Accepted Manuscript

Heme oxygenase-1 mediates the anti-inflammatory effect of molecular hydrogen in LPS-stimulated Raw 264.7 macrophages

Hong-guang Chen, Ke-liang Xie, Huan-zhi Han, Wei-na Wang, Da-quan Liu, Guo-lin Wang, Yong-hao Yu

PII: S1743-9191(13)01080-7

DOI: 10.1016/j.ijsu.2013.10.007

Reference: IJSU 1215

To appear in: *International Journal of Surgery*

Received Date: 10 July 2013

Revised Date: 17 September 2013

Accepted Date: 12 October 2013

Please cite this article as: Chen H-g, Xie K-l, Han H-z, Wang W-n, Liu D-q, Wang G-l, Yu Y-h, Heme oxygenase-1 mediates the anti-inflammatory effect of molecular hydrogen in LPS-stimulated Raw 264.7 macrophages, *International Journal of Surgery* (2013), doi: 10.1016/j.ijsu.2013.10.007.

Heme oxygenase-1 mediates the anti-inflammatory effect of molecular hydrogen in LPS-stimulated Raw 264.7 macrophages

Running Title: HO-1 mediates the anti-inflammatory effect of H_2.

Hong-guang CHEN[1], Ke-liang XIE[1], Huan-zhi HAN[1], Wei-na WANG[1], Da-quan LIU[2], Guo-lin WANG[1], Yong-hao YU[1,*]

[1]Department of Anesthesiology, Tianjin Institute of Anesthesiology, General Hospital of Tianjin Medical University, Tianjin 300052, China

[2]Institute of Acute Abdomen, Tianjin Integrated Chinese and Western Medicine, Tianjin 300100,China

Hong-guang CHEN, Ke-liang XIE and Huan-zhi HAN contributed equally to this work.

*__Correspondence to: Drs. Yong-hao YU and Ke-liang XIE__, Department of Anesthesiology, Tianjin Institute of Anesthesiology, General Hospital of Tianjin Medical University, Tianjin 300052, P. R. China. Tel: +86-22-60361519, Fax: +86-22-27813550, E-mail: yuhonghao@126.com or xiekeliang2009@hotmail.com

Number of words: 4541

Number of pages: 27

Number of figures: 6

Conflict of Interest Statement: The authors have declared that no conflict of interest exists.

Abbreviations:

CO, carbon monoxide; DMEM, Dulbecco's Modified Eagle Medium; ECL, enhanced chemiluminescence; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; H_2, molecular hydrogen; HMGB1, high mobility group box-1; HO-1, heme oxygenase-1; IL-1β, interleukin-1 beta; IL-6, interleukin-6; IL-10, interleukin-10; I/R, ischemia-reperfusion; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; MTT, 3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di-phenytetrazoliumromide; NADPH, nicotinamide adenine dinucleotide phosphate; Nrf2, nuclear factor erythroid 2-related factor 2; PMSF, phenylmethanesulfonyl fluoride; P/S, penicillin/streptomycin solutions; PVDF, polyvinylidene fluoride; RIPA, radioimmunoprecipitation assay; TBST, Tris-Buffered Saline with Tween; TNF-α, tumor necrosis factor-alpha; ZnPP, zinc protoporphyrin.

Abstract.

Background: Molecular hydrogen (H_2) as a new medical gas has an anti-inflammatory effect. In the present study, we investigated whether heme oxygenase-1 (HO-1) contributes to the anti-inflammatory effect of H_2 in lipopolysaccharide (LPS) -stimulated RAW 264.7 macrophages. **Methods**: RAW 264.7 macrophages were stimulated by LPS (1 μg/ml) with presence or absence of different concentrations of H_2. Cell viability and injury were tested by 3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di-phenytetrazoliumromide (MTT) assay and lactate dehydrogenase (LDH) release, respectively. The cell culture supernatants were collected to measure inflammatory cytokines [TNF-α, IL-1β, HMGB1 (high mobility group box-1) and IL-10] at different time points. Moreover, HO-1 protein expression and activity were tested at different time points. In addition, to further identify the role of HO-1 in this process, zinc protoporphyrin (ZnPP)-IX, an HO-1 inhibitor, was used. **Results**: H_2 treatment had no significant influence on cell viability and injury in normally cultured RAW 264.7 macrophages. Moreover, H_2 treatment dose-dependently attenuated the increased levels of pro-inflammatory cytokines (TNF-α, IL-1β, HMGB1), but further increased the level of anti-inflammatory cytokine IL-10 at 3 h, 6 h, 12 h and 24 h after LPS stimulation. Furthermore, H_2 treatment could also dose-dependently increase the HO-1 protein expression and activity at 3 h, 6 h, 12 h and 24 h in LPS-activated macrophages. In addition, blockade of HO-1 activity with ZnPP-IX partly reversed the anti-inflammatory effect of H_2 in LPS-stimulated macrophages. **Conclusions**: Molecular hydrogen exerts a regulating

role in the release of pro- and anti-inflammatory cytokines in LPS-stimulated macrophages, and this effect is at least partly mediated by HO-1 expression and activation.

Keywords: inflammatory cytokines; molecular hydrogen; macrophage; heme oxygenase-1

Introduction

Inflammation is the instinct biological response that protects living organisms from harmful stimuli such as pathogens, damaged cells, or irritants [1], but excessive inflammatory response is the basis for the development of diseases, such as sepsis, ischemia-reperfusion (I/R) injury, cancer, neurodegeneration [2-5]. Recently, more and more researchers demonstrate that molecular hydrogen (H_2) has the anti-inflammatory, antioxidant and anti-apoptotic effects *in vivo* and *in vitro*, and can protect against multiple diseases, such as stroke, sepsis, multiple organ dysfunction syndrome, cisplatin-induced ototoxicity, I/R injury, atherosclerosis, nigrostriatal degeneration diseases, etc [6-17]. Our previous studies have shown that H_2 has a therapeutic effect on sepsis, sepsis-associated organ damage and LPS-induced acute lung injury through reducing inflammatory mediators, such as TNF-α, IL-1β, IL-6 and HMGB1 in serum and tissues [7-9]. Moreover, a recent study has reported that hydrogen saline reduces the TNF-α release in LPS-activated macrophages [17]. So far, however, the specific mechanism underlying the anti-inflammatory role of H_2 is unclear.

Heme oxygenase (HO) catalyzes the rate-limiting step in heme degradation, which can produce bilirubin, iron, and carbon monoxide (CO) [18]. To date, three HO isoforms HO-1, HO-2 and HO-3 have been identified. HO-1, a major heat shock/stress response protein, is ubiquitous as well as its expression and activity can be increased by stimuli that induce cellular stress. HO-1 can contribute to accommodate the release of inflammatory cytokines in intestinal inflammation, sepsis, LPS-stimulated macrophages [19-21]. In addition, H_2 can increase the HO-1 expression

[22]. From above studies, we assumed that HO-1 might mediate the anti-inflammatory effect of H_2.

Macrophages are the first cells to confront microbial pathogens through phagocytosis and cytokine secretion [23]. In the present study, using the LPS-stimulated RAW 264.7 murine macrophage model, we investigated the regulating effect of H_2 on the release of inflammatory cytokines, and then the role of HO-1 in this process was studied.

Materials and Methods

Materials

Cell culture medium (Dulbecco Modified Eagle Medium, DMEM), fetal bovine serum (FBS) and penicillin/streptomycin solutions (P/S) were purchased from Life Technologies Corporation (Grand Island, NY, USA). Cytotoxicity Assay kit was obtained from Roche Applied Science (Roche, IN). HO-1 and β-actin primary antibodies and horseradish peroxidase-conjugated goat anti-rabbit secondary antibody were bought from Abcam (Cambridge, MA, USA). Enzyme-linked immunosorbent assay kits of TNF-α, IL-1β, IL-10 were from R&D Systems Inc (Minneapolis, MN, USA) and HMGB1 was from IBL (Hamburg, Germany). ZnPP-IX was obtained from Porphyrin Products (Logan, UT, USA). All other reagents were purchased from sigma-Aldrich (St. Louis, MO, USA).

Cell culture and LPS stimulation

RAW 264.7 macrophages were purchased from the cell Bank of Shanghai Institutes for Biological Science (Shanghai, China). RAW 264.7 cells were grown in DMEM supplemented with 10% heat-inactivated FBS and 100 U/mL penicillin, 100 μg/mL streptomycin, and cultured at 37 °C with 5% CO_2 in a humidified atmosphere. Confluent cells between the 4^{th} to 6^{th} passages were used for these experiments. The cells were seeded at a density of 1×10^6 cells/mL.

LPS treatment was given by adding 1 μg/mL of LPS into medium. The HO-1 inhibitor ZnPP-IX (20 μmol/L) was added into medium just before the application of hydrogen and LPS.

Hydrogen treatment

According to the previously described method [6], briefly, H_2 was dissolved in DMEM for 4 h under high pressure (0.4 MPa) to a supersaturated level. O_2 was dissolved into the second medium by bubbling O_2 gas at the saturated level, and CO_2 into the third medium by bubbling CO_2 gas. 0.6 mM hydrogen medium was prepared by combining the three medium in the proportion of 90% H_2 medium:5% O_2 medium:5% CO_2 medium. The control medium was prepared by combining the two medium (95% O_2 medium:5% CO_2 medium). The 0.15 mM and 0.3 mM hydrogen complete media were prepared by diluting the 0.6 mM hydrogen complete medium with the control medium. H_2, O_2 and CO_2 concentrations were confirmed with gas chromatography.

Cell viability and cytotoxicity

RAW 264.7 cells (10^4 cells/well) were seeded in a 96-well plate overnight, and then incubated with different concentrations of hydrogen for 24 h. According to the manufacturer's guidelines, cell injury and viability were tested by measuring the LDH release and MTT assay, respectively.

Enzyme-linked immunosorbent assay (ELISA)

The supernatants were collected for measurement of inflammatory cytokines at baseline (0 h) as well as 3 h, 6 h, 12 h and 24 h after LPS stimulation. The supernatants were separated by centrifugation at 3, 000 g for 15 min at 4 °C, aliquoted, and stored at -80 °C until assayed. The levels of TNF-α, IL-1β, HMGB1 and IL-10 were detected by specific ELISA kits with a microplate reader (Molecular Devices,

CA, USA) [8-9]. All standards and samples were run in duplicate.

HO-1 activity assay

HO-1 activity was determined at 0 h, 3 h, 6 h, 12 h and 24 h after LPS stimulation, as described previously [19]. Briefly, cells were harvested and microsomes were prepared. The reaction mixture contained microsomes, cytosolic fraction of rat liver as a source of biliverdin reductase, hemin and NADPH. The reaction mixture was incubated in the dark at 37 ℃ for 1 h and terminated by addition of chloroform. The amount of extracted bilirubin in the chloroform layer was determined by measuring the difference in absorbance between 464 and 530 nm. HO-1 activity was represented as picomoles of bilirubin formed per milligram of protein per hour.

Western blot analysis

The HO-1 expression in Raw 264.7 macrophages was performed at 24 h after LPS stimulation by western blot analysis, as previously described [24]. The collected cells were resuspended in 200 μL RIPA (25 mmol/L Tris-HCl, 150 mmol/L NaCl, 5 mmol/L EDTA, 5 mmol/L EGTA, 1 mmol/L PMSF, 1% Triton X-100, 0.5% Nonidet P40, 10 mg/L aprotinin and 10 mg/L leupeptin) and placed on ice for 30 min. They were centrifuged at 12 000 g for 15 min, and then the supernatants were removed and stored at -80 ℃. The protein samples were denatured at 100 ℃ for 5 min, separated on 10% acrylamide gels, and then electrotransferred to PVDF membranes. The membranes were blocked in a Tris-buffered saline with 0.05% Tween-20 (TBST) solution containing 5% fat-free milk for 2 h, and then incubated under gentle shaking overnight at 4 ℃ with primary antibodies against HO-1 and β-actin (1:2000 dilution).

After being washed three times with TBST (5 min each), the immunoblots were incubated with horseradish peroxidase-conjugated IgG (1:5000 dilution in blocking buffer) for 1 h at room temperature. This was followed by three 5-minute washes with TBST. The protein bands were detected using enhanced chemiluminescence (ECL) reagent, and then visualized and photographed using Gel quantitative Quantity One system (BIO-RAD, Tokyo, Japan). All western blot analyses were carried out at least three times. HO-1 levels were normalized to β-actin.

Statistical analysis

Differences between the groups were analyzed using one-way analysis of variance (ANOVA) followed by LSD comparison. Values were expressed as means ± SD of at least three independent experiments and differences between groups were considered significant at $P < 0.05$.

Results

Effect of hydrogen on cell cytotoxicity and viability in RAW 264.7 macrophages

In the present study, we firstly investigated the effects of H_2 on cell cytotoxicity by measurement of LDH release. The LDH assay signifies membrane integrity and is a direct measurement of cell death. Hydrogen treatment did not cause the damage of normally cultured RAW 264.7 macrophages (Figure 1A). Moreover, the MTT assay was performed to measure mitochondrial activity of cells, which is considered as cell viability. Hydrogen treatment also had no significant effect on the viability of normally cultured RAW 264.7 macrophages (Figure 1B). On the basis of these results, we can clearly find that hydrogen has no effect on cell multiply.

Hydrogen regulates the release of pro- and anti-inflammatory cytokines in LPS-stimulated RAW 264.7 macrophages

As shown in Figure 2, LPS caused the significant production of TNF-α, IL-1β, HMGB1 and IL-10 in macrophages at 3 h, 6 h,12 h and 24 h ($P < 0.05$ vs Con group). Hydrogen treatment attenuated the excessive release of TNF-α, IL-1β and HMGB1 in LPS-stimulated macrophages ($P < 0.05$), while the increased release of IL-10 induced by LPS was further elevated by hydrogen treatment from 3 h to 24 h ($P < 0.05$). In addition, we found that hydrogen treatment could concentration-dependently decrease the TNF-α, IL-1β, HMGB1 levels and increase the IL-10 level in LPS-stimulated macrophages (Figure 3).

Hydrogen increases the HO-1 protein activity and expression in LPS-stimulated RAW 264.7 macrophages

HO-1 is a cytoprotective enzyme that plays a critical role in inflammatory process. We examined the effect of hydrogen on HO-1 activity and expression in LPS-stimulated RAW 264.7 macrophages. As shown in Figure 4, LPS increased the HO-1 activity at 6 h, 12 h and 24 h ($P < 0.05$ vs Con group), while hydrogen treatment further increased the HO-1 activity of LPS-stimulated macrophages in a concentration-dependent manner ($P < 0.05$). Furthermore, hydrogen treatment significantly induced the HO-1 expression of LPS-stimulated macrophages in a concentration-dependent manner ($P < 0.05$, Figure 5). In addition, the HO-1 inhibitor ZnPP-Ⅸ at a dose of 20 μM completely inhibited the HO-1 activity in macrophages with LPS and hydrogen treatment (Figure 4B).

HO-1 inhibition reverses the regulatory effect of hydrogen on inflammatory cytokines in LPS-stimulated RAW 264.7 macrophages

In addition, we further investigated whether the regulatory effect of hydrogen on inflammatory cytokines in LPS-stimulated macrophages was mediated through HO-1. Znpp-□, an inhibitor of HO-1, significantly reversed the regulatory effect of hydrogen on TNF-α, IL-1β, HMGB1 and IL-10 in LPS-stimulated RAW264.7 macrophages (Figure 6). Therefore, HO-1 activation contributes to the regulatory effect of hydrogen on inflammatory cytokines in LPS-stimulated macrophages.

Discussion

In the current study, we investigated the effect of hydrogen on inflammatory cytokines in LPS-stimulated macrophages and the role of HO-1 in this process. Our results showed that 1) treatment with various concentrations of hydrogen had no significant effect on cell viability and injury in normal macrophages; 2) hydrogen could concentration-dependently suppress the release of pro-inflammatory cytokines TNF-α, IL-1β and HMGB1 as well as elevate the level of anti-inflammatory cytokine IL-10 in LPS-stimulated macrophages; 3) hydrogen treatment could increase the HO-1 protein expression and activity of LPS-stimulated macrophages in a concentration-dependent manner; 4) inhibition of HO-1 activity reversed the regulatory effect of hydrogen on inflammatory response in LPS-stimulated macrophages.

Inflammation triggered by noxious stimuli, such as infection and tissue injury, underlies a variety of physiological and pathological processes. Gram-negative bacteria are the main cause of hospital-acquired infections [25]. LPS is a component of the outer envelope of all Gram-negative bacteria. When Gram-negative bacteria invade into the circulation of host, it is recognized by innate immune cells, such as mononuclear, macrophages and neutrophils, which can then release cytokines and chemokines. Macrophages are important cells to confront microbial pathogens in the body's innate resistance to intracellular microbial pathogens through phagocytosis and cytokine secretion [23]. IL-1 and TNF-α are the prototypic inflammatory cytokines that mediate many of the immunopathological features of LPS-induced shock [26]. They

are released during the first 30-90 minutes after exposure to LPS and in turn activate the second level of inflammatory cascades including HMGB1, IL-10, etc [27]. Pro-inflammatory cytokines TNF-α, IL-1β and HMGB1 play an important role in the process of inflammation [28-31]. IL-10, as an anti-inflammatory cytokine, is a potent repressor of pro-inflammatory cytokine expression. Thus, host can maintain a homeostasis via keeping the balance of pro-inflammatory and anti-inflammatory cytokines. The release of pro-inflammatory cytokines outweighed the suppressive effect of anti-inflammatory mediator IL-10, leading to development of inflammatory reaction in LPS-induced macrophages.

Recently, a large number of animal and clinical experiments show that H_2 or hydrogen-rich saline has the property of anti-inflammation, anti-oxidation and anti-apoptosis in different models of diseases [6-16]. We have reported that H_2 has the potent protective effect on sepsis, zymosan-induced organ damage, LPS-induced lung injury and ouabain-induced auditory neuropathy *in vivo* via ameliorating the release of pro-inflammatory cytokines TNF-α, IL-1β, IL-6, HMGB1, macrophage inflammatory protein (MIP)-1, MIP-2 and monocyte chemoattractant protein (MCP)-1 [7-9,32]. Recently, XU *et al* [33] also demonstrates that hydrogen-saline inhibits the secretion of TNF-α in LPS-activated macrophages. In current study, we found that H_2 treatment could suppress the release of pro-inflammatory cytokines, as well as promote the level of anti-inflammatory cytokine in a concentration-dependent and time-dependent manner, suggesting H_2 has an anti-inflammatory effect and makes host to regain the balance of pro- and anti-inflammatory reaction.

HO-1 and its metabolites exert as valuable drug candidates for treatment of sepsis [34,35]. HO-1 is a cytoprotective enzyme that plays a critical role in defending the body against inflammation-induced organ injury [36]. Furthermore, its expression and activity play a significant role in mediating the anti-inflammation in LPS-stimulated macrophages [19]. LI *et al* reports that HO-1 is involved with the release of inflammatory cytokines, and the increase of HO-1 expression and activity can reduce TNF-α secretion [23,19]. Interestingly, H_2 has a protective effect on injured lung tissue via promoting the levels of HO-1 mRNA and protein [22]. H_2 reduces LPS-induced neuroinflammation via augmenting the expression of Nrf2 and HO-1 mRNA [37]. Recent studies have shown that Nrf2 may be one host factor that regulate innate immunity determine susceptibility to sepsis [38]. Once activated by oxidative or xenobiotic stimuli, Nrf2 migrates into the nucleus to induce the expression of phase 2 enzymes, such as HO-1. The specific anti-inflammation mechanism of H_2 may work via Nrf2 in macrophages, activate Nrf2 translocation into the nuclei, and encode the phase 2 enzymes HO-1 expression.

However, there are several limitations in this study. First, we measured the changes of inflammatory cytokines and HO-1 for 24 h after LPS stimulation. We should check the expression and changes for a longer time. Second, we used the RAW 264.7 macrophages. In the future, we might choose primary cultured macrophages. Third, further studies should be conducted to find the underlying mechanisms of H_2 in inflammatory diseases.

In summary, our study clearly provided evidence that H_2 had an anti-inflammatory

effect in LPS-activated macrophages through inhibiting the release pro-inflammatory cytokines and increasing the release of anti-inflammatory cytokine, which was at least mediated by HO-1.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (Nos. 81071533 to Yonghao Yu; 81101409 to Keliang Xie), the Natural Science Foundation of the Tianjin Science Committee (Nos. 11JCYBJC12900 to Yonghao Yu; 13JCQNJC11400 to Keliang Xie), the Foundation of Tianjin Bureau of Public Health (2011KZ108 to Keliang Xie).

Author contribution

Yong-hao YU and Ke-liang XIE designed the research; Hong-guang CHEN, Ke-liang XIE, Huan-zhi HAN, Wei-na WANG and Da-quan LIU performed the research; Guo-lin WANG and Yong-hao YU contributed new analytical reagents and tools. Hong-guang CHEN and Yong-hao YU analyzed the data; Hong-guang CHEN, Ke-liang XIE, Yong-hao YU wrote the paper.

References

[1] Liu J, Liu Y, Zhang H, Chen G, Wang K, Xiao X. Klf4 promotes the expression, translocation, and release of HMGB1in Raw264.7 macrophages in responese to LPS. Shock 2008; 30: 260-66.

[2]Riedemann NC, Guo RF, ard PA. Novel strategies for the treatment of sepsis. Nat med 2003; 9: 517-524.

[3]Arumugam TV, Okun E, Tang SC, Thundyil J, Taylor SM, Woodruff TM. Toll-Like Receptors in Ischemia-Reperfusion Injury. Shock 2009;32:4-16.

[4]Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. Nature 2008; 454: 436-44.

[5]Glass CK, Saijo K, Winner B, Marchetto MC, Gage FH. Mechanisms underlying inflammation in neurodegeneration. Cell 2010; 140: 918-34.

[6] Ohsawa I, Ishikawa M, Takahashi K, Meguni W, Nishimaki K, Yamagata K, *et al*. Hydrogen acts as a therapeutic antioxidant by selectively reducing cytotoxic oxygen radicals. Nat Med 2007; 13: 688-94.

[7] Xie K, Yu Y, Huang Y, Zheng L, LiJ, Chen H, *et al*. Molecular Hydrogen Ameliorates Lipopolysaccharide-Induced Acute Lung Injury in Mice Through Reducing Inflammation and Apoptosis. Shock 2012; 37: 548-55.

[8] Xie K, Yu Y, Pei Y, Hou L, Chen S, Xiong L, *et al*. Prtective effects of hydrogen gas on murine polymicrobial sepsis via reducing oxidative stress and hmgb 1 release. Shock 2010; 34: 90-97.

[9] Xie K, Yu Y, Zhang Z, Liu W, Pei Y, Xiong L, et al. Hydrogen gas iproves

survival rate and organ damage in zymosan-induced generalized inflammation model. Shock 2010; 34: 495-501.

[10] Qu J, Li X, Wang J, Mi W, Xie K, Qiu J. Inhalation of hydrogen gas attenuates cisplatin-induced ototoxicity via reducing oxidative stress. Int J Pediatr Otorhinolaryngol 2012; 76: 111- 5.

[11] Tomohiro I, Nanako H, Riyako T, Ito M , Ohno K, Ichihara M, *et al*. Molecular hydrogen inhibits lipopolysaccharide/interferon γ-induced nitric oxide production through modulation of signal transduction in macrophages. Biochem Biophys Res Commun 2011; 441: 143- 9.

[12] Fukuda K, Asoh S, Ishikawa M, Yamamoto Y, Ohsawa I, Ohta S. Inhalation of hydrogen gas suppresses hepatic injury caused by ischemia/reperfusion through reducing oxidative stress. Biochem Biophys Res Commun 2007; 361:670-74,

[13] Buchholz BM, Kaczorowski DJ, Sugimoto R, Yang R, Wang Y, Billiar TR, *et al.* Hydrogen inhalation ameliorates oxidative stress in transplantation induced intestinal graft injury. Am J Transplant 2008; 8: 2015-24.

[14] Ohsawa I, Nishimaki K, Yamagata K, Ishikawa M, Ohta S. Consumption of hydrogen water prevents atherosclerosis in apolipoprotein E knockout mice. Biochem Biophys Res Commun 2008; 377: 1195-8.

[15] Fu Y, Ito M, Fujita Y, Ichihara M, Masuda A, Suzuki Y, *et al*. Molecular hydrogen is protective against 6-hydroxydopamine-induced nigrostriatal degeneration in a rat model of Parkinson's disease. Neurosci Lett 2009; 453: 81-5.

[16] Cai J, Kang Z, Liu K, Liu W, Li R, Zhang JH, *et al.* Neuroprotective effects of hydrogen saline in neonatal hypoxia-ischemia rat model. Brain Res 2009; 1256: 129-37.

[17] Xu Z, Zhou J, Cai J, Zhu Z, Sun X, Jiang C.Anti-inflammation effects of hydrogen saline in LPS activated macrophages and carrageenan induced paw oedema. J Inflamm 2012; 9: 2-8.

[18] Stefan WR, Augustine MK. Heme oxygenase-1: redox regulation of a stress protein in lung and cell culture models. Antioxid Redox Signal 2005; 7: 80–91.

[19] Li Q, Zhu Y, Jiang H, Jiang H, Xu H, Sun Y. Heme oxygenase-1 mediates the anti-inflammatory effect of isoflurane preconditioning in LPS-stimulated macrophages. Acta Pharmacol Sin, 2009; 30: 228-34.

[20] Dijkstra G, Blokzijl H, Bok L, Homan M, van Goor H, Faber KN, *et al*. Opposite effect of oxidative stress on inducible nitric oxide synthase and heme oxygenase-1 expression in intestinal inflammation: anti-inflammatory effect of carbon monoxide. J Pathol 2004; 204: 296-303.

[21]Chung SW, Liu X, Macias AA, Baron RM, Perrella MA. Heme oxygenase-1-derived carbon monoxide enhances the host defense response to microbial sepsis in mice. J Clin Invest 2008; 118: 239-47.

[22] Huang CS, Kawamura T, Toyoda Y, Nakao A. Recent advances in hydrogen research as a therapeutic medical gas. Free Radic Res 2010; 44: 971-82.

[23] Ha YM, Ham SA, Kim YM, Lee YS, Kim HJ, Seo HG, *et al*. β1-Adrenergic receptor-mediated HO-1 induction, via PI3K and p38 MAPK, by isoproterenol in

RAW 264.7 cells leads to inhibition of HMGB1 release in LPS-activated RAW 264.7 cells and increases in survival rate of CLP-induced septic mice. Biochem Pharmacol 2011; 82: 769-77.

[24] Fang F, Liu GT. Protective effects of compound FLZ, a novel synthetic analogue of squamosamide, on β-amyloid-induced rat brain mitochondrial dysfunction in vitro. Acta Pharmacol Sin 2009; 30: 522-9.

[25] Peleg AY , Hooper DC. Hospital-acquired infections due to gram-negative bacteria. N Eng J Med 2010; 362: 1804- 13.

[26] Dinarello CA.Proinflammatory and anti-inflammatory cytokines as mediators in the pathogenesis of septic shock. Chest 1997; 112: 321S-9S

[27] Cohen J. The immunopathogenesis of sepsis. Nature 2002,420(6917):885-891.

[28] Wang H, Bloom O, Zhang M, Vishnubhakat JM, Ombrellino M, Che J, *et al*. HMGB-1 as a late mediator of endotoxin lethality in mice. Science 1999; 285: 248-51.

[29] Yang H, Ochani M, Li J, Qiang X, Tanovic M, Harris HE, *et al*. Reversing established sepsis with antagonists of endogenous high-mobility group box1. Proc Natl Acad Sci USA 2004; 101: 296-301.

[30] Wang JX, Hou LF, Yang Y, Tang W, Li Y, Zuo JP.SM905, an artemisinin derivative, inhibited NO andpro-infammatory cytokine production by suppressing MAPK and NF-κB pathways in RAW 264.7 macrophages. Acta Pharmacologica Sinica 2009; 30: 1428-35.

[31] Andersson U, Tracey KJ. HMGB1 is a Therapeutic Target for Sterile

Inflammation and Infection. Annual Review of Immunology 2011; 29: 139-62.

[32] Qu J, Gan YN, Xie KL, Liu WB, Wang YF, Hei RY, *et al*. Inhalation of hydrogen gas attenuates ouabain-induced auditory neuropathy. Acta Pharmacologica Sinica 2012; 1: 1-7.

[33] Xu Z, Zhou J, Cai J, Zhu Z, Sun X, Jiang C. Anti-inflammation effects of hydrogen saline in LPS activated macrophages and carrageenan induced pawo edema. J Inflamm 2012; 9: 1-8.

[34] Tsoyi K, Lee TY, Lee YS, Kim HJ, Seo HG, Lee JH, *et al*. Heme-oxygenase-1 induction and carbon monoxide-releasing molecule inhibit lipopolysaccharide (LPS)-induced high mobility group box 1 release in vitro and improve survival of mice in LPS- and cecal ligation and puncture-induced sepsis model in vivo. Mol Pharmacol 2009; 76: 173-82.

[35] Takamiya R, Hung CC, Hall SR, Fukunaga K, Nagaishi T, Maeno T, *et al*. High mobility group box 1 contributes to lethality of endotoxemia in heme oxygenase-1-deficient mice. Am J Respir Cell Mol Biol 2009; 41: 129-35.

[36] Fujii H, Takahashi T, Nakahira K, Uehara K, Shimizu H, Matsumi M, et al. Protective role of heme oxygenase-1 in the intestinal tissue injury in an experimental model of sepsis.Crit Care Med. 2003 Mar;31(3):893-902.

[37] Stefan S ,Karin E, Lie H, Shinkatsu M ,Sanetaka S, Sandra C. Molecular hydrogen reduces LPS-Induced neuroinflammation and promotes recovery fromsickness behaviour in mice. Plos one 2012; 7: e42078.

[38] Kensler TW, Wakabayashi N, Biswal S. Cell survival responeses to

environmental stresses via the keap1-nrf2-are pathway. Annu Rev Pharmacol Toxicol 2007; 47: 89-116.

Figure Legends

Figure 1 Effects of hydrogen treatment on cell viability and injury of RAW 264.7 macrophages. The injury and viability of RAW 264.7 macrophages were measured with LDH release (A) and MTT assay (B). Cells were treated with different concentrations of hydrogen (0, 0.15, 0.30 and 0.60 mM) for 24 h. The relative absorbance is expressed as % of control, and results are presented as mean ± SD (n = 6 each group).

Figure 2. Effects of hydrogen treatment on the production of inflammatory cytokines in LPS-activated Raw 264.7 macrophages. Cells were stimulated with 1μg/mL of LPS or PBS with absence or presence of hydrogen-rich medium (0.6 mM). The levels of TNF-α (A), IL-1β (B), HMGB1 (C) and IL-10 (D) in the culture media were measured at baseline (0 h) as well as 3 h, 6 h, 12 h and 24 h after LPS or PBS administration. Results are presented as mean ±SD (n = 6 each group at every time point). $^{a}P<0.05$ compared with the Con group; $^{b}P<0.05$ compared with the LPS group.

Figure 3. The concentration-dependent effects of hydrogen treatment on inflammatory cytokines production in LPS-stimulated RAW 264.7 macrophages. Cells were challenged with LPS (1μg/mL) for 24 h in the absence or presence of different concentrations of hydrogen. The culture media were collected for

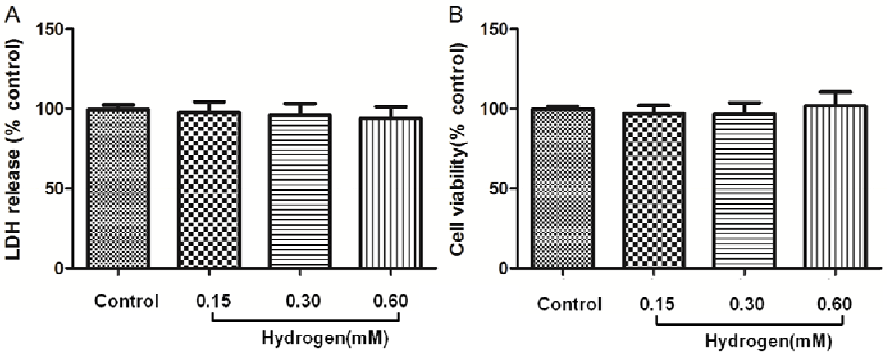
measurement of TNF-α (A), IL-1β (B), HMGB1 (C) and IL-10 (D). Results are presented as mean ± SD (n = 6 each group). $^{a}P<0.05$ compared with the cells without hydrogen and LPS; $^{b}P<0.05$ compared with the cells with LPS stimulation; $^{c}P<0.05$ compared with the cells with 0.15 mM hydrogen and LPS

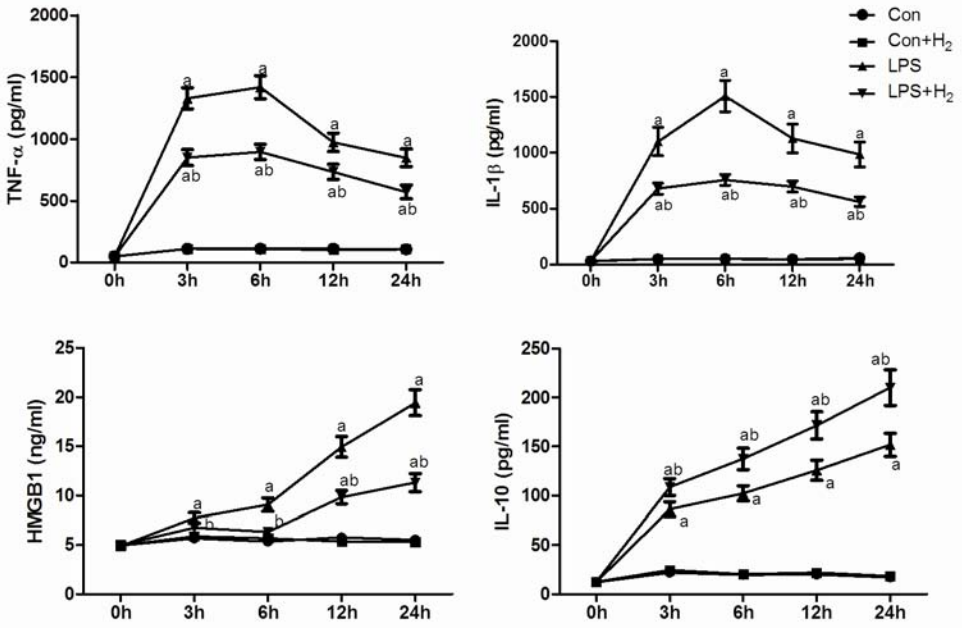
Figure 4. Effect of hydrogen treatment on HO-1 activity in LPS-stimulated Raw 264.7 macrophages. (A) At the indicated time points, the HO-1 activity was detected. (B) Cells were challenged with 1 μg/ml of LPS or PBS in the absence or presence of different concentrations of hydrogen. After incubation for 24 h, HO-1 activity was detected. Results are presented as mean ± SD (n = 6 each group at each time point). $^{a}P<0.05$ compared with the Con group; $^{b}P<0.05$ compared with the LPS group. $^{c}P<0.05$ compared with the cells without hydrogen and LPS; $^{d}P<0.05$ compared with the cells with LPS stimulation. $^{e}P<0.05$ compared with the cells with 0.60 mM hydrogen and LPS.

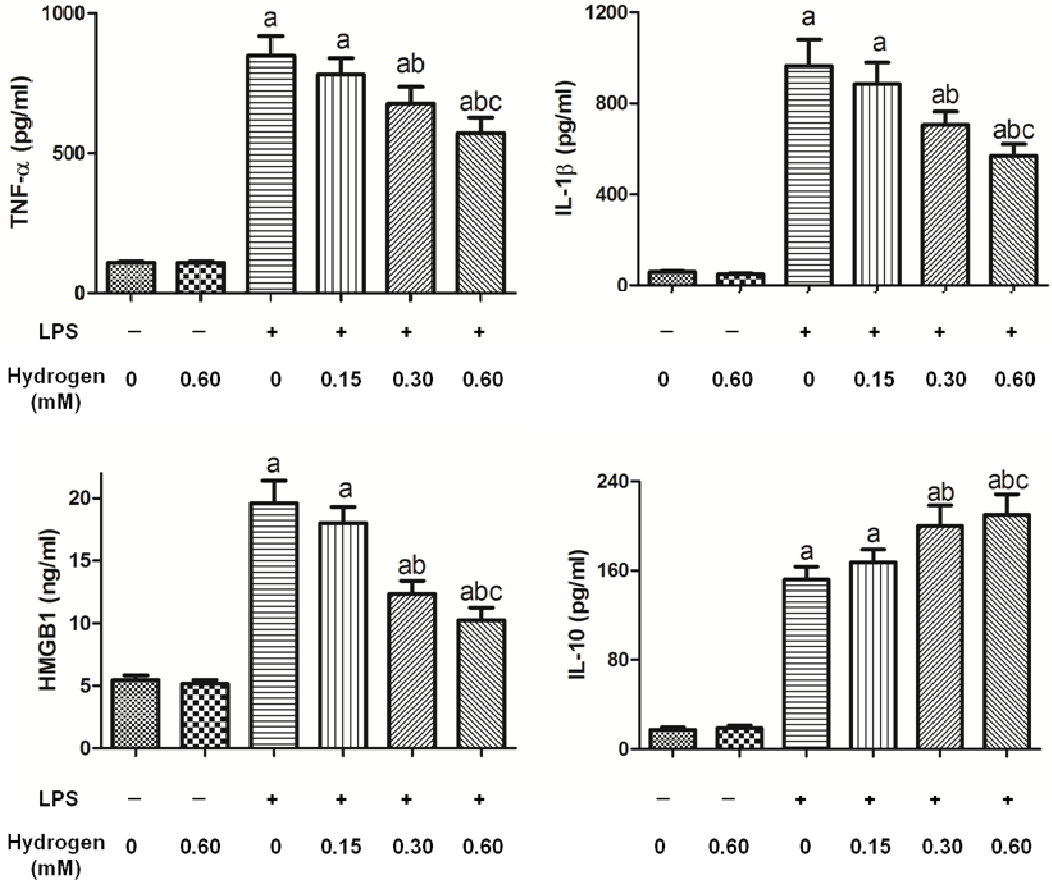
Figure 5. Effect of hydrogen treatment on HO-1 expression in LPS-stimulated Raw 264.7 macrophages. Cells were challenged with 1 μg/ml of LPS or PBS for 24 h in the absence or presence of different concentrations of hydrogen. HO-1 expression was assayed by western blot analysis. Results are presented as mean ± SD (n = 6 each group). $^{a}P<0.05$ compared with the cells without hydrogen and LPS; $^{b}P<0.05$ compared with the cells with LPS stimulation; $^{c}P<0.05$ compared with the cells with 0.15 mM hydrogen and LPS stimulation. $^{d}P<0.05$ compared with the cells with 0.30

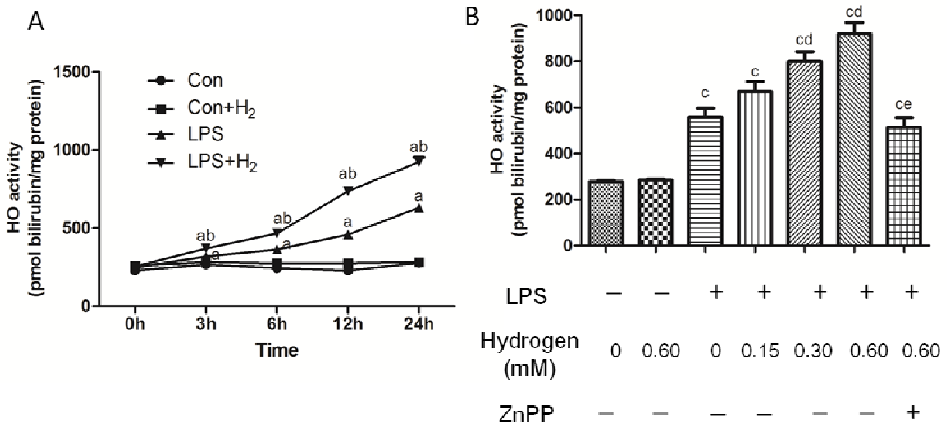
mM hydrogen and LPS.

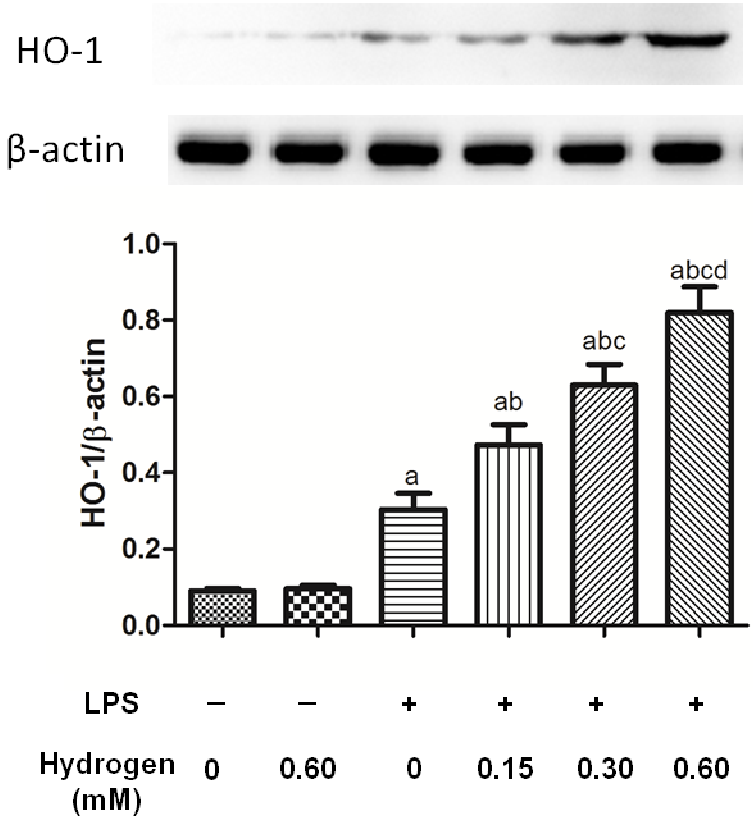
Figure 6. HO-1 inhibitor Znpp could reverse the regulative effect of hydrogen treatment on inflammatory cytokines production in LPS-stimulated Raw 264.7 macrophages. Cells were cultured with 1 μg/ml LPS or PBS under hydrogen treatment for 24 h in the presence or absence of 20 μM of Znpp. Culture media were harvested for measurement of TNF-α (A), IL-1β (B), HMGB1 (C) and IL-10 (D). Results are presented as mean ± SD (n = 6 each group). $^{a}P<0.05$ compared with the cells without hydrogen and LPS; $^{c}P<0.05$ compared with the cells with LPS stimulation. $^{b}P<0.05$ compared with the cells with hydrogen and LPS.

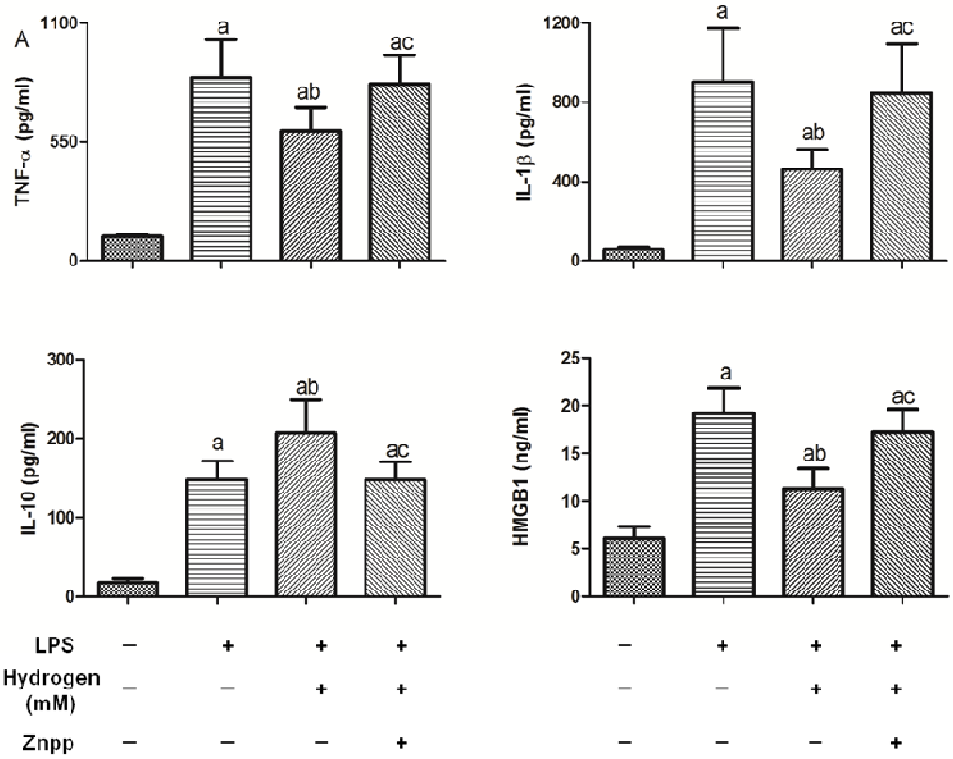





TNF-α (pg/ml)
1000
500
0
a
a
ab
abc
LPS – – + + + +
Hydrogen (mM) 0 0.60 0 0.15 0.30 0.60
IL-1β (pg/ml)
1200
800
400
0
a
a
ab
abc
– – + + + +
0 0.60 0 0.15 0.30 0.60
HMGB1 (ng/ml)
20
15
10
5
0
a
a
ab
abc
LPS – – + + + +
Hydrogen (mM) 0 0.60 0 0.15 0.30 0.60
IL-10 (pg/ml)
240
160
80
0
a
a
ab
abc
– – + + + +
0 0.60 0 0.15 0.30 0.60






A
TNF-α (pg/ml)
1100
550
0
a
ab
ac
IL-1β (pg/ml)
1200
800
400
0
a
ab
ac
IL-10 (pg/ml)
300
200
100
0
a
ab
ac
HMGB1 (ng/ml)
25
20
15
10
5
0
a
ab
ac
LPS – + + + – + + +
Hydrogen (mM) – – + + – – + +
Znpp – – – + – – – +