



Molecular hydrogen protects against oxidative stress-induced RAW 264.7 macrophage cells through the activation of Nrf2 and inhibition of MAPK signaling pathway

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Abstract

Background Oxidative stress is involved in the development of many inflammatory, metabolic and aging diseases.

Objective In this study we investigated, the protective effects of H₂ on RAW 264.7 macrophage cell against LPS-and H₂O₂-induced oxidative stress by the inhibition of MAPK pathway and also activate the Nrf2 pathway.

Results Our results showed H₂ increased the macrophage cell proliferation and generated ROS and NO against LPS stimulation to exert an active immune response. Similarly, H₂ protected the macrophage cell from H₂O₂-induced oxidative stress. H₂ reduced the LPS-and H₂O₂-induced inflammatory cytokine production and intracellular calcium influxes. H₂ inhibited the LPS-and H₂O₂-induced phosphorylation of MAPK pathway and its downstream signaling molecules. Furthermore, H₂ protected the macrophage cell from mitochondrial apoptosis. H₂ increased Nrf2 protein expression indicating its strong anti-oxidative effects against oxidative stress.

Conclusion Collectively, our results indicate the strong antioxidant role of H₂ against LPS-and H₂O₂-induced oxidative stress on macrophage cells by activating the Nrf2 pathway and inhibiting the MAPK-signaling pathway.

Graphic Abstract

Our results clearly showed that LPS increased the cellular ROS by recognizing the TLR4 and H₂O₂ rapidly increased the cellular (1) and mitochondrial (2) oxidative stress. Excessive ROS/NO molecules cause intracellular calcium influxes (3) As a results imbalance the cellular membrane homeostasis and activate the stress response MAPK signaling pathway with its downstream signaling protein and mitochondrial caspase protein (4) that collapse the anti-oxidant mechanisms and induced the inflammatory cytokine secretion; leads to cell apoptosis (5) Whether H₂ reduced the cellular and mitochondrial oxidative stress, intracellular calcium influxes and inhibits the stress response MAPK, caspase cell signaling pathway through the activation of Nrf2/ARE signaling pathway (6) Consequently, increased the antioxidant enzymes and reduced the inflammatory cytokine that influences the macrophage cell proliferation (7) to protect the cell from apoptosis. The different effects of ROS and H₂ used in this study are indicated in red and green, red arrow depicts ROS effects in the cell, green arrow depicts H₂ effects.

Rahima Begum and Cheol-Su Kim equally contributed to this work.

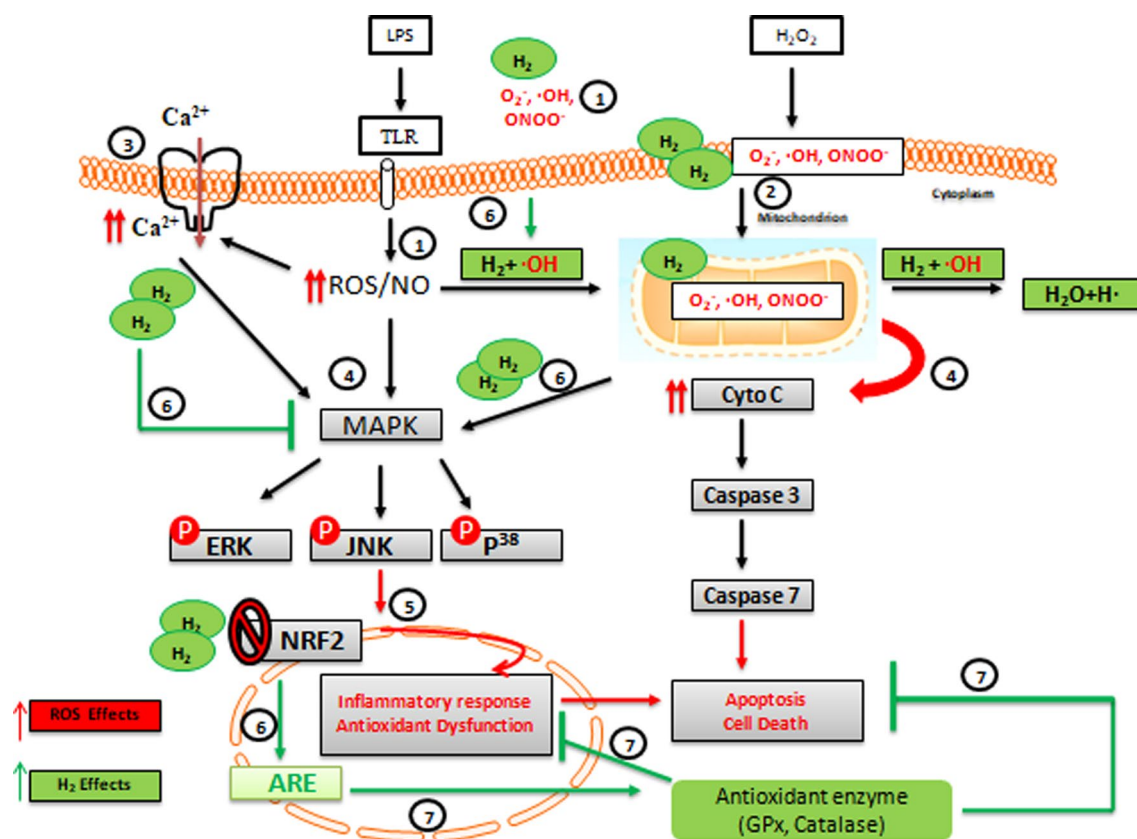
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Keywords Molecular hydrogen · Oxidative stress · Anti-oxidant · Macrophage · MAPK signaling · Nrf2

Introduction

Oxidative stress is defined as the excessive production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in the cells and tissues, wherein antioxidant system fails to neutralize them (Liguori et al. 2018). Under normal physiological condition, reactive oxidants, such as ROS and RNS, are produced in low amounts, where they can act as cellular signaling molecules such as growth factor signaling, mitochondrial autophagy, and nuclear cell division. ROS and RNS are also important in immune function, inflammation and other important cellular and tissue responses (Di Meo et al. 2016). Excessive oxidative stress and imbalance of the protective mechanisms may contribute to the etiology or progression of various inflammatory, metabolic deregulations, and aging associated disease by damaging the cells (Liguori et al. 2018; Di Meo et al. 2016). During the disease progression, various stimuli like lipopolysaccharide (LPS), pro-inflammatory cytokines, growth factors, hormones or oxidative stress may over stimulate the mitogen-activated protein kinase (MAPK) and the mitochondrial caspase pathway, resulting to cell death by apoptotic process (Nagai et al. 2007). MAPK signaling pathway is closely related to

cell proliferation and apoptosis (Nagai et al. 2007; Kim and Choi 2010; Son et al. 2013). In this study, we examined the effects of molecular hydrogen (H₂) in the response of LPS and hydrogen peroxide (H₂O₂) on MAPK and mitochondrial caspase signaling pathway in RAW 264.7 macrophage cells. Macrophage cell is the first line of defense mechanisms in the response of pathogens and oxidative stress (Muñoz Carrillo et al. 2017). To exert the protective mechanisms, macrophage cell secretes the anti-inflammatory cytokines and thus maintain the tissue homeostasis (Muñoz Carrillo et al. 2017; Arango Duque and Descoteaux 2014). Therefore, macrophage cells will be a novel immune cell candidate for therapeutic approach to improve the oxidative stress and inflammatory disease prognosis by H₂. Natural antioxidant and anti-oxidative enzymes has pivotal role in reduction of cellular ROS/RNS and thus terminate the oxidative chain reaction by activating the anti-oxidative nuclear factor erythroid-2-related factor 2 (Nrf2) signaling pathway (Birben et al. 2012; Niture et al. 2014). Over the last decades, H₂ has been a great attention in medical science as a nonfunctional gas which is considered a safe and effective application without any known side effects (Ohsawa et al. 2007). H₂ selectively neutralizes hydroxyl radicals (·OH)

and peroxynitrite (ONOO^-), especially $\cdot\text{OH}$, which are the strongest of the oxidant species, reacts with nucleic acids, lipids and proteins and there is no known endogenous detoxification system for them in the human body (Ohsawa et al. 2007; Ohno et al. 2012; Ohta 2011, 2014). H_2 can protect the cell from oxidative stress and inflammation by increasing the antioxidant enzymes such as catalase, superoxide dismutase or heme-oxygenase-1 (HO-1) (Sun et al. 2009; Xie et al. 2010). In addition, the anti-apoptotic, anti-inflammatory and antioxidant properties of H_2 have also been revealed in various disease models (Kawamura et al. 2010; Shao et al. 2016; Tian et al. 2017). A recent review paper discussed about the biological effects and potential mechanisms of action of H_2 in various diseases, and also stated several problems that are still unresolved such as the absence of studies on concentration–response effects and in signaling pathway (Ge et al. 2017). Although most of the studies addressed effects of H_2 on LPS-induced inflammation of macrophage cell in vitro at different diseases and disease models, the time-dependent macrophage response and its effect in both of LPS- and H_2O_2 -induced oxidative stress and inflammatory response, and the signaling mechanisms are still a concern in the hydrogen research area. Therefore, we performed a time-dependent treatment of H_2 on LPS- and H_2O_2 -induced oxidative stress and confirmed Nrf2 cell signaling mechanisms in RAW 264.7 macrophage cells through the inhibition of MAPK cell signaling pathway.

Materials and methods

Reagents and antibodies

Dulbecco's Modified Eagle Medium (DMEM) and 10% fetal bovine serum (FBS) were purchased from Hyclone Laboratories, Inc. (South Logan, USA). 1% penicillin/streptomycin solution was obtained from Gibco™, Invitrogen Corporation (Auckland, N.Z., USA), Cell counting kit-8 (CCK-8) was obtained from Dojindo Molecular technologies, Inc. (Rockville, MD USA), 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) was obtained from Sigma-Aldrich (St. Louis, MO, USA), Nitric Oxide reagent (Griess reagent kit) was purchased from iNtRON Biotechnology (Sungnam, South Korea). Catalase assay kit, Glutathione Peroxidase (GPx) assay kit and Calcium Colorimetric assay kit were obtained from BioVision Inc. (Milpitas, CA, USA), Bio-plex® Multiple Bead Suspension array kit [tumor necrosis factor (TNF)- α , interleukin (IL)-6, and IL-12] was obtained from Bio-Rad Laboratories (San Diego, CA, USA), Lipopolysaccharide (LPS, serotype O111:B4) was purchased from Sigma Aldrich (Spruce Street, St. Louis, USA) and 30% H_2O_2 was from DAEJUNG (Siheung-si, Gyeonggi-do, Korea), TakaRa BCA protein assay kit was purchased from TAKARA BIO

Inc. (Japan). Antibodies recognizing phospho-extracellular signal-related kinase (p-ERK) 1 and 2, phospho-c-Jun N-terminal kinase (p-JNK), phospho-p38 (p-p38), cytochrome C, cleaved caspase-3, cleaved caspase-7, Nrf2 and β -actin (dilution 1:2000) were rabbit monoclonal IgM and secondary antibodies (dilution 1:5000) were horseradish peroxidase-linked anti-rabbit IgG which were obtained from Cell Signaling Technology (Massachusetts, USA).

Cell culture and LPS/ H_2O_2 stimulation

Murine macrophage RAW 264.7 cells (American Type Cell Culture Collection, USA) were grown in DMEM supplemented with 10% (v/v) heat-inactivated FBS and 100 $\mu\text{g}/\text{mL}$ streptomycin, and cultured in 35 mm dishes at 37 °C with 5% CO_2 in a humidified atmosphere. Confluent cells between 17th to 19th passages were used in these experiments. The cells were seeded at a density of 3×10^5 cells/mL. After 80–90% confluence, the RAW 264.7 cells were treated with LPS (5 $\mu\text{g}/\text{mL}$) for 1 h and H_2O_2 (100 $\mu\text{M}/\text{mL}$) for 30 min by adding 10% serum-free DMEM medium just before the application of H_2 . Before H_2 treatment, cells were washed with cold PBS and replaced with DMEM culture medium supplemented with 10% FBS and 1% antibiotic.

H_2 treatment procedure

The H_2 treatment system was designed following a previous study with some modifications (Murakami et al. 2017). Cell culture dishes or multi-well plates were placed in an airtight sealed acrylic box ($17.1 \times 10^3 \text{ cm}^3$). Then, the box was filled with H_2 gas using H_2 gas generator (HN-IPEM-100, Human Nature Company, Bucheon, Korea) under 0.2 MPa pressure (Fig. 1a). When H_2 gas flowed into the box, the presence of dissolved hydrogen (DH) concentration in the whole box was measured indirectly from water using the DH analyzer (MARK-509, Russia). The concentration of H_2 was maintained within the duration of time (Fig. 1b). The overall H_2 treatment system was illustrated in Fig. 1.

After the treatment with H_2 gas, the culture plates were properly wrapped with aluminium foil, which helped to preserve the H_2 gas for long time (Kurokawa et al. 2015). Then, the plates were incubated at 37 °C at 5% CO_2 incubator for 24 h. After 24 h, the cells were harvested and used for the experiment.

Cell proliferation assay

Cell viability and proliferation were assessed by a CCK-8 reagent (Rockville, MD USA) according to the manufacturer's protocol. In brief, RAW 264.7 murine macrophage cells (1×10^4 cells/well) were plated in a 96-well plate and the plate was kept at 37 °C in 5% CO_2 incubator for 24 h.

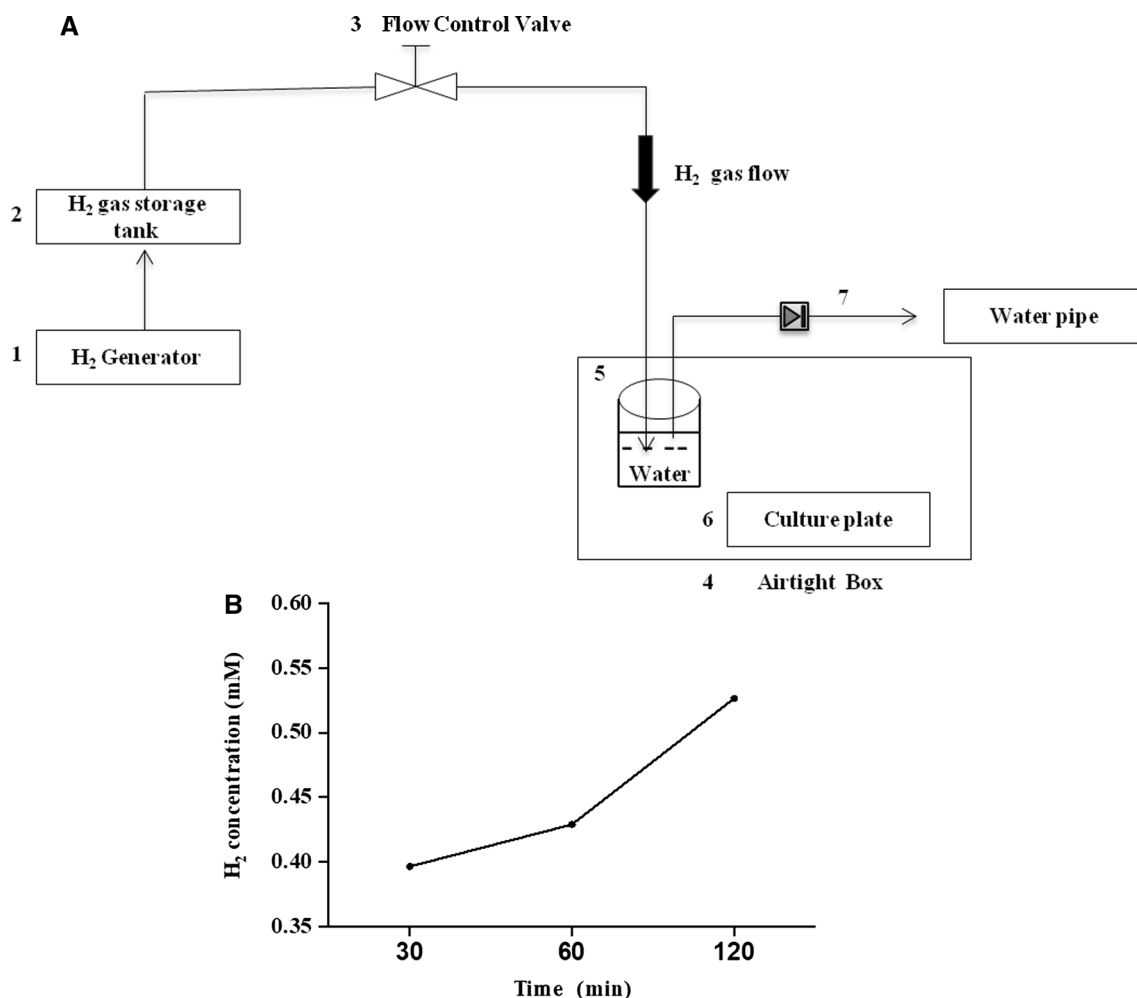


Fig. 1 Illustration of the overall H₂ treatment set-up and procedure. **a** Schematic representation of the H₂ treatment system. (1) H₂ generator (generating H₂ gas at 0.2 MPa pressure). (2) H₂ Gas storage tank (stored gas at a 0.2 MPa pressure approximately about 16 L). (3) Flow control valve. (4) Airtight acrylic box. (5) Bubbler bottle (filled with water, where dissolved H₂ was directly mixed and diffused into the whole box). (6) Cell culture dish 7. Water drawn out line (Water

was drawn out in this line using syringe, to measure the presence of DH concentration into the box or cell culture medium. **b** H₂ concentration in cell culture medium was indirectly measured by dissolved H₂ m (MARK-509, Russia) at a concentration of 1.6 ppm or 0.8 mM concentration. The final H₂ concentration measured at 30, 60 and 120 min were 0.3 mM, 0.4 mM and 0.5 mM, respectively

After treatment of LPS and H₂O₂, the media was discarded and replaced with complete DMEM media and then treated with different doses of H₂ at indicated time. After 12 h of H₂ treatment 10 µL/well CCK-8 solutions were added into each well and incubated for 1 h at 37 °C. The absorbance was measured at 380 nm using a DTX multi-mode micro plate reader (Beckman Counter Inc., USA).

Intracellular total ROS assay

The intracellular ROS levels were determined using a cell-permeable fluorescent DCFH-DA reagent (Sigma-Aldrich; St, Louis, MO, USA) according to the manufacturer's instruction. After treatment of H₂, cells were washed twice

with ice-cold PBS and incubated with 100 µL of 10 µM DCFH-DA in the dark place for 1 h at 37 °C. The intracellular fluorescent product was measured at 380 nm in the DTX-880 multi-mode micro plate reader (Beckman Counter Inc., USA).

Measurement of NO production

The nitrite (NO₂⁻) present in the cell was measured using the Griess reagent (iNtRON). The cells were seeded into 24-well plates (5 × 10⁵ cells/well) and then treated with H₂. After treatment, the cell culture media was centrifuged at 4 °C for 5 min and the supernatant was collected to measure the amount of nitrite. Following the manufacturer's protocol,

50 μ L supernatant of sample was mixed with an equal volume of Griess reagent in a 96-well micro titer plate and incubated at room temperature for 15 min. The absorbance was read at 540 nm using a DTX-880 multimode micro plate reader (Beckman Counter Inc. USA). The results were expressed in μ M/mL.

Intracellular calcium level

The intracellular calcium level was measured using the calcium colorimetric assay kit (BioVision, California, USA). The cells were seeded into 35 mm culture dishes (2×10^4 cells/well) and cell lysate was prepared with calcium assay buffer and was then centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant was collected to measure intracellular calcium level according to manufacturer's protocol. In brief, 50 μ L samples was added into 96-well micro plates and incubated for 30 min. The optical density of each well was analyzed at 590 nm. The results were expressed in mg/dL.

Endogenous antioxidant enzyme activities

The endogenous antioxidant enzymes (catalase and GPx) in cells were measured using the Biovision kit (California, USA). The cells were seeded into 35 mm culture dishes (1×10^6 cells/well) and cell lysate was prepared with assay buffer and was centrifuged at 10,000 rpm for 15 min at 4 °C. Cell supernatant was used to measure the GPx and catalase activities according to the manufacturer's instruction. Briefly, 73 μ L samples for catalase assay and 50 μ L samples for GPx assay were added into 96 well micro plates and incubated for 30 min. The optical density of catalase (570 nm) and GPx (340 nm) were measured using a DTX multi-mode micro plate reader (Beckman Counter Inc., USA). Both enzyme activities results were expressed in nmol/mL.

Cytokine analysis

The inflammatory cytokines such as IL-6, IL-12 and TNF- α were analyzed using Bio-Plex Cytokine Assay (Bio-Rad, California, USA) according to the manufacturer's instructions. Standard curves for each cytokine were generated using the reference concentrations provided in the kits. Mean fluorescence intensity was acquired on a Luminex technology by Bio-Rad's Bio-Plex 200 system Multiplex Bead Array System™ (California, USA) and analyzed with associated software using a 5-parameter logistic method. The results were expressed in pg/mL.

Western blot analysis

The cell supernatant with the normalized protein concentration was equally loaded and separated by SDS-polyacrylamide gel electrophoresis and was transferred

electrophoretically to nitrocellulose membranes (Sartorius, USA). The membrane was then blocked with protein free blocking buffer (TaKaRa, BIO INC., Japan) at room temperature for 2 h and incubated with the following primary antibodies: p-JNK, p-p38, p-ERK1/2, Nrf2, cytochrome *c*, cleaved caspase-3, cleaved caspase-7 and β -actin (dilution: 1:2000; Cell Signaling Technology, Massachusetts, USA) in tris-buffered saline/tween 20 (1X TBST) containing 5% bovine serum albumin overnight in 4 °C. The secondary antibody used was anti-rabbit (dilution 1:2000; Cell Signaling Technology), and then incubated at room temperature for 2 h. Bound antibodies were detected using an enhanced chemiluminescence (ECL Pierce Biotechnology) UVP Biospectrum 600 Imaging System (UVP, LLC, Upland, CA, USA). β -actin (dilution 1: 2000, Cell Signaling Technology) was used as loading controls for the total protein content. Band intensity was analyzed with Image J software (Version 150 win Java, USA).

Data management and statistical analysis

Data values were expressed as the mean value \pm standard deviation (SD). The mean values among the groups were analyzed and compared using one-way analysis of variance (ANOVA) followed by subsequent multiple comparison test (Tukey) with GraphPad Prism 5.0 software package (GraphPad, LAJolla, CA, USA). Differences were considered statistically significant at $p < 0.05$.

Results

H₂ increased cell viability of LPS- and H₂O₂-induced oxidative damage in RAW 264.7 macrophages

In this study, we firstly evaluated the cytoprotective effect of H₂ by measuring the cell viability of LPS- and H₂O₂-induced damaged cells, which was assayed by the CCK-8 assay. Upon induction of LPS and H₂O₂, the cell viability was decreased as compared to normal control cell (Fig. 2a, b). However, after H₂ treatment, the cell viability of LPS-induced oxidative damaged macrophage cells were slightly increased in H₂ in time-dependent manner, but not significantly changed as compared to only LPS-induced group (Fig. 2a). In case of H₂O₂-induced group, the cell viability increased at 120 min of H₂ treatment as compared to only H₂O₂-induced group, but not significant (Fig. 2b).

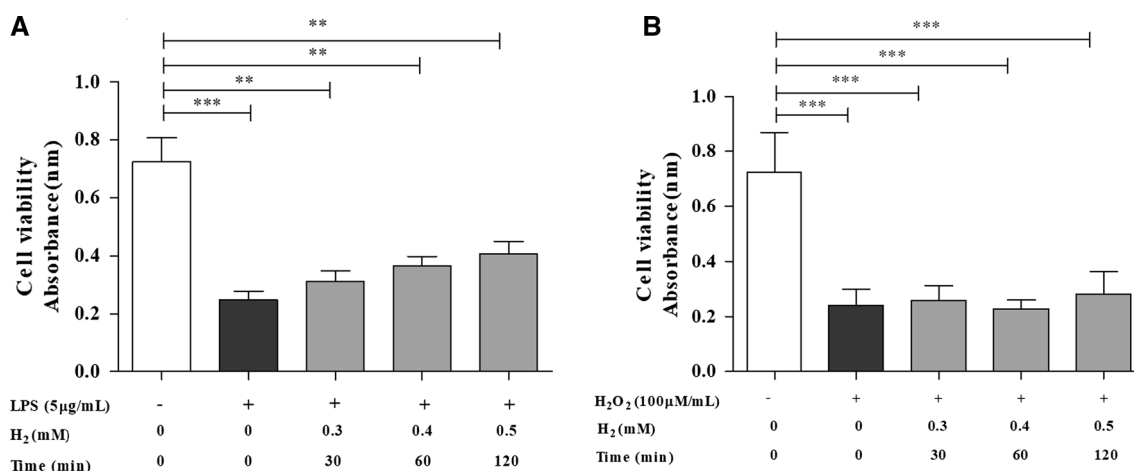


Fig. 2 Effects of H₂ treatment on cell viability and proliferation in LPS- and H₂O₂-induced oxidative stress of murine macrophage RAW 264.7 cells. **a** The cells were pre-treated with or without LPS (5 µg/mL) for 1 h and **b** H₂O₂ (100 µM/mL) for 30 min. Then the cells were treated in presence or absence of different concentrations (0.3 mM, 0.4 mM and 0.5 mM) of H₂ containing environment at different time interval (30, 60 and 120 min). After 24 h of incubation

the cell viability was compared with H₂ treated group and non-treated group through CCK-8 assay reagents. The results are expressed as the viable cell of the control group without and with H₂ treatment and the data are expressed as the mean \pm SD ($n=3$ each group). The significant difference was analyzed with ANOVA Tukey's test, $^{**}p<0.01$ and $^{***}p<0.001$

Effects of H₂ on macrophage cells polarization to exert an active immune function by releasing the ROS and NO molecule after exposure of LPS

To study the H₂ response in the macrophage cell against LPS, ROS and NO generation was evaluated. Macrophage is the first-line defense in the body's immunity. Macrophage polarization started after a response to LPS and inflammatory cytokines resulting to ROS and NO release, which has strong antibacterial activities (Di Meo et al. 2016; Arango Duque and Descoteaux 2014). To confirm these active effects of H₂ on macrophage cell polarization after exposure to LPS, we observed the ROS and NO generation level. Our results demonstrated that H₂ significantly promoted the polarization of macrophage cells to produce intracellular ROS levels over time and H₂ concentration compared to the normal control group, not only significantly increased compared to the LPS-induced group (Fig. 3a). The levels of NO also significantly increased in the 120 min H₂ treatment group compared with the LPS-stimulated group ($p<0.05$) (Fig. 3b).

H₂ attenuated the H₂O₂-induced oxidative stress in RAW 264.7 macrophages cells

Next, we evaluated the H₂ effects in H₂O₂ induced cellular oxidative stress on macrophage cells. H₂O₂ significantly

increased the cellular ROS and NO levels as compared to the normal control group ($p<0.01$ and $p<0.001$). However, H₂ treatment significantly reduced the cellular ROS and NO level on time-dependent manner as compared to only H₂O₂ induction group (Fig. 4a, b).

H₂ reduced the intracellular calcium level in LPS- and H₂O₂-induced RAW 264.7 macrophages cells

Then, we investigated the effects of H₂ in the reduction of intracellular calcium level. We showed that the LPS- and H₂O₂-induction significantly increased the intracellular calcium levels as compared to the normal control group ($p<0.001$ and $p<0.05$) (Fig. 5a, b). After H₂ treatment, the intracellular calcium levels of LPS- and H₂O₂-induced group were significantly decreased at 60 and 120 min as compared to only LPS- and H₂O₂-induced group ($p<0.001$, respectively).

H₂ increased the endogenous antioxidant enzymes (GPx and catalase) level in LPS- and H₂O₂-induced RAW 264.7 macrophages cells

Next, we studied whether H₂ restored the endogenous GPx and catalase levels in the LPS- and H₂O₂-induced oxidative damaged cells. Our results showed that, H₂ treatment significantly increased the GPx activities at 120 min in LPS

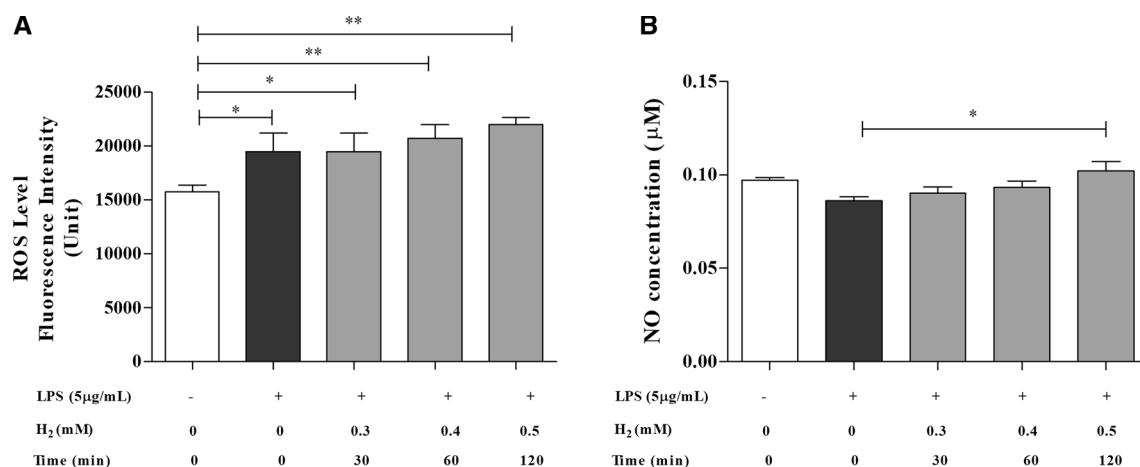


Fig. 3 Effects of H₂ on the LPS- induced macrophage cell polarization in ROS and NO production of RAW 264.7 macrophage cells. **a** The total intracellular ROS level was determined after 1 h addition of LPS and was compared with H₂ treated groups and control groups through fluorescence probe DCFH-DA assay. **b** The level of NO was

determined by Griess reagent after 1 h LPS stimulation and H₂ treatment. Data values are expressed as mean ± SD (*n* = 3) each group. The significant difference was analyzed with ANOVA Tukey's test, **p* < 0.05 and ***p* < 0.01

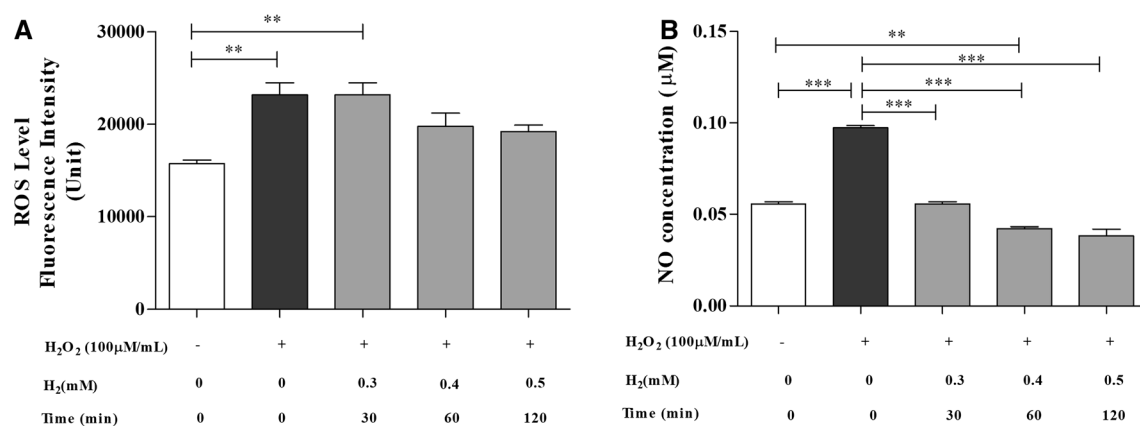


Fig. 4 Effects of H₂ on the reduction of oxidative stress in H₂O₂-induced RAW 264.7 macrophage cells. **a** The total intracellular ROS level was determined after 30 min addition of H₂O₂ and was compared with H₂ treated groups and control groups through fluores-

cence probe DCFH-DA assay. **b** The level of NO was determined by Griess reagent. Data values are expressed as mean ± SD (*n* = 3) each group. The significant difference was analyzed with ANOVA Tukey's test, ***p* < 0.01 and ****p* < 0.001

group (*p* < 0.05 vs. 30 min and *p* < 0.01 vs. 0 min) and H₂O₂ group (*p* < 0.05 vs. 0 min) (Fig. 6a, b).

Similarly, H₂ also significantly increased the catalase production as compared to normal control (Fig. 6c, d). Significant catalase levels were increased at 60 and 120 min of H₂ treatment in LPS group (*p* < 0.01 vs. 0 min, respectively) and in H₂O₂ group (*p* < 0.05 vs. 0 min, respectively) (Fig. 6c, d).

Effects of H₂ on reduction of inflammatory cytokine in the response of LPS- and H₂O₂-induced macrophage RAW 264.7 cells

Next, we evaluated the effects of H₂ treatment on inflammatory-related cytokines such as TNF-α, IL-6, and IL-12 in macrophage cells after stimulation of LPS and H₂O₂. Our results showed that, higher concentration of TNF-α and IL-6 were increased in cells treated with LPS, whereas only TNF-α were found increased with H₂O₂ treated cells as

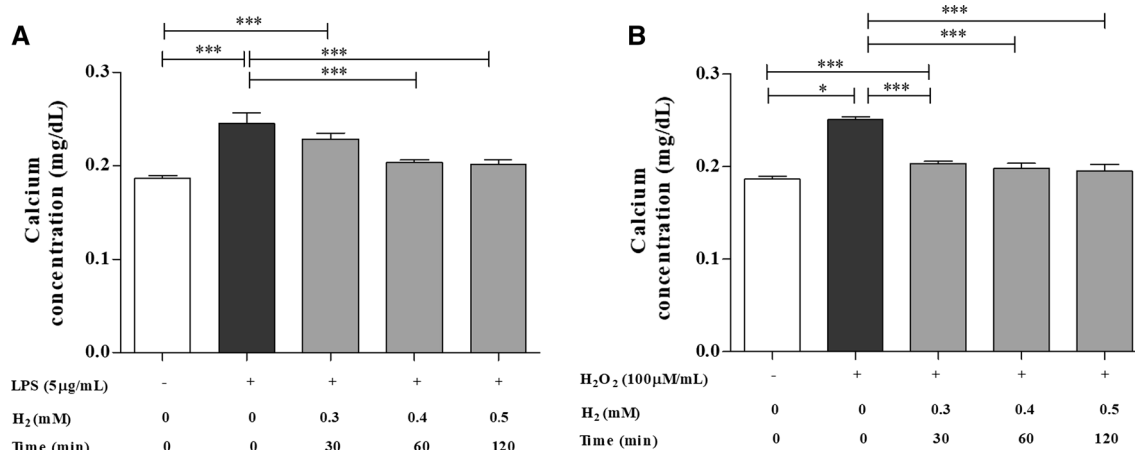


Fig. 5 Effects of H₂ in LPS- and H₂O₂-induced intracellular calcium level on RAW 264.7 macrophage cells. **a** The RAW 264.7 cells were pre-treated with or without LPS (5 μg/mL) and **b** H₂O₂ (100 μM/mL). After stimulation, the cells were treated with H₂ at the indicated

period of time. After H₂ treatment the total intracellular calcium level was analyzed through calcium colorimetric assay. Data values are expressed as the mean ± SD (*n* = 3 each group). Significant difference was analyzed with ANOVA Tukey's test, **p* < 0.05 and ****p* < 0.001

compared to normal control group, but without significant difference (Fig. 7a–c). In the LPS-induced group, there was significant decrease of these inflammatory cytokines at 30 and 120 min H₂ treatment in TNF-α (*p* < 0.05 vs. 0 min), IL-6 (*p* < 0.01 vs. 0 min), and IL-12 as compared to only LPS induction group. However, at 60 min we found significant increment as compared to 30 min (*p* < 0.05, *p* < 0.01 and *p* < 0.001, respectively) (Fig. 7a, c, e). In H₂O₂-induced group, H₂ reduced the TNF-α concentration at 30 and 120 min treatment, whereas increased in 60 min without any significant difference. In addition, IL-6 did not show any distinct significant changes with H₂ treatment in H₂O₂-induced group (Fig. 7d). In line with this result, IL-12 was found reduced at 30, 60 and 120 min H₂ treatment, but not statistically significant (Fig. 7f).

H₂ inhibited the phosphorylation of LPS- and H₂O₂-induced MAPK protein expression in RAW 264.7 macrophages cells

To assess the LPS- and H₂O₂-induced intracellular oxidative stress-mediated pathway, we measured the phosphorylation of ERK, JNK, and p38. Our results showed that both LPS- and H₂O₂-induced cells increased the MAPK protein phosphorylation. In addition, the expressions of p-ERK, p-JNK, and p-p38 were significantly inhibited with the increasing of H₂ treatment time as compared to 0 min (Fig. 8a, b). In the normal control group, the MAPK protein phosphorylation was not significantly expressed (Supplementary Figure 1A).

H₂ attenuated the mitochondrial apoptotic protein expression in LPS- and H₂O₂-induced RAW 264.7 macrophages cells

Next, we investigated the inhibition effects of H₂ in response to the oxidative stress-induced mitochondrial apoptotic pathway on macrophage cells. As shown in Fig. 9a, b, LPS- and H₂O₂-induction increased the higher mitochondrial cytochrome c, cleaved caspase-3, and cleaved caspase-7 protein phosphorylation in RAW 264.7 macrophage cells. H₂ treatment on LPS-induced groups significantly reduced apoptotic proteins phosphorylation especially on cleaved caspase-3 and cleaved caspase-7 at 120 min treatment (Fig. 9a). In H₂O₂-induced group, H₂ treatment significantly reduced the cytochrome c protein phosphorylation at 60 min treatment as compared to 30 min (*p* < 0.05), while cleaved caspase-3 significantly reduced in time-dependent manner (Fig. 9b). In normal control groups, the cleaved caspase 3 and 7 protein phosphorylation was not significantly expressed (Supplementary Figure 1b).

H₂ activated the anti-oxidative Nrf2 pathway in LPS and H₂O₂-oxidative stress induced RAW 264.7 macrophages cells

To investigate whether the antioxidant activity of H₂ in LPS and H₂O₂ stress-induced macrophage cells correlates with the activation of Nrf2 signaling, protein expression of Nrf2 was examined. As shown in Fig. 10a, H₂ increased the Nrf2 protein phosphorylation time-dependently. In LPS- and

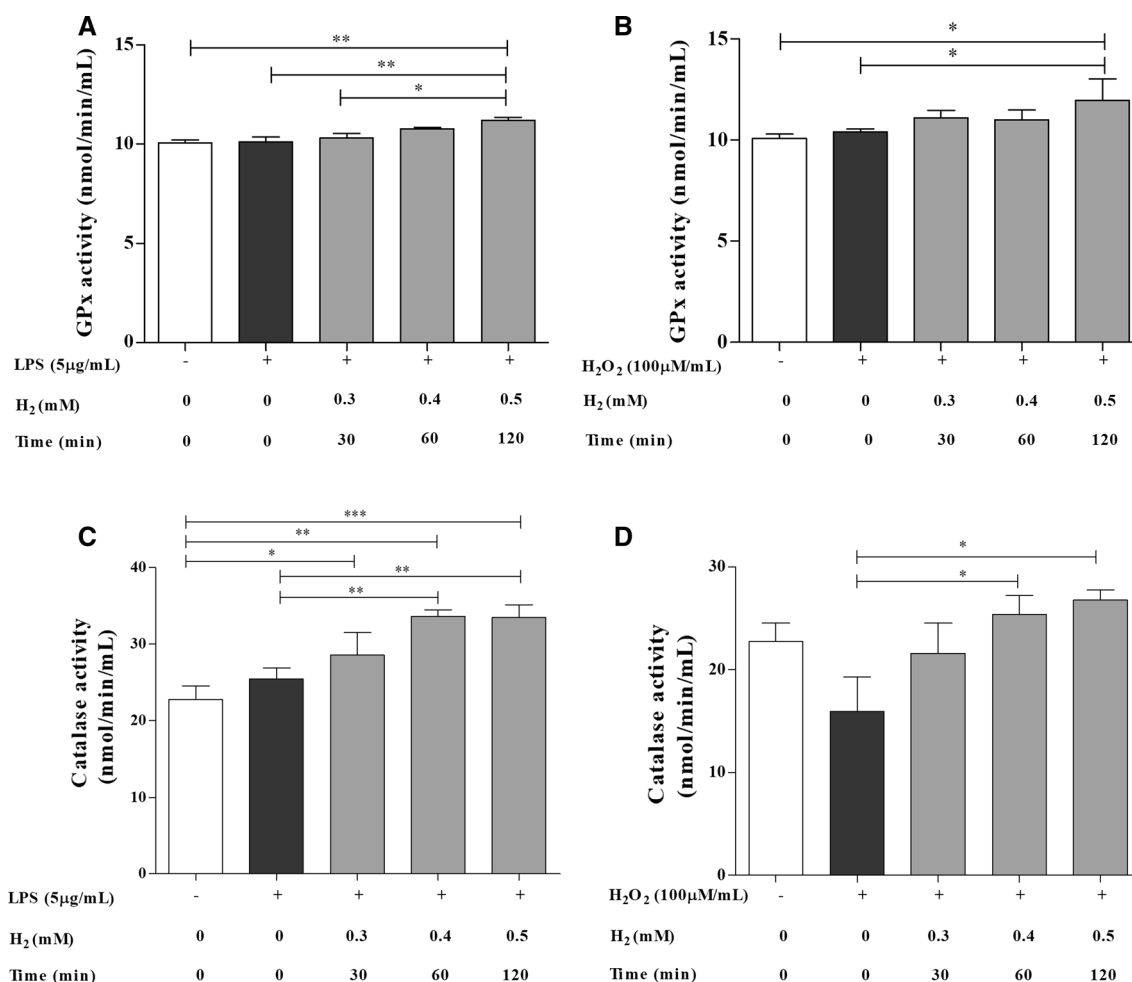


Fig. 6 Effects of H₂ in antioxidant enzyme GPx and catalase production in LPS- and H₂O₂-induced oxidative stress on RAW 264.7 cells. The GPx activity after **a** LPS- and **b** H₂O₂-induction, and the catalase activity after **c** LPS- and **d** H₂O₂-induction. The RAW 264.7 cells were pre-treated with LPS (5 μg/mL) and H₂O₂ (100 μM/mL) for 1 h and 30 min. After stimulation, the cells were treated with H₂

at the indicated period of time. After 24 h of H₂ treatment the GPx and catalase levels were measured by GPx and catalase assay kit. Data values are expressed as the mean ± SD (*n* = 3 each group). Significant difference was analyzed with ANOVA Tukey's test, **p* < 0.05, ***p* < 0.01

H₂O₂-induced macrophage cell, H₂ also increased the Nrf2 protein phosphorylation, concentration and time-dependently, especially in 60 and 120 min compared to 0 min (Fig. 10b, c). These data strongly suggested that H₂ inhibits the cellular oxidative stress and activate the antioxidant Nrf2 signaling pathway.

Discussion

Since the initial discovery of its antioxidant properties, H₂ has been investigated in a wide range of human diseases for potential beneficial and therapeutic effects (Ohsawa et al. 2007; Ohno et al. 2012; Ohta 2011, 2014). Although, many studies clearly showed that H₂ has effects in LPS-induced inflammation in macrophage cells (Chen et al. 2013; Itoh

et al. 2011), studies on H₂O₂-induced oxidative stress in macrophage cells are not clearly found in H₂-related studies. Therefore, the aim of this present study was to find out the protective effects of H₂ in both of LPS- and H₂O₂-induced oxidative stress and inflammatory responses in RAW 264.7 macrophage cells and analyzes the H₂ role in the inhibition of stress response MAPK cell signaling pathway and the activation of antioxidant Nrf2 pathway. This study clearly demonstrated that H₂ increased the macrophages cell viability and reduced the stress induced MAPK signaling protein and apoptosis with increasing the time and H₂ concentration by the activation of the anti-oxidant Nrf2 pathway.

In this study, a murine macrophage RAW 264.7 cell for the in vitro model of oxidative stress and inflammatory response was used. Macrophage is the most important cells to confront bacterial pathogens in the body's immunity to

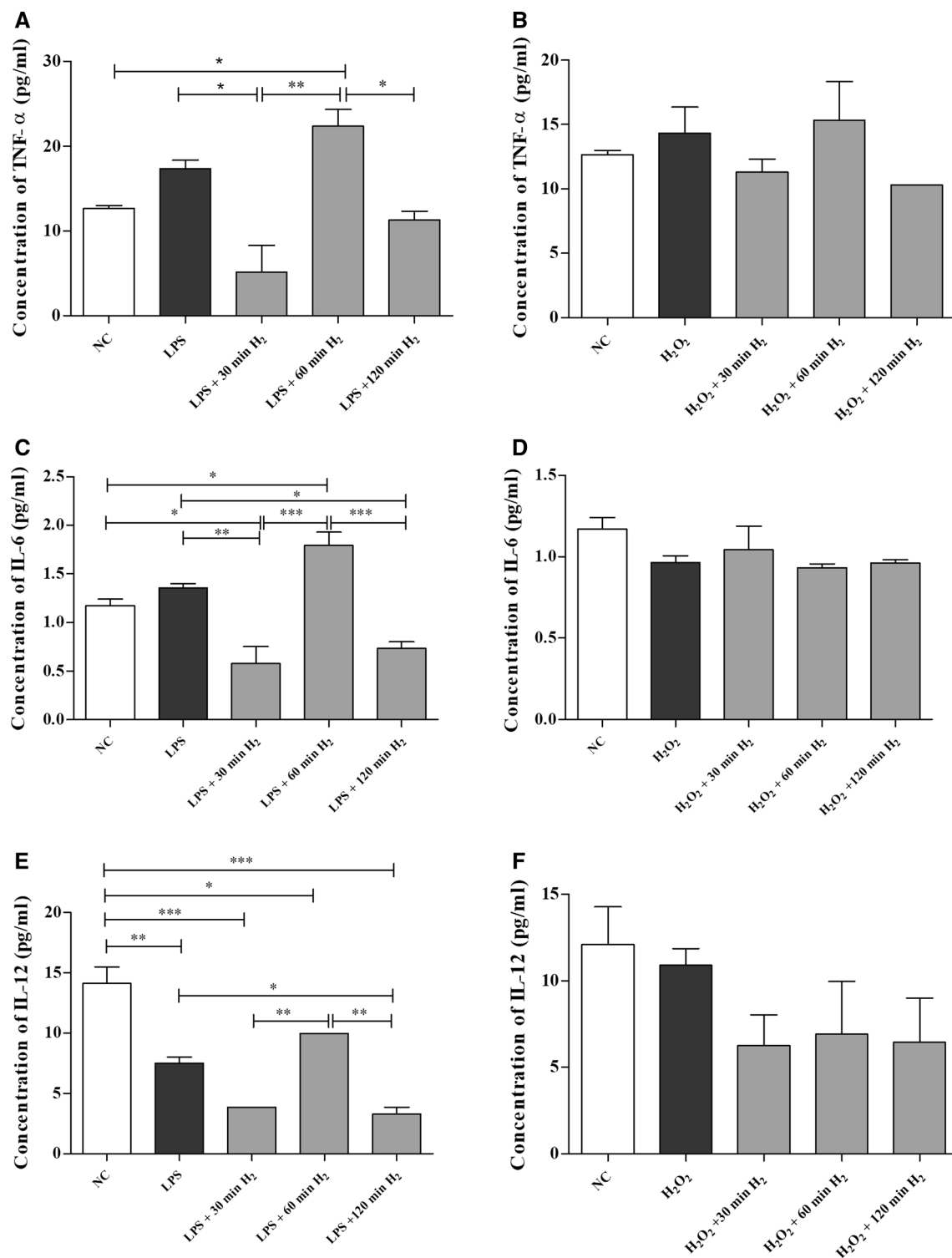


Fig. 7 Effects of H₂ in inflammatory cytokine reduction in LPS- and H₂O₂-induced oxidative stress on murine macrophage RAW 264.7 cells. **a, c, e** RAW 264.7 cells were pre-treated with and without LPS (5 µg/mL) for 1 h; **b, d, f** H₂O₂ (100 µM/mL) for 30 min. After stimulation, the cells were treated with different concentration of H₂ at the indicated period of time and then incubated for 24 h. Cell cul-

ture supernatants were collected for cytokine analysis. TNF-α (**A, B**), IL-6 (**c, d**) and IL-12 (**e, f**) were measured using Bioplex multiplex immunoassay. Data values are expressed as the mean ± SD (*n* = 3 each group). Significant difference was analyzed with ANOVA Tukey's test, **p* < 0.05, ***p* < 0.01, and ****p* < 0.001

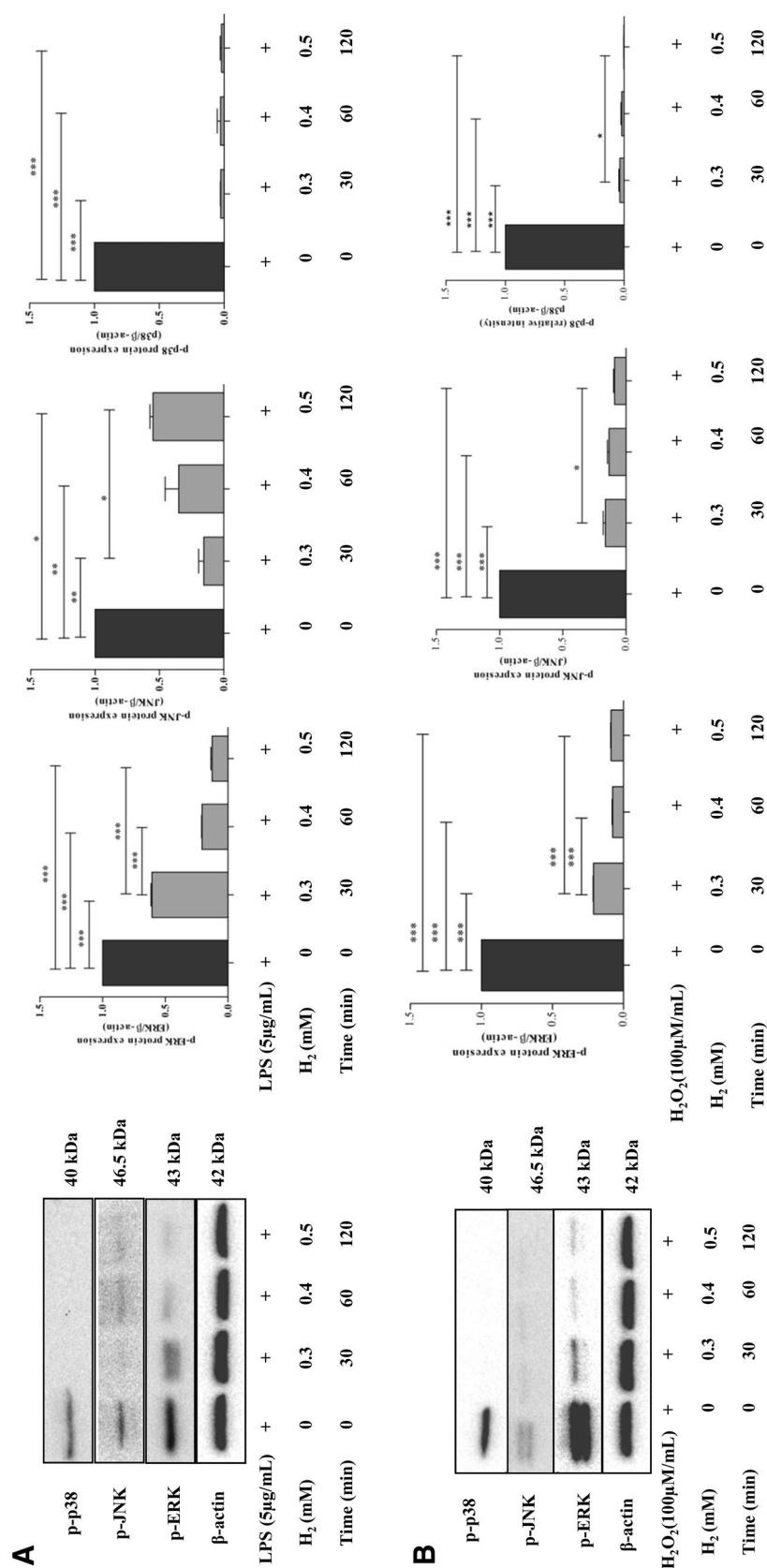


Fig. 8 Effects of H₂ on MAPK signal transduction pathway against LPS- and H₂O₂-induced RAW 264.7 macrophage cells. **a** Cells were pretreated with 5 μg/mL LPS and **b** 100 μM/mL H₂O₂. After treatment with LPS and H₂O₂ cell were treated with H₂ for indicated time periods. Then the cell lysates were harvested and subjected to western blot analysis for indicated proteins. Data values are expressed as the mean ± SD (*n* = 3 each group). Significant difference was analyzed with ANOVA Tukey's test, **p* < 0.05, ***p* < 0.01 and ****p* < 0.001

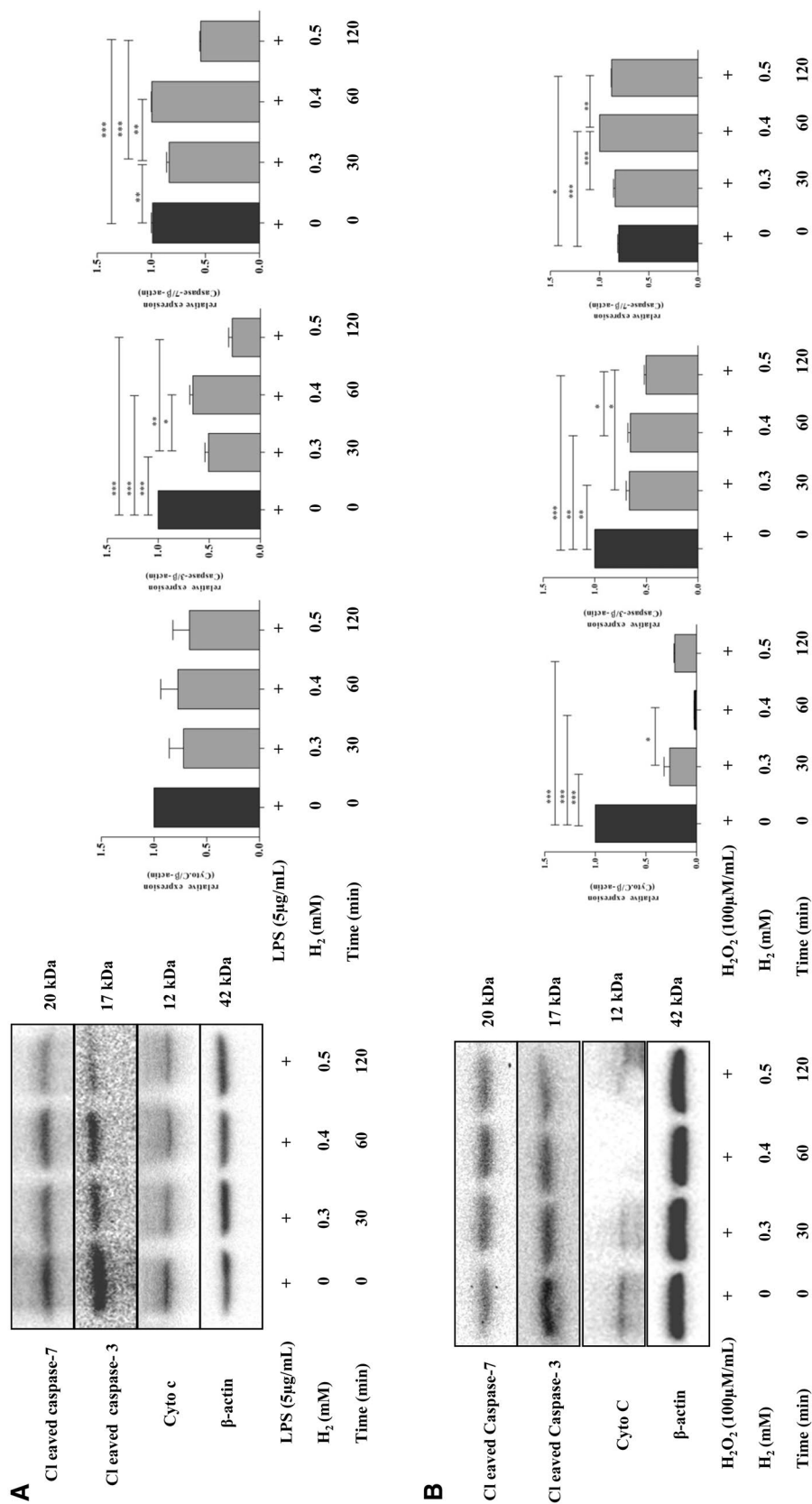


Fig. 9 Inhibition effects of H₂ on mitochondrial apoptotic protein expression against LPS- and H₂O₂-induced RAW 264.7 macrophage cells. Cells were pretreated with **a** 5 μg/mL LPS- and **b** 100 μM/mL H₂O₂. After treatment with LPS and H₂O₂ cells were treated with H₂ for indicated time periods. Then the cell lysate was harvested and subjected to western blot analysis for indicated proteins. Data values are expressed as the mean ± SD (*n* = 3 each group). Significant difference was analyzed with ANOVA Tukey's test, **p* < 0.05, ***p* < 0.01 and ****p* < 0.001

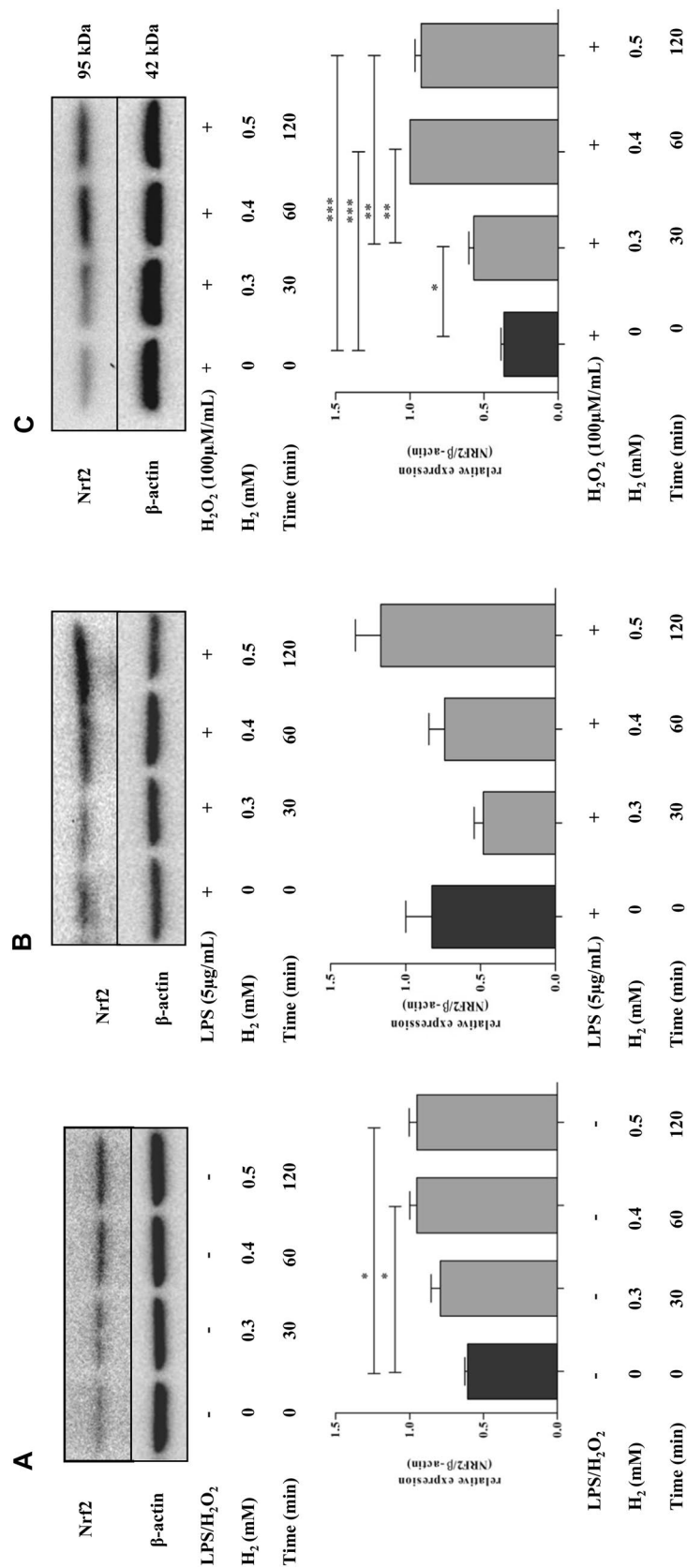


Fig. 10 Effects of H₂ on Nrf2 protein phosphorylation against LPS- and H₂O₂-induced RAW 264.7 macrophage cells. **a** Normal control cell, **b** cells were pretreated with 5 μg/mL LPS and **c** 100 μM/mL H₂O₂. After treatment with LPS and H₂O₂ cell were treated with H₂ for indicated time periods. Then the cell lysate was harvested and subjected to western blot analysis for indicated proteins. Data values are expressed as the mean ± SD (*n* = 3 each group). Significant difference was analyzed with ANOVA Tukey's test, **p* < 0.05, ***p* < 0.01 and ****p* < 0.001

intracellular microbial pathogens through phagocytosis and cytokine secretion. A number of reports already showed the anti-inflammatory effects of H_2 (Sun et al. 2009; Xie et al. 2010). However, in present study we also tried to investigate this anti-inflammatory mechanism after LPS and H_2O_2 exposure. Our results showed that ROS and NO were significantly increased after treatment with H_2 in response to LPS stimulation. ROS and NO are primarily released by activated M1 macrophages cell into LPS (McCoy and O'Neill 2008), which exerts strong antimicrobial activities. A number of studies have shown that bacterial infection induces the polarization of M1 macrophages through phagocytosis and cytokine secretion (Shaughnessy and Swanson 2007; Benoit et al. 2008). In addition, our result also showed that H_2 significantly reduced the inflammatory-related cytokines. In particular, compared with the levels in LPS stimulated group, the inflammatory cytokines such as TNF- α , IL-6, and IL-12 displayed marked reduction at various time points (30 and 120 min), while at 60 min showed increment in cytokines production after treatment with H_2 in response to LPS stimulation. It is possible that the alterations of cytokines release induced by LPS with hydrogen treatment maybe differ in different time point. However, the specific mechanism behind this phenomenon requires further investigation. It is known that TNF- α , IL-6, and IL-12 are the inflammatory cytokines that mediate many of the immunopathological features of LPS-induced shock (Dinarello 1997). They are released during the first 30–90 min after exposure to LPS and play an important role in the process of inflammation (Dinarello 1997; Cohen 2002). These results suggested that, macrophage cell time-dependently responded to H_2 after LPS exposure in the reduction of cytokine. With this observation, we can imply that H_2 might promote the macrophage M1 polarization against bacterial infection. Alternatively, the overproduction of ROS including H_2O_2 induction may act as a significant and adverse participant in abnormal inflammatory disease (Di Marzo et al. 2018). Among the ROS molecules, the hydroxyl radical ($\bullet OH$) has most toxic effects in cell. The discovery of H_2 treatment (at 1.6 ppm or 0.6 mM) as a selective scavenger of hydroxyl radical ($\bullet OH$) and peroxynitrite ($ONOO^-$) inside the cell proves its action as a strong antioxidant to protect the cells against oxidative stress and cell death (Ohsawa et al. 2007). Our results imply that H_2 significantly reduced the H_2O_2 -induced cellular ROS and NO levels and protect the cell from death, which were also consistent with the decrease of the inflammatory response cytokines (IL-6, IL-12 and, TNF- α).

Additionally, we investigated the effects of H_2 in case of intracellular calcium which has a pivotal role in many body functions and plays a dual role in the pathology of

many inflammatory and oxidative stress diseases (Ermak and Davies 2002). Hence, the inhibition of intracellular calcium production is essential for the prevention of inflammatory diseases. Generally, ROS, NO, and calcium have a pivotal role in maintaining the cellular physiological function as a secondary messenger. However, higher oxidative stress causes the influx of intracellular calcium level that modifies the normal physiological function including MAPK cell signaling pathways and causes cell death. Impaired redox regulation and altered calcium signaling lead to depleting the cellular antioxidant enzyme which may stimulate the macrophage cell for the inflammatory response (Chudeland and Seger 2008; Orrenius et al. 2015). In this study, we found that H_2 reduced the intracellular calcium levels with increasing the exposure time as compared to non-treated cell especially at 60 min of 0.5 mM concentration. Moreover, we observed the H_2 effects in restoring the endogenous antioxidant enzymes (GPx and catalase). GPx and catalase are the most important cellular first line defense antioxidants (Ighodaro and Akinloye 2018) and our result shows that H_2 increased the GPx and catalase enzyme in both LPS and H_2O_2 induction groups, which proves the antioxidant effect of H_2 . In addition, our result suggests that H_2 treatment attenuate the oxidative stress induced intracellular calcium level influxing which might control the redox homeostasis as a strong antioxidant and also may influence the stress-induced MAPK downstream signaling targets.

The MAPK signaling pathway is activated by various stress and stimuli such as ROS, TNF- α , LPS, and calcium influx (Nagai et al. 2007). LPS activates the inflammatory response MAPK signaling pathway and suppressed the inflammation by modulating the production of cytokines production (Nagai et al. 2007; Kim and Choi 2010) and also through ROS production with the addition of cellular receptor known as TLR4, which in turns activate the JNK and p38-MAPK (Son et al. 2013). From our results we observed that H_2 strongly, time-dependently inhibits the ROS-induced activation of ERK, JNK, and p38 signaling network that might prevent cell death and significantly increased the cell viability. This might be due to the strong anti-oxidant role of H_2 . Studies also have shown that the prevention of ROS accumulation by antioxidants blocks the MAPK activation after cell stimulation with cellular stimuli (Son et al. 2011), indicating the involvement of ROS in the activation of MAPK pathways. Another study also showed that antioxidant enzymes were expressed by the activation of Nrf2, which may further prevent the activation of MAPK (Ding et al. 2010).

Accumulated evidence indicates that the Nrf2 plays an important role in protecting the cell from oxidative damage by promoting the expression of antioxidant enzymes (Niture

et al. 2014; Senger et al. 2016; Kensler et al. 2007). Recently, several studies have shown that activation of the Nrf2 pathway regulate the host innate immunity and found to prevent a large number of chronic inflammatory diseases, sepsis and protected the cell from apoptosis (Senger et al. 2016; Kensler et al. 2007). Nrf2 is a redox-sensitive transcription factor that binds to antioxidant response elements (ARE) to regulate the expression of antioxidant enzymes that protect against oxidative damage triggered by injury and inflammation (Niture et al. 2014). Once activated by oxidative stimuli, Nrf2 migrates into nucleus and to induce antioxidant enzymes (Kensler et al. 2007). Subsequent studies indicate that hydrogen activates the Nrf2 signaling pathway in oxidative stress (Ichihara et al. 2015). In this experiment we observed that 120 min of H₂ treatment significantly activated the Nrf2, which was consistent with the increased levels of GPx and catalase enzymes. It has been reported that molecular hydrogen elevated the antioxidant enzymatic activities by regulation of Nrf2 in radiation and traumatic brain injury (Kura et al. 2018; Yuan et al. 2018). From these results, it consistently proved that H₂ acted as a strong anti-oxidant in the reduction of cellular oxidative stress and inflammatory cytokine that might help to replenish the antioxidant level to protect the macrophage cell from apoptosis. Studies also have shown that H₂ might have beneficial effects through the activation of Nrf2 pathway that enhances the antioxidant activities and reduces apoptosis and inflammation (Kura et al. 2018). The specific anti-inflammation mechanisms of H₂ may work via Nrf2 in macrophages (Chen et al. 2013).

Lastly, we investigate the ROS-induced mitochondrial apoptosis signaling pathway. In general, excessive ROS, NO and calcium level causes an imbalance the cellular membrane homeostasis and collapses the anti-oxidative mechanisms which then lead to intrinsic mitochondrial apoptosis pathway by the activation cytochrome c and the caspase proteins including cleaved caspase 3 and cleaved caspase 7, that destroy the various substrate protein necessary for cell survival and eventually induce apoptosis (Redza-Dutordoir and Averill-Bates 2016). Our results showed that H₂ treatment decreased the LPS- and H₂O₂-induced apoptotic protein phosphorylation that might influence the macrophage cell proliferation to increase the viability and protect the cell and mitochondria against LPS- and H₂O₂-induced oxidative damage or inflammation. H₂ treatment showed the greatest cell viability in 0.5 mM concentration at 120 min treatment than other exposure times, which is in contrast to the report that showed there was no cell proliferation effect of H₂ at the concentration of 0.6 mM (Chen et al. 2013). It is very well known that the free radical scavenging properties of H₂ can easily diffuse into all cellular organelles especially in mitochondria (Ohsawa et al. 2007), by increasing the other antioxidants enzymes, resulting to protect the cellular cytoplasm.

Conclusion

Collectively, this study provided evidence that H₂ reduces oxidative stress and inflammatory response MAPK signaling pathway, based on the measurement of intracellular ROS, calcium, TNF- α , IL-6, IL-12, GPx, catalase, and Nrf2 signaling pathway. It also suggests the possible crosstalk among anti-oxidative, anti-inflammatory, and anti-apoptotic pathways of H₂ treatment. Identifying the direct targets of H₂ as a strong antioxidant, anti-inflammation and anti-apoptotic will help to realize the potential therapeutic agent of many inflammatory, metabolic and aging diseases.

Author contributions RB and CSK contributed equally to this work; RB performed most of the experiments, analyzed the results, interpreted the data, wrote and revised the whole manuscript; CSK planned, supervised the whole study, performed the experiment, interpreted the results and revised the manuscript; AF and JB performed the experiments, analyzed the results, and revised the manuscript; XJ performed the experiment and revised the manuscript; DHK revised the manuscript; SKK suggested the plan and revised the manuscript; KJL conceived, supervised and supported the whole study, and revised the whole manuscript.

Compliance with ethical standards

Conflict of interest Rahima Begum, Cheol-Su Kim, Ailyn Fadriquel, Johny Bajgai, Xingyu Jing, Dong-Heui Kim, Soo-Ki Kim and Kyu-Jae Lee that they have no conflict of interest.

Ethical approval The article does not contain any studies with human and animal and this study was performed following institutional and national guidelines.

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