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Plumbagin Mediates Cardioprotection Against Myocardial Ischemia/Reperfusion Injury Through Nrf-2 Signaling

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Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: Plumbagin is a potent antioxidant with anti-inflammatory and anti-carcinogenic action. Myocardial ischemia/reperfusion injury results in organ damage through oxidative stress and inflammatory mechanisms. In this study, we analyzed the potential role of plumbagin against myocardial I/R injury in Wistar rats.

Material/Methods: Oxidative stress was measured through ROS, lipid peroxide content, and antioxidant enzyme activities. The expression of redox signaling and inflammatory proteins was analyzed through Western blotting. Inflammatory cytokine expressions were determined through ELISA.

Results: Oxidative stress status was reduced by plumbagin by decreasing ROS and lipid peroxide levels in rats with myocardial I/R (MI/R) injury. Plumbagin regulated redox imbalance induced by I/R injury by modulating the transcription factors NF-κB and Nrf-2. Further, downstream targets of NF-κB (COX-2, iNOS) and Nrf-2 (HO-1, NQO1 and GST) expression were significantly downregulated by plumbagin treatment. Pro-inflammatory cytokine expressions were significantly abrogated by plumbagin treatment.

Conclusions: This study shows the protective role of plumbagin against myocardial I/R injury by regulating antioxidant and inflammatory mechanisms.

MeSH Keywords: Inflammation • Myocardial Ischemia • Oxidative Stress • Plumbaginaceae

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Background

Ischemia is a condition of poor oxygen supply to the tissues; prolonged ischemia and subsequent reperfusion causes severe irreversible damage to the myocardium [1,2]. Consequences of myocardial ischemia reperfusion (MI/R) injury include thrombolysis, angioplasty, coronary by-pass, and heart transplantation [3–6]. Mediation of reperfusion injury is multifactorial and includes increased production of oxygen free radicals, Ca^{2+} levels, loss of membrane phospholipids, and endothelial dysfunction [7,8]. These factors lead to changes in myocardial functional status. Oxidative stress is one of the key mediators of MI/R injury. Increased reactive oxygen and nitrogen species target cellular protein and lipid moieties. These reactive species arise from the arachidonic acid pathway, mitochondrial electron transport chain, and through neutrophil activation [9]. In recent years, antioxidants have gained much importance in preventing I/R injury because these compounds acts as redox balancers. Antioxidant molecules directly scavenge the free radicals and also induce antioxidant enzyme activities to mediate protective effects.

Plumbagin (5-hydroxy-2-methyl-1, 4-naphthoquinone) is a potent antioxidant. Plants rich is this compound were used in ancient times for protection against heart and liver diseases and for their neuroprotective properties [10]. Plumbagin had been reported to mediate anti-inflammatory, analgesic, and anti-arthritic activities [11,12]. Various studies have reported a potential role in anti-cancer effects, including cancers in the breasts [13] and lungs [14], as well as leukemia [15], melanoma [16], prostate cancer [17], and osteosarcoma [18]. Plumbagin modulates redox status through targeting oxidative stress and redox-sensitive transcription factor (NF-κB) [19,20]. Further, inflammation-associated cytokine expression was significantly reduced by plumbagin and it effectively reduces inflammation and prevents endotoxemia [21]. The potential role of plumbagin in prevention of oxidative stress-associated diseases and other important biological functions [22], as well as antifungal [23] and anti-atherosclerotic [24] action have been reported. In the present study, we aimed at understanding the protective role of plumbagin against MI/R injury and its mechanism.

Material and Methods

Animals and myocardial ischemia-reperfusion (I/R) treatment

Male C57BL6/J mice 8–12 weeks of age were used for the present study. All experimental procedures were approved and followed the guidelines of the Institute for Animal Care and Use Committee at the Chinese Ministry of Education and Chinese Ministry of Health, Qilu Hospital, China. The animals were maintained in separate cages with controlled conditions of temperature (22±1°C) and relative humidity (70–72%) with alternate dark and light cycles. The animals were acclimatized to their environment for 1 week and fed with standard rat pellets and water ad libitum. Surgical ligation of the left coronary artery (LCA) was performed as described previously [25]. The rats were randomly divided into 4 groups with 10 animals in each (n=10): Group 1 (sham); Group 2 (plumbagin); Group 3 (MI/R injury); and Group 4 (plumbagin+ MI/R group) subjected to 45 min of myocardial ischemia followed by 4 h of reperfusion. Animals with MI/R injury received plumbagin (5 mg/kg) with i.p. injection 1 h before the reperfusion. A preliminary study was carried out with different plumbagin concentrations (2.5, 5, and 10 mg/kg) (data not shown); however, 5 mg/kg showed better cytoprotection. Therefore, further studies were performed with this dose. After the treatment, the hearts were removed and the LV was frozen in liquid nitrogen and stored at –80°C.

Oxidative stress parameters

Lipid Peroxidation

The lipid peroxidation content was determined as described by Ohkawa et al. [26]. Thiobarbituric acid reactive substance (TBARS) was measured spectrophotometrically at 532 nm.

Reactive oxygen species

The tissue samples were incubated in 50 µl of a 30-µM c-H_2DCFDA stock solution for 30 min. The reaction mixture was centrifuged and fluorescent intensity was measured using a 485/520-nm filter set. The results are expressed as percentage of ROS generation [27].

Antioxidant status

Non-enzymic antioxidant – Glutathione

The total GSH content was determined using Cayman's GSH assay kit. The principle involves the reaction between the sulfhydryl group of GSH and DTNB (5, 5'-dithiobis-2-nitrobenzoic acid) in the presence of glutathione reductase. The formation of 5-thio-2-nitrobenzoic acid (TNB) in reaction with Ellman's reagent was measured at 412 nm.

Enzymic antioxidant activity – GST

The principle involves measurement of the conjugation product of 1-chloro-2, 4-dinitro benzene (CDNB) with reduced glutathione, which is measured 340 nm. One unit of GST activity is the amount of enzyme producing 1 mmol of CDNB-GSH conjugate/min [28].

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GPx

The principle involves reduction of oxidized glutathione (GSSG) formed during GPx reaction, which is reduced by nicotinamide adenine dinucleotide phosphate (NADPH) and glutathione reductase. Thus, the rate of NADPH consumption is proportional to GSSG formation. The kinetic change was measured at 340 nm (37°C) for 3 min. GPx activity was expressed as mmol of NADPH oxidized/minute/mg protein (U/mg protein) [29].

Catalase

The CAT activity was measured as described by Clairborne (1985) [30]. The principle involves the measurement of CAT activity by the rate of H_2O_2 degradation, which was measured at 230 nm. The results are expressed as H_2O_2 consumed/min/mg protein.

SOD

The SOD activity was estimated as described by Kakkar et al. (1985) [31]. Superoxide dismutase (SOD) activity is based on the inhibition of the formation of (NADH-PMS-NBT) complex. 1 U of SOD activity is calculated as a 50% reduction in NBT/1 min. The results are expressed as U/mg of protein.

ELISA: MCP-1, TNF-α, IL-6, and IL-8 levels

The serum cytokines were measured using Cayman's EIA kit (Cayman Chemicals, Ann Arbor, MI). The protocol was performed as described in the manufacturer's instructions. The interleukin levels are expressed as pg/ml.

Western blot analysis

The tissues (20 mg) were placed in pre-chilled glass petri dishes and minced on ice using sharp scissors followed by addition of 200 µL of cytoplasm isolation buffer (10 mM HEPES-KOH, pH 7.9, at 4°C, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, and 0.2 mM PMSF) incubated on ice for 15 min and centrifuged at 13 000 rpm for 20 min. The supernatant containing the cytoplasm was discarded. Nuclear extract was isolated from the remaining pellets. We added 50 µL of nuclear fraction isolation buffer (20 mM HEPES-KOH, pH 7.9 at 4°C, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, and 0.2 mM EDTA) incubated for 40 min in ice and vortexed at 10-min intervals. The mixture was centrifuged at 13 000 rpm for 5 min. The supernatant containing the nuclear extract was aliquoted in separate tubes and stored at –80°C for further analysis. Approximately 50 µg of protein was loaded on 12% SDS-PAGE gels and resolved at 100 V for 2 h. The proteins were transferred into PVDF membranes. Membranes were blocked with skimmed milk for 1 h. Later, the blots were washed and incubated with primary antibodies directed against NF-κB-p65 and COX-2 (1: 1000, Calbiochem, La Jolla, CA) and Nrf-2, HO-1, NQO1, and GST (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA) proteins overnight at 4°C. After washing in TBST, we added secondary antibody (horseradish peroxidase-coupled rabbit IgG) and incubated for 1 h. The immunoreactive proteins were detected with the ECL-Western blot system (Amersham Pharmacia, Braunschweig, Germany) and subsequent autoradiography for 2 min.

Statistical analysis

Data are expressed as mean ± standard deviation. One-way analysis of variance [ANOVA] followed by Tukey's multiple comparison test and the *t* test were used for statistical analysis. $p<0.05$ was considered significant. All experiments were performed 3 times to ensure reproducibility.

Results

Plumbagin prevents oxidative stress during myocardial I/R injury

Reactive oxygen species generation and lipid peroxide content was significantly higher ($p<0.05$) during myocardial I/R injury in Wistar rats compared to control. Plumbagin treatment reduced the oxidative stress by decreasing ROS and lipid peroxide content ($p<0.05$) compared to myocardial I/R injury rats (Figure 1).

Plumbagin improved antioxidant defense system: Upregulation of antioxidant enzyme levels

Myocardial I/R injury was significantly less ($p<0.05$) in the enzymic and non-enzymic antioxidant defense system of GSH, SOD, CAT, GPX, and GST activities compared to that of sham rats. Rats treated with plumbagin followed by MI/R injury had better ($p<0.05$) antioxidant status than myocardial I/R injury rats (Figure 2).

Plumbagin induces Nrf-2 activation and exerts anti-inflammation

Myocardial I/R injury showed improved oxidative stress and inflammation by upregulation ($p<0.05$) of NF-κB and downregulation of Nrf-2 and their target gene expression compared to that of controls. Plumbagin treatment followed by MI/R injury induced Nrf-2 activation with concomitant increases ($p<0.05$) in HO-1, NQO1, and GST protein expression. Further, plumbagin reduced inflammatory markers such as NF-κB, COX-2, and iNOS protein expression ($p<0.05$) compared to that of MI/R injury rats (Figure 3).

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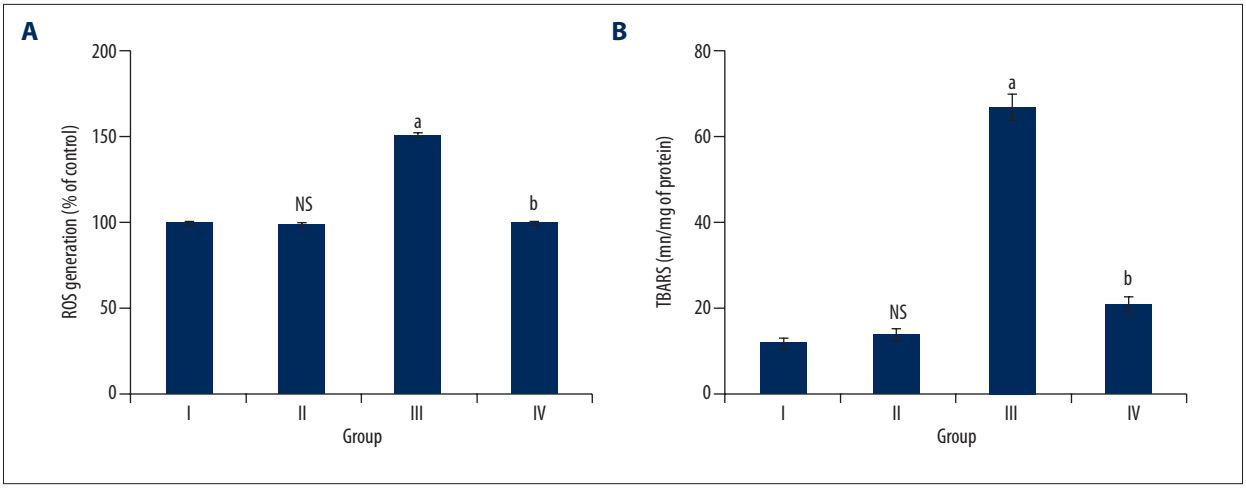

Figure 1. Plumbagin reduces oxidative stress in rats with cardiac I/R injury. (**A**) Plumbagin inhibits ROS generation: The results are expressed as ROS generated (%) when compared to sham rats. (**B**) Plumbagin inhibits Lipid peroxidation: The results are expressed as nanomoles of TBARS formed/mg of protein. Results are expressed in nM/mg of protein. a. p<0.05, when compared to sham group. b. p<0.05, when compared to I/R rats. Group I (sham); Group II (Plumbagin); Group III (MI/R rats); Group III (Plumbagin +MI/R). Results are given as the mean ±SEM for 10 rats in each group. (One-way ANOVA followed by Tukey's multiple comparison).

Plumbagin induced anti-inflammatory effect by decreasing cytokine levels

Figure 4 shows significant upregulation (p<0.05) of pro-inflammatory cytokine expression (MCP-1, IL-6, IL-8, and TNF-α) in MI/R injury compared to sham rats. Cytokine expressions were downregulated (p<0.05) by plumbagin treatment in MI/R injury rats, showing its anti-inflammatory action against myocardial injury (Figure 4).

Discussion

In the present study, we determined that plumbagin provides significant cytoprotection against MI/R injury. Myocardial injury showed a significant increase in reactive oxygen species and modulated oxidative stress and inflammation through Nrf-2 and NF-κB pathways. Plumbagin treatment effectively protected against myocardial I/R injury by upregulating Nrf-2 and downregulating NF-κB expressions.

Myocardial I/R injury impairs the blood supply to the myocardium, leading to irreversible cardiac damage, which is mainly mediated through cell death mechanisms, including apoptosis and necrosis [32]. However, oxidative stress and inflammation are early events, ultimately leading to cell death. Ischemic myocardium converts the reintroduced molecular oxygen during reperfusion and increases the reactive oxygen species [33]. Treatment with antioxidants against myocardial I/R injury might regulate the overall protection mechanism against initiation and progression of myocardial I/R-induced effects. In the present study, plumbagin significantly prevented myocardial oxidative stress by decreasing generation of reactive oxygen species and lipid peroxide content. Plumbagin has 5 functional hydroxyl and 2 methyl groups, it shows potential antioxidant effects and anti-mutagenic effects against gamma radiation-induced oxidative stress [34]. Plumbagin modulated LPS-induced redox status and prevented endotoxic shock and oxidative stress in macrophages [21]. Thus, the antioxidant potential of plumbagin might be involved in the protective effect against myocardial I/R injury through reducing oxidative stress.

The central mechanism in regulating oxidative stress and inflammatory mechanism is redox balance [35]. Cells are equipped with enzymic and non-enzymic antioxidant defence mechanisms to scavenge the free radicals and check the balance, thereby preventing the activation of inflammatory mediators. NF-κB is activated under redox imbalance and regulates the transcription of inflammation-associated genes, including COX-2 [36,37]. In the present study, we found that myocardial I/R injury activated NF-κB and expression of its downstream targets, COX-2 and iNOS [37]. Treatment with plumbagin suppressed the oxidative stress levels and prevented expression of inflammatory proteins. Further, pro-inflammatory cytokine expressions such as TNF-α, MCP-1, IL-6, and IL-8 levels were significantly reduced by plumbagin treatment compared to that of MI/R injury rats. Similar results were reported earlier in studies in which LPS-induced redox status and inflammation was suppressed by plumbagin treatment by downregulating NF-κB, MAPK, and pro-inflammatory cytokines TNF-α and IL-6 [21]. The anti-inflammatory effect of plumbagin was shown to be

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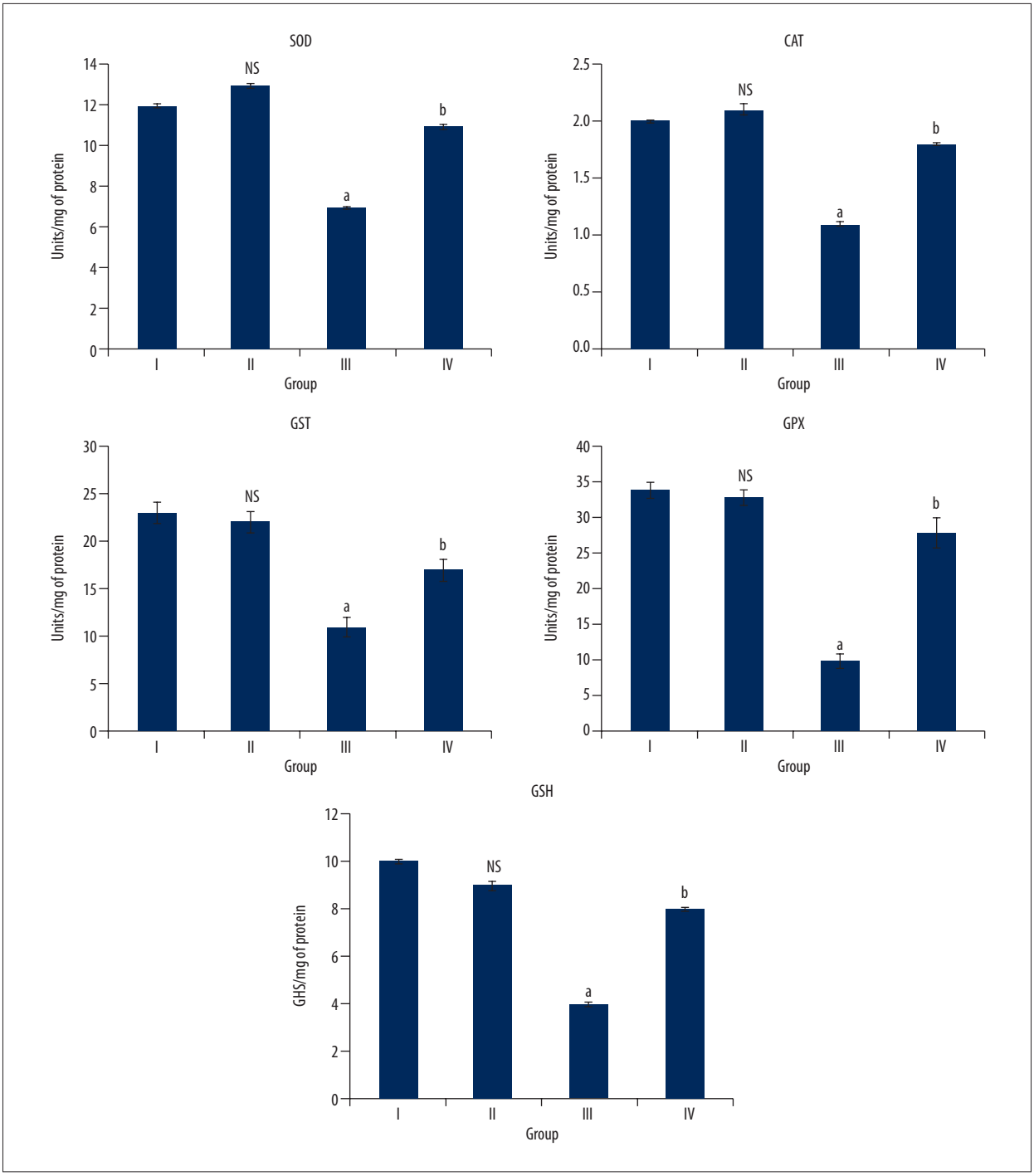

Figure 2. Plumbagin enhances antioxidant status. Antioxidant enzyme activities -SOD, CAT, GST, and GPx are expressed in units/mg protein. GSH is expressed as nmol of GSH/mg of protein. Results are given as the mean ±SEM for 10 rats in each group. a. $p<0.05$, when compared to sham group. b. $p<0.05$, when compared to MI/R rats. Group I (sham); Group II (Plumbagin); Group III (MI/R rats); Group III (Plumbagin + MI/R). (One-way ANOVA followed by Tukey's multiple comparison).

mediated through inhibition of NF-κB, COX-2, and iNOS and pro-inflammatory cytokine expression in rat paw edema models [12]. Thus, plumbagin might regulate inflammatory protein expressions of NF-κB and their downstream targets (COX-2, iNOS), as well as pro-inflammatory cytokine expressions, by regulating ROS levels.

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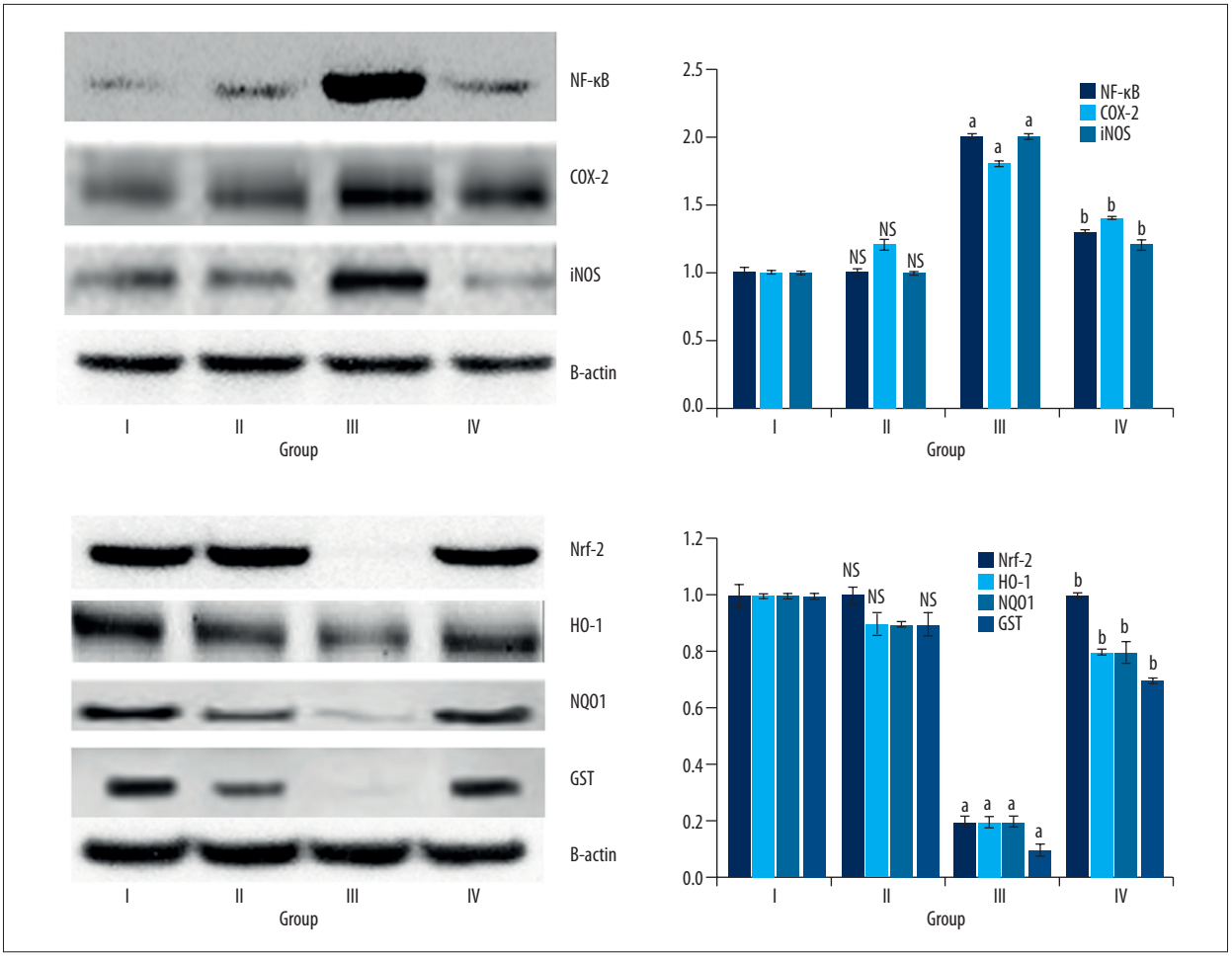

Figure 3. Plumbagin modulates NF-κB and Nrf-2 signaling pathway. NF-κB and Nrf-2 target proteins were determined by Western Blot. a. p<0.05, when compared to sham group. b. p<0.05, when compared to MI/R rats. Group I (sham); Group II (plumbagin); Group III (MI/R rats); Group III (plumbagin +MI/R). Results are given as the mean ±SEM for 10 rats in each group. (One-way ANOVA followed by Tukey's multiple comparison).

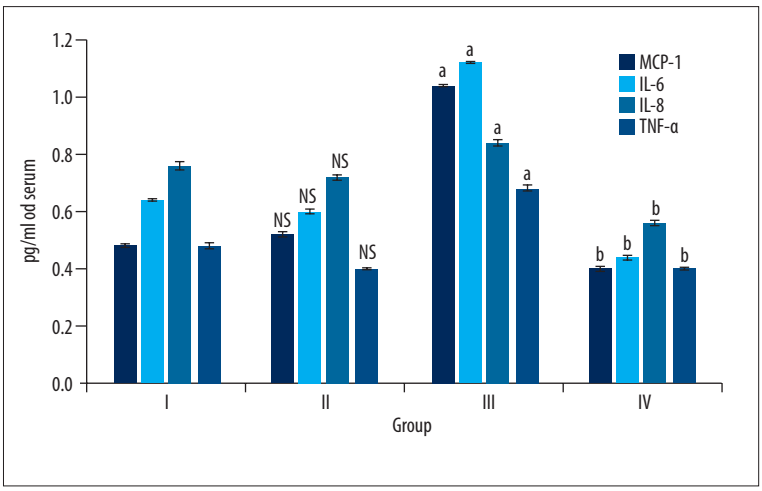

Figure 4. Plumbagin reduces expression of Pro-inflammatory cytokines. Results are expressed as pg/ml of serum. a. p<0.05, when compared to sham group. b. p<0.05, when compared to MI/R rats. Group I (sham); Group II (plumbagin); Group III (MI/R rats); Group III (Plumbagin + MI/R). Results are given as the mean ±SEM for 10 rats in each group. (One-way ANOVA followed by Tukey's multiple comparison).

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One of the main regulators of redox status in the cells is Nrf-2, a redox-sensitive transcription factor. Nrf-2 maintains basal and induced levels of antioxidant status and protects against cellular oxidants. Under basal or un-stressed conditions, Nrf-2 is bound to Kelch-like ECH-associated protein1 (Keap1), which is an endogenous inhibitor that prevents Nrf-2 activation. During stress, Nrf-2 -Keap-1 cysteine interaction is modified, leading to Nrf-2 activation. The Nrf-2 then translocates into the nucleus and binds with other transcription factors and induces antioxidant gene expression through ARE (Antioxidant Response Element) binding [38,39]. Nrf-2 regulates expression of about 200 genes related to the cellular antioxidant defense system, some of which include (NAD(P)H quinone oxyreductase, glutathione, and heme oxygenase-1 (HO-1). Disruption of Nrf-2 levels results in excessive oxidative stress and inflammation, leading to the onset of various diseases [39]. In the present study, myocardial I/R injury induced increased oxidative stress and inflammation, which might be related to downregulation in Nrf-2 levels and antioxidant enzyme activities. Plumbagin improved the antioxidant defense mechanism through activation and expression of Nrf-2. Further, Nrf-2 downstream targets (NQO1, GST, and HO-1) expression were significantly upregulated by plumbagin compared to rats with MI/R injury. Previous studies on plumbagin showed that cerebral ischemia was effectively ameliorated by translocation and expression of Nrf-2 and HO-1 proteins [40]. Thus, plumbagin reduces oxidative stress and inflammation in MI/R injury rats through upregulating Nrf-2 expression and improving antioxidant status.

Conclusions

The present study shows novel evidence of the cardioprotective role of plumbagin against myocardial I/R injury by modulating important mechanisms of oxidative stress and inflammation through Nrf-2 expression.

Conflict of interest

The authors declare no conflict of interest.

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