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

Wang Kuan-Hong[1], Li Bai-Zhou[2]

[1]Department of Neurology, Xinxiang Central Hospital, Henan, China
[2]Department of Pathology, the Second Affiliated Hospital of Zhejiang University Medical College, Hangzhou, China

Submitted: 16 April 2016
Accepted: 23 August 2016

Arch Med Sci 2018; 14, 5: 1112–1118
DOI: https://doi.org/10.5114/aoms.2016.64768

Corresponding author:
Wang Kuan-Hong
Department of Neurology
Xinxiang Central Hospital
56 Jinsui Av.
Xinxiang
453000 Henan, China
Phone/fax: +86 37 2048937
E-mail: wkuanhong@gmail.com

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 H_2O_2-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 H_2O_2-induced cell death in PC12 cells. Plumbagin effectively ameliorated H_2O_2-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 H_2O_2-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 H_2O_2-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 H_2O_2-induced neurotoxicity and its associated inflammatory responses.

Key words: H_2O_2, 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), Ca^{2+} 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-

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 H_2O_2-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 H_2O_2 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, H_2O_2 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 IC_{50} value was determined. Protective effect of plumbagin: After attachment, the cells were pre-treated with plumbagin for 24 h (5–25 µM) followed by H_2O_2 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 H_2O_2. For determining individual effects, the cells were treated with H_2O_2 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 H_2O_2 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 H_2O_2, 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 H_2O_2 in PC12 neuronal cells. Half maximal inhibitory concentration (IC_{50}) value was shown to be 28 µM. Further, the cytoprotective effect of plumbagin was determined by pre-treatment with plumbagin followed by H_2O_2 treatment. Plumbagin at a concentration of 20 µM showed a protective effect against H_2O_2-induced neurotoxicity (Figures 1 A, B).

Plumbagin prevents H_2O_2-induced oxidative stress

H_2O_2-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 H_2O_2-treated cells. However, plumbagin

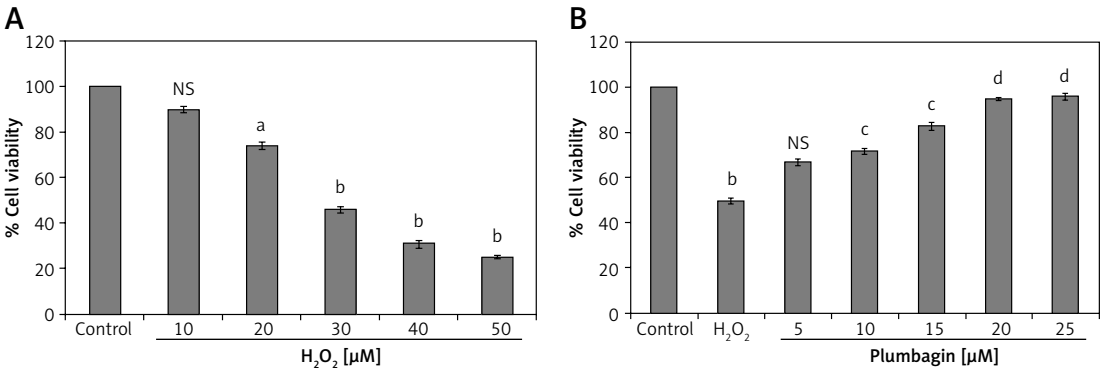

Figure 1. Plumbagin protects against H_2O_2-induced neurotoxicity. Cell viability in the presence of H_2O_2 (**A**) and plumbagin (**B**) was analyzed by MTT assay. Results show cell viability (%)

Data are presented as mean ± SD. [a]p < 0.05, [b]p < 0.01, compared to control, [c]p < 0.05, [d]p < 0.01, compared to H_2O_2 treatment.

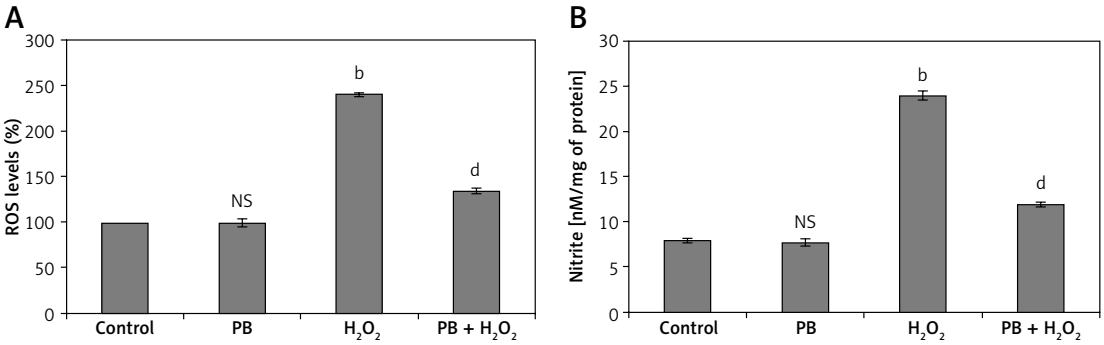

Figure 2. Plumbagin reduces ROS and nitrite levels. **A** – Plumbagin reduces H_2O_2-induced ROS generation: ROS is expressed in (%) compared to that of control (100%). **B** – Plumbagin inhibits nitrite levels: the results are expressed in nanomoles of nitrite formed/mg of protein

Data are presented as mean ± SD. [b]p < 0.01, compared to control, [d]p < 0.01, compared to H_2O_2 treatment.

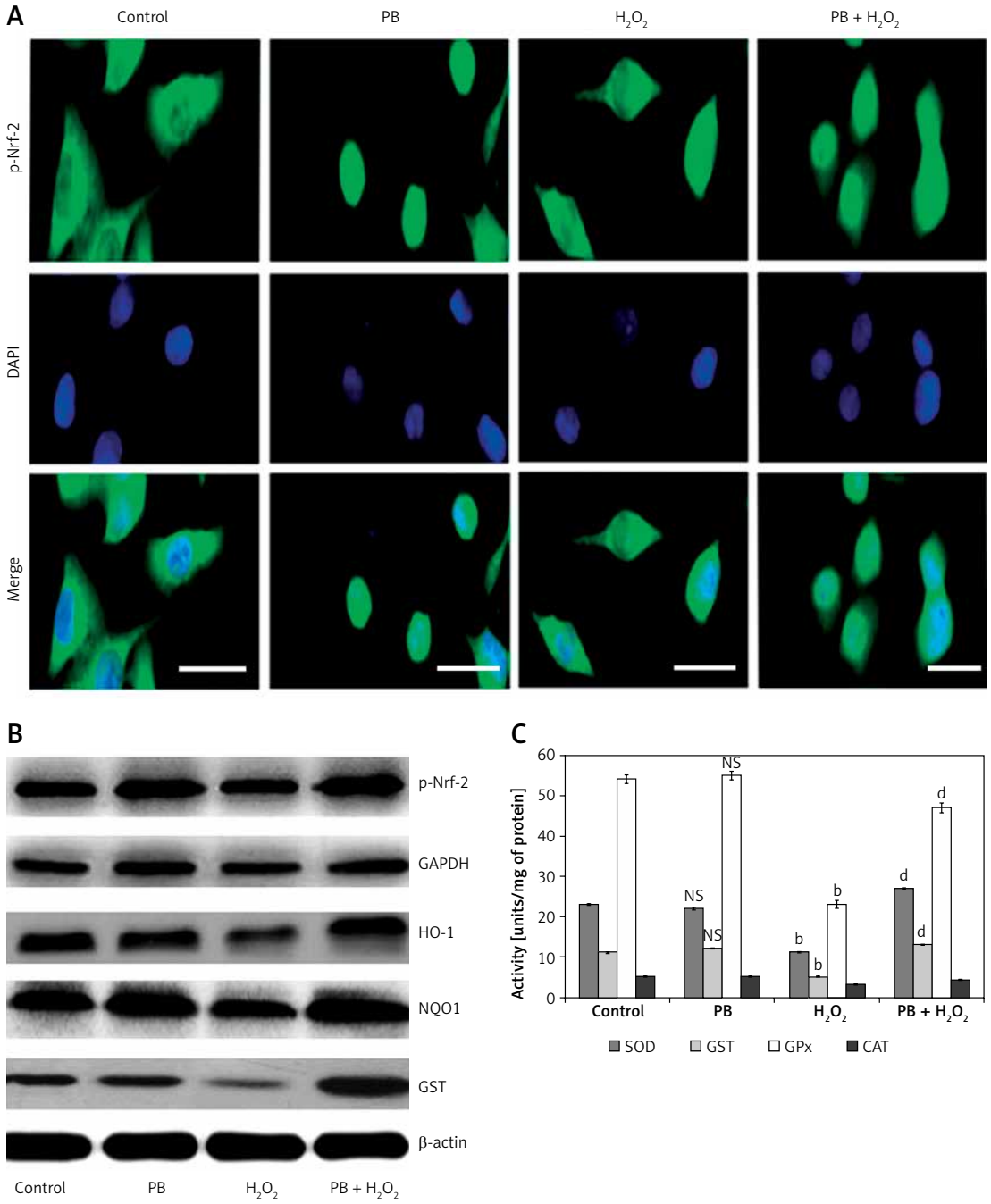

Figure 3. Plumbagin improves antioxidant defense: activation of Nrf-2 target proteins. **A** – Immuno fluorescence of Nrf-2 levels. (Nrf-2-FITC; nucleus stained with DAPI). Scale bar = 100 µm. **B** – Western blot of Nrf-2 and downstream proteins. **C** – Antioxidant enzyme activities

Data are presented as mean ± SD. [b]p < 0.01, compared to control, [d]p < 0.01, compared to H_2O_2 treatment.

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_2O_2 treatment; however, plumbagin + H_2O_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_2O_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-

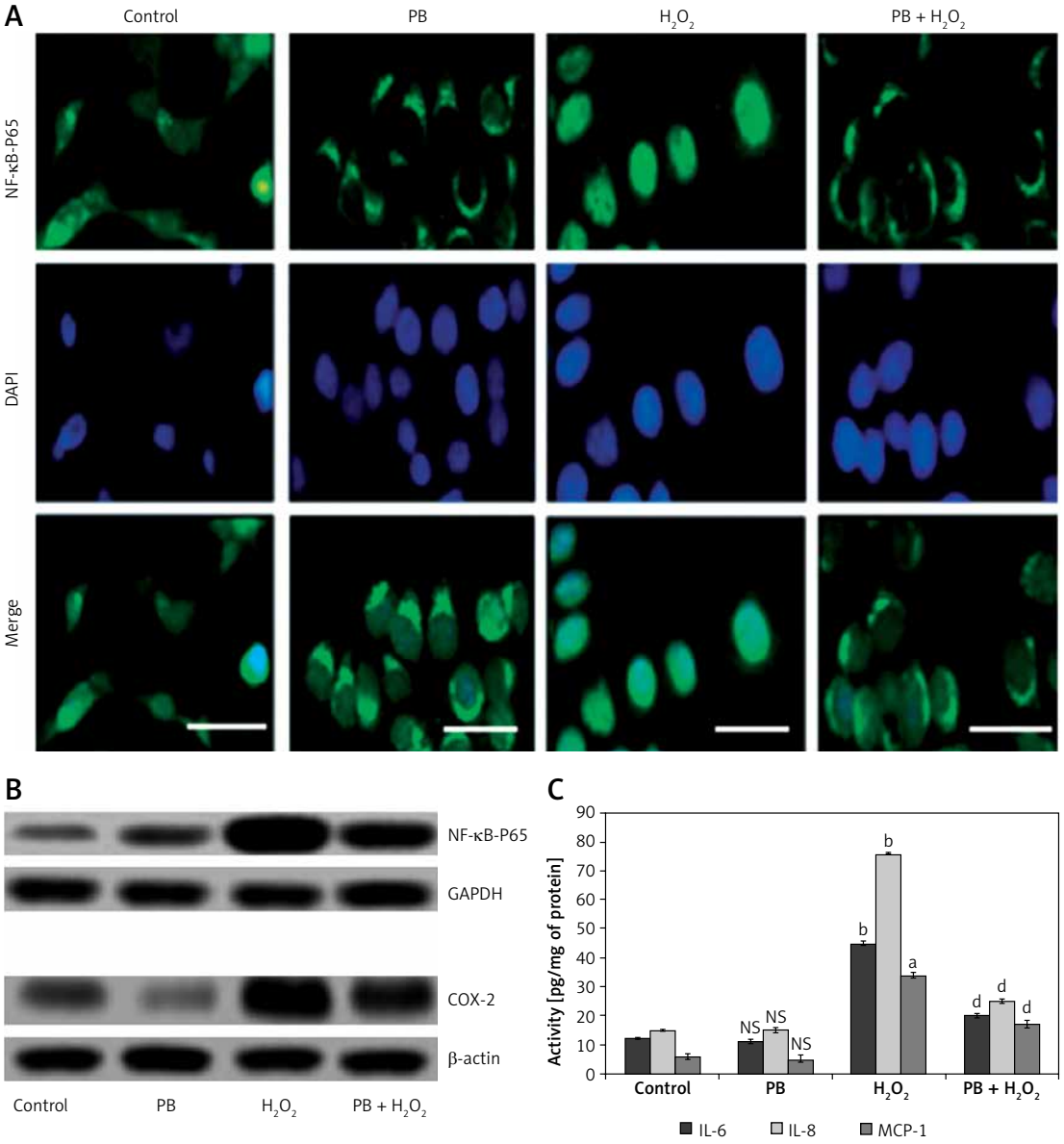

Figure 4. Anti-inflammatory effect of plumbagin against H_2O_2-induced neurotoxicity. **A** – Immunofluorescence of NF-κB levels (NF-κB -FITC; nucleus stained with DAPI). Scale bar = 100 µm. **B** – Western blot of NF-κB and COX-2 expression. **C** – Interleukin expression. The data are expressed in pg/mg of protein

Data are presented as mean ± SD. [a]p < 0.05, [b]p < 0.01, compared to control, [d]p < 0.01, compared to H_2O_2 treatment.

sults showed that H_2O_2 increased nuclear migration of NF-κB p65 (Figure 4 A) and further increased expression of NF-κB p65 and COX-2. Treatment with plumbagin followed by H_2O_2 downregulated NF-κB 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_2O_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_2O_2-induced neurotoxicity by reducing redox signaling and inflammation in PC12 cells. Plumbagin significantly reduced H_2O_2-induced cell death and offered cytoprotection by improving the cell viability. H_2O_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-κB 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_2O_2 in PC12 cells. H_2O_2 dose-dependently induced cell death with an IC_{50} value of

28 μM. Plumbagin pre-treatment significantly improved the cell viability at a concentration ranging from 10 to 25 μM, from which the lowest concentration with the maximum effect was chosen for further studies. Thus, preliminary studies revealed the significant cytoprotective role of plumbagin. Similar neurotoxicity studies have demonstrated that H_2O_2 has a prime role in inducing cell death through apoptotic induction in rat cerebral cortex neurons [14, 15]. Next, we studied the detailed molecular mechanism of plumbagin-induced cellular protective mechanisms. Plumbagin prevented H_2O_2-induced oxidative stress by reducing the generation of ROS levels and nitrite levels. Further, plumbagin prevented redox signaling through increasing the activation of Nrf-2 and the expression of antioxidant defense system proteins such as HO-1, NQO1, and GST. Plumbagin increased the overall antioxidant status by enhancing the activities of glutathione levels and various enzymic antioxidants: SOD, CAT, GST, GPx. Nrf-2 (nuclear factor erythroid 2 (NF-E2)-related factor 2) belongs to the Cap 'n' Collar (CNC) family of proteins involved in cytoprotective defense and survival. In normal conditions, it is associated with Keap-1 (Kelch-like erythroid cell-derived protein with CNC homology (ECH)-associated protein 1) in the cytoplasm, and upon oxidative insult Nrf-2 localizes into the nucleus, thereby escaping Keap-1 mediated ubiquitination. Inside the nucleus, it associates with small Maf proteins, binds to the antioxidant response element (ARE) and induces transcription of NAD(P)H-quinone oxidoreductase 1 (NQO1), heme oxygenase 1 (HO-1), glutamate-cysteine ligase (GCL) and glutathione-S-transferases (GSTs) [16, 17]. In the present study, we found that H_2O_2 prevented the nuclear translocation of p-Nrf-2 and its expression. However, plumbagin significantly prevented these events by inducing nuclear translocation of p-Nrf-2 and increased its expression. In addition, expression of its downstream target proteins such as HO-1, NQO1 and GST was significantly increased during plumbagin pre-treatment. Previous *in vivo* and *in-vitro* studies on plumbagin mediated effects on cerebral ischemia demonstrated the protective role by inducing Nrf-2/ARE signaling and thereby reduced the oxidative insult [18]. Thus, plumbagin offers neuroprotection by increasing the antioxidant defense mechanisms.

Down-regulation of Nrf-2 activates inflammatory signaling [19]; thus oxidative stress and inflammatory activation are interrelated. In neurodegenerative diseases, activation and sustained inflammatory responses lead to dysfunction of neurons, resulting in disease progression [20, 21]. NF-κB p65 activation induces pro-inflammatory signaling, which results in nuclear translocation and activation of various downstream target proteins including COX-2 and inducible nitric oxide synthase. In the present study, H_2O_2 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 H_2O_2-induced neuronal toxicity and damage.

Conflict of interest

The authors declare no conflict of interest.

References

1. Lin MT, Beal MF. Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. Nature 2006; 443: 787-95.
2. Halliwell, B. Oxidative stress, and neurodegeneration: where are we now? J Neurochem 2006; 97: 1634-58.
3. Sumathi T, Shobana C, Thangarajeswari M, Usha R. Protective effect of L-theanine against aluminium-induced neurotoxicity in the cerebral cortex, hippocampus and cerebellum of rat brain – histopathological, and biochemical approach. Drug Chem Toxicol 2015; 38: 22-31.
4. Assaf N, Shalby AB, Khalil WK, Ahmed HH. Biochemical and genetic alterations of oxidant/antioxidant status of the brain in rats treated with dexamethasone: protective roles of melatonin and acetyl-L-carnitine. J Physiol Biochem 2012; 68: 77-90.
5. Sandur SK, Ichikawa H, Sethi G, Ann KS, Aggarwal BB. Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) suppresses NF-kappaB activation and NF-kappaB-regulated gene products through modulation of p65 and IkappaBalpha kinase activation, leading to potentiation of apoptosis induced by cytokine and chemotherapeutic agents. J Biol Chem 2006; 281: 17023-33.
6. Checker R, Sharma D, Sandur SK, Khanam S, Poduval TB. Anti-inflammatory effects of plumbagin are mediated by inhibition of NF-kappaB activation in lymphocytes. Int Immunopharmacol 2009; 9: 949-58.
7. Kuo PL, Hsu YL, Cho CY. Plumbagin induces G2-M arrest and autophagy by inhibiting the AKT/mammalian target of rapamycin pathway in breast cancer cells. Mol Cancer Ther 2006; 5: 3209-21.

8. Hsu YL, Cho CY, Kuo PL, Huang YT, Lin CC. Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) induces apoptosis and cell cycle arrest in A549 cells through p53 accumulation via c-Jun NH2-terminal kinase-mediated phosphorylation at serine 15 in vitro and in vivo. J Pharmacol Exp Ther 2006; 318: 484-94.
9. Nair S, Nair RR, Srinivas P, Srinivas G, Pillai MR. Radiosensitizing effects of plumbagin in cervical cancer cells is through modulation of apoptotic pathway. Mol Carcinog 2008; 47: 22-33.
10. Checker R, Patwardhan RS, Sharma D, et al. Plumbagin, a vitamin K3 analogue, abrogates lipopolysaccharide-induced oxidative stress, inflammation and endotoxic shock via NF-kappaB suppression. Inflammation 2014; 37: 542-54.
11. Checker R, Gambhir L, Sharma D, Kumar M, Sandur SK. Plumbagin induces apoptosis in lymphoma cells via oxidative stress mediated glutathionylation and inhibition of mitogen-activated protein kinase phosphatases (MKP1/2). Cancer Lett 2015; 357: 265-78.
12. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983; 65: 55-63.
13. Royall JA, Ischiropoulos H. Evaluation of 2,7-dichlorofluorescein and dihydrorhodamine 123 as fluorescent probes for intracellular H2O2 in cultured endothelial cells. Arch Biochem Biophys 1993; 302: 348-55.
14. Wang JY, Shum AY, Ho YJ, Wang JY. Oxidative neurotoxicity in rat cerebral cortex neurons: synergistic effects of H2O2 and NO on apoptosis involving activation of p38 mitogen-activated protein kinase and caspase-3. J Neurosci Res 2003; 72: 508-19.
15. Wang W, Gao C, Hou XY, Liu Y, Zong YY, Zhang GY. Activation and involvement of JNK1/2 in hydrogen peroxide-induced neurotoxicity in cultured rat cortical neurons. Acta Pharmacol Sin 2004; 25: 630-6.
16. Jaramillo MC, Zhang DD. The emerging role of the Nrf2-Keap1 signaling pathway in cancer. Genes Dev 2013; 27: 2179-91.
17. Kansanen E, Jyrkkänen HK, Levonen AL. Activation of stress signaling pathways by electrophilic oxidized and nitrated lipids. Free Radic Biol Med 2012; 52: 973-82.
18. Son TG, Camandola S, Arumugam TV, et al. Plumbagin, a novel Nrf2/ARE activator, protects against cerebral ischemia. J Neurochem 2010; 112: 1316-26.
19. Qin WS, Deng YH, Cui FC. Sulforaphane protects against acrolein-induced oxidative stress and inflammatory responses: modulation of Nrf-2 and COX-2 expression. Arch Med Sci 2016; 12: 871-80.
20. Amor S, Puentes F, Baker D, van der Valk P. Inflammation in neurodegenerative diseases. Immunology 2010; 129: 154-69.
21. Glass CK, Saijo K, Winner B, Marchetto MC, Gage FH. Mechanisms underlying inflammation in neurodegeneration. Cell 2010; 140: 918-34.
22. Wang T, Wu F, Jin Z, et al. Plumbagin inhibits LPS-induced inflammation through the inactivation of the nuclear factor-kappa B and mitogen activated protein kinase signaling pathways in RAW 264.7 cells. Food Chem Toxicol 2014; 64: 177-83.