

Hydrogen-Rich Medium Ameliorates Lipopolysaccharides-Induced Mitochondrial Fission and Dysfunction in Human Umbilical Vein Endothelial Cells (Huvecs) via Up-Regulating HO-1 Expression

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Research

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Abstract

Background

Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection. It has been showed that the change of mitochondrial dynamics has been proved to be one of the main causes of death in patients with severe sepsis. And hydrogen has been proved to exert its protective effects against sepsis via heme oxygenase-1 (HO-1). This study was designed to demonstrate that whether the benefit effects of hydrogen can maintain the dynamic process of mitochondrial fusion/fission to mitigate human umbilical vein endothelial cells (HUVECs) injury exposed to endotoxin through HO-1.

Methods

HUVECs cells cultured with medium which contained Lipopolysaccharides (LPS), Saline, hydrogen, Mdivi-1 (a dynamin-related protein 1 [Drp1] inhibitor) or zinc protoporphyrin IX (Znpp) (a HO-1 inhibitor) were also used in the research. Cell death and apoptosis were assessed using FITC annexin V and PI. Mitochondria were stained with Mitotracker orange and observed by confocal microscope. Oxygen consumption rate was assessed by seahorse xf24 extracellular analyzer. Mitochondrial membrane potential monitored by JC-1 dye. The expressions of Drp1 and HO-1 were tested by Western blot. The co-localization of Drp1 and mitochondria was determined by immunofluorescence.

Results

LPS caused a decrease in ATP content, mitochondrial membrane potential, and maximal respiration rate. At the same time, increased expression of Drp1 were observed in LPS-stimulated HUVECs, concomitantly with excessive mitochondrial fission. We found that hydrogen-rich medium can increase ATP content, mitochondrial membrane potential and maximal respiration rate, and decrease the expression of Drp1 in LPS-treated HUVECs. Meanwhile, hydrogen can ameliorate excessive mitochondrial fission caused by LPS. Furthermore, hydrogen-rich medium had a similar effect to Mdivi-1, a mitochondrial fission blocker. Both of them rescued the up-regulation of Drp1 and mitochondrial fission induced by LPS, then normalized mitochondrial shape after LPS stimulation. But after Znpp pretreatment, HO-1 expression was inhibited and the protective effects of hydrogen were abrogated.

Conclusions

Hydrogen-rich medium can alleviate the LPS-induced mitochondrial fusion/fission and dysfunction in HUVECs via HO-1 up-regulation.

Introduction

Sepsis is a common complication after burns, trauma and major surgery. It is the main cause of death in acute and critical patients [1]. Sepsis has caused enormous global burden in terms of incidence rate and mortality. Lipopolysaccharide / endotoxin (LPS), which exists in the outer membrane of Gram-negative bacteria, binds to receptors on the surface of endothelial cells, resulting in microcirculation disturbance, septic shock and multiple organ failure [2, 3]. Endothelial cell injury has been considered as one of the important pathological features of sepsis [4]. So far, the potential mechanism of LPS induced endothelial cell injury has not made a breakthrough, which is a medical problem to be solved.

Mitochondria is a highly mobile organelle, which frequently occurs division / fusion, biosynthesis and autophagy. The dynamic process of mitochondrial division and fusion is called mitochondrial dynamics [5–8]. Its balance is a necessary condition for maintaining the normal morphology and function of mitochondria. Mitochondrial dysfunction has been proved to be one of the main causes of death in patients with severe sepsis, and the key cause of mitochondrial dysfunction is the change of mitochondrial dynamics. The disorder of mitochondrial dynamic balance plays an important role in the pathogenesis of sepsis. The prognosis of sepsis is closely related to the early activation of mitochondrial biosynthesis [9].

Heme oxygenase (HO) is recognized as a core component of the mammalian stress response and antioxidant stress defense [10–13]. As an inducible stress response protein, HO-1 is highly concentrated in tissues closely related to heme protein catabolism, and can degrade heme into carbon monoxide (CO), free iron and biliverdin [14]. The protein is also induced in response to various stimuli, such as reactive oxygen species (ROS) production [15]. In the process of oxidative stress, induction of HO-1 exerts powerful antioxidant, anti-inflammatory and anti-apoptotic properties [16]. Previous studies have shown that the endogenous defense effect of HO-1 pathway on sepsis may be related to mitochondrial dynamics [17]. Mitochondrial fission is regulated by dynein related protein 1 (Drp1), which is a highly conserved dynamin-related GTPases [18]. However, the overall regulatory mechanism remains unclear.

Molecular hydrogen (H_2), the smallest gas molecule with molecular weight, is colorless, odorless, insoluble in water. It has a strong ability to penetrate and enter cells and an extremely high biological safety profile, which can react with ROS [19]. In recent years, it has been demonstrated that molecular hydrogen, with selective antioxidant, anti-inflammatory and anti-apoptotic properties, has therapeutic effects on a variety of diseases [20–24]. Hydrogen has been reported to ameliorate sepsis induced damage of important organs [25], but the specific mechanism by which it exerts protective effects remains to be revealed.

Herein, we tested the hypothesis that HO-1 played a critical role in maintaining the dynamic process of mitochondrial fusion/fission to mitigate human umbilical vein endothelial cells (HUVECs) injury exposed to endotoxin.

Materials And Methods

Cell culture

The HUVECs were purchased from the American Type Culture Collection (ATCC) and incubated with Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA) with 10% fetal bovine serum (FBS) (Gibco, USA) and 1% Penicillin-Streptomycin Solution (Solarbio, China) at 37 °C in 5% CO₂ and 95% air.

Drug treatment

Cells were cultured to 80~90% confluence and treated for 3, 12, 24 hours with LPS (Sigma, USA) (10 ug/ml) or hydrogen-rich medium (0.6 mmol/L), then the cells were harvested for the following experiments. 25 uM Mdivi-1 (Sigma, USA), a selective inhibitor of Drp1, was joined to the medium 1 h before LPS or hydrogen-rich medium treatment. HUVECs were pretreated for 6 hours with the HO-1 inhibitor zinc protoporphyrin IX (Znpp) (Sigma, USA) (10 μM).

Cell apoptosis

HUVECs were cultured on 10 mm glass dishes. After stimulation with or without LPS and hydrogen-rich medium. The cell death was tested by Dead Cell Apoptosis Kit with FITC annexin V and PI (Invitrogen, USA) and was performed by following the manufacturer's instruction. The cells were concentrated, and washed twice with cold PBS. Basically, collected individual cells were stained with FITC annexin V and PI, then analyzed by confocal microscope (Nikon). Cytoplasm with bright green fluorescence was apoptotic cell.

Mitochondria imaging

HUVECs were cultured on 10 mm glass dishes. After stimulation with or without LPS, hydrogen-rich medium and Znpp, cells were incubated with 500 nM MitoTracker Orange (Molecular Probes-Invitrogen, USA) for 5 min, then washed with PBS, and were examined under confocal microscope (Nikon). HUVECs in 3 independent experiments were used for quantification of mitochondrial aspect ratio (length/width) using ImageJ-3D Object counter plug-in. Each experiment was done at least four times and each time 16–25 cells per condition were quantified. Aspect ratio was defined as length/width.

Mitochondrial activity

The rates of oxygen consumption in hybrid cell lines were measured with a Seahorse Bioscience XF-24 extracellular flux analyzer (Seahorse Bioscience) [26, 27]. XF24 creates a transient, 7 ul-chamber in specialized microplates that allows for the determination of oxygen and proton concentrations in real time. To allow comparison between different experiments, data are expressed as the rate of oxygen consumption in pmol/min. Normalized to cell protein in individual wells determined by the Bradford protein assay (Bio-Rad). A density of 60 000 cells per well in 24-well plate was coated with Cell-

TakTM adhesive. The rates of O₂ were determined under basal condition and the addition of oligomycin (1.0 μM), carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone (FCCP) (0.3 μM), rotenone (1.0 μM) and antimycin A (1 μM) [26, 27].

Mitochondrial membrane potential

HUVECs were cultured on 10 mm glass dishes. After stimulation with LPS, cells were incubated with 500 nM JC-1 for 20 min (Biyuntian, Nanjing, China), washed with PBS, and were examined under confocal microscope (Nikon). HUVECs in 3 independent experiments were used for quantification. Each experiment was done at least four times.

Western blot analysis

Equal amounts of protein from cells were separated by SDS-PAGE (10% polyacrylamide gels) and electrotransferred to nitrocellulose. Membranes were blocked with 5% defatted milk in Tris-buffered saline, pH 7.6, containing 0.1% (v/v) Tween 20 (TBST). Membranes were incubated with primary antibodies at 4 °C, total Drp1 1:1000 (Cell Signalling, USA), HO-1 1:1000 (Abcam, USA), β-actin 1:1000 (Sigma, USA) and re-blotted with horseradish peroxidase-linked secondary antibody 1:5000 (Proteintech Group, USA). The bands were detected using ECL (Perkin Elmer, USA) with exposure to Kodak film and quantified by scanning densitometry. Protein content was normalized by β-actin.

Drp1 and mitochondria colocalization

HUVEC cells cultured on 10 mm glass dishes were stimulated with LPS for 12h. During the last 5 min of treatment 500 nM MTO was added. Cells were washed with PBS and fixed with PBS containing 4% paraformaldehyde and incubated for 10 min in ice-cold. 0.3% Triton X-100 (Sigma-Aldrich, USA) were used for 10 min for permeabilization. Nonspecific sites were blocked with 5% Goat Serum in PBS for 1 h and then the cells were incubated with anti-Drp1 (1:50) antibody (Cell Signalling, USA). Secondary antibody was anti-rabbit (Molecular Probes-Invitrogen, USA). For the colocalization analysis only one focal plane was analyzed with a Zeiss LSM-5 Pascal 5 Axiovert 200 microscope. Images obtained were deconvolved and background was subtracted using the ImageJ software. Colocalization between the Drp1 and mitochondria was quantified using the Manders' algorithm, as detailed elsewhere [28, 29].

Statistical analysis

Data are shown as mean ± SD (standard deviation). Data were analyzed by one-way ANOVA and differences among groups was detected using a Dunnett's test. Statistical significance was defined as $P < 0.05$.

Results

Hydrogen-rich medium restrained LPS-induced cell death in HUVECs

Exposure to 10 µg/ml LPS for 12h induced a significant increase in Annexin V-FITC-positive cells compared with control cells ($P < 0.05$), indicating that LPS induced cell apoptosis in HUVECs. However, hydrogen-rich medium attenuated LPS-induced cell apoptosis in HUVECs ($P < 0.05$) (Fig. 1).

Hydrogen-rich medium attenuated LPS-induced mitochondrial dysfunction in HUVECs

To assess the changes in mitochondrial oxidative phosphorylation, we measured oxygen consumption rates (OCR) at baseline and after maximal uncoupling. Basal OCR was observed no significantly change after LPS or hydrogen-rich medium treatment (Fig. 3a-c). Decline in OCR was due to a decreased mitochondrial oxidative capacity, because FCCP-stimulated maximal OCR was significantly decreased with LPS treatment, which was improved by hydrogen-rich medium (Fig. 3d-f). Furthermore, LPS treatment decreased mitochondrial membrane potential (Fig. 2) and ATP content (Fig. 3g-i) while hydrogen-rich medium ameliorated these damages. These last data suggest that hydrogen-rich medium attenuates LPS-induced mitochondrial dysfunction in HUVECs.

Hydrogen-rich medium inhibits LPS induced mitochondrial fission

Treatment of HUVEC with LPS stimulated the appearance of short fragment mitochondria while hydrogen-rich medium elongated the mean length of mitochondria and decreased its fragmentation after LPS stimulation (Fig. 4). Taken together, these results suggest that LPS stimulates mitochondrial fission in HUVECs while hydrogen-rich medium inhibits these changes.

Hydrogen-rich medium depressed Drp1 expression level and activity

Treatment with LPS increased mitochondrial fission protein Drp1 expression and hydrogen-rich medium restrained LPS-induced Drp1 over expression (Fig. 5a-d). Mdivi-1 is a selective inhibitor of Drp1. We used Mdivi-1 to understand the role of Drp1 in LPS-treated HUVECs. LPS induced Drp1 over expression were decreased by pretreatment with 25 µM Mdivi-1 and hydrogen-rich medium played a similar role to Mdivi-1 in this experiment (Fig. 5e-f). Previous studies have established the migration of Drp1 from the cytosol to the mitochondrial surface as an initial step in mitochondrial fission [30]. Therefore, we next evaluated whether mitochondrial fission triggered by LPS was associated with changes in the subcellular distribution of Drp1. Considered that LPS stimulated Drp1 express level was highest at 12 h (Fig. 5b), we chose 12 h after LPS stimulation to observe the co-localization of Drp1 and mitochondria. Immunofluorescence studies showed that the Drp1 was recruited to mitochondria after 12 h of treatment with LPS. Both hydrogen-rich medium and Mdivi-1 restrained the recruitment of Drp1. Hydrogen-rich medium and Mdivi-1 used together showed no significance compared with used hydrogen-rich medium or Mdivi-1 alone (Fig. 5e-g). Taken together, these data suggest that LPS induced Drp1 over expression and recruitment to mitochondria triggered mitochondrial fission. Hydrogen-rich medium played a similar role to Mdivi-1, both of them could depressed Drp1 expression and recruitment.

Hydrogen-rich medium attenuates LPS-induced Drp1 activation, mitochondrial dysfunction and mitochondrial fission by up regulating HO-1 expression

HO-1, namely, heat shock protein [31], is reported to impede oxidative cellular injury [32]. To further study the role of HO-1 in LPS-stimulated HUVEC, we detected the expression of HO-1 by western blot. The result showed that HO-1 expression was higher in LPS group compared with sham group and in LPS+H₂ group, HO-1 increased more percent compared with LPS group (Fig. 6a-b). HO-1 expression didn't be influenced by Mdivi-1 (Fig. 6c-d). Pretreatment of 10 μM Znpp decreased LPS induced up-regulation of HO-1 and reversed the effects of hydrogen-rich medium on HO-1 content (Fig. 6e-f). Znpp also reversed the inhibition effect of hydrogen-rich medium on total Drp1 level (Fig. 6g-h). Based on these data, we got a conclusion that hydrogen-rich medium down-regulate Drp1 by increasing HO-1 expression. To confirm if HO-1 have protective effects on mitochondrial activities, Znpp also had been used to in mitochondrial activity experiment and mitochondrial morphology experiment. The results were consistent with previous, Znpp restricted the protective effects of hydrogen-rich medium on mitochondrial membrane potential (Fig. 7a-b), ATP content (Fig. 7c), maximal respiration rate (Fig. 7d) and aggravated mitochondrial fission (Fig. 7e-f). Znpp had no influence on basal respiration rate.

Discussion

Sepsis is a systemic inflammatory response syndrome induced by infection, which is the leading cause of deaths in intensive care patients, and there is no effective treatment. In sepsis, as the target cells and the effect cells of the inflammatory response, vascular endothelial cells were activated continuously, and were severe damaged, eventually leading to organs damage and failure [33]. Therefore, vascular endothelial cells as a targets for sepsis are of great significance. Based on this, we used LPS to induce an inflammatory model in HUVECs and successfully prepared the inflammatory state of the cells to perform subsequent relevant experiments from the cellular level.

Hydrogen is a small molecular gas, has antioxidative, anti-inflammatory, and antiapoptotic effects. A large amount of research show that H₂ have beneficial effects to multi-organ injury induced by severe sepsis [20–24]. Consistent with our experiment, after LPS stimulation, the V-FITC positive cells increased significantly ($P < 0.05$), the maximum OCR, mitochondrial membrane potential and ATP content decreased significantly ($P < 0.05$), the mitochondrial fragments increased significantly ($P < 0.05$), and the expressions of Drp1 also increased significantly ($P < 0.05$), while the hydrogen-rich medium can reverse the above changes of various indexes caused by LPS treatment ($P < 0.05$) at 3 h, 12 h and 24 h. The trend of HO-1 at 12 h was same to that of Drp1.

Mitochondria are dynamic organelles, which undergo continuous fission and fusion [34]. Fission events are regulated by Drp1 and fusion events are regulated by the large dynamin-related GTPases known as mitofusin 1 and 2 (Mfn1 and 2) as well as optic atrophy1 (OPA1) [35, 36]. Alterations in mitochondrial dynamics significantly impact mitochondrial numbers and shape, oxygen consumption rate and ATP production [37]. A large amount of research find that Imbalances of mitochondrial fission and fusion can induces mitochondrial dysfunction (impaired respiration, mitochondrial memberian potential and ATP production) in cardio vascular disease, diabetes, neuropaphy and other disease [28, 29, 32, 38, 39]. As can also be seen from our experiments, LPS treatment of HUVECs caused changes in intracellular

mitochondrial dynamics, resulting in a significant decrease in maximal OCR, while also affecting mitochondrial membrane potential and ATP content, demonstrating that LPS induces mitochondrial dysfunction (Fig. 2, 3). Another indicator of altered mitochondrial dynamics, the shape of mitochondria, was also changed after LPS treatment of HUVECs, which stimulated mitochondrial fission with increased mitochondrial fragmentation (Fig. 4), again demonstrating that LPS causes mitochondrial dysfunction.

Drp1 is one of the most important proteins involved in the regulation of mitochondrial fission. Drp1 translocates from the cytosol to the mitochondria and interacts with mitochondrial outer membrane proteins (Fis-1, Caf-4, MDV-1 in yeast and Mff in metazoans) [30, 40]. After generating a ring around the organelle, the GTP hydrolysis causes their constriction triggering the mitochondrial fission [40]. Our data support the idea that LPS-induced mitochondrial fission depends on Drp1 activation. This view was further demonstrated using Mdivi-1. Mdivi-1 is a selective inhibitor of Drp1. LPS induced Drp1 over-expression was decreased by pretreatment of 25 μ M Mdivi-1. Previous studies have determined that the migration of Drp1 from cytoplasm to mitochondrial surface is the first step of mitochondrial division [38]. Therefore, the relationship between LPS induced mitochondrial division and the change of Drp1 subcellular distribution was evaluated through the co-localization of Drp1 and mitochondria. It was found that Drp1 was recruited to mitochondria after LPS treatment, which was inhibited by hydrogen-rich medium and Mdivi-1. However, the simultaneous treatment of hydrogen and Mdivi-1 did not produce superposition effect (Fig. 5). It is proved that LPS induced Drp1 over-expression and mitochondrial recruitment triggered mitochondrial division, which could be inhibited by hydrogen.

Previous studies have shown that the endogenous defense effect of HO-1 pathway on sepsis may be related to mitochondrial dynamics [17]. HO-1 is reported to impede oxidative cellular injury [32]. Consistent with the method of verifying Drp1, Znpp, an inhibitor of HO-1, was used for verification. LPS induced increased HO-1 expression, while in LPS + H₂ group, HO-1 expression increased more than that in LPS group (Fig. 6a-b). Pretreatment of 10 μ M Znpp decreased LPS induced HO-1 up-regulation and reversed the effect of hydrogen-rich medium on HO-1 content (Fig. 6e-f). ZnPP also reversed the inhibitory effect of hydrogen-rich medium on total Drp1 level (Fig. 6g-h). It was proved that hydrogen could down-regulate Drp1 by increasing HO-1 expression. At the same time, we explored whether HO-1 was protective or harmful to mitochondria. ZnPP limited the protective effect of hydrogen-rich medium on mitochondrial membrane potential, ATP content, maximum respiratory rate and increased mitochondrial division (Fig. 7), which inversely proved the protective effect of HO-1 on mitochondria and the protective effects of H₂ is relevant for HO-1 gene regulation.

Conclusions

In summary, our results showed that the protective effect of H₂ on LPS-treated HUVECs could be involved in the mitochondrial dynamics dependent mechanism mediated by HO-1 and Drp1.

Abbreviations

HUVECs: Human umbilical vein endothelial cells; HO-1: Heme oxygenase-1; HO: Heme oxygenase; LPS: Lipopolysaccharides; Drp1: Dynamin-related protein 1; Znpp: Zinc protoporphyrin Zn^{2+} ; CO: Carbon monoxide; H_2 : Molecular hydrogen; ATCC: American type culture collection; DMEM: Dulbecco's modified eagle medium; FBS: fetal bovine serum; OCR: Oxygen consumption rates;

Declarations

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

Ethics approval and consent to participate

Not applicable.

Consent for publication

All listed authors consent to the submission, and all data are used with the consent of the person generating the data.

Availability of data and materials

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

KLX, XM and NQL contributed equally to this work. KLX and XM contributed to the conception and design. XM performed the experiments, analyzed data. NQL wrote the manuscript. YYW, HGC and YZW participated in the animal experiments. YYH and WGL helped to revise the manuscript. All authors read and approved the final manuscript.

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Figures

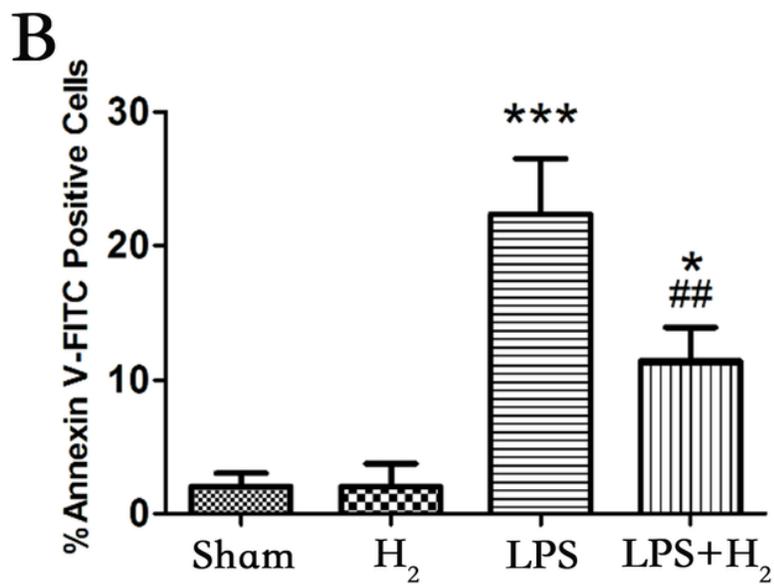
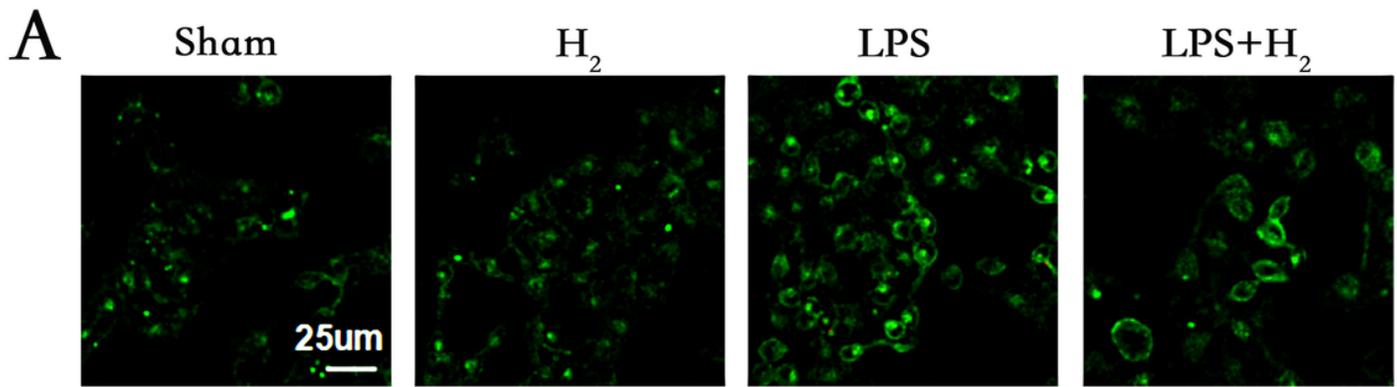


Figure 1

Effects of LPS and H₂ on cell apoptosis. HUVECs were stimulated with or without 10 ug/ml LPS for 12 h and hydrogen-rich medium was given as soon as LPS joined. HUVECs were stained by Annexin V-FITC and PI. a LPS treatment increased Annexin V-FITC positive cells and hydrogen-rich medium decreased LPS induced cell apoptosis. b Percentage of Annexin V-FITC positive cells in different group. *P<0.05; **P<0.01 vs. group Sham; #P < 0.05; ##P < 0.01vs. group LPS.

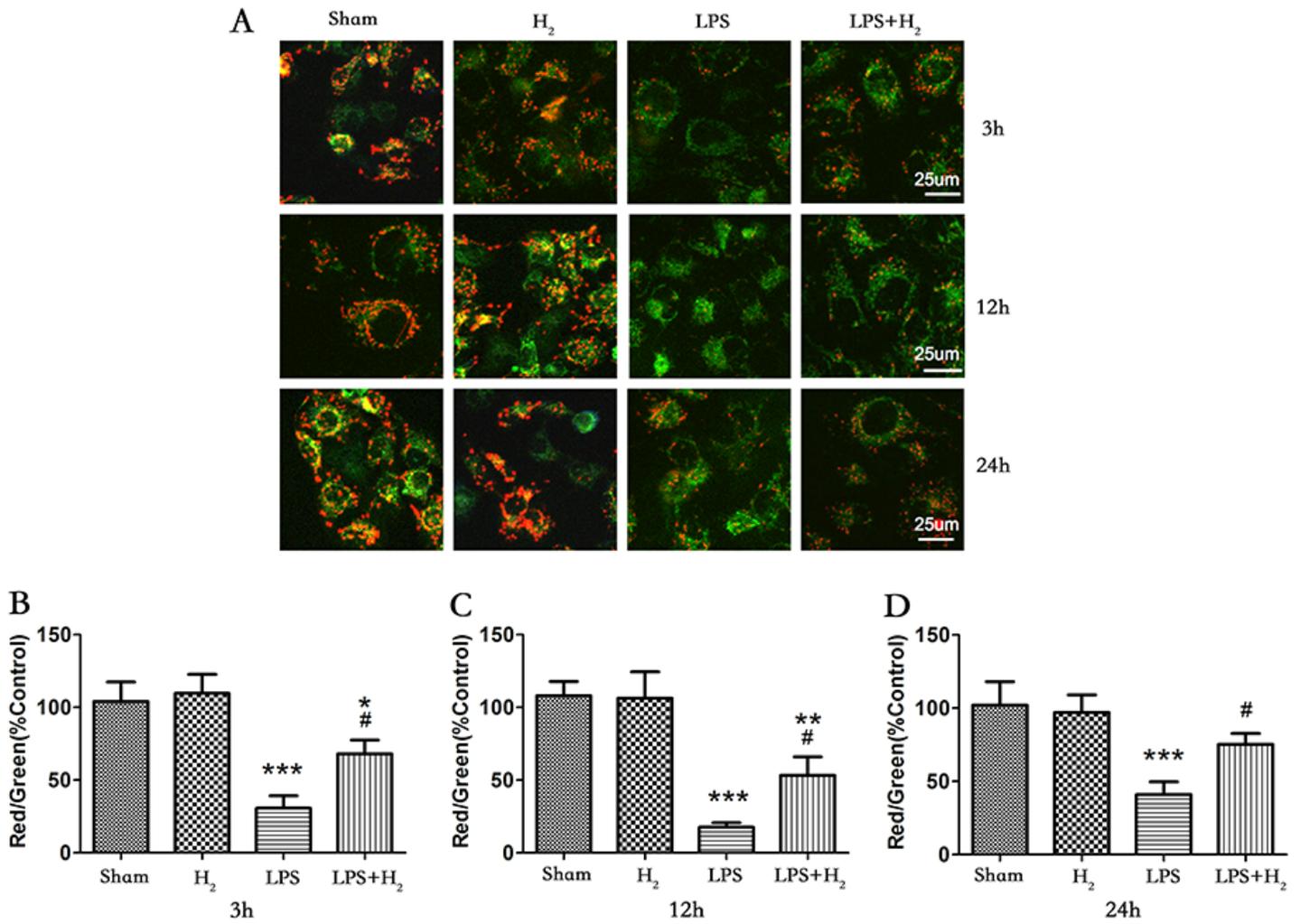


Figure 2

Effects of LPS and H₂ on mitochondrial member potential($\Delta\Psi_m$). HUVECs were stimulated with or without 10ug/ml LPS and 0.6 mM hydrogen-rich medium. After 3 h, 12h and 24 h of stimulation, cells were incubated with 500 nM JC-1 for 20 min and then observed by confocal scope. a Exposure to LPS decreased $\Delta\Psi_m$ and hydrogen-rich medium alleviated LPS induced $\Delta\Psi_m$ injury. b-d Red/Green fluorescence content result after 3 h, 12 h and 24 h treatment. *P<0.05; **P<0.01 vs. group Sham; #P < 0.05; ##P < 0.01vs. group LPS.

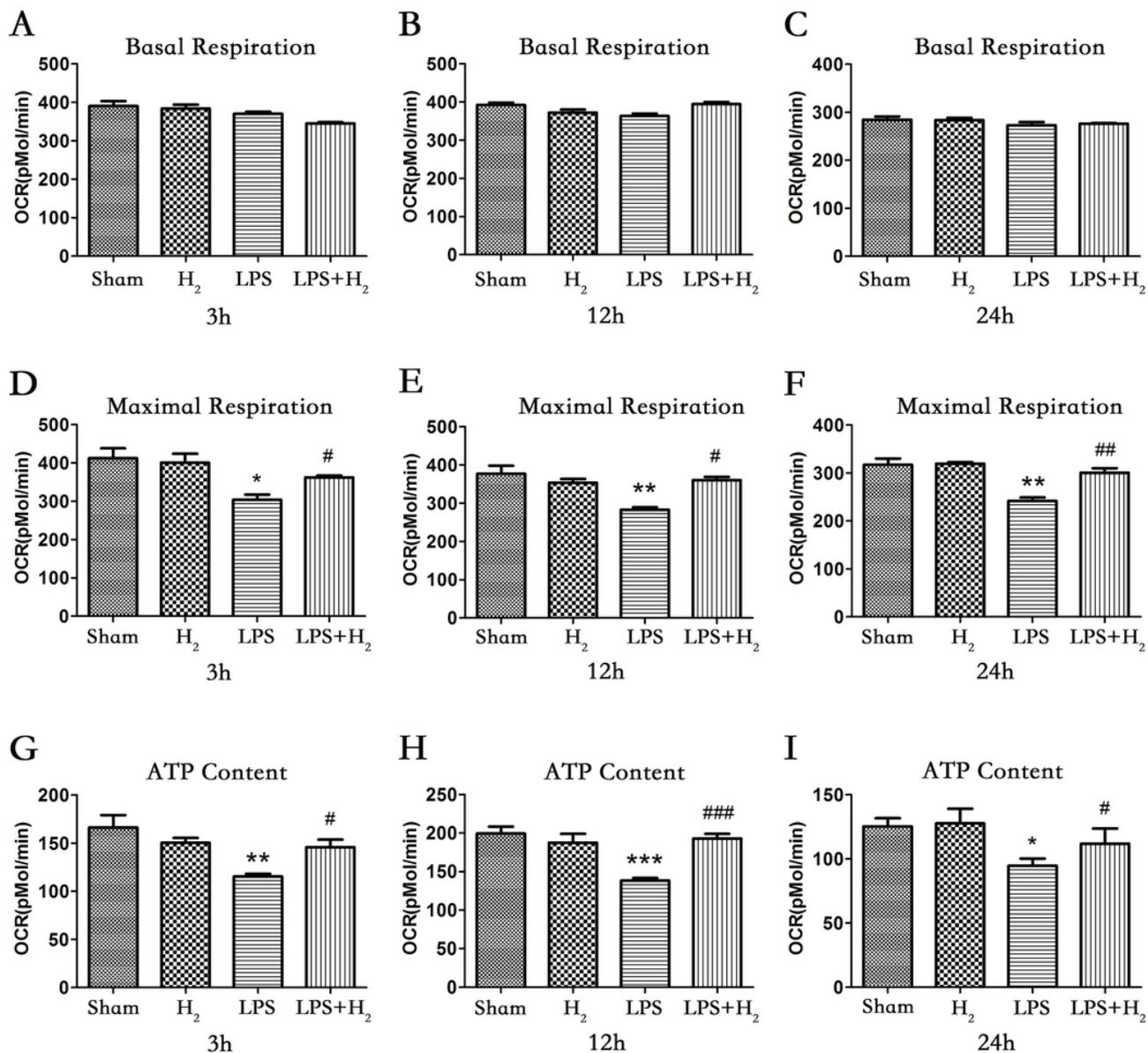


Figure 3

Effects of LPS and H₂ on oxygen consumption rate (OCR). HUVECs were stimulated with or without 10 ug/ml LPS and 0.6 mM hydrogen-rich medium. After 3 h, 12 h and 24 h of stimulation, OCR were tested by seahorse xf24 extracellular flux analyzer. Analysis result of basal respiration rate (a-c), maximal respiration rate (d-f) and ATP content (g-i). Maximal respiration rate and ATP content decreased in LPS group compared with sham group. Hydrogen-rich medium alleviated LPS induced mitochondria respiration dysfunction. Basal respiration rate didn't be influenced by LPS or hydrogen-rich medium. *P<0.05; **P<0.01 vs. group Sham; #P < 0.05; ###P < 0.01 vs. group LPS.

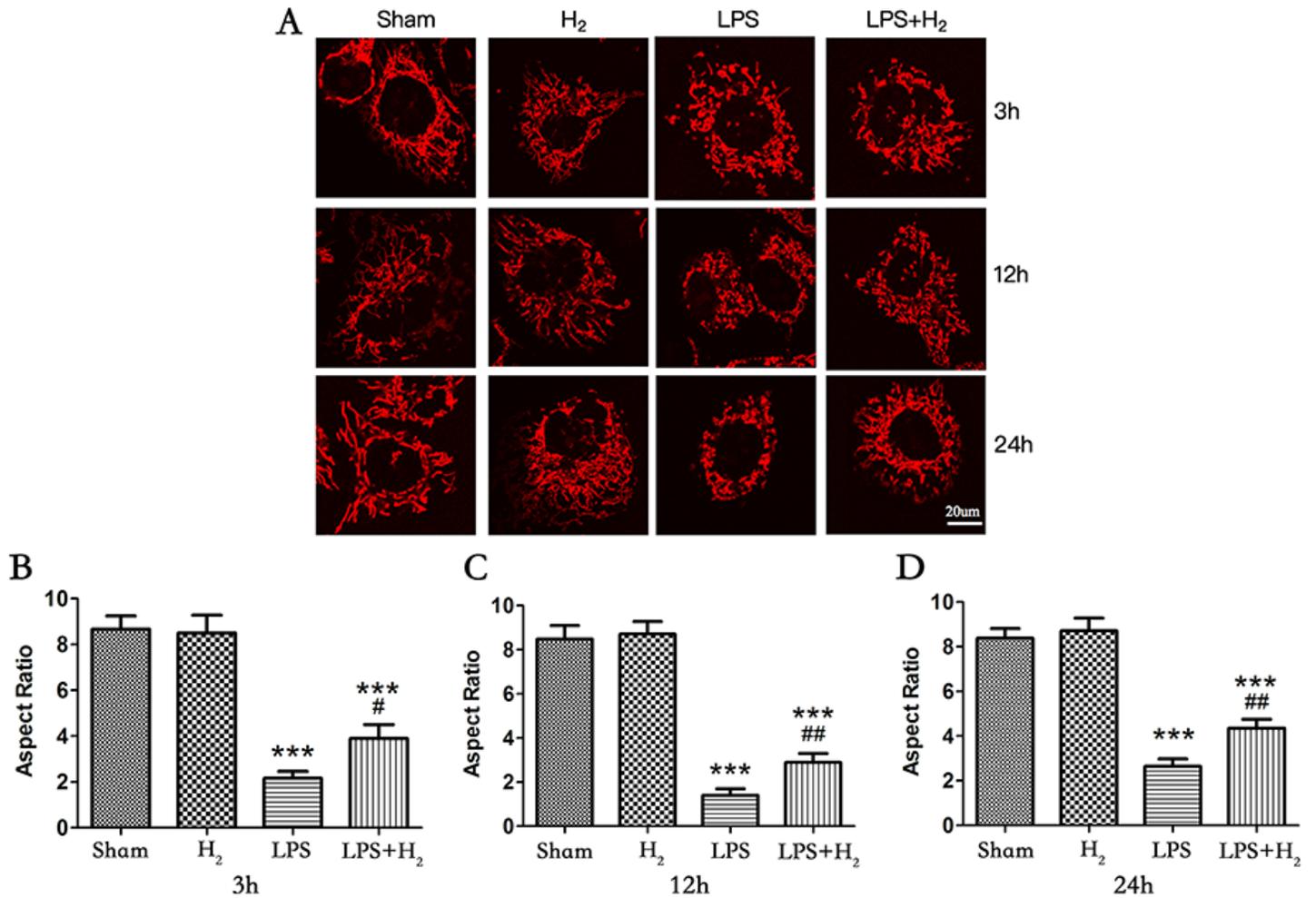


Figure 4

Effects of LPS and H₂ on mitochondrial morphology. HUVECs were stimulated with or without 10 µg/ml LPS and 0.6 mM hydrogen-rich medium. a After 3 h, 12 h and 24 h of stimulation, cells were incubated with 500 nM mitotracker orange for 5 min and then photographed by confocal scope. b-d Analysis result of aspect ratio (length/width). Exposure to LPS induced mitochondrial fragmentation in HUVECs.

*P<0.05; **P<0.01 vs. group Sham; #P < 0.05; ##P < 0.01 vs. group LPS.

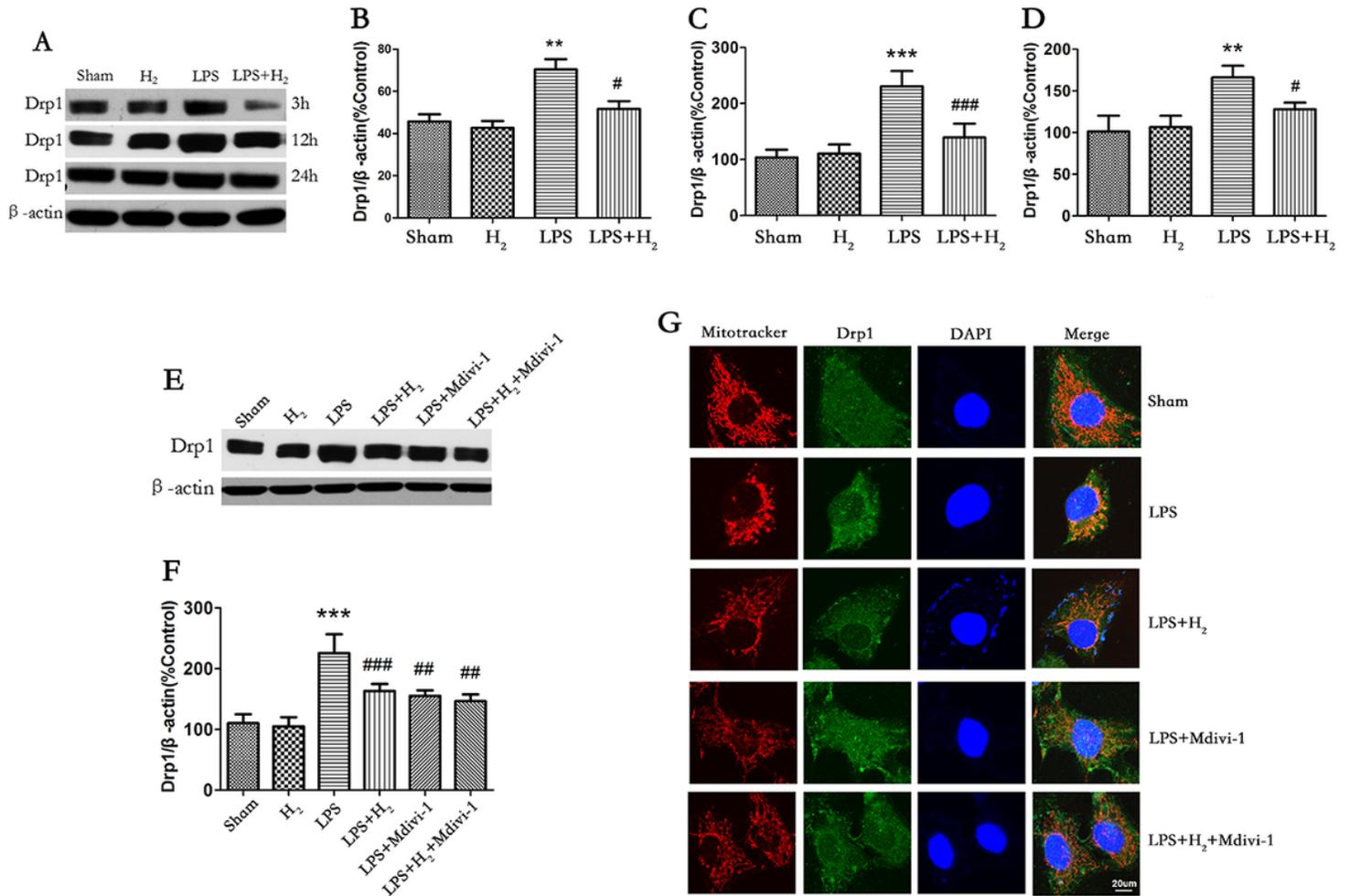


Figure 5

Exposure of HUVECs to LPS altered the expression of the mitochondrial fission protein Drp1. a-d LPS stimulated Drp1 expression while hydrogen-rich medium inhibited it. HUVECs were pretreated for 1 h with 25 μM mdivi-1. e-f LPS and hydrogen-rich medium incubated 12 h. Western blot showed hydrogen-rich medium had a similar effect to Mdivi-1, both of them could inhibit Drp1 expression. g Immunofluorescence also been used to confirm the activity of Drp1. Exposure to LPS activated Drp1 and promoted Drp1 recruitment to mitochondria. Hydrogen-rich medium and Mdivi-1 inhibit Drp1 activity. *P<0.05; **P<0.01 vs. group Sham; #P < 0.05; ###P < 0.01 vs. group LPS.

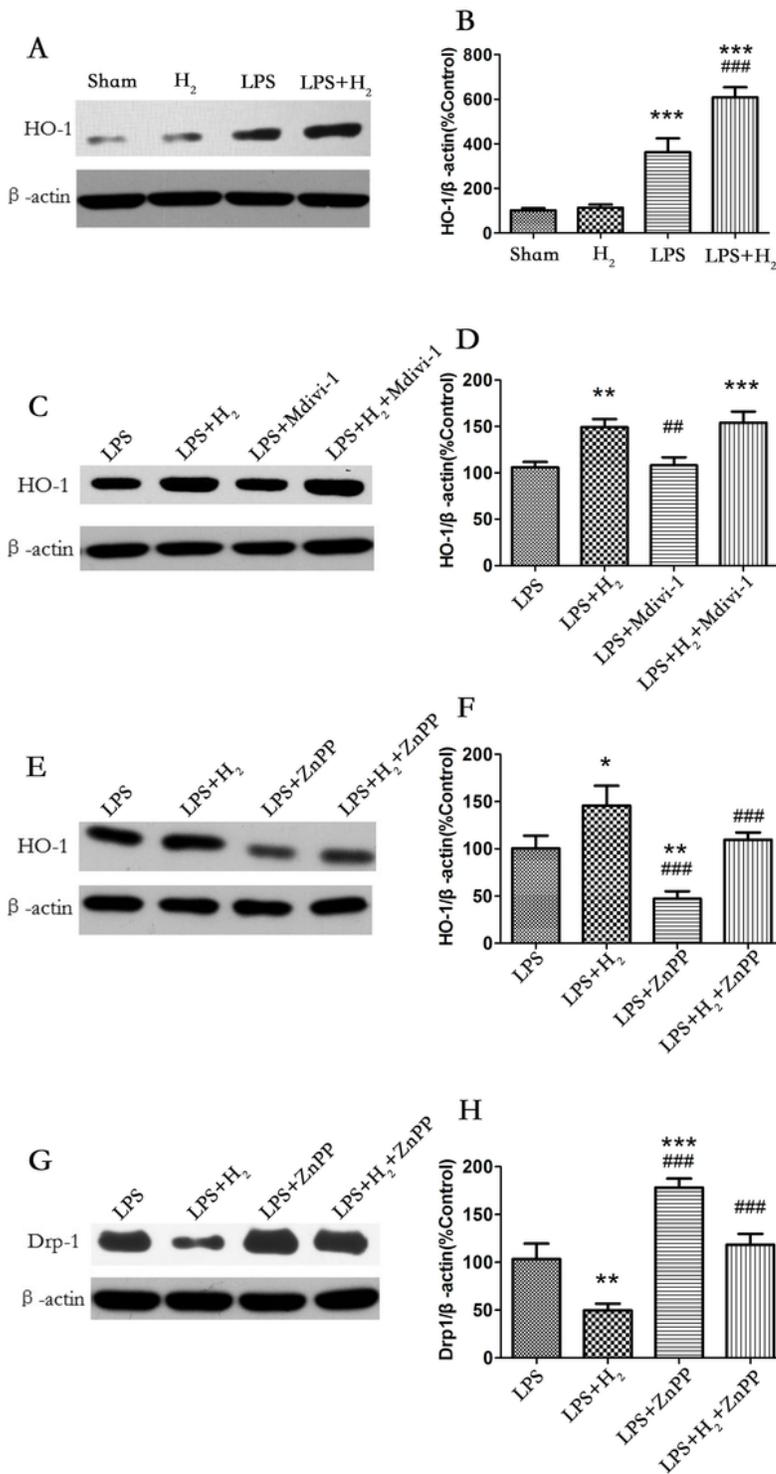


Figure 6

a-b HO-1 expression after 12 h of 10ug/ml LPS and 0.6 mM hydrogen-rich medium treatment. * $P < 0.05$; ** $P < 0.01$ vs. group Sham; # $P < 0.05$; ### $P < 0.01$ vs. group LPS. c-d HUVECs were pretreated for 1 h with 25 μ M Mdivi-1. Mdivi-1 had no influence to HO-1 expression. e-f Pretreatment with the HO-1 inhibitor Znpp (10uM) for 6 hours reversed the effect of hydrogen-rich medium to up-regulating HO-1. g-h Znpp also

reversed the effect of hydrogen-rich medium to inhibit Drp1 expression. *P<0.05; **P<0.01 vs. group LPS; #P < 0.05; ##P < 0.01 vs. group LPS+H₂.

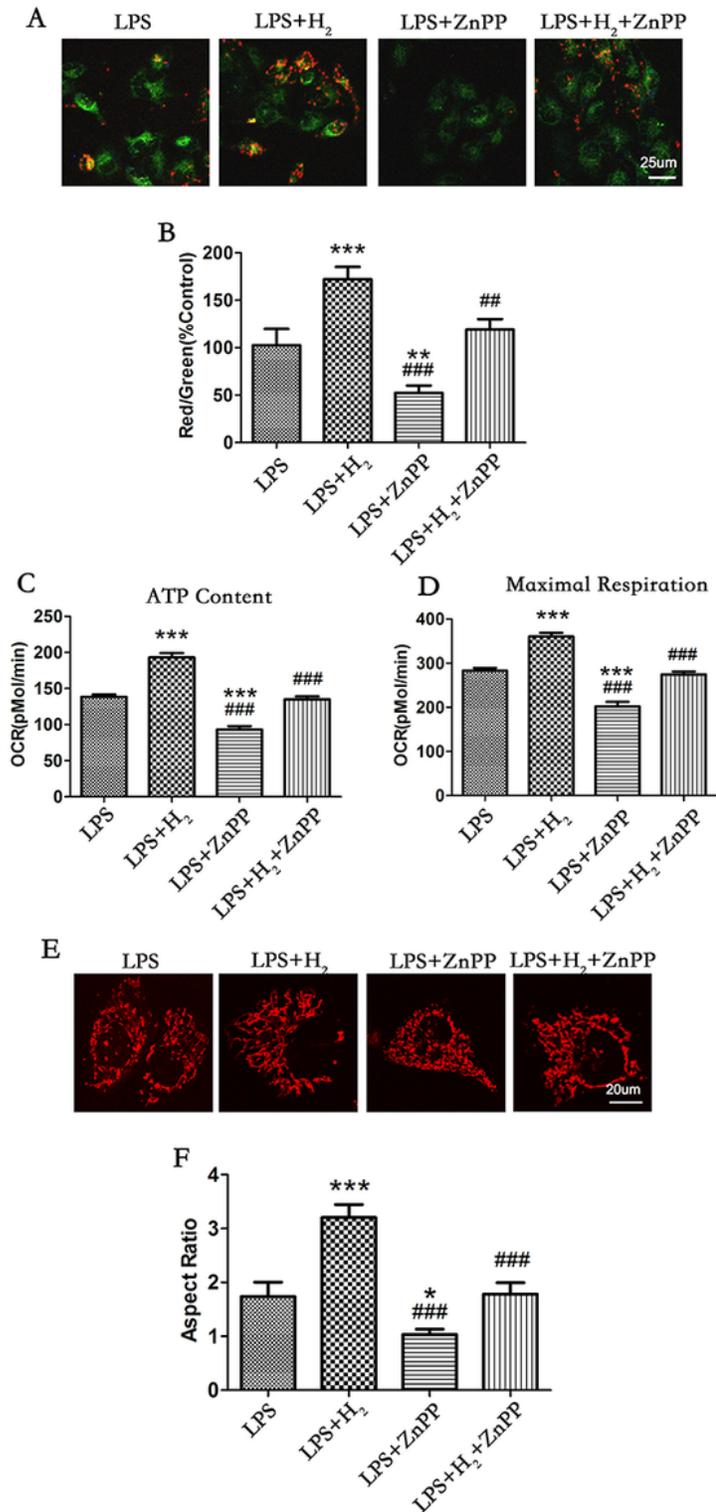


Figure 7

Pretreatment with the HO-1 inhibitor Znpp (10uM) for 6 hours reversed the protective effects of hydrogen-rich medium on $\Delta\Psi_m$ (a-b), mitochondrial respiratory function (c-d) and mitochondrial morphology (e-f). *P<0.05; **P<0.01 vs. group LPS; #P < 0.05; ##P < 0.01 vs. group LPS+H₂.