

# Activation of protein kinase C- $\alpha$ /heme oxygenase-1 signaling pathway improves mitochondrial dynamics in lipopolysaccharide-activated NR8383 cells

XIANGYUN LI<sup>1\*</sup>, YUAN ZHANG<sup>1\*</sup>, JIANBO YU<sup>1\*</sup>, RUI MU<sup>1</sup>, LILI WU<sup>1</sup>,  
JIA SHI<sup>1</sup>, LIRONG GONG<sup>1</sup> and DAQUAN LIU<sup>2</sup>

<sup>1</sup>Department of Anesthesiology, Tianjin Nankai Hospital, Tianjin Medical University; <sup>2</sup>Department of Pharmacology, Institute of Integrated Traditional Chinese and Western Medicine for Acute Abdominal Diseases, Tianjin 300100, P.R. China

Received August 24, 2017; Accepted April 20, 2018

DOI: 10.3892/etm.2018.6290

**Abstract.** Mitochondrial function and morphology are dynamically regulated by fusion and fission. Heme oxygenase-1 (HO-1), which may be upregulated by protein kinase C- $\alpha$  (PKC- $\alpha$ ), improves mitochondrial dynamics by controlling the balance between fusion and fission *in vivo* and *in vitro*. However, whether the PKC- $\alpha$ /HO-1 signaling pathway is one of the underlying mechanisms in adjusting mitochondrial dynamics in lipopolysaccharide (LPS)-activated macrophages has remained elusive. To explore this, NR8383 cells were pre-treated with PKC- $\alpha$  inhibitor Go6976 or PKC- $\alpha$  activator phorbol-12-myristate-13-acetate for 30 min and then stimulated with LPS for 24 h. Next, the expression of PKC- $\alpha$ , HO-1, mitofusin 1 (Mfn1) and mitofusin 2 (Mfn2), optic atrophy 1 (OPA1), dynamin-related protein 1 (Drp1) and fission 1 (Fis1) was detected to evaluate the possible implication of the PKC- $\alpha$ /HO-1 signaling pathway in the LPS-induced NR8383 cells. The results indicated that activation of the PKC- $\alpha$ /HO-1 signaling pathway increased superoxide dismutase activities and the respiratory control ratio (RCR), decreased the levels of malondialdehyde, reactive oxygen species (ROS), Drp1 and Fis1, and simultaneously enhanced the levels of Mfn1,

Mfn2 and OPA1. In contrast, the PKC- $\alpha$  inhibitor decreased the expression of RCR, Mfn1, Mfn2 and OPA1, and increased the expression of MDA and ROS in NR8383 cells. The results suggest that activation of the PKC- $\alpha$ /HO-1 signaling pathway is necessary for the balance of mitochondrial dynamics and oxidative stress in macrophages, which provides clues for probing novel strategies against the detrimental effects of sepsis and other disease states.

## Introduction

Mitochondria are dynamic organelles, which are continuously remodeled through the dynamic processes, including fusion, fission and mitophagy (1). Fusion and fission are opposing actions that are crucial for maintaining the number, size, shape and function of mitochondria (2,3). Mitochondrial fusion is regulated by 3 GTPases, namely mitofusin 1 (Mfn1), mitofusin 2 (Mfn2) and optic atrophy 1 (OPA1), while dynamin-related protein 1 (Drp1) and fission 1 (Fis1) have roles in mitochondrial fragmentation (4,5). Mitochondrial fusion contributes to the formation of mitochondrial networks and facilitates the exchange of proteins, lipids and nucleic acids among defective mitochondria (6,7). Conversely, mitochondrial fission divides the tubular mitochondrial network to ensure that a sufficient number of normally functional mitochondria is present (8). Therefore, balanced mitochondrial dynamics are not only crucial for maintaining proper mitochondrial morphology, but also regulate its functions inside the eukaryotic cells (9).

Heme oxygenase (HO)-1, a low-molecular weight heat shock protein, is thought to protect against pro-oxidant heme release caused by numerous agents, including lipopolysaccharides (LPS), cytokines and reactive oxygen species (ROS) (10,11). *In vitro* and *in vivo*, the beneficial effects of HO-1 through regulating apoptosis and inflammation, protection against oxidative injury as well as contribution to angiogenesis have been demonstrated (12-14). As one of the cytoprotective enzymes, HO-1 eliminates heme and produces carbon monoxide (CO), biliverdin and free iron. In this decade, intensive investigations have demonstrated that endogenous CO also conveys a protective effect through the modulation of antioxidative, anti-apoptotic, anti-proliferative

**Correspondence to:** Professor Jianbo Yu, Department of Anesthesiology, Tianjin Nankai Hospital, Tianjin Medical University, 6 Chang Jiang Road, Tianjin 300100, P.R. China  
E-mail: yujianbo11@126.com

\*Contributed equally

**Abbreviations:** ALI, acute lung injury; CO, carbon monoxide; Drp1, dynamin-related protein 1; Fis1, fission 1; HO-1, heme oxygenase-1; LPS, lipopolysaccharide; MDA, malondialdehyde; Mfn1, mitofusin 1; OPA1, optic atrophy 1; PKC- $\alpha$ , protein kinase C- $\alpha$ ; RCR, respiratory control ratio; ROS, reactive oxygen species; SOD, superoxide dismutase

**Key words:** heme oxygenase-1, mitochondrial dynamics, protein kinase C- $\alpha$

and anti-inflammatory processes (15-17). Beyond these, CO increases mitochondrial ROS leakage by binding to cytochrome oxidase, which promotes the expression of genes associated with mitochondrial biogenesis (10,18).

Of note, previous studies by our group have confirmed that the induction of HO-1/CO by LPS protects organs from septic shock in rats (18-20). Furthermore, *in vivo* and *in vitro* experiments by our group have also demonstrated that the HO-1/CO system contributes to the attenuation of LPS-induced acute lung injury by increasing the expression of mitochondrial fusion proteins and decreasing the levels of mitochondrial fission proteins (20,21). Therefore, it has been known that mitochondrial dynamics may be regulated by the HO-1/CO system during septic shock, but the precise mechanisms have remained to be systematically demonstrated.

The protein kinase C (PKC) family comprises a group of multi-functional serine/threonine kinases that have a key role in signal transduction (22). The PKC family may be classified into three major groups (conventional, novel and atypical PKCs) according to their modes of activation and primary structures (23,24). PKC- $\alpha$  belongs to the conventional classical PKCs (cPKCs; types  $\alpha$ ,  $\beta$ I,  $\beta$ II and  $\gamma$ ), which depend on calcium and diacylglycerol/phorbol esters for activation (23). It has been demonstrated that PKC- $\alpha$  is an important modulator in the upregulation of HO-1 stimulated by LPS in human monocytic cells (25). However, whether the PKC- $\alpha$ /HO-1 signaling pathway is involved in the adjustment of mitochondrial dynamics in LPS-activated macrophages has remained to be elucidated.

Therefore, in the present study, it was hypothesized that HO-1 may protect cells from oxidative stress and improve mitochondrial dynamics in rat macrophages stimulated by LPS via the PKC- $\alpha$ /HO-1 signaling pathway. To the best of our knowledge, the results of the present study are the first to support the notion that the PKC- $\alpha$ /HO-1 signaling pathway regulates mitochondrial dynamics by altering the expression of fusion and fission proteins.

## Materials and methods

**Reagents.** The murine NR8383 macrophage cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). LPS (*Escherichia coli* serotype O111:B4), Go6976 and phorbol-12-myristate-13-acetate (PMA) were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Antibodies specific for PKC- $\alpha$  (cat. no. ab32376), HO-1 (cat. no. ab13248),  $\beta$ -actin (cat. no. ab8227) were obtained from Abcam (Cambridge, UK). Antibodies specific for Mfn1 (cat. no. sc-50330), Mfn2 (cat. no. sc-50331), OPA1 (cat. no. sc-367890), Drp1 (cat. no. sc-32898), Fis1 (cat. no. sc-376447) were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The superoxide dismutase (SOD; cat. no. A001-1) and malondialdehyde (MDA; cat. no. A003-1) kits were supplied by Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

**Cell culture.** Alveolar macrophages (AMs) were incubated in Ham's F-12K medium containing 15% heat-inactivated fetal bovine serum (both Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml penicillin and 100 pg/ml

streptomycin at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Cells were seeded in 6-well plates at a density of 1x10<sup>6</sup> cells/ml. The medium was replaced every 2-3 days. Cell viability was assessed by trypan blue exclusion.

**Experimental grouping.** The wells containing AMs were randomly divided into 7 groups (n=5 each): The control (group C), LPS (group L), LPS+Go6976 (group LG), LPS+PMA (group LP), LPS+DMSO (group LD), Go6976 (group G) and PMA (group P) groups. Group L was stimulated with 10  $\mu$ g/ml LPS (L2630; Sigma-Aldrich; Merck KGaA) to establish the experimental endotoxemia model. The cells in group C received an equal amount of normal saline. To block the PKC- $\alpha$ /HO-1 signaling pathway, group LG was pre-treated with 5  $\mu$ M Go6976 (an inhibitor of PKC- $\alpha$ ) for 30 min prior to stimulation with LPS. Conversely, PMA, a direct PKC activator, was used to active PKC- $\alpha$ ; 100 nM PMA was added for 30 min prior to the incubation of LPS in group LP. Group LD was pre-treated with an equivalent amount of drug vehicle dimethylsulfoxide (DMSO) instead. Group G and group P were pre-treated with Go6976 and PMA respectively. Go6976 and PMA were dissolved in 0.1% DMSO in normal saline.

**Measurement of intracellular ROS production.** Intracellular ROS levels were detected by using the dichloro-dihydro-fluorescein diacetate (DCFH-DA) probe (Beyotime Institute of Biotechnology, Nanjing, China). In brief, after treatment with LPS for 24 h, macrophages were incubated with 10  $\mu$ M DCFH-DA at 37°C for 20 min and then washed twice in PBS (26). DCF fluorescence was monitored with excitation and emission wavelengths of 485 and 535 nm. The results were recorded as the differences in fluorescence relative to the initial one.

**Assessment of SOD and MDA.** The activities of SOD and contents of MDA were measured using commercial reagent kits (Nanjing Jiancheng Bioengineering Institute). All of the measurements and calculations were performed according to the manufacturer's protocols. The content of MDA and the activity of SOD were expressed as millimoles per milligram and U per milligram of protein respectively.

**Mitochondrial respiratory control ratio.** According to the methods described by Carlson *et al* (27), mitochondria were isolated and stored at 0°C in a buffer containing 250 mM sucrose, 10 mM Tris-hydrochloric acid (HCl), 0.5 mM EDTA, and 0.5 g/l fatty acid-free bovine serum albumin (BSA; Invitrogen; Thermo Fisher Scientific, Inc.) (pH 7.2) (21). The respiratory control ratio (RCR) was measured by a Clark-type oxygen electrode (Hansatech, King's Lynn, United Kingdom) at 37°C (28). The respiration medium contained 110 mM sucrose, 60 mM K-lactobionate, 0.5 mM EGTA, 1 g/l BSA essentially fatty acid-free, 3 mM MgCl<sub>2</sub>, 20 mM taurine, 10 mM KH<sub>2</sub>PO<sub>4</sub>, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.1) and 280 IU/ml catalase (Nanjing Jiancheng Bioengineering Institute) (28). A 60- $\mu$ l mitochondrial suspension was incubated in the chamber for 10 min at 25°C. Subsequently, 0.1 or 0.2 mM K-ADP (Nanjing Jiancheng Bioengineering Institute) was added

for the determination of the State 3 respiration (27). Next, 2  $\mu$ M carboxyatractyloside was added to induce a State 4 respiration. Finally, the RCR was calculated as  $\text{RCR} = \text{State 3 respiration} / \text{State 4 respiration}$ .

**RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was extracted from the cells using TRIzol (Thermo Fisher Scientific, Inc.). Complementary DNA was synthesized using the Reverse Transcription Kit (Takara, Otsu, Japan) and Real-time PCR was performed using a SYBR Premix Ex Taq Kit (cat. no. DRR041; Takara) according to the manufacturer's protocols. The expression of PKC- $\alpha$ , HO-1, Mfn1, Mfn2, OPA1, Drp1 and Fis1 was assessed at the same time. The primers were as follows:  $\beta$ -actin, forward: 5'-TGTGTCCGTCGTGGA TCTGA-3' and reverse: 5'-TTGCTGTTGAAGTCGCAG GAG-3' (149 bp); PKC $\alpha$ , forward: 5'-TGGCAAGGTCAT GCTCTCAG-3' and reverse: 5'-GGAAGCAGGAATGGA GCTGA-3' (133 bp); HO-1, forward: 5'-GAATCGAGCAGA ACCAGCCT-3' and reverse: 5'-CTCAGCATTTCTCGGCTTG GA-3' (135 bp); Mfn1, forward: 5'-ACTGTAGGAGGAAGC GGACT-3' and reverse: 5'-CACAACTCTCCGCAAGGCA TC-3' (102 bp); Mfn2, forward: 5'-ACTTCTCCTCTGTTC CAGTTGT-3' and reverse: 5'-GTGCTTGAGAGGGGAAGC AT-3' (181 bp); OPA1, forward: 5'-ACCTTGCCAGTTTAG CTCCC-3' and reverse: 5'-ACCTAACAAGAGAAGGGC CTC-3' (131 bp); Drp1, forward: 5'-GCCTCAGATCGTCGT AGTGG-3' and reverse: 5'-TGCTTCAACTCCATTTTCTTC TCC-3' (187 bp); and Fis1, forward: 5'-TACCCCGAGGCT GTCCTAAG-3' and reverse: 5'-CAGGACATTAGGCCCA GA GC-3' (147 bp). PCR was performed in a final volume of 20  $\mu$ L. The PCR products were amplified using the following thermocycling conditions: 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec and 60°C for 34 sec. An ABI-7500 Sequence Detection System (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used for product detection. The  $\Delta\Delta C_q$  method was used for determining the mRNA levels. Finally, based on the equation  $F = 2^{-\Delta\Delta C_q}$ , the quantity of mRNA was calculated (29).

**Western blot analysis.** The proteins of cell lysates were extracted using a total and nuclear protein isolation kit (Thermo Fischer Scientific, Inc.). Furthermore, the protein concentration was determined using a bicichoninic acid assay kit (Thermo Fischer Scientific, Inc.). Equal amounts of extracted protein (50  $\mu$ g) were fractionated by 12% SDS-PAGE and then transferred to a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA). The membranes were blocked with blocking buffer (PBS with 5% skimmed milk and 0.05% Tween 20) and incubated overnight at 4°C with primary antibodies against PKC- $\alpha$  (1:800 dilution), HO-1 (1:800 dilution), Mfn1 (1:800 dilution), Mfn2 (1:800 dilution), OPA1 (1:500 dilution), Drp1 (1:800 dilution) and Fis1 (1:500 dilution). Next, the blots were incubated at 37°C for 1 h with the horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (1:3,000 dilution; cat. no. CW0156S; Beijing ComWin Biotech Co., Ltd., Beijing, China) after three washes for 10 min each with Tris-buffered saline containing 0.05% Tween-20. The blots were visualized by enhanced chemiluminescence (30) and quantified by densitometry (Molecular Analyst image

analysis software; version 3.0; both Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**Statistical analysis.** Values are expressed as the mean  $\pm$  standard deviation. Parametric data were analyzed by one-way analysis of variance, followed by Dunnett's post-hoc test to determine significant differences between groups. The analyses were performed using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA).  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Effect of PKC- $\alpha$  activity on HO-1 in LPS-activated NR8383 cells.** To evaluate the effect of PKC- $\alpha$  activity on the expression of HO-1, NR8383 cells were pre-treated with 5  $\mu$ M Go6976 and 100 nM PMA for 30 min prior to incubation with 10  $\mu$ g/ml LPS for 24 h. As presented in Fig. 1, compared with those in the control group, the gene and protein expression of PKC- $\alpha$  and HO-1 were increased by treatment with LPS. Furthermore, the protein and mRNA expression of PKC- $\alpha$  and HO-1 exhibited a reduction when cells were pre-treated with Go6976 (Fig. 1). Conversely, the expression of PKC- $\alpha$  and HO-1 was increased when the LPS-activated NR8383 macrophages were pre-treated with PMA (Fig. 1). Of note, Go6976 or PMA alone had no effect on the expression of PKC- $\alpha$  and HO-1 (Fig. 1).

**Levels of ROS and MDA, as well as the RCR and the SOD activity in LPS-induced NR8383 cells.** To investigate the effect of PKC- $\alpha$ /HO-1 signaling on oxidative stress, the levels of ROS and MDA, the RCR and the activity of SOD were analyzed (Fig. 2). Compared with group C, groups L, LG, LP and LD exhibited an apparent increase in the ROS (Fig. 2B) and MDA (Fig. 2A) content and a decline of SOD activity (Fig. 2C) and the RCR (Fig. 2D) after administration of 10  $\mu$ g/ml LPS to NR8383 cells. Pre-treatment with 100 nM PMA, a direct PKC- $\alpha$  activator, increased the RCR (Fig. 2D), improved the SOD activity (Fig. 2C) and decreased the generation of MDA (Fig. 2A) and ROS (Fig. 2B) in the LP group compared with that in the L group. In comparison, pre-treatment with 5  $\mu$ M Go6976 in the LG group had the opposite effect, while the effect on the activity of SOD was not significant.

**The activation of PKC- $\alpha$ /HO-1 signaling pathway improves mitochondrial dynamics in LPS-activated NR8383 cells.** To further address the effect of the PKC- $\alpha$ /HO-1 signaling pathway on mitochondrial dynamics, western blot and RT-qPCR were used to measure the mRNA and protein contents of Mfn1, Mfn2, OPA1, Drp1 and Fis1 in all groups. Compared with those in the control group, LPS significantly induced the mRNA and protein expression of Drp1 and Fis1, while concurrently decreasing the expression of Mfn1, Mfn2 and OPA1 (Figs. 3 and 4). Therefore, the mitochondrial dynamic equilibrium was disturbed by endotoxin in macrophages of rats. To examine whether the activation of the PKC- $\alpha$ /HO-1 signaling pathway has any effect on mitochondrial dynamics in NR8383 cells, cells were pre-treated



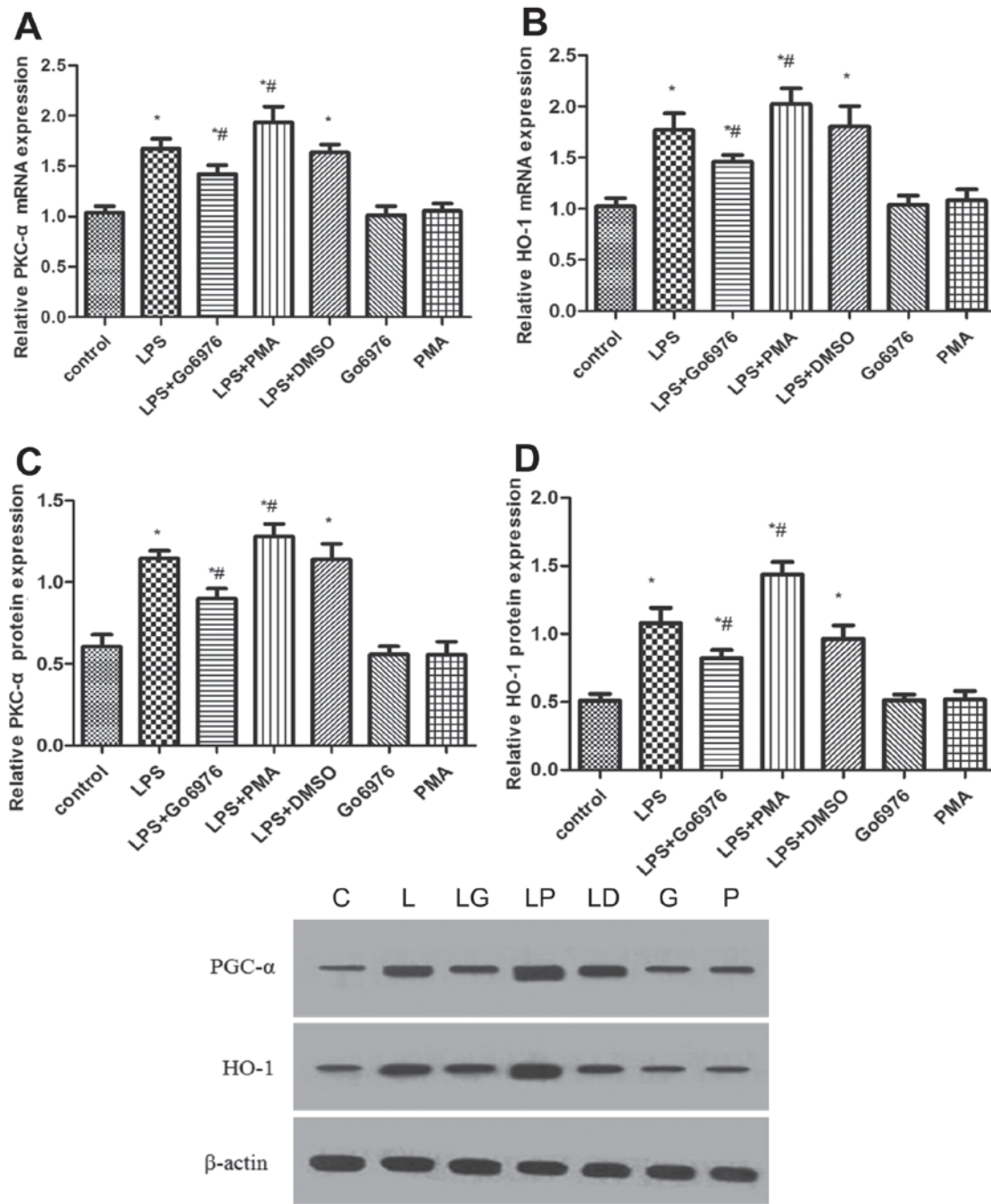


Figure 1. Role of PKC- $\alpha$  in the LPS-induced mRNA and protein expression of HO-1 in NR8383 cells. Reverse transcription-quantitative polymerase chain reaction analysis was used to assess the mRNA levels of (A) PKC- $\alpha$  and (B) HO-1, while western blot analysis was used to assess the protein levels of (C) PKC- $\alpha$  and (D) HO-1. A representative western blot image is presented in the lower panel. After LPS treatment, the mRNA and protein levels of PKC- $\alpha$  and HO-1 were increased, which was attenuated by Go6976 but enhanced in the presence of PMA. \* $P < 0.05$  vs. control, <sup>#</sup> $P < 0.05$  vs. LPS group; analysis of variance followed by Dunnett's post-hoc test ( $n = 5$ ). HO, heme oxygenase; LPS, lipopolysaccharide; PMA, phorbol-12-myristate-13-acetate; PKC, protein kinase C; DMSO, dimethylsulfoxide.

with 5  $\mu$ M Go6976 or 100 nM PMA for 30 min, followed by incubation with 10  $\mu$ g/ml LPS for 24 h. The results indicated that Go6976 blocked the mRNA and protein expression of Mfn1, Mfn2 and OPA1, while increasing Drp1 and Fis1 in the LG group compared with those in the L group (Figs. 3 and 4). However, pre-treatment with PMA caused an elevation of the mRNA and protein levels of Mfn1, Mfn2 and OPA1, and a downregulation of Drp1 and Fis1 in the LP group vs. those in the L group (Figs. 3 and 4).

## Discussion

In the present study, it was demonstrated that activation of the PKC- $\alpha$ /HO-1 signaling pathway in LPS-activated NR8383 cells markedly increased the expression of Mfn1, Mfn2 and OPA1, while decreasing the levels of Drp1 and Fis1, in parallel with an increased expression of HO-1 and PKC- $\alpha$  proteins. Furthermore, activation of the PKC- $\alpha$ /HO-1 signaling pathway significantly increased the SOD activity and the RCR,

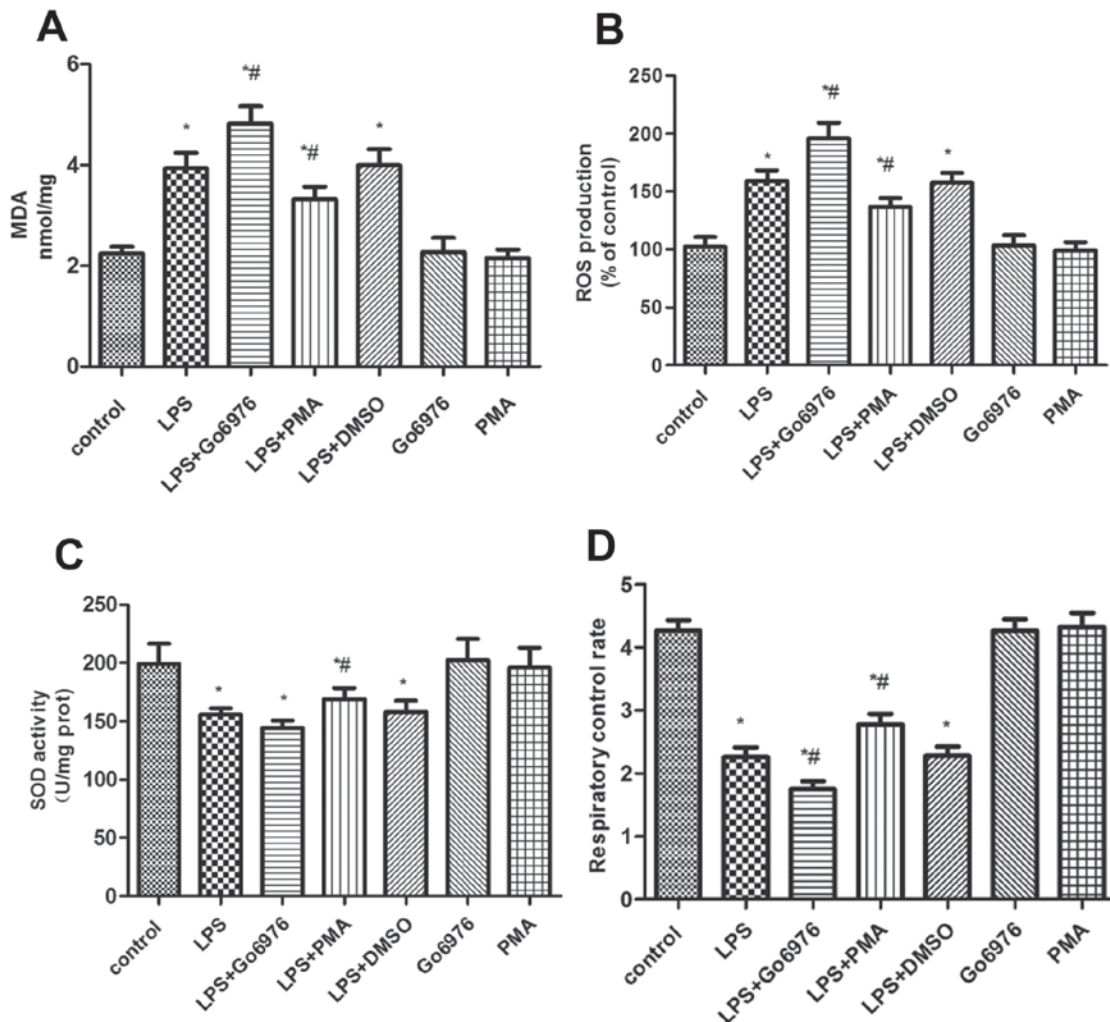


Figure 2. Induction of oxidative stress in NR8383 cells. (A) Levels of MDA, (B) ROS content, (C) SOD activity and (D) RCR levels in the NR8383 cell line following stimulation with LPS. \*P<0.05 vs. control, #P<0.05 vs. LPS group; analysis of variance followed by Dunnett's post-hoc test (n=5). MDA, malondialdehyde; ROS, reactive oxygen species; SOD, superoxide dismutase; RCR, respiratory control rate; PMA, phorbol-12-myristate-13-acetate; DMSO, dimethylsulfoxide; LPS, lipopolysaccharide.

and diminished the MDA and ROS content in LPS-activated NR8383 cells. However, blockade of the PKC- $\alpha$ /HO-1 signaling pathway by pre-treatment with Go6976, a specific inhibitor of PKC- $\alpha$ , had the opposite effect to that of the activator PMA on LPS-activated macrophages.

The RCR is a crucial and versatile indicator of mitochondrial health due to its close association with oxidative phosphorylation (OXPHOS). State 3 respiration reflects the amount of oxygen utilized for ATP production, which is measured following the addition of ADP and phosphoric acid groups. State 4 respiration is obtained after ADP is completely phosphorylated to ATP during OXPHOS. Therefore, the mitochondrial RCR expresses the ratio between the oxygen consumption rate in State 3 vs. the oxygen consumption rate in State 4 (31). A high RCR (>2.5) may be regarded as an indicator of good-quality mitochondrial respiration (31). Consistent with a previous study by our group (21), the present results indicated that NR8383 cells induced by LPS had a lower RCR compared with that of a vehicle-treated control. However, the induction of the PKC- $\alpha$ /HO-1 signaling pathway by PMA effectively attenuated the LPS-induced depression

of mitochondrial function via restoring the RCR. By contrast, exposure to Go6976 significantly reduced the RCR, thereby aggravating the effect of LPS.

HO-1 is an important antioxidant enzyme and exerts a crucial cytoprotective effect in various disease states and organ systems (32-34). In addition, the protective effect of HO-1 is closely associated with the normal function of mitochondria. A recent study has also indicated that HO-1 partially mediates cardiac protection by regulating mitochondrial quality control, comprising mitochondrial dynamics, biogenesis and mitophagy (33). Importantly, overexpression of HO-1 abrogated increases in the expression of Fis1 and elevated the expression of Mfn1 and Mfn2 in mice with doxorubicin-induced dilated cardiomyopathy (33). In line with these results, preceding studies by our group indicated that the HO-1/CO system exerted anti-oxidant effects via increasing the expression of mitochondrial fusion proteins and decreasing the levels of mitochondrial fission proteins in an endotoxin-induced acute lung injury (ALI) model and in LPS-activated RAW 264.7 cells (21). According to previous studies, the experimental model of LPS-activated NR8383 cells was applied in the

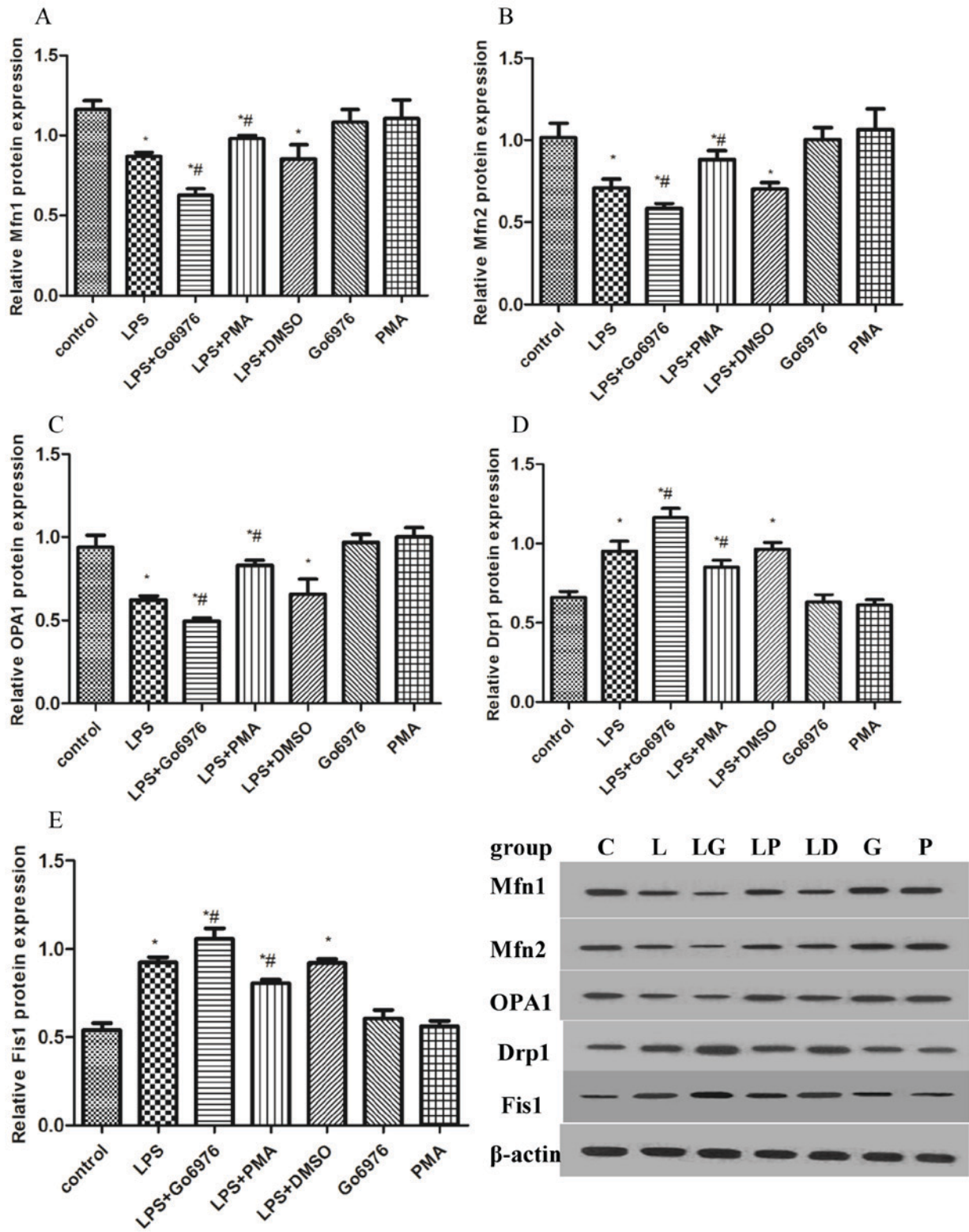


Figure 3. Effects of PKC- $\alpha$ /HO-1 signaling pathway on mitochondrial fusion/fission proteins in response to LPS in NR8383 cells. To evaluate the implication of the PKC- $\alpha$ /HO-1 signaling pathway in the effects of LPS on mitochondrial dynamic markers, NR8383 cells were pre-treated with 5  $\mu$ M Go6976 and 100 nM PMA for 30 min prior to incubation with 10  $\mu$ g/ml LPS for 24 h. The protein levels of the mitochondrial dynamic markers (A) Mfn1, (B) Mfn2, (C) OPA1, (D) Drp1 and (E) Fis1 were assessed by western blot analysis. \* $P$ <0.05 vs. control, # $P$ <0.05 vs. LPS group; analysis of variance followed by Dunnett's post-hoc test ( $n$ =5). PMA, phorbol-12-myristate-13-acetate; DMSO, dimethylsulfoxide; LPS, lipopolysaccharide; Drp1, dynamin-related protein 1; Fis1, fission 1; Mfn, mitofusin; OPA1, optic atrophy 1.

present study. HO-1 expression was induced by stimulation of NR8383 cells with LPS. Furthermore, after pre-treatment with Go6976, low HO-1 expression coincided with low expression

of Mfn1, Mfn2 and OPA1, as well as high levels of Drp1 and Fis1, which supports the protective effect of HO-1 in improving mitochondrial dynamics.



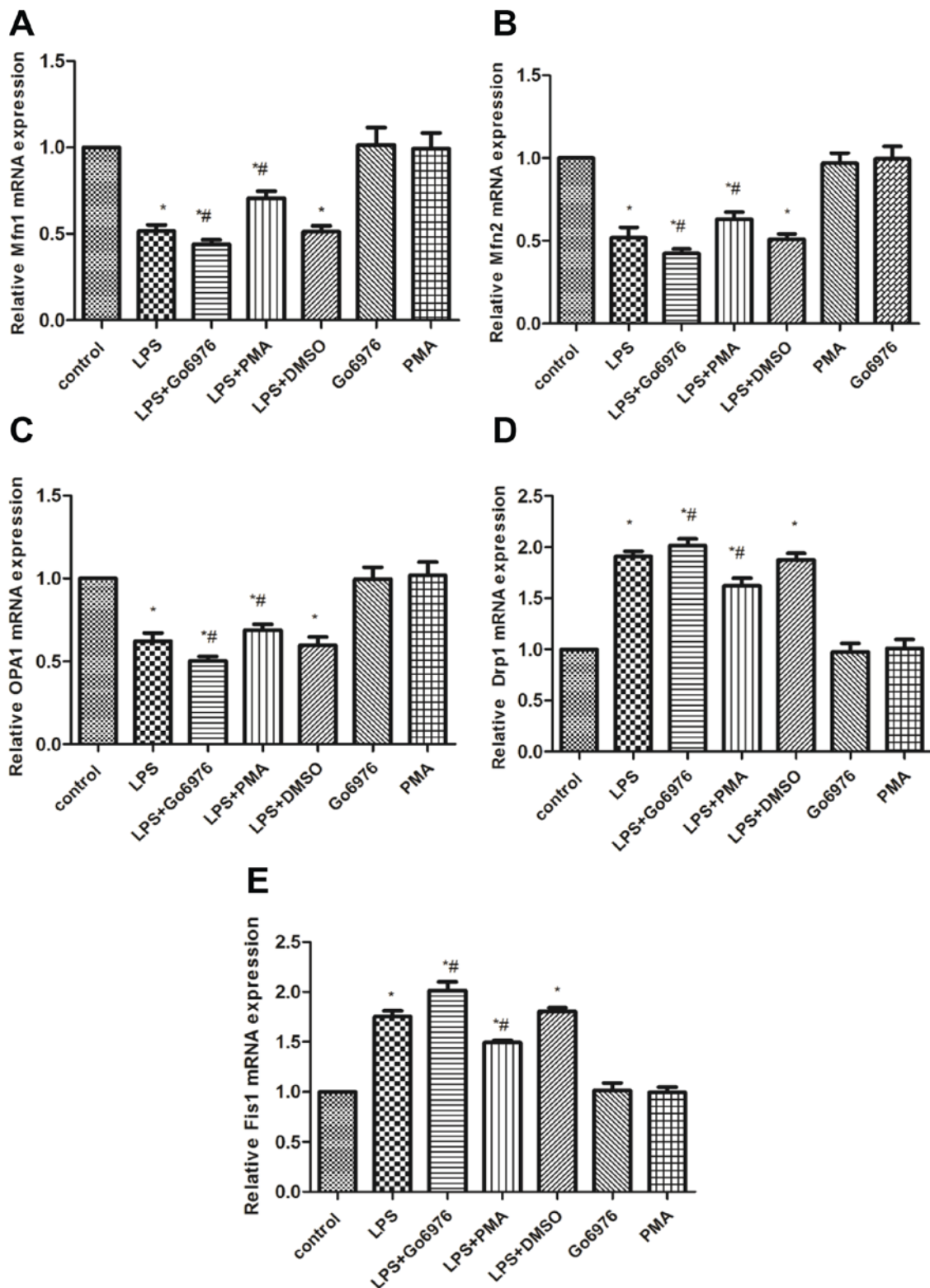


Fig 4. Effects of p PKC- $\alpha$ /HO-1 signaling pathway on the mRNA levels of mitochondrial fusion/fission markers in NR8383 cells induced with LPS. To evaluate the effect of PKC- $\alpha$ /HO-1 signaling pathway on mitochondrial dynamic markers, NR8383 cells were pretreated with 5  $\mu$ M Go6976 and 100 nM phorbol-12-myristate-13-acetate for 30 min prior to the incubation of 10  $\mu$ g/ml LPS for 24 h. The mRNA levels of the mitochondrial dynamic markers (A) Mfn1, (B) Mfn2, (C) OPA1, (D) Drp1 and (E) Fis1 were assessed by reverse transcription-quantitative polymerase chain reaction analysis. \* $P$ <0.05 vs. control, # $P$ <0.05 vs. LPS group; analysis of variance followed by Dunnett's post-hoc test ( $n$ =5). LPS, lipopolysaccharide; Drp1, dynamin-related protein 1; Fis1, fission 1; Mfn, mitofusin; OPA1, optic atrophy 1.

ALI associated with sepsis is the leading cause of mortality in intensive care units. During the course of ALI, alveolar macrophages generate and release a variety of mediators once

activated by bacteria and/or viruses, which in turn promotes excessive recruitment of leukocytes (35). Furthermore, accompanied with an uncontrolled inflammatory response,

numerous kinases and signaling pathways may be stimulated in LPS-activated monocytes (36,37). The activation of PKC, particularly in the PKC- $\alpha$ /HO-1 signaling pathway, has been reported to be involved in the protection against oxidative stress, inflammation and apoptosis (38-40). However, whether HO-1 has any effect on mitochondrial dynamics through PKC- $\alpha$  pathways has remained to be elucidated. In the present study, Go6976, a specific PKC- $\alpha$  inhibitor, decreased the mRNA and protein expression of PKC- $\alpha$  and HO-1 in LPS-induced NR8383 cells, along with the depression of Mfn1, Mfn2 and OPA1, and the increase of Drp1 and Fis1. Furthermore, suppression of PKC- $\alpha$  and HO-1 led to increases in the levels of ROS and MDA, and a reduction of the RCR. Therefore, the present study hypothesized and experimentally verified that the PKC- $\alpha$ /HO-1 signaling pathway is a candidate mechanism via which cells may be protected from oxidative stress injury and mitochondrial function may be regulated by improving mitochondrial dynamics in LPS-activated macrophages.

In summary, the present study indicated that the activation of PKC- $\alpha$  by LPS stimulated the expression of HO-1 protein, which in turn ameliorated mitochondrial injury by increasing the expression of Mfn2 and OPA1, and decreasing the levels of Drp1 and Fis1 in NR8383 cells. Furthermore, PKC- $\alpha$ /HO-1 signaling pathway was identified to be implicated in the anti-oxidant and cytoprotective effects against LPS-induced activation in macrophages. Based on the present results, it may be concluded that the PKC- $\alpha$ /HO-1 signaling pathway constitutes at least one critical signal transduction pathway for modulating mitochondrial dynamics in LPS-activated macrophages. Therefore, the present results provide an approach for further investigation into the function of HO-1 in improving the quality of mitochondrial dynamics as well as the underlying mechanisms, which may be a potential and efficient strategy to protect cells against sepsis.

### Acknowledgements

The authors would like to thank Dr Donghua Li (Department of Pharmacology, Institute of Integrated Traditional Chinese and Western medicine for Acute Abdominal Disease, Tianjin, China) for the technical assistance.

### Funding

The present study was supported by the National Natural Science Foundation of China (grant number 81772106; Beijing, China).

### Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

### Authors' contributions

XL and YZ designed the study and drafted the manuscript. XL, RM, LW, JS and DL conducted the experiments. JY, LG and YZ conceived and supervised the study, and revised the manuscript. All of the authors read and approved the final manuscript, and each author believes that the manuscript represents honest work.

### Ethical approval and consent to participate

Not applicable.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

### References

- DeBalsi KL, Hoff KE and Copeland WC: Role of the mitochondrial DNA replication machinery in mitochondrial DNA mutagenesis, aging and age-related diseases. *Ageing Res Rev* 33: 89-104, 2017.
- Sheridan C and Martin SJ: Mitochondrial fission/fusion dynamics and apoptosis. *Mitochondrion* 10: 640-648, 2010.
- Kieper N, Holmström KM, Ciceri D, Fiesel FC, Wolburg H, Ziviani E, Whitworth AJ, Martins LM, Kahle PJ and Krüger R: Modulation of mitochondrial function and morphology by interaction of Omi/HtrA2 with the mitochondrial fusion factor OPA1. *Exp Cell Res* 316: 1213-1224, 2010.
- Yu J, Wu J, Xie P, Maimaitili Y, Wang J, Xia Z, Gao F, Zhang X and Zheng H: Sevoflurane postconditioning attenuates cardiomyocyte hypoxia/reoxygenation injury via restoring mitochondrial morphology. *PeerJ* 4: e2659, 2016.
- Zhuang X, Maimaitijiang A, Li Y, Shi H and Jiang X: Salidroside inhibits high-glucose induced proliferation of vascular smooth muscle cells via inhibiting mitochondrial fission and oxidative stress. *Exp Ther Med* 14: 515-524, 2017.
- Zhang Q, Tamura Y, Roy M, Adachi Y, Iijima M and Sesaki H: Biosynthesis and roles of phospholipids in mitochondrial fusion, division and mitophagy. *Cell Mol Life Sci* 71: 3767-3778, 2014.
- Lin L, Zhang M, Yan R, Shan H, Diao J and Wei J: Inhibition of Drp1 attenuates mitochondrial damage and myocardial injury in Cocksackievirus B3 induced myocarditis. *Biochem Biophys Res Commun* 484: 550-556, 2017.
- Han XJ, Tomizawa K, Fujimura A, Ohmori I, Nishiki T, Matsushita M and Matsui H: Regulation of mitochondrial dynamics and neurodegenerative diseases. *Acta medica Okayama* 65: 1-10, 2011.
- Hu C, Huang Y and Li L: Drp1-dependent mitochondrial fission plays critical roles in physiological and pathological progresses in mammals. *Int J Mol Sci* 18: pii: E144, 2017.
- Rayamajhi N, Kim SK, Go H, Joe Y, Callaway Z, Kang JG, Ryter SW and Chung HT: Quercetin induces mitochondrial biogenesis through activation of HO-1 in HepG2 cells. *Oxid Med Cell Longev* 2013: 154279, 2013.
- Yu JB, Zhou F, Yao SL, Tang ZH, Wang M and Chen HR: Effect of heme oxygenase-1 on the kidney during septic shock in rats. *Transl Res* 153: 283-287, 2009.
- Constantin M, Choi AJ, Cloonan SM and Ryter SW: Therapeutic potential of heme oxygenase-1/carbon monoxide in lung disease. *Int J Hypertens* 2012: 859235, 2012.
- Loboda A, Damulewicz M, Pyza E, Jozkowicz A and Dulak J: Role of Nrf2/HO-1 system in development, oxidative stress response and diseases: an evolutionarily conserved mechanism. *Cell Mol Life Sci* 73: 3221-3247, 2016.
- Lee SE, Yang H, Son GW, Park HR, Park CS, Jin YH and Park YS: Eriodictyol protects endothelial cells against oxidative stress-induced cell death through modulating ERK/Nrf2/ARE-Dependent heme oxygenase-1 expression. *Int J Mol Sci* 16: 14526-14539, 2015.
- Lin YT, Chen YH, Yang YH, Jao HC, Abiko Y, Yokoyama K and Hsu C: Heme oxygenase-1 suppresses the infiltration of neutrophils in rat liver during sepsis through inactivation of p38 MAPK. *Shock* 34: 615-621, 2010.
- Fredenburgh LE, Perrella MA and Mitsialis SA: The role of heme oxygenase-1 in pulmonary disease. The role of heme oxygenase-1 in pulmonary disease 36: 158-165, 2007.



17. Liu XM, Peyton KJ and Durante W: Ammonia promotes endothelial cell survival via the heme oxygenase-1-mediated release of carbon monoxide. *Free Radic Biol Med* 102: 37-46, 2017.
18. MacGarvey NC, Suliman HB, Bartz RR, Fu P, Withers CM, Welty-Wolf KE and Piantadosi CA: Activation of mitochondrial biogenesis by heme oxygenase-1-mediated NF-E2-related factor-2 induction rescues mice from lethal *Staphylococcus aureus* sepsis. *Am J Respir Crit Care Med* 185: 851-861, 2012.
19. Yu JB and Yao SL: Effect of heme oxygenase-endogenous carbon monoxide on mortality during septic shock in rats. *Ir J Med Sci* 178: 491-496, 2009.
20. Yu J, Wang Y, Li Z, Dong S, Wang D, Gong L, Shi J, Zhang Y, Liu D and Mu R: Effect of heme oxygenase-1 on mitofusin-1 protein in LPS-induced ALI/ARDS in rats. *Sci Rep* 6: 36530, 2016.
21. Yu J, Shi J, Wang D, Dong S, Zhang Y, Wang M, Gong L, Fu Q and Liu D: Heme oxygenase-1/carbon monoxide-regulated mitochondrial dynamic equilibrium contributes to the attenuation of endotoxin-induced acute lung injury in rats and in lipopolysaccharide-activated macrophages. *Anesthesiology* 125: 1190-1201, 2016.
22. Wang G, Chen Z, Zhang F, Jing H, Xu W, Ning S, Li Z, Liu K, Yao J and Tian X: Blockade of PKC $\beta$  protects against remote organ injury induced by intestinal ischemia and reperfusion via a p66shc-mediated mitochondrial apoptotic pathway. *Apoptosis* 19: 1342-1353, 2014.
23. Hug H and Sarre TF: Protein kinase C isoenzymes: Divergence in signal transduction? *Biochem J* 291: 329-343, 1993.
24. Zhou H, Wang Y, Zhou Q, Wu B, Wang A, Jiang W and Wang L: Down-regulation of protein kinase C- $\epsilon$  by prolonged incubation with PMA inhibits the proliferation of vascular smooth muscle cells. *Cell Physiol Biochem* 40: 379-390, 2016.
25. Rushworth SA, Chen XL, Mackman N, Ogborne RM and O'Connell MA: Lipopolysaccharide-induced heme oxygenase-1 expression in human monocytic cells is mediated via Nrf2 and protein kinase C. *J Immunol* 175: 4408-4415, 2005.
26. Yuan ZH, Liang ZE, Wu J, Yi JE, Chen XJ and Sun ZL: A potential mechanism for the anti-apoptotic property of koumine involving mitochondrial pathway in LPS-mediated RAW 264.7 macrophages. *Molecules* 21: pii: E1317, 2016.
27. Carlson DE, Pumplin DW, Ghavam S, Fiedler SM, Chiu WC and Scalea TM: ATP accelerates respiration of mitochondria from rat lung and suppresses their release of hydrogen peroxide. *J Bioenerg Biomembr* 37: 327-338, 2005.
28. Labieniec-Watala M, Siewiera K and Jozwiak Z: Resorcyldiene aminoguanidine (RAG) improves cardiac mitochondrial bioenergetics impaired by hyperglycaemia in a model of experimental diabetes. *Int J Mol Sci* 12: 8013-8026, 2011.
29. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25: 402-408, 2001.
30. Balogun E, Hoque M, Gong P, Killeen E, Green CJ, Foresti R, Alam J and Motterlini R: Curcumin activates the haem oxygenase-1 gene via regulation of Nrf2 and the antioxidant-responsive element. *Biochem J* 371: 887-895, 2003.
31. Jacoby RP, Millar AH and Taylor NL: Assessment of respiration in isolated plant mitochondria using Clark-type electrodes. *Methods Mol Biol* 1305: 165-185, 2015.
32. Chi X, Guo N, Yao W, Jin Y, Gao W, Cai J and Hei Z: Induction of heme oxygenase-1 by hemin protects lung against orthotopic autologous liver transplantation-induced acute lung injury in rats. *J Transl Med* 14: 35, 2016.
33. Hull TD, Boddu R, Guo L, Tisher CC, Traylor AM, Patel B, Joseph R, Prabhu SD, Suliman HB, Piantadosi CA, *et al*: Heme oxygenase-1 regulates mitochondrial quality control in the heart. *JCI insight* 1: e85817, 2016.
34. Zhong ZY and Tang Y: Upregulation of periostin prevents high glucose-induced mitochondrial apoptosis in human umbilical vein endothelial cells via activation of Nrf2/HO-1 signaling. *Cell Physiol Biochem* 39: 71-80, 2016.
35. Ding X, Jin S, Tong Y, Jiang X, Chen Z, Mei S, Zhang L, Billiar TR and Li Q: TLR4 signaling induces TLR3 up-regulation in alveolar macrophages during acute lung injury. *Sci Rep* 7: 34278, 2017.
36. Guha M and Mackman N: LPS induction of gene expression in human monocytes. *Cell Signal* 13: 85-94, 2001.
37. Lee TY, Lee KC, Chen SY and Chang HH: 6-Gingerol inhibits ROS and iNOS through the suppression of PKC- $\alpha$  and NF-kappaB pathways in lipopolysaccharide-stimulated mouse macrophages. *Biochem Biophys Res Commun* 382: 134-139, 2009.
38. Zhang Z, Cui W, Li G, Yuan S, Xu D, Hoi MP, Lin Z, Dou J, Han Y and Lee SM: Baicalein protects against 6-OHDA-induced neurotoxicity through activation of Keap1/Nrf2/HO-1 and involving PKC $\alpha$  and PI3K/AKT signaling pathways. *J Agric Food Chem* 60: 8171-8182, 2012.
39. Lin M, Zhai X, Wang G, Tian X, Gao D, Shi L, Wu H, Fan Q, Peng J4, Liu K and Yao J: Salvianolic acid B protects against acetaminophen hepatotoxicity by inducing Nrf2 and phase II detoxification gene expression via activation of the PI3K and PKC signaling pathways. *J Pharmacol Sci* 127: 203-210, 2015.
40. Lee H, Park YH, Jeon YT, Hwang JW, Lim YJ, Kim E, Park SY and Park HP: Sevoflurane post-conditioning increases nuclear factor erythroid 2-related factor and haemoxygenase-1 expression via protein kinase C pathway in a rat model of transient global cerebral ischaemia. *Br J Anaesth* 114: 307-318, 2015.