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Metformin Alleviates Lipopolysaccharide-induced Acute Lung Injury through Suppressing Toll-like Receptor 4 Signaling

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ABSTRACT

Signaling AMP-activated protein kinase (AMPK), an energy sensing enzyme, has been implicated in controlling inflammation. In this study we investigated whether activation of AMPK by metformin could protect the lung from lipopolysaccharide (LPS)-induced acute injury by inhibitingng TLR4 pathway.

Male Wistar rats were randomly divided into 3 groups (n=6): control group received normal saline (0.5 mL), LPS group received LPS (0.5 mg/kg), and metformin-treated group received LPS and metformin (100 mg/kg). Nine hours later nuclear factor-κB (NF-κB), phosphorylated, and non-phosphorylated AMPK using western blot, and the rate of TLR4 mRNA expression using real-time PCR were assessed in the lung tissue. To evaluate neutrophil infiltration, the myeloperoxidase (MPO) activity was measured. The severity of the lung damage was assessed by histological examinations.

It was found that the ratio of p-AMPKα to AMPKα was significantly upregulated by 22% ($p<0.05$) in the lungs obtained from the metformin group. In LPS-treated rats, we observed a high expression of TLR4 in the tissue along with increased levels of MyD88, NF-κB, and TNFα. Metformin considerably reduced all these parameters. Histological examinations revealed that metformin remarkably attenuated congestion and inflammatory cellular infiltration into the alveolar walls and also decreased MPO activity by 37% ($p<0.05$).

We conclude that metformin could protect the lung tissue against LPS-evoked TLR4 activation and the protective effect can be related to the activation of AMPK.

Keywords: AMP-activated protein kinase; Lipopolysaccharide; Lung; Metformin; Sepsis; Toll-like receptors

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INTRODUCTION

Acute lung injury and respiratory stress caused by sepsis is associated with a high risk of morbidity and mortality.[1] lipopolysaccharide (LPS) derived from

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gram-negative bacteria plays a critical role in the pathogenesis of sepsis-induced lung injuries characterized by recruitment of leukocytes to the lung and release of inflammatory mediators.[2] LPS induces inflammatory cell signaling by a cascade of LPS receptors and accessory proteins, LPS binding protein (LBP), CD14, and the Toll-like receptor4 (TLR4)–MD-2 complex. This process activates cells such as macrophages and endothelial cells to produce pro-inflammatory mediators, cytokines, and chemokines leading to expression of adhesion molecules and transient neutrophil infiltration into the lung.[3, 4]Inflammatory responses to foreign agents are largely mediated through TLRs[5] and different studies have confirmed the role of TLR4 in LPS-induced inflammation.[3,6] TLRs activate different signaling pathways, among them activation of the adaptor protein, myeloid differentiation primary response protein (MyD88) promotes the nuclear factor kappa-B (NF-κB) translocation into the nucleus and therefore induction of gene transcription of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α).[7]

Metformin is one of the most widely prescribed drugs and it is the first-line medication for treating type 2 diabetes. Activation of AMP-activated protein kinase (AMPK) in the cells is the main molecular target of metformin. Recent studies have demonstrated that AMPK plays an important role in limiting inflammation and therefore, metformin can serve as a potential drug to treat inflammatory disorders.[8-10] It has been reported that metformin reduces neutrophil infiltration, indexed as myeloperoxidase activity, and TNF-α mRNA content in the liver as well as decreasing the blood level of TNF-α following LPS-induced hepatic injury.[11] It has also been indicated that metformin noticeably reduces the acute activation and recruitment of neutrophils through an AMPK-dependent pathway in carrageenan-induced peritonitis in mice.[12] In a mouse model of endotoxemia, metformin decreased serum levels of TNF-α, interleukin-1β, and myeloperoxidase activity in lung.[13] At the moment, different pharmacological and ventilatory interventions are used in the management of sepsis; however, most of them failed to effectively treat the sepsis-induced acute lung injury. Therefore, performingresearchs to investigatean effective and safe treatment is highly necessary.

Recent studies have shown that activation of AMPK attenuates TLRs activity associated with myocardial infarction and indicate a link between AMPK and TLRs.[14] Furthermore, we observed in a recent study that metformin with AMPK activating function afforded an effective cardioprotection against LPS-stimulated TLR4 signaling in heart tissue.[15] Therefore, we hypothesize that AMPK could be a potential target in the treatment of clinical manifestations of sepsis. In this regard, we investigated the effect of metformin on TLR4 expression and activity in lung tissue in LPS-induced endotoxemia in rats.

MATERIALS AND METHODS

Animals

Male Wistar rats were supplied by the Laboratory Animal Center, Tabriz University of Medical Sciences, Iran. Animals were housed under specific conditions of 12-h light/12-h dark cycle in an air conditioned room at 23±2 °C with 50±10% relative humidity. Normal rat pellet diet and water were supplied *ad libitum*. Rats were randomly allocated to different experimental groups. All animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals of Tabriz University of Medical Sciences, Tabriz, Iran (National Institutes of Health Publication No. 85-23, revised 1985).

Chemical Reagents

Metformin (1, 1-dimethylbiguanide hydrochloride) was a generous gift from Osveh Pharmaceutical Inc. (Tehran, Iran). LPS (lipopolysaccharide, Escherichia coli serotype K235) were purchased from Sigma (Missouri, USA). Rabbit monoclonal antibodies against phospho-AMPKα (Thr172), AMPKα, and MyD88 were obtained from Cell Signaling Technology (Danvers, MA. Mouse monoclonal antibodies against glyceraldehyde phosphate dehydrogenase (GAPDH), NF-κB, β-actin, peroxidase-conjugated goat anti-rabbit, and rabbit anti-mouse secondary antibodies were obtained from Abcam (Cambridge, MA, USA). ELISA kit for TNF-α was purchased from Bender Med Systems Inc. (Vienna, Austria).

Experimental Protocol

The animals were randomized into three groups of control, LPS and LPS+metformin (n=6 in each group). The control group received saline (0.5 mL) intraperitoneally (IP). The sepsis was established by IP injection of LPS (0.5 mg/kg) in LPS group. The rats in

LPS+metformin group were treated with a single dose of metformin (100 mg/kg, IP.) 30 min before LPS injection. Nine hours later, the rats were anesthetized with ketamine/xylasin. Subsequent to anesthetizing, the lung tissues were removed. Tissues were snap frozen in liquid nitrogen and stored at -70 °C for myeloperoxidase assay, real time-polymerase chain reaction (RT-PCR) and western blotting.

Histopathological Examinations

For the histopathological examinations, 9 hours after LPS injections the lungs were removed under anesthesia and serial 5µm-thick, transverse, 10% buffered formalin-fixed lung pieces were embedded in paraffin and stained with hematoxylin and eosin (H&E) for specifying different tissue structures and Masson's trichrome (MT) stain for distinguishing connective tissue, muscle and collagen fibers.[16, 17]

Myeloperoxidase Assay

Myeloperoxidase (MPO) was assessed in the lung samples for quantifying the activity of neutrophils in lung tissue, as previously described by Mullane et al.[18]In brief, the samples were homogenized (IKA Hemogenizer, Staufen, Germany) in phosphate buffer (50 mM, pH=6) containing 0.5% hexa-decyl trimethyl-ammonium bromide (HTAB) for 45 s repeated 5 times with 1 minute-intervals at 7600 ×g. The homogenates intermittently were sonicated for 20 s and frozen-thawed 3 times, and then centrifuged at 2100 ×g, in 4 °C for 20 min. An aliquot of the supernatant (0.1 mL) or standard was then allowed to react with 2.9 mL of phosphate buffer (50 mM; pH=6) containing 0.167 mg/mL of O-dianisidin dihydrochloride and 0.0005% hydrogen peroxide. After 5 minutes, the reaction was stopped by adding 0.1 mL of 1.2 M hydrochloric acid. The absorbance was measured spectrophotometrically (Cecil 9000, Cambridge, UK) at 460 nm. The concentrations were calculated by using calibration curve and were expressed as mille units of MPO activity per gram weight of wet tissue (mU/g).

RT-PCR and Quantitative RT-PCR

Total RNA was isolated from snap frozen lung tissue using RNX-Plus Solution (SinaClon, Iran) according to the manufacturer's instructions. After confirming the integrity and purity of the extracted RNA, 1 µg of each extracted RNA sample was used for cDNA synthesis. Reverse transcriptase PCR was performed by dNTP mix, random hexamer primer, ribonuclease inhibitor, and moloney murine leukemia virus (M-MuLV) reverse transcriptase (SinaClon, Iran) according to the supplier's recommendations. All reactions were performed in a total volume of 20 µLcontaining: 1 µL cDNA, 0.6 µL primer (300 nM each primer), 10 µL 2X qPCR Green-Master Mix (EvaGreen, Jena Bioscience, Germany) and up to 20 µL PCR-grade water. All reactions were performed in triplicate and a mock PCR (without cDNA) was included to exclude contamination. The thermocycling conditions were as follow:

1 cycle at 94 °C for 10 min, 40 cycles at 95 °C for 15 s, annealing temperature (AT) for 30 sand 72 °C for 25 s. The cDNA samples were standardized based on the content of β-actin cDNA as housekeeping gene. The PCR primers were designed as shown in Table 1 and the interpretation of the result was performed using the Pfaffle Method.[19]

Western Blotting

Western blot analyses were performed according to assay described by Omar et al.[20] and Kewalramani et al.[21] with minor modifications. Briefly, frozen lung tissue was lysed in ice-cold lysis buffer, pH 7.4, containing 50 mM Tris–HCl, 150 mM NaCl, 5 mM Sodium Pyrophosphate (NaPPi), 50 mM NaF, 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.1% SDS (w/v), 1% TXT-100 (v/v), and protease inhibitor cocktail (Roche, Mannheim, Germany). Subsequently, the tissue homogenate was prepared for protein Assay.[22] The samples were subjected to SDS-Polyacrylamide gel electrophoresis using Bio-Rad Mini-P (Hercules, CA, USA).

After separation in polyacrylamide gel, the aliquots were transferred to an Immobilon (Millipore, Billerica, MA, USA). The membranes were then probed using a range of primary antibodies raised against phospho-AMPKα (Thr172), AMPKα, NF-κB, β-actin, as well as GAPDH. With the use of peroxidase-conjugated goat anti-rabbit and rabbit anti-mouse secondary antibodies (1:5000 dilution, at rotator for 60 min, bound antibodies were detected by BM Chemiluminescence kit (Roche, Mannheim, Germany). The molecular weights of proteins were confirmed according to their protein markers (PageRuler Unstained Protein Ladder, Fermentas, Lithuania). Quantification of band intensities by densitometry was carried out using ImageImageJj software (Wayne Rasband, National

Table 1. The primer sequences and anneling tempreture (AT) of Toll like receptor 4 (TLR4) and the standard gene of β-actin

	Sense primer	Antisense primer	AT(°C)
TLR4	5′-AAGTTATTGTGGTGGTGTCTAG-3′	5′-GAGGTAGGTGTTTCTGCTAAG-3′	60
β-actin	5′-GAGGTAGGTGTTTCTGCTAAG-3′	5′-ATC GTA CTC CTG CTT GCT GA-3′)	58

Institute of Health, USA). The densitometric values for phosphorylated AMPKα were normalized to AMPKα and in the case of MyD88 and NF-κB, the values were normalized to GAPDH and β-actin, respectively.

Cytokine Assay

The levels of TNF-a was measured by commercially available enzyme-linked immunosorbent assay (ELISA) kits from Bender Med Systems (Vienna, Austria) according to the manufacturer's instructions. Briefly, the samples were homogenized in lysis buffer containing protease inhibitor cocktail (Roche, Mannheim, Germany) and then centrifuged twice at 10,600 ×g for 10 min at 4 °C and the supernatants were used for assay. The optical density of each well was measured at 450 nm using a microplate reader. The concentration of the cytokine was expressed as pg per 100 mg of lung tissue.

Statistical Analysis

Data are expressed as mean ± standard error of mean (SEM). Differences between data sets were assessed by one-way analysis of variance followed by LSD post-hoc test using SPSS software (version 16.0, SPSS Inc., USA). For the RT- PCR, Pair Wise Fixed reallocation randomization test using REST software (version 2009, QIAGEN, Munich, Germany) was used to make comparisons between the groups. A p value<0.05 was considered as statistically significant.

RESULTS

The Effects of Metformin on Histology of Lung Tissue Following LPS-induced Injury

Histological images of sections obtained from the lung tissues stained with H & E are demonstrated in Figure 1. Sections from control group revealed normal lung histology with no inflammatory infiltration and degeneration (Figure 1A). However, inflammatory cellular infiltration and aggregation of polymorphonuclear cells in alveolar walls and congestion were observed in LPS group (Figure 1B). Metformin treatment attenuated the LPS-induced inflammatory cellular infiltration and congestion (Figure 1C).

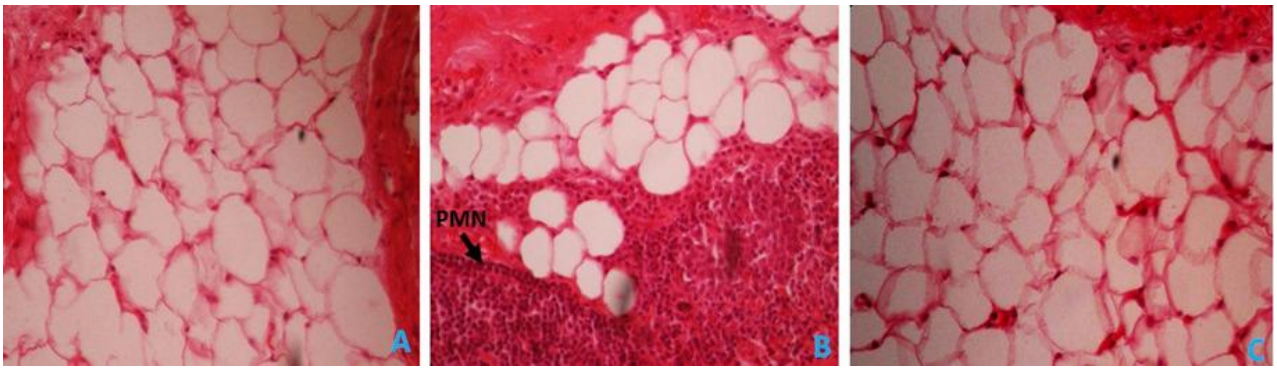

Figure 1. Histological examination of the left lungs in different groups of control, LPS and metformin plus LPS by hematoxylin and eosin, magnification: x40. Lipopolysaccharide (LPS, 0.5 mg/kg, IP) was given 9 hours before lung resection and metformin (100 mg/kg, IP) was injected 30 minutes before LPS (0.5 mg/kg, IP) injection. A: Control group reveals normal lung histology; B: LPS group with interstitial edema, congestion, polymorphonuclear cells (PMN) infiltration and aggregation; C: The metformin receiving group with less leukocytes infiltration.

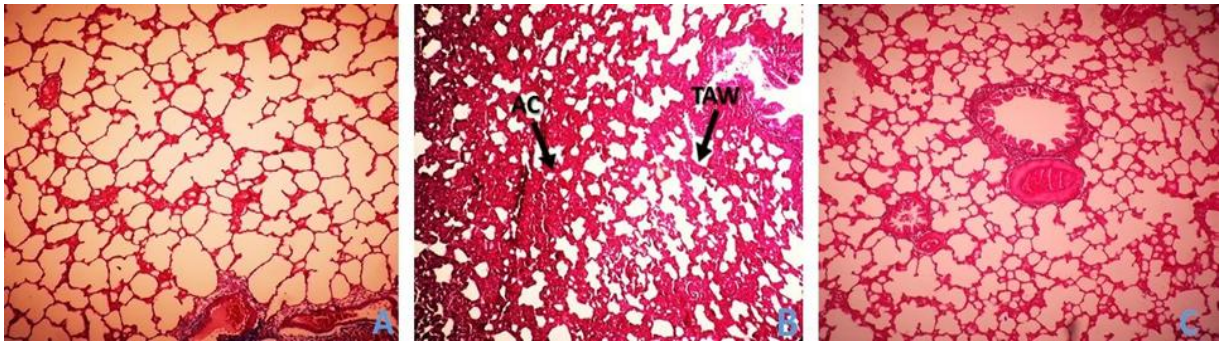

Figure 2. Histological examination of the left lungs in different groups of control, LPS and metformin plus LPS by Masson's trichrome staining, magnification: x40. Lipopolysaccharide (LPS, 0.5 mg/kg, IP) was given 9 hours before lung resection and metformin (100 mg/kg, IP) injected 30 minutes before LPS (0.5 mg/kg, IP) injection. A: Control group with normal alveolar structure; B: LPS group revealed alveolar collapse (AC) and thickening of alveolar walls (TAW); C: Metformin treated group displayed an alveolar structure more similar to control group with less thickening of the alveolar walls.

The Masson's trichrome stained sections are shown in Figure 2. Control group displayed normal alveolar structure (Figure 2A); while acute inflammation and thickness of the alveolar walls and alveolar collapse were observed in LPS group (Figure 2B). Metformin-treated group showed an alveolar structure more similar to the control group with less thickening of the alveolar epithelium (Figure 2C).

The Effects of Metformin on Lung Tissue MPO Activity following LPS-induced Injury

As shown in Figure 3, a significant increase (p<0.001) in MPO activity was observed in rats receiving LPS (15380 ± 248 mU/g) compared to the control group (7805 ± 205 mU/g). At the same time, administration of metformin to the rats that had receivedLPS significantly lowered the MPO activities to 11211 ± 261 mU/g (p<0.001).

The Effects of Metformin on AMPK Phosphorylation in Lung Tissue Following LPS-induced Injury

To verify whether metformin activates AMPK in our experimental model, the phosphorylation levels of the residue Thr172 of the catalytic α-subunit of AMPK, a requirement for activation of the kinase was assessed. Our data demonstrated that LPS did not induce a significant increase in AMPK activation.

However, there was an intrinsic increase in AMPK phosphorylation in the lung tissues of metformin-treated group compared with LPS group (0.98 ± 0.05 vs. 0.8±0.03 relative density; p<0.05), as demonstrated in Figure 4.

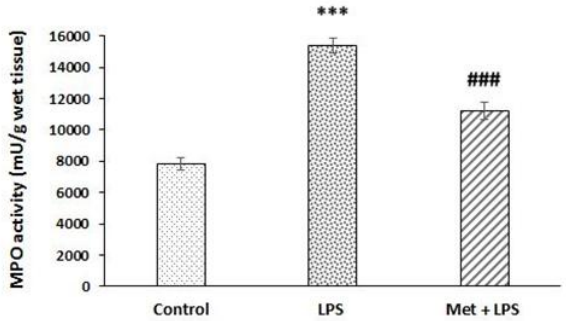

Figure 3. Effects of metformin on the myeloperoxidase (MPO) activity in different groups of control, LPS and metformin plus LPS. LPS (0.5 mg/kg, IP) were given 9 hours before lung resection and metformin (100 mg/kg, IP) was injected 30 minutes before LPS (0.5 mg/kg, iIP) injection. LPS: Lipopolysaccharide; Met: Metformin. Results are presented as mean ± SEM (n=6). $^{***}p$<0.001 in LPS group compared to control group, $^{###}p$<0.001 in metformin plus LPS group compared to LPS group using one-way ANOVA with LSD post-hoc test.

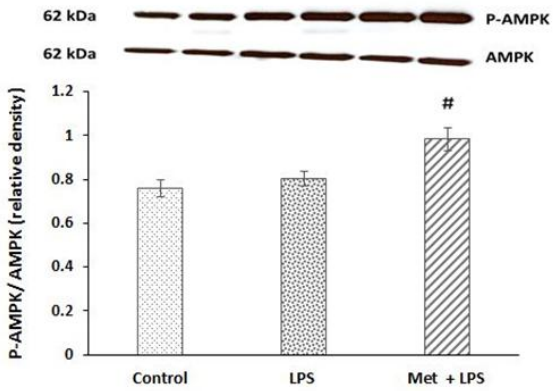

Figure 4. Effects of metformin on AMPK phosphorylation in lung tissue of different groups of control, LPS plus metformin and LPS. LPS (0.5 mg/kg, IP) were given 9 hours before lung resection and metformin (100 mg/kg, IP) was injected 30 minutes before LPS (0.5 mg/kg, IP) injection. LPS: Lipopolysaccharide; Met: Metformin. Results are presented as mean±SEM (n=6). $^{\#}p$<0.05 vs. LPS group using one-way ANOVA with LSD post-hoc test.

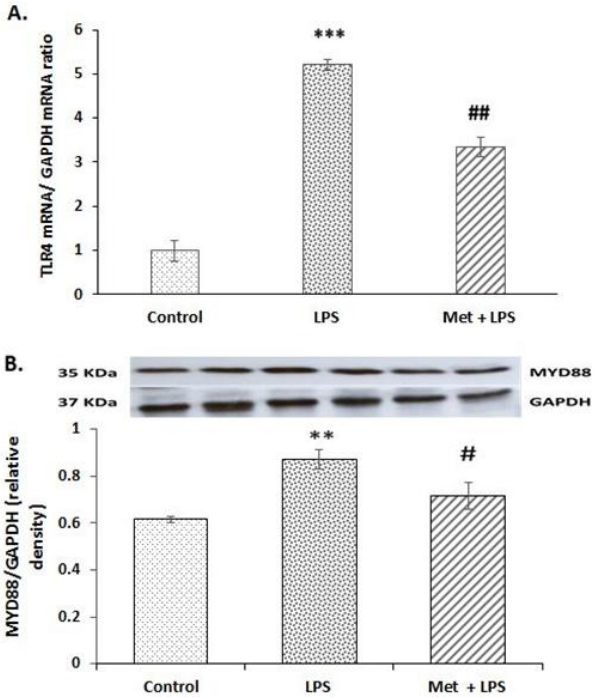

Figure 5. Effects of metformin on the levels of TLR4 mRNA expression (A) and MyD88 protein content (B) in different groups of control, LPS and metformin plus LPS lung tissues. LPS (0.5 mg/kg, IP) were given 9 hours before lung resection and metformin (100 mg/kg, IP) was injected 30 minutes before LPS (0.5 mg/kg, IP) injection. LPS: Lipopolysaccharide; Met: Metformin. Results are presented as mean±SEM (n=6).

For TLR4: $^{***}p$<0.001 in LPS group compared to control group, $^{\#\#}p$<0.01 in metformin plus LPS compared to LPS group.

For MYD88: $^{**}p$<0.01 in LPS group compared to control group, $^{\#}p$<0.05 in metformin plus LPS group compared to LPS group. The PCR data was analyzed using Pair Wise Fixed Reallocation Randomization Test and for western blotting data the one-way ANOVA with LSD post-hoc test was used.

The Effect of Metformin on the Lung Tissue Level of TLR4 mRNA and MYD88 Protein Following LPS-induced Injury

The RT-PCR technique was performed to investigate the TLR4 mRNA expression. As demonstrated in Figure 5A, the TLR4 mRNA level at 9 hours after LPS injection was profoundly increased (p<0.001) approximately 5.2 fold as compared to the control group (Figure 5A). Administration of metformin significantly reduced the LPS-stimulated TLR4 expression compared to LPS group (p<0.01). Furthermore, we examined MYD88 protein expression with western blotting on lung homogenates to determine whether the elevation of TLR4 mRNA is associated with the increase in the level of MyD88 protein. As demonstrated in Figure 5B, MyD88 protein content in the lungs of LPS group was up-regulated when compared to control group (+41%, p<0.01). The group treated with metformin displayed a significant (p<0.05) reduction in the lung content of MyD88 by 21% in comparison to LPS group (Figure 5B).

The Effect of Metformin on the Lung Tissue Level of NF-κBand TNF-α Following LPS-induced Injury

To further confirm the involvement of the NF-κB pathway, we investigated the effects of metformin on LPS-induced NF-κB expression in the rat lung tissue. As shown in Figure 6A, LPS treatment significantly increased NF-κB content approximately 1.65 fold compared to the control group (p<0.05). Western-blot analysis showed that the LPS-stimulated increase of NF-κB levels was approximately reduced to the control levels in the presence of metformin treatment (p<0.05 compared to LPS group). The potential effect of metformin on the level of TNFα, which is a key cytokine released following TLRs activation, is shown

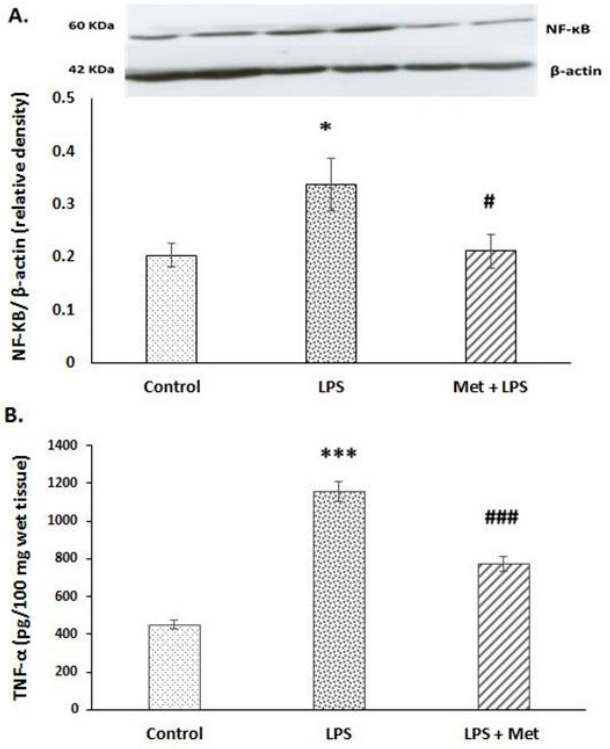

Figure 6. Effects of metformin on NF-κB protein expression (A) and TNF-α level (B) in lung tissue of different groups of control, LPS and metformin lus LPS. LPS (0.5 mg/kg, IP) were given 9 hours before lung resection and metformin (100 mg/kg, IP) was injected 30 minutes before LPS (0.5 mg/kg, i.p.) injection. LPS: Lipopolysaccharide; Met: Metformin. Results are presented as mean±SEM (n=6). For NF-κB *p<0.05 vs. control group and, #p<0.05 vs. LPS group. For TNF-α ***p<0.001 vs. control group and ###p<0.001 vs. LPS group using one-way ANOVA with LSD post-hoc test.

in the Figure 6B. The lungs obtained from rats exposed to LPS demonstrated enhanced level of TNF-α (1156±52 pg per 100 mg of lung tissue), whereas TNF-α level was low in rats treated with saline without metformin (450±21 pg per 100 mg of lung tissue). Treatment with metformin significantly decreased TNF-α level of the tissue to 772±37 pg per 100 mg of lung tissues compared to LPS group (p<0.001).

DISCUSSION

The key finding of the present study is that metformin protected the lung tissue from LPS-induced acute injury in rats by reducing inflammatory cellular infiltration, reducingg tissue congestion, and decreasing thickness of the alveolar epithelium. These effects were associated with a marked suppression of TLR4 expression in the lung tissue along with a significant decrease of protein content of intracellular TLRs adapter protein (MyD88) which was elevated following LPS injection. The protective effect of metformin was evidenced by reduction in the MPO activity and decrease in the level of NF-κB as well as by decline in the TNF-α concentration in the lung tissues.

Acute lung injury is a common complication of sepsis and major cause of death in this pathological condition without many effective pharmacological interventions. That is why the search for an effective treatment of this disease is essential. Nowadays, the TLRs and their relationship in different lung disease have a great importance in studies.[23] The main element in the development of sepsis is microbes-derived LPS. Generally, LPS by activating of TLRs produces inflammatory reactions which in the first stage are necessary to recruit neutrophils into the inflammatory site to phagocyte foreign agents. However, in some cases these defensive reactions are over-stimulated and cause an acute hyper-inflammation. Hence, to manage sepsis-induced acute tissue injuries the use of a drug able to regulate inflammation is critical. AMPK in addition to providing intracellular energy during stress can affect production of inflammatory cytokines and also suppress immunological factors. Several studies have indicated that AMPK can act as an intracellular anti-inflammatory enzyme.[24, 25] Moreover, it is also demonstrated that AMPK has the ability to promote macrophage polarization to an anti-inflammatory functional phenotype by differential regulation of transcription factors like NF-κB and cytokines like IL-10.[26]Metformin is a well-known antidiabetic drug that has been shown to activate AMPK by phosphorylation of it at α subunit.[27] The results of the present study showed an increased ratio of phosphorylated AMPKα to non-phosphorylated form in the lung tissues of rats treated with metformin. Metformin is reported to reduce intercellular adhesion molecule-1 (ICAM-1)[28]and it is also demonstrated that ICAM-1 expression can be regulated by NF-κB.[29] In a study on the effects of metformin on endothelial cells, decrease in TNF-α-induced ICAM-1 by inhibiting NF-κB activation has been reported.[9] Considering the decrease of NF-κB activation and TNF-α level after metformin treatment detected in our study, it can be concluded that

metformin conferred resistance to LPS-induced damages partly through decreasing ICAM-1expression.

Our results in this study also demonstrated that metformin considerably reduced the LPS-elevated MPO activity in the lungs. MPO is a lysosomal protein stored in azurophilic granules of the neutrophils and MPO activity assay is widely used in the researches for evaluating tissue neutrophil, LPS caused extensive leukocytes (especially neutrophils) infiltration into the lung tissue which was inhibited by metformin. Additionally, histology evaluation showed that metformin attenuated the LPS-induced inflammation in alveolar walls, inflammatory cells infiltration and aggregation and pulmonary congestion. In accordance to this study, in several studies the protective role of metformin on LPS-induced histopathological changes in other tissues such as heart and liver have been reported.[15, 30]Aggregation of neutrophils containing potent compounds including toxic oxygen radicals and granular proteins can be one of the tissue damaging reasons in such inflammatory conditions.[31] Accordingly, metformin by diminishing neutrophil infiltration, which was confirmed by MPO assay, may play a protective role against lung injury.

It was reported that TLR4 is involved in the recruitment of neutrophils in the inflamed tissue.[32] Therefore, increased expression of TLR4 documented in this study may facilitate neutrophil migration into the LPS-stimulated lungs. The increase in TLR4 expression in our experiment coincides with well-established increase in the adaptor protein MYD88, transcription factor of NF-κB, and cytokine of TNF-α in LPS-induced lung inflammation. The minimal tissue expression of TLR4, MYD88, NF-κB, and TNF-α in rats receiving metformin was in agreement with our recent observations on heart tissue.[15] Taken together, decline in TLR4 signaling elements along with AMPK activation by metformin may indicate the critical role of AMPK in protecting the lung from LPS-triggered inflammation. Since in the present study, only the MYd88 dependent signaling was assessed, the effect of metformin on the other pathways should also be in further studies considered.

To conclude, the present study shows that TLR4 signaling pathway including adaptor protein of MYD88, transcription factor NF-κB, and inflammatory cytokine TNF-α are involved in the LPS-induced lung injury in rat. Furthermore, metformin as an AMPK activator negatively regulated the TLR4 mediated signaling pathway in this model. The present study reveals a new insight into the critical role AMPK in regulating acute inflammatory conditions such as sepsis. Suppression of toll-like receptor 4 signaling by metformin can be a useful therapeutic target to protect the lung tissue from LPS-induced injury.

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REFERENCES

1. Russell JA. Management of sepsis. N Engl J Med 2006; 355(16):1699-713.
2. Garber BG, Hebert PC, Yelle JD, Hodder RV, McGowan J. Adult respiratory distress syndrome: a systemic overview of incidence and risk factors. Crit Care Med 1996; 24(4):687-95.
3. Janardhan KS, McIsaac M, Fowlie J, Shrivastav A, Caldwell S, Sharma RK, et al. Toll like receptor-4 expression in lipopolysaccharide induced lung inflammation. Histol Histopathol 2006; 21(7):687-96.
4. Reaves TA, Chin AC, Parkos CA. Neutrophil transepithelial migration: role of toll-like receptors in mucosal inflammation. Mem Inst Oswaldo Cruz 2005; 100 Suppl 1:191-8.
5. Takeda K, Kaisho T, Akira S. Toll-like receptors. Annu Rev Immunol 2003; 21:335-76.
6. Rameshrad M, Maleki-Dizaji N, Vaez H, Soraya H, Nakhlband A, Garjani A. Lipopolysaccharide Induced Activation of Toll Like Receptor 4 in Isolated Rat Heart Suggests a Local Immune Response in Myocardium. Iran J Immunol 2015; 12(2):104-16.
7. Newton K, Dixit VM. Signaling in innate immunity and inflammation. Cold Spring Harb Perspect Biol 2012; 4(3).
8. Nath N, Khan M, Paintlia MK, Singh I, Hoda MN, Giri S. Metformin attenuated the autoimmune disease of the central nervous system in animal models of multiple sclerosis. J Immunol 2009; 182(12):8005-14.
9. Hattori Y, Suzuki K, Hattori S, Kasai K. Metformin inhibits cytokine-induced nuclear factor kappaB

activation via AMP-activated protein kinase activation in vascular endothelial cells. Hypertension 2006; 47(6):1183-8.
10. Kim J, Kwak HJ, Cha JY, Jeong YS, Rhee SD, Kim KR, et al. Metformin suppresses lipopolysaccharide (LPS)-induced inflammatory response in murine macrophages via activating transcription factor-3 (ATF-3) induction. J Biol Chem 2014; 289(33):23246-55.
11. Yuan H, Li L, Zheng W, Wan J, Ge P, Li H, et al. Antidiabetic drug metformin alleviates endotoxin-induced fulminant liver injury in mice. Int Immunopharmacol 2012; 12(4):682-8.
12. Hafezzadeh H, Soraya H, Banani A, Adibpour M, Rameshrad M, Garjani A, et al. The Effect of Metformin on Neutrophil Recruitment in Carrageenan- Induced Peritonitis in Mice. Pharma Sci 2012; 17(4):209-18.
13. Tsoyi K, Jang HJ, Nizamutdinova IT, Kim YM, Lee YS, Kim HJ, et al. Metformin inhibits HMGB1 release in LPS-treated RAW 264.7 cells and increases survival rate of endotoxaemic mice. Br J Pharmacol 2011; 162(7):1498-508.
14. Soraya H, Farajnia S, Khani S, Rameshrad M, Khorrami A, Banani A, et al. Short-term treatment with metformin suppresses toll like receptors (TLRs) activity in isoproterenol-induced myocardial infarction in rat: are AMPK and TLRs connected? Int Immunopharmacol 2012; 14(4):785-91.
15. Vaez H, Rameshrad M, Najafi M, Barar J, Barzegari A, Garjani A. Cardioprotective effect of metformin in lipopolysaccharide-induced sepsis via suppression of toll-like receptor 4 (TLR4) in heart. Eur J Pharmacol 2016; 772:115-23.
16. Zhu MY, Lu YM, Ou YX, Zhang HZ, Chen WX. Dynamic progress of 2,4,6-trinitrobenzene sulfonic acid induced chronic colitis and fibrosis in rat model. J Dig Dis 2012; 13(8):421-9.
17. Liu Y, Yang G, Bu X, Liu G, Ding J, Li P, et al. Cell-type-specific regulation of raft-associated Akt signaling. Cell Death Dis 2011; 2:e145.
18. Mullane KM, Kraemer R, Smith B. Myeloperoxidase activity as a quantitative assessment of neutrophil infiltration into ischemic myocardium. J Pharmacol Methods 1985; 14(3):157-67.
19. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001; 29(9):e45.
20. Omar MA, Verma S, Clanachan AS. Adenosine-mediated inhibition of 5'-AMP-activated protein kinase and p38 mitogen-activated protein kinase during reperfusion enhances recovery of left ventricular mechanical function. J Mol Cell Cardiol 2012; 52(6):1308-18.
21. Kewalramani G, An D, Kim MS, Ghosh S, Qi D, Abrahani A, et al. AMPK control of myocardial fatty acid metabolism fluctuates with the intensity of insulin-deficient diabetes. J Mol Cell Cardiol 2007; 42(2):333-42.
22. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72:248-54.
23. Zhang Q, Qian FH, Yin XW, Cao Q, Bai JL, Du Q, et al. Associations of Toll-like receptor 7 and 8 Polymorphisms with Asthma and Asthma-related Phenotypes in a Chinese Han Population. Iran J Allergy Asthma Immunol 2015; 14(6):569-80.
24. Nath N, Giri S, Prasad R, Salem ML, Singh AK, Singh I. 5-aminoimidazole-4-carboxamide ribonucleoside: a novel immunomodulator with therapeutic efficacy in experimental autoimmune encephalomyelitis. J Immunol 2005; 175(1):566-74.
25. Paintlia AS, Paintlia MK, Singh I, Singh AK. Immunomodulatory effect of combination therapy with lovastatin and 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside alleviates neurodegeneration in experimental autoimmune encephalomyelitis. Am J Pathol 2006; 169(3):1012-25.
26. Sag D, Carling D, Stout RD, Suttles J. Adenosine 5'-monophosphate-activated protein kinase promotes macrophage polarization to an anti-inflammatory functional phenotype. J Immunol 2008; 181(12):8633-41.
27. Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, et al. Role of AMP-activated protein kinase in mechanism of metformin action. J Clin Invest 2001; 108(8):1167-74.
28. Diamanti-Kandarakis E, Paterakis T, Alexandraki K, Piperi C, Aessopos A, Katsikis I, et al. Indices of low-grade chronic inflammation in polycystic ovary syndrome and the beneficial effect of metformin. Hum Reprod 2006; 21(6):1426-31.
29. Tak PP, Firestein GS. NF-kappaB: a key role in inflammatory diseases. J Clin Invest 2001; 107(1):7-11.
30. Bergheim I, Luyendyk JP, Steele C, Russell GK, Guo L, Roth RA, et al. Metformin prevents endotoxin-induced liver injury after partial hepatectomy. J Pharmacol Exp Ther 2006; 316(3):1053-61.
31. Iraz M, Iraz M, Esrefoglu M, Aydin MS. Protective effect of beta-glucan on acute lung injury induced by lipopolysaccharide in rats. Turk J Med Sci 2015; 45(2):261-7.

32. Andonegui G, Bonder CS, Green F, Mullaly SC, Zbytnuik L, Raharjo E, et al. Endothelium-derived Toll-like receptor-4 is the key molecule in LPS-induced neutrophil sequestration into lungs. J Clin Invest 2003; 111(7):1011-20.