

Metformin suppresses intrahepatic coagulation activation in mice with lipopolysaccharide/D-galactosamine-induced fulminant hepatitis

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Abstract. Metformin is a widely-used antidiabetic drug with hypoglycemic activity and previously described anti-inflammatory properties. Previous studies have demonstrated that metformin attenuates endotoxic hepatitis, however the mechanisms remain unclear. Inflammation and coagulation are closely associated pathological processes, therefore the potential effects of metformin on key steps in activation of the coagulation system were further investigated in endotoxic hepatitis induced by lipopolysaccharide/D-galactosamine (LPS/D-Gal). The current study demonstrated that treatment with metformin significantly suppressed the upregulation of tissue factor and plasminogen activator inhibitor-1 in LPS/D-Gal-exposed mice. In addition, a reduction in the expression of interleukin 6 and inhibition of nuclear translocation of nuclear factor- κ B were observed. These data indicate that the LPS/D-Gal-induced elevation of the stable protein level of hypoxia inducible factor 1 α , the mRNA level of erythropoietin, vascular endothelial growth factor and matrix metalloproteinase-3, and the hepatic level of lactic acid were also suppressed by metformin. The current study indicates that the suppressive effects of metformin on inflammation-induced

coagulation may be an additional mechanism underlying the hepatoprotective effects of metformin in mice with LPS/D-Gal-induced fulminant hepatitis.

Introduction

Fulminant hepatitis has been reported to result from a variety of causes, such as viral infection and drug poisoning, and is commonly associated with a poor prognosis (1,2). In the early stages of fulminant hepatitis, the excessive inflammatory reactions serve a critical role in mediating the rapid loss of hepatocytes (3,4). Therapies targeting the pro-inflammatory mediators are suggested to provide protective benefits in experimental studies (5,6). In addition to the direct detrimental effects of pro-inflammatory mediators, inflammation may also activate the coagulation system (7,8). The inflammation-induced activation of coagulation cascades markedly potentiates liver damage via interrupting the blood supply and propagating further inflammation (9,10).

It has been well documented that inflammation and inflammation-induced coagulation are crucial pathological mechanisms underlying the progression of critical disorders, including sepsis and multiple organ dysfunction syndrome (11,12). The rapid induction of tissue factor (TF) under inflammatory circumstances is the pivotal step associating inflammation with coagulation (13). TF is the essential initiator of the extrinsic pathway of blood coagulation (14). The inflammatory stimuli and inflammatory cytokines may markedly induce the expression of TF and activate the extrinsic coagulation pathway (15). In addition to enhanced activation of coagulation cascades, inflammation may induce the dysregulation of fibrin removal via the upregulation of the plasminogen activator inhibitor-1 (PAI-1) (16,17).

A previous study demonstrated that the antidiabetic drug, metformin, attenuated lipopolysaccharide/D-galactosamine (LPS/D-Gal)-induced fulminant hepatitis (18). LPS/D-Gal-induced fulminant liver damage predominantly depends on the induction of the pro-inflammatory cytokine, tumor necrosis factor α (TNF- α), which has been proposed as a key factor propagating the progression of hepatitis induced by toxic insults (4,19). In a previous study, metformin significantly

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Abbreviations: D-Gal, D-galactosamine; EPO, erythropoietin; HIF-1 α , hypoxia inducible factor 1 α ; IL-6, interleukin 6; I κ B, inhibitory κ B; LA, lactic acid; LPS, lipopolysaccharide; MMP-3, matrix metalloproteinase-3; NF- κ B, nuclear factor κ B; PAI-1, plasminogen activator inhibitor-1; TF, tissue factor; TNF- α , tumor necrosis factor α ; VEGF, vascular endothelial growth factor

Key words: lipopolysaccharide, metformin, coagulation, tissue factor, hepatitis

suppressed the expression of TNF- α , alleviating the liver lesions and improving the survival rate of LPS/D-Gal-exposed mice (18).

Due to evidence indicating that the coagulation system is involved in the pathogenesis of LPS/D-Gal-induced fulminant hepatitis (20,21), the current study investigated whether metformin is able to modulate the activation of the coagulation response. In addition, the potential effects of metformin on LPS/D-Gal-induced upregulation of TF and PAI-1 and the subsequent metabolic disturbances were investigated.

Materials and methods

Materials. Metformin, LPS (from *Escherichia coli*, 055:B5) and D-Gal were purchased from Sigma-Aldrich (St. Louis, MO, USA). The lactic acid (LA) assay kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The Total Protein Extraction kit was purchased from Beyotime Institute of Biotechnology (Jiangsu, China). The mouse interleukin 6 (IL-6) Enzyme-Linked Immunosorbent Assay (ELISA) kit was obtained from NeoBioscience Technology Company (Shenzhen, China). The rabbit anti-mouse monoclonal nuclear factor κ B (NF- κ B) p65 antibody (D14E12; 1:1,000; cat. no. 8242), rabbit anti-mouse hypoxia inducible factor 1 α (HIF-1 α) monoclonal antibody (D2U3T; 1:1,000; cat. no. 14179), rabbit anti-mouse PAI-1 monoclonal antibody (D9C4; 1:1,000; cat. no. 11907), rabbit anti-mouse β -actin monoclonal antibody (D6A8; 1:1,000; cat. no. 8457) and Histone 3: rabbit anti-mouse Histone 3 monoclonal antibody (D1H2; 1:500; cat. no. 4499) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). The rabbit anti-mouse TF monoclonal antibody (EPR8986; 1:1,000; cat. no. ab151748) was purchased from Abcam (Cambridge, UK). The bicinchoninic acid (BCA) protein assay kit, horse-radish peroxidase-conjugated goat anti-rabbit polyclonal secondary antibody (1:5,000; cat. no. G21234) and enhanced chemiluminescence (ECL) reagents were obtained from Pierce Biotechnology, Inc. (Rockford, IL, USA).

Animals. A total of 64 male Balb/c mice (16 in each group), weighing 20–25 g, were obtained from the Experimental Animal Center of Chongqing Medical University (Chongqing, China). All animals were fed with a standard laboratory diet and water *ad libitum*. They were housed in a specific pathogen-free room at a temperature of 20–25°C and 50 \pm 5% relative humidity under a 12 h dark/light cycle and had acclimatized for a minimum of 1 week prior to use. All experimental procedures involving animals were approved by the Animal Care and Use Committee of Chongqing Medical University.

Induction of fulminant hepatitis. Balb/c mice were intraperitoneally injected with vehicle or metformin (400 mg/kg, dissolved in normal saline), administered 0.5 h prior to the injection of LPS (10 μ g/kg) combined with D-Gal (700 mg/kg). The dose of metformin was selected based on previous experimental data (18). Subsequent to injection, the animals were returned to their cages and allowed food and water *ad libitum*. The experimental animals were allocated to four groups: Control (CON), mice received

vehicle administration only; metformin (MET), mice treated with metformin without LPS/D-Gal exposure; LPS/D-Gal, mice exposed to LPS/D-Gal; LPS/D-Gal + MET, mice treated with metformin and exposed to LPS/D-Gal. Mice were sacrificed by intraperitoneal injection of pentobarbital (50 mg/kg, Sigma-Aldrich) at 1.5 h (n=8 per group) or 6 h (n=8 per group) subsequent to LPS/D-Gal challenge, and blood samples and liver tissues were harvested and stored at -80°C until required.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from liver samples using TRIzol reagent (Takara Bio, Inc., Otsu, Japan). First-strand complementary DNA was synthesized from 1 μ g total RNA using oligo-dT primer (Takara Bio, Inc.) and the M-MLV reverse transcriptase (Takara Bio, Inc.). RT-qPCR was performed with SYBR green PCR Master Mix (Takara Bio, Inc.). The sequences of the primers used to amplify the target genes are presented in Table I. PCR was performed on a CFX96 Touch (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using the following PCR conditions: Denaturing at 95°C for 10 sec, annealing at 58°C for 20 sec and elongation at 72°C for 20 sec. The mRNA levels of TF, PAI-1, IL-6, erythropoietin (EPO), vascular endothelial growth factor (VEGF) and matrix metalloproteinase-3 (MMP-3) were normalized to levels of β -actin.

Western blot analysis. Total proteins from liver samples were prepared using the Total Protein Extraction kit according to the manufacturer's instructions. The total protein concentration was determined using the BCA protein assay kit. Protein extracts were fractionated on a 10% polyacrylamide-sodium dodecyl sulfate gel (Beyotime Institute of Biotechnology) and then were transferred to nitrocellulose membranes (Pierce Biotechnology, Inc.). The membranes were blocked with 5% (w/v) nonfat milk in Tris-buffered saline containing 0.05% Tween-20 (Enzo Life Sciences, Farmingdale, NY, USA), prior to incubation with primary antibodies overnight at 4°C, followed by incubation with secondary antibodies for 1 h at 37°C. Antibody binding was visualized using an ECL system and a short exposure of the membrane to X-ray films (Kodak, Rochester, NY, USA).

ELISA. The protein levels of IL-6 in the plasma were determined using the ELISA kits according to the manufacturer's instructions (NeoBioscience Technology Company).

LA measurement. The LA contents in the liver tissue were determined with an LA assay kit according to the manufacturer's instructions. The LA values were assessed according to the absorbance measured at 530 nm (Varioscan Flash; Thermo Fisher Scientific, Waltham, MA, USA). The levels of hepatic LA were normalized to the total protein concentration in the same sample.

Statistical analysis. All data were presented as the mean \pm standard deviation. Statistical significance was determined by Student's t-test for the comparison of two groups. Data were analyzed using Statistical Package for Social Sciences (SPSS) software (version 16; SPSS, Inc., Chicago,

Table I. Sequences of the primers for reverse transcription-quantitative polymerase chain reaction.

Target gene	Forward primer	Reverse primer
TF	5-CTTATCGGAAAGGCTCAA-3	5-CACCACTGCTCCCACAAT-3
PAI-1	5-CATGTTTAGTGCAACCCTGGC-3	5-TGAGATGACAAAGGCTGTGGAG-3
IL-6	5-AGTTGCCTTCTTGGGACTGATG-3	5-TCTCATTTCACGATTTCCAG-3
EPO	5-CAGCCACCAGAGACCCTTCA-3	5-TGTGAGTGTTCCGGAGTGGAGC-3
VEGF	5-ACGATGAAGCCCTGGAGTGC-3	5-GCTCATCTCTCTATGTGCTGGC-3
MMP-3	5-CCACAGACTTGTCCCGTTTCC-3	5-GTGCTGACTGCATCAAAGAACAA-3
β -actin	5-CTGAGAGGGAATCGTGCGT-3	5-CCACAGGATTCCATACCCAAGA-3

TF, tissue factor; PAI-1, plasminogen activator inhibitor-1; IL-6, interleukin 6; EPO, erythropoietin; VEGF, vascular endothelial growth factor; MMP-3, matrix metalloproteinase-3.

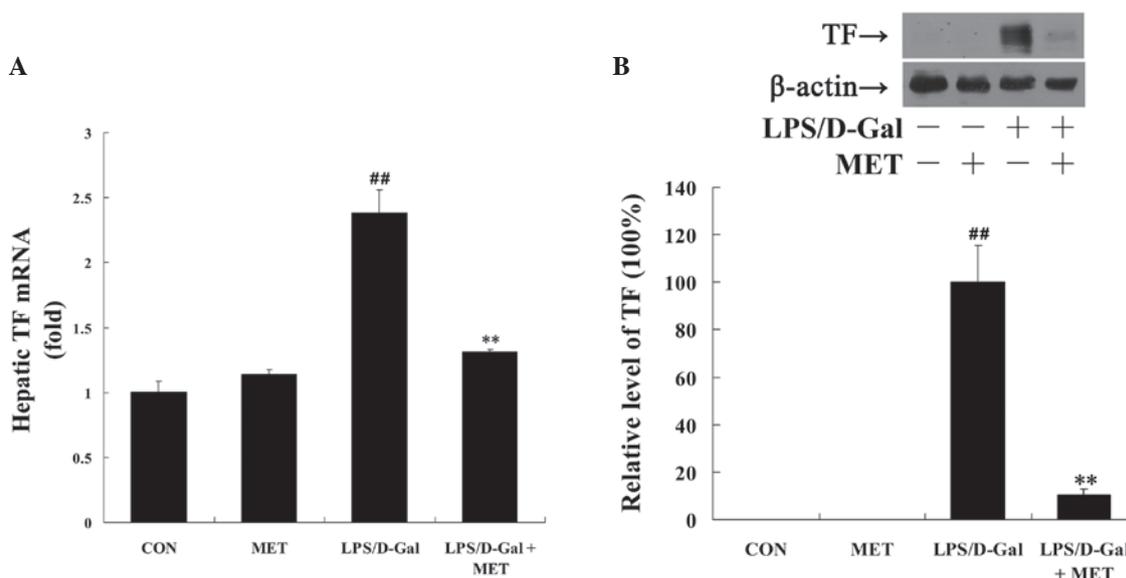


Figure 1. MET suppressed LPS/D-Gal-induced upregulation of TF. Mice were treated with the vehicle or MET (400 mg/kg) in the presence or absence of LPS/D-Gal. (A) The mRNA level of TF 1.5 h subsequent to LPS/D-Gal exposure and (B) the protein levels of TF 6 h subsequent to LPS/D-Gal exposure were determined. The bands of TF and β -actin are indicated by arrows. The blots underwent densitometric analysis and the data are presented as relative intensity units. Data are presented as the mean \pm standard deviation, n=8. ^{**}P<0.01 vs. LPS/D-Gal group; ^{##}P<0.01, vs. CON group. MET, metformin; LPS, lipopolysaccharide; D-Gal, D-galactosamine; TF, tissue factor; CON, control.

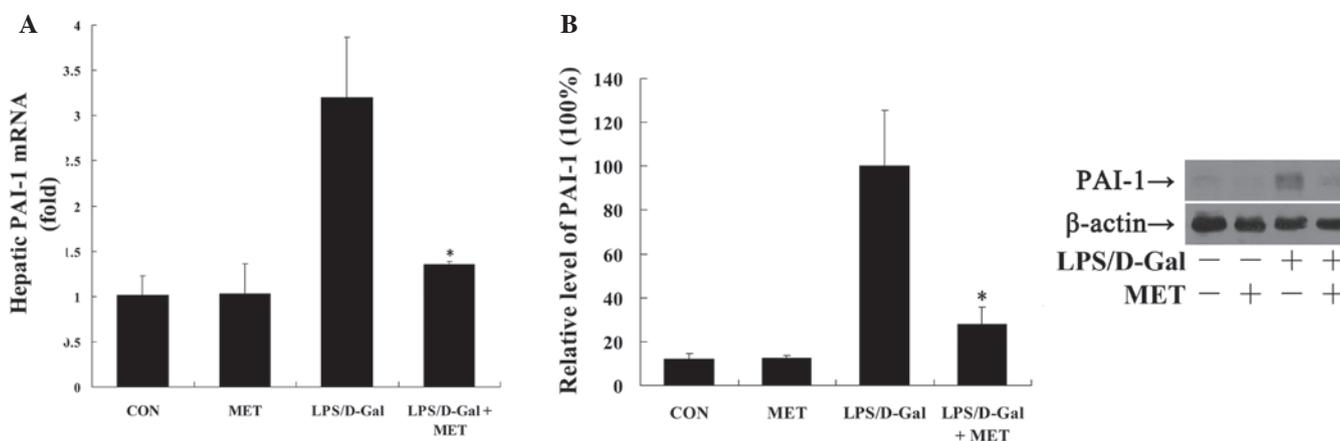


Figure 2. MET suppressed LPS/D-Gal-induced upregulation of PAI-1. Mice were treated with vehicle or MET (400 mg/kg) in the presence or absence of LPS/D-Gal. (A) The mRNA level of PAI-1 1.5 h subsequent to LPS/D-Gal exposure and (B) the protein levels of PAI-1 6 h subsequent to LPS/D-Gal exposure were determined. The bands of PAI-1 and β -actin are indicated by arrows. The blots underwent densitometric analysis and the data are presented as relative intensity units. Data are presented as the mean \pm standard deviation, n=8. ^{*}P<0.05 vs. LPS/D-Gal group. MET, metformin; LPS, lipopolysaccharide; D-Gal, D-galactosamine; PAI-1, plasminogen activator inhibitor type 1; CON, control.

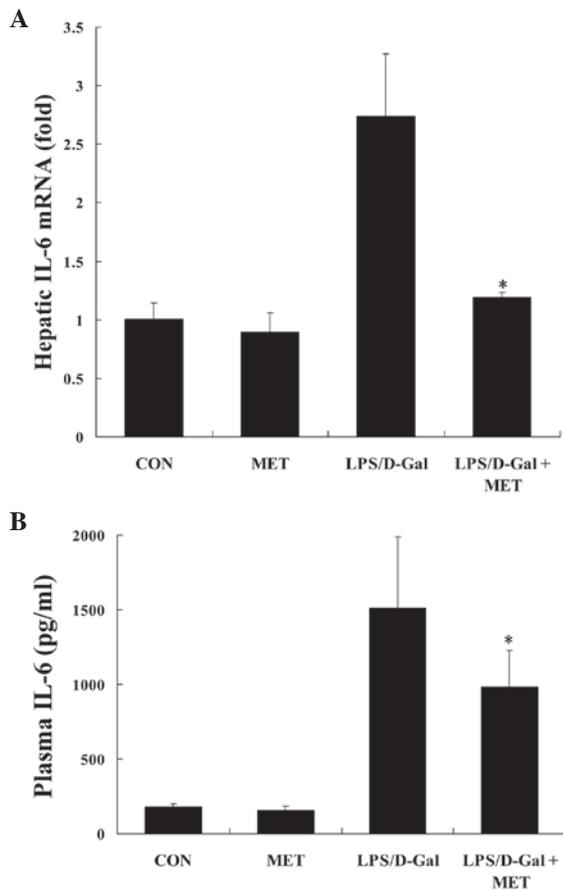


Figure 3. MET suppressed LPS/D-Gal-induced expression of IL-6. Mice were treated with vehicle or MET (400 mg/kg) in the presence or absence of LPS/D-Gal exposure. (A) The mRNA levels of IL-6 in the liver and (B) the protein levels of IL-6 in the plasma were determined 1.5 h subsequent to LPS/D-Gal exposure. Data are presented as the mean \pm standard deviation, n=8. * P <0.05 vs. LPS/D-Gal group. MET, metformin; LPS, lipopolysaccharide; D-Gal, D-galactosamine; IL-6, interleukin 6; CON, control.

IL, USA). P <0.05 was considered to indicate a statistically significant difference.

Results

Metformin suppresses LPS/D-Gal-induced TF and PAI-1 expression. TF is the initiator of the coagulation cascade, which is rapidly induced by inflammatory stimuli (13). In the present study, LPS/D-Gal exposure was demonstrated to markedly upregulate the mRNA and protein levels of TF, compared with the control, which was significantly reversed by metformin (P <0.01; Fig. 1). In addition, the LPS/D-Gal-induced expression of PAI-1, an inflammation-induced factor suppressing the fibrinolysis process (17), was also significantly inhibited by metformin (P <0.05; Fig. 2).

Metformin suppresses LPS/D-Gal-induced IL-6 expression. IL-6 is a crucial pro-inflammatory cytokine linking inflammation to coagulation (22). In the current study, it was demonstrated that the challenge with LPS/D-Gal induced the upregulation the mRNA levels of IL-6 in liver tissue, whereas treatment with metformin significantly reduced the level of IL-6 (P <0.05; Fig. 3A). In addition, similar inhibitory

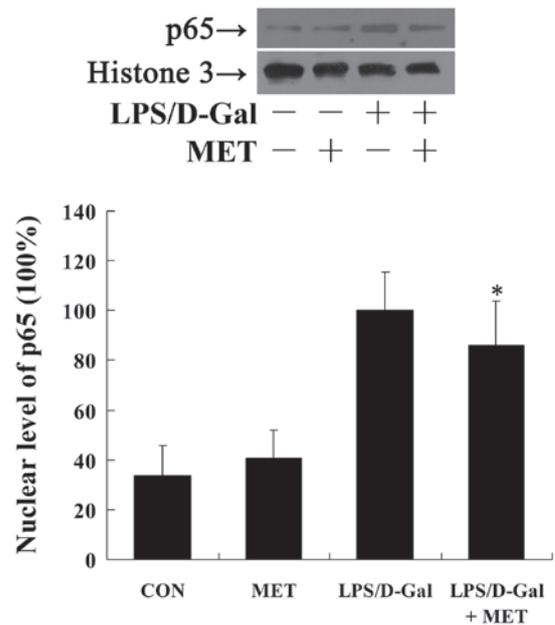


Figure 4. MET blocked LPS/D-Gal-induced nuclear translocation of NF- κ B p65. Mice were treated with vehicle or MET (400 mg/kg) in the presence or absence of LPS/D-Gal. Liver samples were harvested 1.5 h subsequent to LPS/D-Gal exposure. The levels of p65 in nuclear proteins were determined by immunoblot analysis. The bands of p65 and histone 3 are indicated by arrows. The blots underwent densitometric analysis and the data are presented as relative intensity units. Data are presented as the mean \pm standard deviation, n=8. * P <0.05, vs. LPS/D-Gal group. MET, metformin; LPS, lipopolysaccharide; D-Gal, D-galactosamine; NF- κ B, nuclear factor- κ B; CON, control.

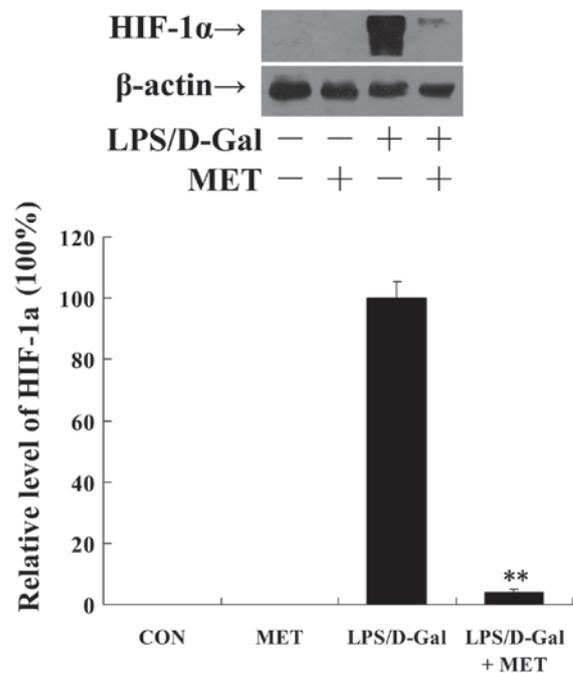


Figure 5. MET suppressed LPS/D-Gal-induced upregulation of HIF-1 α . Mice were treated with vehicle or MET (400 mg/kg) in the presence or absence of LPS/D-Gal exposure. Liver samples were harvested 6 h subsequent to LPS/D-Gal exposure and the protein levels of HIF-1 α were determined. The bands of HIF-1 α and β -actin are indicated by arrows and the blots underwent densitometric analysis and the data are presented as relative intensity units. Data are presented as the mean \pm standard deviation, n=8. ** P <0.01 vs. LPS/D-Gal group. MET, metformin; LPS, lipopolysaccharide; D-Gal, D-galactosamine; HIF-1 α , hypoxia inducible factor 1 α ; CON, control.

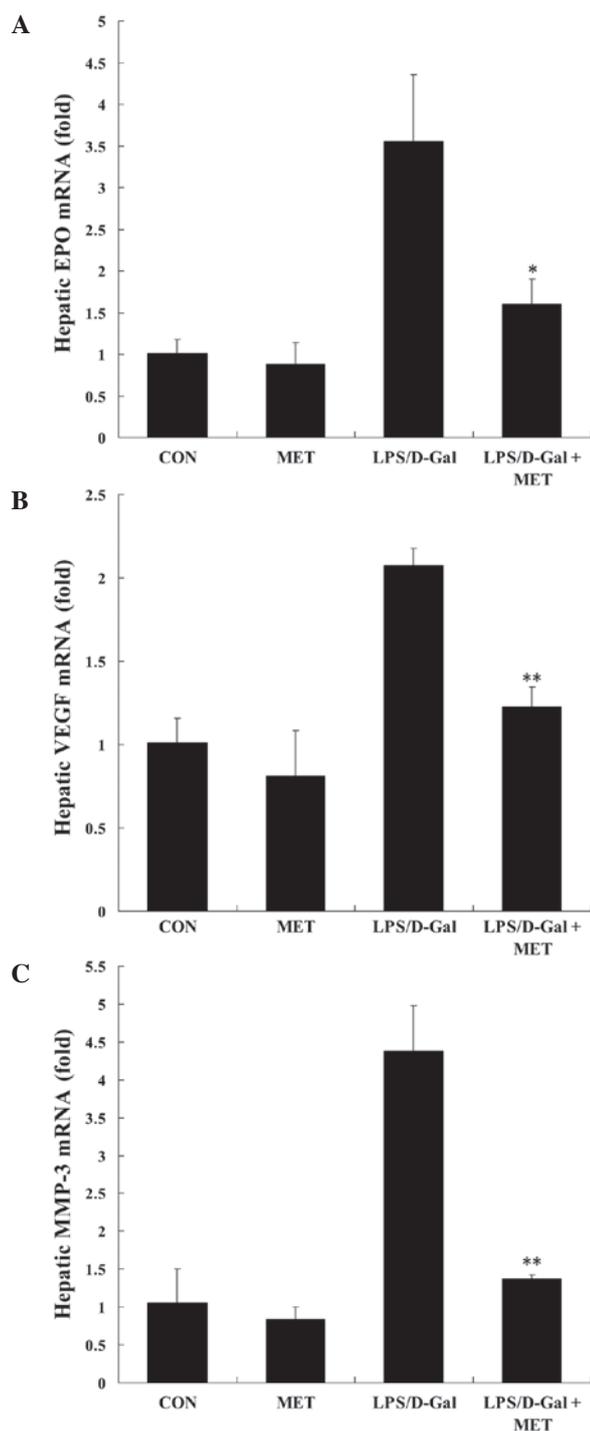


Figure 6. MET suppressed LPS/D-Gal-induced upregulation of EPO, VEGF and MMP-3. Mice were treated with the vehicle or MET (400 mg/kg) in the presence or absence of LPS/D-Gal exposure. Liver samples were harvested 6 h subsequent to LPS/D-Gal exposure and the mRNA levels of (A) EPO, (B) VEGF and (C) MMP-3 were determined. Data are presented as the mean \pm standard deviation, n=8. *P<0.05, **P<0.01 vs. LPS/D-Gal group. MET, metformin; LPS, lipopolysaccharide; D-Gal, D-galactosamine; EPO, erythropoietin; VEGF, vascular endothelial growth factor; MMP-3, matrix metalloproteinase-3; CON, control.

effects of metformin on the plasma protein levels of IL-6 in LPS/D-Gal-exposed mice were observed (P<0.05; Fig. 3B).

Metformin suppresses LPS/D-Gal-induced NF- κ B translocation. NF- κ B is a pivotal transcriptional factor involved in the

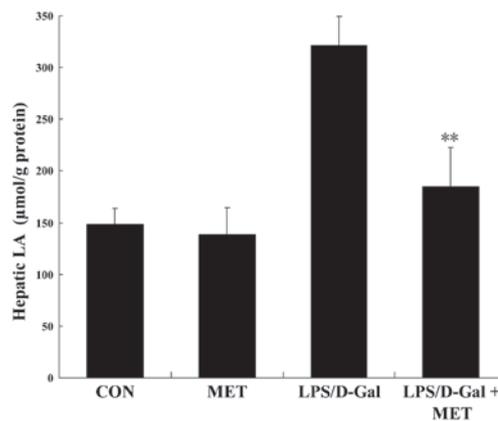


Figure 7. MET suppressed LPS/D-Gal-induced upregulation of LA. Mice were treated with the vehicle or MET (400 mg/kg) in the presence or absence of LPS/D-Gal exposure. Liver samples were harvested 6 h subsequent to LPS/D-Gal exposure and the hepatic levels of LA were determined. Data are presented as the mean \pm standard deviation, n=8. **P<0.01 vs. LPS/D-Gal group. MET, metformin; LPS, lipopolysaccharide; D-Gal, D-galactosamine; LA, lactic acid; CON, control.

transcriptional regulation of IL-6 and TF, and the nuclear translocation of NF- κ B is crucial for its activation (23,24). In the present study, the exposure to LPS/D-Gal markedly induced the translocation of NF- κ B p65 into the nuclei indicated by the increased nuclear level of p65 following LPS/D-Gal challenge, whilst treatment with metformin significantly reduced the nuclear level of p65 (P=0.023; Fig. 4).

Metformin alleviates LPS/D-Gal-induced hypoxia. Tissue hypoperfusion and hypoxia occur as a consequence of coagulation activation (12). In the present study, the degree of intrahepatic hypoxia was evaluated via the detection of the level of HIF-1 α . These data demonstrate that metformin significantly suppressed the upregulation of HIF-1 α protein levels (P<0.01; Fig. 5). In agreement with this, HIF-1-targeted genes, including EPO, VEGF and MMP-3 (25,26), were upregulated following LPS/D-Gal exposure, however this upregulation was suppressed by metformin (Fig. 6).

Metformin alleviates LPS/D-Gal-induced metabolic disturbances. The metabolic disturbance resulting from hypoxia was determined by detecting the level of LA, an anaerobic metabolic product. The data collected indicate that LPS/D-Gal exposure significantly increased the hepatic level of LA, which was inhibited by metformin (Fig. 7).

Discussion

Metformin is used in the clinical treatment of diabetes due to its hypoglycemic activity (27). In addition, studies have identified the anti-inflammatory properties of metformin *in vitro* and *in vivo*. In an LPS/D-Gal-induced hepatitis mouse model (28-30), it was demonstrated that treatment with the antidiabetic drug metformin provided therapeutic benefits. This suggested that these protective effects may result from the reduced production of the pro-inflammatory cytokine, TNF- α (18), a major detrimental and proapoptotic factor in this model of hepatitis (19). In the present study, it

was demonstrated that metformin significantly suppressed LPS/D-Gal-induced coagulation activation, as indicated by the reduced expression levels of TF and PAI-1, reduced level of HIF-1 and reduced content of LA in the liver.

The induction of TF serves a role in the activation of coagulation under inflammatory circumstances (8). TF is constitutively expressed in the majority of tissues, however is not expressed in cells in direct contact with the blood (14). Once the integrity of a vessel wall is disrupted, TF comes into contact with other coagulation factors in the blood and initiates coagulation (14). Additionally, the expression of TF may be induced by inflammatory stimuli. Therefore, the cells in direct contact with the blood, such as monocytes and endothelial cells, may be induced to express TF under inflammatory circumstances (13). Several pro-inflammatory cytokines have been suggested to mediate the induction of TF expression, however IL-6 may be the most important mediator, as inhibition of IL-6 reverses TF-dependent thrombin generation in experimental endotoxemia (22,31). In the present study, the level of IL-6 and TF increased significantly in mice challenged with LPS/D-Gal, however this upregulation was suppressed by metformin, suggesting that the key steps in the activation of the coagulation system were inhibited by metformin.

The formation of thrombin may be counter-regulated by the plasmin-catalyzed fibrinolysis process (32). Plasmin is generated from its precursor plasminogen and this conversion is stimulated by two activators, urokinase-type and tissue-type plasminogen activator (32), which are inhibited by PAI-1 (33). Additionally, PAI-1 is associated with inflammation due to the previously reported potential upregulation of expression of PAI-1 by inflammatory stimuli (34). In the present study, the LPS/D-Gal-induced expression of PAI-1 was significantly reduced by metformin, suggesting that the suppression of fibrinolysis may be reversed by metformin. Previous studies have demonstrated that TNF- α and IL-6 contribute to the increased expression of PAI-1 under inflammatory conditions (34). In addition, the induced production of TNF- α has been demonstrated to be significantly inhibited by metformin (18), with the present study indicating that the expression of IL-6 was also suppressed. These anti-inflammatory effects of metformin may result in reduced expression of PAI-1.

The transcription factor NF- κ B controls the expression of numerous pro-inflammatory genes including TNF- α and IL-6 (35). In unstimulated cells, NF- κ B is sequestered by its inhibitor, inhibitory κ B (I κ B) (36). Inflammatory stimuli may induce the degradation of I κ B and the release of NF- κ B, resulting in the translocation of NF- κ B into the nucleus, where it activates the transcription of target genes (36). In the present study, the increased level of nuclear NF- κ B in LPS/D-Gal-challenged mice was partially reversed by metformin, which may contribute to the anti-inflammatory effect on TNF- α and IL-6 expression. Additionally, there is evidence indicating that NF- κ B is involved in the transcriptional regulation of TF and PAI-1 (24,34). Thus, the inhibitory effects on NF- κ B activation may underlie the reduced induction of TF and PAI-1.

The activation of the coagulation system may result in the reduction of blood flow, which leads to tissue hypoperfusion and hypoxia (12). In the present study, the degree of hepatic hypoxia was determined via the detection of the stable protein

level of HIF-1 α . HIF is an oxygen-sensitive transcription factor containing an oxygen-labile α -subunit and a constitutively expressed β -subunit (37). Under normoxic conditions, the hydroxylation of HIF-1 α by the prolyl-4-hydroxylase domain-containing enzymes (PHD) targets HIF-1 α for polyubiquitination and proteosomal degradation (38). In hypoxic conditions, PHD activity is inhibited and HIF-1 α accumulates and binds to the hypoxia-responsive element sequences of target gene promoters (38). In mice exposed to LPS/D-Gal, levels of the stable protein of HIF-1 α were markedly increased, suggesting the presence of severe hypoxia in the liver tissues. Treatment with metformin significantly reduced the level of HIF-1 α protein in LPS/D-Gal-challenged mice. Additionally, the LPS/D-Gal-induced expression of HIF-1-targeted genes, including EPO, VEGF and MMP-3 (25-26), was suppressed following metformin treatment. These data were consistent with the reduced level of TF. The HIF-1-driven expression of harmful products such as MMP-3 may represent one of the mechanisms involved in hypoxia-induced tissue damage (39).

Furthermore, tissue hypoxia and hypoperfusion may lead to metabolic disturbance. Hypoxia may block mitochondrial oxidative phosphorylation resulting in the generation of ATP from the anaerobic metabolic pathway. In the anaerobic metabolic pathway, pyruvate is converted into LA instead of acetyl-coenzyme A (40). In the present study, the level of LA increased significantly following LPS/D-Gal exposure, however treatment with metformin reduced the level of LA. Increased levels of LA have been suggested to be a pathological factor responsible for liver injury via the stimulation of the release of hydrolytic lysosomal enzymes into the cytosol and other mechanisms, including the induction of endothelial dysfunction (41,42).

Taken together, the results of the present study further indicate that metformin significantly suppresses LPS/D-Gal-induced NF- κ B activation, and IL-6, TF and PAI-1 expression. These effects are suggested to be associated with alleviated hepatic hypoxia and metabolic disturbance. Therefore, the present study identified an additional mechanism underlying the hepatoprotective effects of metformin in mice with LPS/D-Gal-induced fulminant hepatitis. The suppressive effects of metformin on inflammation and inflammation-induced coagulation may contribute to the protective benefits of metformin.

Acknowledgements

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