-κB p65, cyclooxygenase-2 (COX-2) and interleukin (IL-6, IL-8, and MCP-1) expression.

Results: The results showed that plumbagin increased (p < 0.01) the cell viability against H₂O₂-induced cell death in PC12 cells. Plumbagin effectively ameliorated H₂O₂-induced oxidative stress through reducing oxidative stress (p < 0.01) and activating p-Nrf-2 levels. Further, plumbagin up-regulated antioxidant enzyme activities (p < 0.01) against H₂O₂-induced oxidative stress. Plumbagin showed anti-inflammatory effect by suppressing NF-κB p65 activation and down-regulating NF-κB p65 and COX-2 expression. In addition, plumbagin modulated (p < 0.01) inflammatory cytokine expression against H₂O₂-induced neurotoxic effects.

Conclusions: Together, our results show that plumbagin modulated NF-κB and Nrf-2 signaling. Thus, plumbagin might be an effective compound in preventing H₂O₂-induced neurotoxicity and its associated inflammatory responses.

Key words: H₂O₂, oxidative stress, inflammation, Nrf-2, neurotoxicity.

Introduction

Oxidative stress is a common mediator in the development of neurodegenerative diseases, including Alzheimer’s disease (AD), Parkinson’s disease (PD), Huntington’s disease, multiple sclerosis and Creutzfeldt-Jacob disease. Increased generation of reactive oxygen species (ROS), Ca²⁺ deregulation, loss of mitochondrial permeability transition pore (MPP), and improper clearance of mitochondria are the major factors which ultimately lead to mitochondrial dysfunction and neuronal oxidative dam-
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Age. These post-mitotic neurons are extremely sensitive to ROS and thus oxidative stress is associated with apoptosis [1, 2]. Thus, an imbalance in protective mechanisms against such oxidative stress signaling is the main reason for initiation of neuro-toxic effects and degeneration. Endogenous antioxidant defense mechanisms are regulators of oxidative stress. Decrease in antioxidant capacity in neurotoxicity is well established [3, 4]. Improving antioxidant-mediated defense mechanisms is important in modulating oxidative stress and cytoprotection.

Plumbagin is a yellow pigmented secondary metabolite isolated from the roots of Plumbaginaceae, Ancestrocladaceae and Dioncophyllaceae families. The compound is used in treatment of various diseases from ancient times and is identified with multiple functional properties [5]. Plumbagin is a potential antioxidant with anti-inflammatory, anti-cancer and radiosensitizing properties [6–9]. Numerous studies have reported its role in ameliorating redox signaling and thereby preventing oxidative stress associated damage [10, 11]. In this study, we demonstrate the important role of plumbagin in oxidative stress and inflammatory responses against H2O2-induced neurotoxic effects in PC12 cells. In order to evaluate the effect, we identified various oxidative stress markers including ROS, nitrite levels, antioxidant status, nuclear localization of transcription factors NF-κB p65 and p-Nrf-2, expression of redox regulators and inflammatory proteins such as nuclear factor κB (NF-κB) p65, cyclooxygenase-2 (COX-2), p-Nrf-2, NAD(P)H quinone dehydrogenase 1 (NQO1), glutathione-s-transferase (GST) and heme oxygenase-1 (HO-1). Further, anti-inflammatory responses were evaluated through pro-inflammatory cytokine levels.

Chemicals

RPMI-1640, Fetal Calf Serum, Antibiotic and Antimycolytic solution, DCF-DA and interleukins (IL-6, IL-8 and MCP-1) were purchased from Sigma-Aldrich, China. Primary antibodies were: Nrf-2 (Anti-Nrf2 (phospho S40) antibody, Abcam ab76026), GST (Anti-Glutathione S-Transferase antibody, Abcam ab53942), NQO1 (Anti-NQO1 antibody Abcam (ab34173), HO-1 (Abcam-ab13248), NF-κB p65 (Cell Signaling Technology-Phospho-NF-κB p65 (Ser536)), COX-2 (Abcam-ab15191). Secondary antibodies were purchased from Cell Signaling Technology, Beverly, USA.

Cell culture

PC12, pheochromocytoma derived from rat adrenal medulla was procured from American Type Culture Collection (ATCC-CRL-1721). The cells were grown in RPMI-1640 medium supplemented with fetal bovine serum.

Cell viability

The cytotoxic dose of H2O2 was determined through MTT assay. The cells were cultured and cells in log phase were trypsinised and seeded into a 96-well plate. After overnight attachment of the cells, H2O2 at different concentrations (10–50 µM) was added and incubated for 24 and 48 h. The cells after the respective treatment schedule were treated with DMSO and dissolved formazan crystals were measured at 570 nm [12]. The cell viability was calculated and the IC50 value was determined. Protective effect of plumbagin: After attachment, the cells were pre-treated with plumbagin for 24 h (5–25 µM) followed by H2O2 treatment. The appropriate cytoprotective dose selected was used to study the molecular mechanism involved.

Oxidative stress markers

Intracellular ROS generation

Increased ROS levels initiate oxidative stress. The ROS levels were determined by DCF-DA as described previously [13]. PC12 cells were pre-treated with plumbagin (24 h), after which cells were washed with PBS and treated with H2O2. For determining individual effects, the cells were treated with H2O2 and plumbagin as a separate group. After the respective treatment schedule, ROS levels were determined spectrophotometrically (480 nm and 520 nm).

Nitrite estimation

The nitrite levels were determined using the Nitrite/Nitrate Assay Kit, Sigma-Aldrich (23479).

Antioxidant enzyme activities

The specific activity of the antioxidant enzymes was determined using: Superoxide Dismutase Activity Colorimetric Assay Kit (ab65354); Catalase Specific Activity Assay Kit (ab118184), GST Activity Assay Kit (Fluorometric) (ab65325), GPx activity Kit ab102530. The specific activity was calculated and results were expressed as U/mg of protein.

Western blot

After the respective treatment schedule, nuclear and whole cell extracts were isolated and used for protein expression through western blot analysis. 30 µg of protein were separated on precast 12% SDS-PAGE gels and transferred to NC membrane. After blocking, primary antibodies (p-Nrf-2,
GST, NQO1, HO-1, NF-κB p65, COX-2) were added and incubated overnight. Following TBST wash, appropriate secondary antibodies were added and bands were visualized by the enhanced chemiluminescence (ECL) system. Image J software was used for densitometric analysis of western blots.

**Immunofluorescence**

The cells were grown on cover slips and coated with lysine. The cells were allowed to attach to the cover slips and treatment was carried out. Plumbagin was administered for 24 h followed by H₂O₂ for 3 h. After PBS wash, cells were treated with primary antibody (1:50) overnight at 4°C followed by secondary antibody (1:2000). Immunofluorescence was carried out for 2 different antibodies, NF-κB-p65 and p-Nrf-2. The images were acquired and nuclear localization was analyzed through Lumi Vision Imager software.

**Interleukin expression**

Following treatment with plumbagin and H₂O₂, the supernatant was determined for interleukin expression (IL-6, IL-8, and MCP-1); R&D Systems China Co., Ltd.

**Statistical analysis**

The data obtained were statistically analyzed using the t-test. All the experiments were repeated three times in triplicate.

**Results**

**Plumbagin increases cell viability**

Figure 1 A shows dose-dependent cell death induced by H₂O₂ in PC12 neuronal cells. Half maximal inhibitory concentration (IC₅₀) value was shown to be 28 µM. Further, the cytoprotective effect of plumbagin was determined by pre-treatment with plumbagin followed by H₂O₂ treatment. Plumbagin at a concentration of 20 µM showed a protective effect against H₂O₂-induced neurotoxicity (Figures 1 A, B).

**Plumbagin prevents H₂O₂-induced oxidative stress**

H₂O₂-induced oxidative stress is well established. In the present study, we identified that plumbagin treatment significantly ameliorated oxidative stress by reducing reactive oxygen species generation and nitrite levels compared to those of H₂O₂-treated cells. However, plumbagin

![Figure 1](image1.png)

*Figure 1. Plumbagin protects against H₂O₂-induced neurotoxicity. Cell viability in the presence of H₂O₂ (A) and plumbagin (B) was analyzed by MTT assay. Results show cell viability (%). Data are presented as mean ± SD. *p < 0.05, **p < 0.01, compared to control, ***p < 0.001, compared to H₂O₂ treatment.*

![Figure 2](image2.png)

*Figure 2. Plumbagin reduces ROS and nitrite levels. A – Plumbagin reduces H₂O₂-induced ROS generation: ROS is expressed in (%) compared to that of control (100%). B – Plumbagin inhibits nitrite levels: the results are expressed in nanomoles of nitrite formed/mg of protein. Data are presented as mean ± SD. *p < 0.05, **p < 0.01, compared to control, ***p < 0.001, compared to H₂O₂ treatment.*
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and control cells showed non-significant levels of oxidative stress markers (Figures 2 A, B).

**Plumbagin induces p-Nrf-2 levels and antioxidant defense**

Figure 3 A shows p-Nrf-2 protein localization in the cytoplasm during H$_2$O$_2$ treatment; however, plumbagin + H$_2$O$_2$ treatment resulted in increased nuclear levels compared to the control cells. The expression of p-Nrf-2 and target genes (NQO1, GST, and HO-1) and antioxidant enzyme activities were significantly increased during pre-treatment with plumbagin compared to H$_2$O$_2$ treatment alone (Figures 3 B, C).

**Plumbagin reduces NF-κB p65, COX-2 and cytokine levels**

We next determined inflammatory effects through nuclear levels of NF-κB p65. Immunofluorescence re-

*Figure 3. Plumbagin improves antioxidant defense: activation of Nrf-2 target proteins. A – Immuno fluorescence of Nrf-2 levels. (Nrf-2-FITC; nucleus stained with DAPI). Scale bar = 100 µm. B – Western blot of Nrf-2 and downstream proteins. C – Antioxidant enzyme activities

Data are presented as mean ± SD. bp < 0.01, compared to control, dp < 0.01, compared to H$_2$O$_2$ treatment.*
Results showed that H$_2$O$_2$ increased nuclear migration of NF-$\kappa$B p65 (Figure 4 A) and further increased expression of NF-$\kappa$B p65 and COX-2. Treatment with plumbagin followed by H$_2$O$_2$ downregulated NF-$\kappa$B p65 nuclear levels and expression. Further, the downstream target COX-2 expression was suppressed by plumbagin pre-treatment (Figure 4 B). Figure 4 C shows significant up-regulation of interleukin levels (IL-6, IL-8, and MCP-1) upon H$_2$O$_2$ treatment; the increased levels were downregulated by plumbagin treatment.

Discussion

In this study, we showed that plumbagin acted as a potent regulator of H$_2$O$_2$-induced neurotoxicity by reducing redox signaling and inflammation in PC12 cells. Plumbagin significantly reduced H$_2$O$_2$-induced cell death and offered cytoprotection by improving the cell viability. H$_2$O$_2$-induced oxidative stress was reduced by decreasing the ROS levels with a subsequent decline in antioxidant defense mechanisms. Further, plumbagin prevented oxidative stress-induced inflammation by downregulating NF-$\kappa$B signaling and pro-inflammatory cytokine expressions and up-regulating Nrf-2 driven gene expressions.

In order to understand the neuroprotective properties of plumbagin, we first evaluated the cell death induced by H$_2$O$_2$ in PC12 cells. H$_2$O$_2$ dose-dependently induced cell death with an IC$_{50}$ value of...
Plumbagin protects against hydrogen peroxide-induced neurotoxicity by modulating NF-κB and Nrf-2 proteins including COX-2 and inducible nitric oxide synthase. In the present study, H₂O₂ induced NF-κB p65 nuclear translocation and expression with subsequent up-regulation of the inflammatory protein COX-2. However, plumbagin pre-treatment suppressed the inflammation by preventing NF-κB p65 translocation. Further, expression of COX-2 and pro-inflammatory cytokines such as IL-6, IL-8 and MCP-1 was down-regulated. The immunomodulatory effect of plumbagin by suppressing NF-κB signaling was previously reported in ConA-induced inflammation in lymphocytes [6]. Plumbagin prevented cellular invasion by activation of the NF-κB pathway and tumor necrosis factor (TNF-α) mediated apoptosis [5]. Anti-inflammatory effects of plumbagin were demonstrated in lipopolysaccharides-induced inflammation through suppression of NF-κB and MAPK signaling in Raw 264.7 cells [22].

In conclusion, in the present study we demonstrated that plumbagin acts as a neuroprotectant by regulating mechanisms involving both redox signaling and inflammation. Thus, plumbagin might potentially target early activation of ROS and inflammatory proteins, thereby preventing H₂O₂-induced neuronal toxicity and damage.

Conflict of interest

The authors declare no conflict of interest.

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