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ORIGINAL ARTICLE

Modulation of diabetes-related liver injury by the HMGB1/TLR4 inflammatory pathway

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Abstract
Chronic inflammation plays an essential role in the development of diabetic complications. Understanding the molecular mechanisms that support inflammation is a prerequisite for the design of novel anti-inflammatory therapies. These would take into consideration circulating levels of cytokines and damage-associated molecular patterns (DAMPs) that include the high mobility group box 1 (HMGB1) protein which, in part, promotes the inflammatory response through TLR4 signaling. The liver, as the source of circulating cytokines and acute-phase proteins, contributes to the control of systemic inflammation. We previously found that liver injury in streptozotocin-induced diabetic rats correlated with the level of oxidative stress, increased expression of HMGB1, and with the activation of TLR4-mediated cell death pathways. In the present work, we examined the effects of ethyl pyruvate (EP), an inhibitor of HMGB1 release/expression, on the modulation of activation of the HMGB1/TLR4 inflammatory cascade in diabetic liver. We observed that increased expression of inflammatory markers, TNF-α, IL-6, and haptoglobin in diabetic liver was associated with increased HMGB1/TLR4 interaction, activation of MAPK (p38, ERK, JNK)/NF-κB p65 and JAK1/STAT3 signaling pathways, and with decreased expression of Nrf2-regulated antioxidative enzymes. The reduction in HMGB1 expression as the result of EP administration reduced the pro-inflammatory activity of HMGB1 and exerted a protective effect on diabetic liver, which was observed as improved liver histology and antioxidant and inflammatory statuses. Our results suggest that prevention of HMGB1 release and blockage of the HMGB/TLR4 axis represents a potentially effective therapeutic strategy aimed at ameliorating diabetes-induced inflammation and ensuing liver injury.

Keywords Diabetes · Ethyl pyruvate · HMGB1/TLR4 signaling · Inflammatory response · Liver

Introduction

Diabetes mellitus is a metabolic disease caused by defects in insulin secretion, insulin action, or both. It is also an inflammatory disease given that one of its major features is chronic low-grade inflammation, characterized by elevated circulating pro-inflammatory cytokines and acute-phase (AP) proteins [8, 12]. While inflammation is primarily a beneficial response activated to restore tissue homeostasis, when the magnitude or duration of inflammation is excessive, inflammation leads to tissue injury. In diabetes, the presence of chronic, low-grade inflammation increases the risk of development of insulin resistance, cardiovascular complications, and kidney problems [12]. As the major source of circulating cytokines and AP proteins, the liver is an essential constituent of the immune system contributing to the control of systemic inflammation [5]. In diabetes, chronic hyperglycemia creates conditions that promote hepatocyte injury, and inflammation can increase the risk of development of liver disease [27]. In our previous study, we showed that in streptozotocin (STZ)-induced diabetic rats, the serum levels of interleukin (IL)-6 and tumor necrosis factor (TNF)-α are increased and that these cytokines upregulate in the liver, in a concentration-dependent manner haptoglobin (Hp), an AP protein with anti-inflammatory, immunomodulatory, and antioxidant properties [2]. We also showed that the presence of these cytokines was strongly correlated with the extent of diabetes-related oxidative stress and liver damage. Depending on its concentration, TNF-α can either have protective or damaging effects. This has been

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demonstrated in acute liver injury [11] where its elevated presence triggers a series of intracellular events that result in the activation of apoptosis, a type of cell death which is increased in diabetic rat liver [33]. Since inflammation is one of the main etiological factors in the development and progression of diabetes-related tissue injury, it is important to investigate diabetes-induced signaling pathways that maintain the inflammatory environment and to identify endogenous participants that would be suitable therapeutic targets for controlling inflammation.

According to Dasu and Jialal [6], high glucose level in diabetes induces inflammation followed by cytokine release, through increasing in Toll-like receptor (TLR) expression and activity, and activation of nuclear factor-kappa B (NF-κB) transcription factors. Healthy liver contains low levels of a variety TLRs, and their expression under pathological conditions is upregulated. Increased expression of TLR4 was reported in liver injury models, such as partial hepatectomy and ischemia-reperfusion, where it is involved in the clearance of endotoxins, in the production of pro-inflammatory and anti-inflammatory cytokines, and the generation of reactive oxidative species (ROS) [14, 36]. In our previous study, we described the involvement of TLR4 in the induction of the intrinsic apoptotic cell death pathway in diabetic rat liver through interaction with exogenous high mobility group box 1 (HMGB1) protein [33].

The innate immune system can sense danger signals from damaged hepatocytes during non-infection-related liver injury, resulting in an inflammatory response [10, 14]. HMGB1, originally described as a chromatin-associated protein, functions as a key endogenous danger signal molecule that extracellularly exerts a cytokine-like effect on immune cells by promoting pro-inflammatory signaling and release of cytokines [35, 44]. HMGB1, which belongs to damage-associated molecular pattern molecules (DAMPs), is released passively from stressed, damaged, or dying cells and actively from immune cells. ROS are important mediators in HMGB1 release from both immune and nonimmune cells. In studies of hepatic ischemia-reperfusion, it was shown that this protein mediates liver injury by acting as an endogenous activator of TLR4 [40]. Activated TLR4 leads to activation of several signaling pathways, including the mitogen-activated protein kinase (MAPK), NF-κB, and Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathways that determine the transcriptional activities of genes for the pro-inflammatory cytokines IL-6 and TNF-α, and also for Hp and other immune-related proteins [31]. Recently, Yang et al. [46] established that Hp, or its β subunit, forms a complex with HMGB1, inhibits HMGB1-mediated induction of pro-inflammatory mediators, and elicits an anti-inflammatory response by stimulating the production of the antioxidative enzyme heme oxygenase-1 (HO-1) and anti-inflammatory cytokines such as IL-10. Previously, we noted a significant increase in the expression of both HMGB1 and Hp, in the serum and liver of diabetic rats [2, 16].

The above data indicate that HMGB1 could play an important role in mediating diabetes-related liver injury by balancing pro- and anti-inflammatory activities through a paracrine-like pro-inflammatory signaling mechanism. By interacting with TLR4, exogenous HMGB1 could be involved in a vicious cycle of enhanced cytokine production, ROS generation, and HMGB1 release that together with diabetes-associated oxidative stress, additionally aggravate liver functionality and lead to its damage. However, through the same TLR4 signaling pathways, HMGB1 could mediate anti-inflammatory responses by stimulating the production of anti-inflammatory factors. Thus, it is expected that the pathological role of HMGB1 in diabetic liver depends on its extracellular presence. To test this hypothesis, we investigated whether inhibition of HMGB1 secretion by the simple aliphatic ester of pyruvic acid, ethyl pyruvate (EP), decreases diabetes-related hepatocellular damage, suppresses activation of inflammatory cascades from TLR4, and modulates the inflammatory status. By virtue of its ability to ameliorate multiple acute inflammatory organ injuries in experimental animal models, EP acts as an anti-inflammatory agent [45]. More importantly, a part of its anti-inflammatory properties include it capability to downregulate HMGB1 release [37].

Materials and methods

Animals Animal care and animal procedures were in accordance with the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (Institute for Laboratory Animal Research 1996) and approved by the Ethical Committee for the Use of Laboratory Animals of the Institute for Biological Research "SinišaStanković," Belgrade (No. 2-15/10). Male albino rats of Wistar strain, weighing 200–220 g at the beginning of the experiment, were used. The rats were housed three per cage in a room with constant temperature and humidity (22 ± 2 °C, 50 ± 5%, 12 h dark/light intervals) and could freely take food and tap water.

Treatments Experimental animals were separated randomly into the following four experimental groups: control group (C), control group treated with EP (C + EP), diabetic group (D), and diabetic group treated with EP (D + EP). Diabetes was induced by a single intraperitoneal (i.p.) injection of STZ (65 mg/kg; Sigma-Aldrich) that was dissolved in freshly prepared 0.01 mol/L sodium citrate, pH 4.5. Blood glucose was monitored using a hand-held glucometer (GlucoSure Plus, Apex Biotechnology Corp). Blood was obtained from the tail tip. Rats with a fasting blood glucose concentration greater than 20 mmol/L were considered as diabetic. EP

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(Sigma-Aldrich) was freshly prepared in Ringer's solution (130 mM NaCl, 4 mM KCl, 2.7 mM $CaCl_2$, pH 7.0). According to Liang et al. [22], EP at a dose of 80 mg/kg is effective in inhibiting HMGB1 release/secretion. Therefore, this dose of EP was intraperitoneally administered daily, with the first dose injected 10 days after diabetes induction. EP administration continued until the end of the study, 4 weeks after initiation of the STZ treatment, when the rats were weighed and killed by decapitation. Control rats were injected only with the solvent.

Tissue preparation Immediately after decapitation, the blood was rapidly collected and the serum was obtained by centrifugation at 2000*g* in an Eppendorf 5415 R centrifuge (Sigma-Aldrich) for 15 min. The liver was removed and weighed and a liver section was processed for microscopic examination. For measuring the activities of antioxidative enzymes in the liver, a 10% homogenate of fresh liver (500 mg) was prepared in sucrose buffer (250 mM sucrose, 1 mM EDTA, 50 mM Tris-HCl, pH 7.4). The homogenate was centrifuged at 100,000*g* for 90 min at 4 °C and the resulting supernatant was aliquoted and stored at − 80 °C. The rest of the liver was snap-frozen in liquid nitrogen and kept at − 80 °C.

Preparation of the whole liver homogenate and subcellular fractions For whole liver homogenate preparation, frozen rat liver tissue (200–400 mg) was homogenized at 4 °C in a Dounce tissue grinder in ice-cold homogenization buffer (250 mM sucrose, 10 mM Tris pH 7.6, 1 mM EDTA) supplemented with phosphatase inhibitor Mix I and protease inhibitor Mix G (Serva). The homogenate was centrifuged at 9700*g* for 20 min at 4 °C, and the resulting supernatant was aliquoted, snap-frozen in liquid nitrogen, and stored at − 80 °C. For preparation of the cytosolic and nuclear protein extracts, the commercial Subcellular Protein Fractionation Kit for Tissues (Cat. no 87790, Pierce) was used. Liver tissue (200 mg) was homogenized in 10 volumes of ice-cold cytoplasmic extraction buffer containing protease inhibitor Mix G (Serva) and the homogenate was centrifuged at 500*g* for 5 min at 4 °C. The resulting supernatant (cytoplasmic extract) was cleared by centrifugation, divided into aliquots, and stored at − 80 °C. The 500*g* pellet was resuspended in ice-cold membrane extraction buffer containing protease inhibitors, vortexed on the highest setting, incubated at 4 °C for 10 min with gentle mixing, and centrifuged at 3000*g* for 5 min. The resulting pellet was resuspended in ice-cold nuclear extraction buffer supplemented with protease inhibitor Mix G, vortexed on the highest setting for 15 s, incubated at 4 °C for 30 min, and centrifuged at 5000*g* for 5 min at 4 °C. The resulting supernatant (nuclear extract) was separated into aliquots and stored at − 80 °C. Protein concentration was determined by the method of Lowry.

Determination of serum glucose, insulin, and alanine aminotransferase concentrations Serum glucose was measured by the glucose oxidase procedure using a glucose GOD/PAP test kit (Randox Laboratories, detection limit 0.4–41 mmol/L on Shimadzu UV-160 spectrophotometer). Serum insulin was measured by the rat insulin ELISA kit purchased from Mercodia AB (Uppsala). Serum alanine aminotransferase (ALT) was measured with an ILab-600 Analyzer (International Laboratory).

Immunohistochemistry Liver samples were fixed in Bouin's solution and embedded in paraffin. For examination of diabetes-related hepatocyte deterioration, 5-μm-thick sections of rat liver were deparaffinized, rehydrated, stained with hematoxylin/eosin (H&E), and examined using standard light microscopy (Opton, Zeiss). Representative liver sections were stained by the immunohistochemical method. After deparaffinization and rehydration of sections, endogenous peroxidase activity was blocked by incubation in methanol containing 0.3% H_2O_2 for 15 min at room temperature. Heat-induced antigen retrieval was performed using 0.1 mol/L sodium citrate buffer pH 6.0 and then heated at 750 W in a microwave oven three times for 7 min. Non-specific background staining was reduced by incubation with normal porcine serum (Dakopatts) diluted 1:10 *v*/*v* in PBS for 45 min. The sections were incubated with polyclonal rabbit anti-HMGB1 antibody in PBS (1:500 *v*/*v*; ab18256, Abcam) overnight at 4 °C, followed by washing in PBS, three times for 5 min. For the negative control, liver sections were incubated in PBS without primary antibody. The secondary antibody, polyclonal bovine anti-rabbit IgG (1:100 *v*/*v* in PBS; sc 2379, Santa Cruz Biotechnology) was applied and incubated for another 60 min and rinsed in PBS for 10 min. Antibody localization was visualized by a 3,3-diaminobenzidine tetrachloride (DAB) liquid substrate chromogen system (Serva). The sections were thoroughly washed in distilled water, counterstained with hematoxylin, and mounted on DPX (Sigma-Aldrich). All light microscopy images of the liver sections were made using a DM RB Photomicroscope (Leica) with a DFC 320 CCD Camera (Leica) for the image acquisition.

Antioxidant enzyme assays To measure the activities of antioxidative enzymes in the liver, a 10% homogenate of fresh liver, prepared in sucrose buffer, was used. Total activity of superoxide dismutase (SOD) was determined by the epinephrine method [30] which is based on the measurement of inhibition of epinephrine auto-oxidation by the SOD contained in the sample at 480 nm. The reaction was performed in a reaction medium containing 3 mM epinephrine dissolved in 0.1 N HCl and 50 mM Na_2CO_3 with 1 mM EDTA, pH 10.2. SOD activity was expressed as SOD units per milligram of protein. One unit is defined as the amount of enzyme that inhibits the rate of epinephrine auto-oxidation by 50%. Catalase (CAT)

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activity was measured by the method of Beutler [4]. The reaction is based on the rate of H_2O_2 degradation by CAT contained in the analyzed samples. The reaction was performed in an incubation mixture containing 50 μl buffer (1 M Tris HCl pH 8.0, 5 mM EDTA), 5–10 μl sample, and 1 ml 10 mM H_2O_2 liver homogenate, and 10 mM H_2O_2 (A = 0.800–0.860). The rate of decrease in absorbance was measured at 230 nm. CAT activity was expressed as micromoles of H_2O_2 per minute per milligram of protein (U/mg of protein) in liver homogenates, with one CAT activity unit defined as 1 μmol of H_2O_2.

Western immunoblotting Protein samples of serum (1 μl), whole liver homogenates (30 μg), cytosolic (30 μg), or nuclear (25 μg) extracts were separated by 12% SDS-PAGE, transferred onto polyvinylidene difluoride (PVDF) membrane (Hybond-P, Amersham Pharmacia Biotech), which was blocked in a solution of 0.2% Tween 20, 50 mM Tris–HCl pH 7.6, 150 mM NaCl containing 5% non-fat condensed milk. After protein transfer, the PVDF membrane was incubated with a primary antibody for 1.5 h at room temperature. The following primary antibodies were used for the Western blotting: Abcam—rabbit polyclonal antibodies for CAT (ab1877), Hp (ab131236), HMGB1 (ab18256), Nrf2 (ab 31163), NF-κB p65 (Ab 7970), TLR4 (Ab 83444), β-actin (Ab 8227), mouse monoclonal antibody for TNF-α (ab1793); Santa Cruz Biotechnology—rabbit polyclonal antibodies for MnSOD/SOD-2 (sc 30080), p38 (sc 535), ERK1(sc-94), p-p38 (sc 7975-R), p-NF-κB p65 (sc 33039), mouse monoclonal for JNK (sc 1648), goat polyclonal antibodies for IL-6 (sc 1266), p-ERK (sc 16982), p-JNK (sc 12882), p-JAK1 (sc 16773), CuZnSOD/SOD-1 (sc8637), HO-1 (sc 1796), Lamin B (sc6217); Cell Signaling Technology—rabbit polyclonal antibody for p-STAT3-ser (9134S). The blots were probed with horseradish peroxidase-conjugated secondary antibody purchased from Santa Cruz Biotechnology—bovine anti-rabbit IgG (sc 2379), bovine anti-goat IgG (sc 2378); Abcam—goat anti-rabbit IgG (ab 97051); Cell Signaling—anti-mouse IgG (7076S). For reprobing, the membranes were incubated in 2% SDS, 100 mM β-mercaptoethanol, and 62.5 mM Tris–HCl pH 6.8 for 35 min at 50 °C, and then rinsed three times, blocked, and probed again with another antibody. Immunoreactive bands were identified by an enhanced chemiluminescence (ECL) detection system (Santa Cruz Biotechnology). The results were analyzed using TotalLabPhoretix electrophoresis software (ver. 1.10).

Immunoprecipitation Immunoprecipitation was performed as previously described [33]. The whole liver homogenate (500 μg) was precleared by incubation with control IgG and protein A/G agarose (Santa Cruz Biotechnology) and the precleared supernatant was incubated overnight at 4 °C with 2 μg of anti-HMGB1 antibody (ab18256) in TEG buffer (10 mM TrisHCl, pH 7.6, 4 mM EDTA, 10% glycerol, 50 mM NaCl). A/G-agarose-coupled beads were added and incubated on a shaker for an additional 30 min at 4 °C. The beads were pelleted by centrifugation (9300*g*, 1 min, 4 °C.) and washed five times in TEG buffer. The immunoprecipitated proteins were eluted from the beads by heating in sample buffer at 95 °C for 10 min and separated by 12% SDS-PAGE. The obtained immunoprecipitate was analyzed by Western immunobloting. For background elimination of heavy and light chains during immunoprecipitation, Clean-Blot™ IP Detection Reagent (HRP) was used according to the manufacturer's instructions (21230, Thermo Fisher Scientific).

Statistical analysis All experiments were repeated at least three times. The data presented as the means ± SE. For intergroup comparison between two means, one-way analysis of variance (ANOVA) followed by the Mann-Whitney U test was applied. Values of $P < 0.05$ were considered statistically significant.

Results

Effects of EP on the diabetic status and liver injury

Four weeks after completion of the STZ treatment, the rats developed diabetes. The animals exhibited a 30% decrease in body weight ($P < 0.05$), a sixfold increase ($P < 0.05$) in serum glucose concentration, and a threefold decrease ($P < 0.05$) in serum insulin concentration (Table 1, $n = 15$). A disturbance in liver functioning was manifested as a 2.3-fold ($P < 0.05$) increase in serum ALT activity (Table 1, $n = 15$). Histological examination of the livers of diabetic rats (Fig. 1, $n = 9$) revealed loss of the usual concentric arrangement of hepatocytes and the presence of hydropic degeneration of hepatocytes, hepatocellular necrosis, reduction in glycogen content, and prominent Kupffer cells. Diabetic rats treated with EP (80 mg/kg, 18 days, $n = 15$) displayed less weight loss ($P < 0.05$) and improved liver injury, manifested by a 35% decrease ($P < 0.05$) in serum ALT activity in comparison to diabetic rats (Table 1, $n = 15$). In addition, liver architecture was normalized, the cytoplasm was homogeneous, exhibiting minimal vacuolization, and the number of deteriorated and necrotic cells was strongly reduced (Fig. 1, $n = 9$). Kupffer cells were less prominent. In EP-treated diabetic rats, the serum glucose concentration was not significantly changed and while insulin concentration was increased 1.6-fold ($P < 0.05$, $n = 15$) in comparison to diabetic rats, it remained decreased (by 47%; $P < 0.05$) in comparison to control animals. The EP treatment of control rats did not elicit significant changes in the examined biochemical and histological parameters.

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Table 1 The effects of EP on body weight and serum concentration of glucose, insulin, and ALT in diabetic rats

	C	D	C + EP	D + EP
Body weight (g)	315.5 ± 12.5[a]	222 ± 9.9[b]	278 ± 13.2[a]	268 ± 11.8[c]
Glucose (mmol/l)	6.3 ± 0.3[a]	37.3 ± 3.2[b]	8.6 ± 0.6[a]	35.6 ± 3.1[b]
Insulin (mIU/l)	61.5 ± 7.2[a]	20.9 ± 2.4[b]	50.8 ± 5.4[a]	32.7 ± 3.6[c]
ALT (U/l)	66.7 ± 7.4[a]	151.9 ± 10.1[b]	58.4 ± 5.3[a]	98.7 ± 8.8[c]

C control rats, *D* diabetic rats, *C + EP* control rats treated with EP, *D + EP* diabetic rats treated with EP. Values are means ± SE from three independent experiments performed in triplicate, $n = 15$. [a,b,c] Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$)

Antioxidative and anti-inflammatory effect of EP on the liver of diabetic rats

As EP possesses antioxidative and anti-inflammatory activities [37], we examined whether it changed the antioxidative and inflammatory responses in the liver of STZ-induced diabetic rats. The activities of CAT and total SOD were decreased by about 24 and 22%, respectively (Fig. 2a, $P < 0.05$, $n = 15$) in diabetic liver. In the diabetic group treated with EP, the activities of both CAT and SOD were increased 1.2- and 1.4-folds, respectively $P < 0.05$, $n = 15$) when compared to diabetic rats (Fig. 2a). Thus, EP restored the activity of SOD to the control level while the activity of CAT remained about 11% ($P < 0.05$) below the control level.

Relative changes in CuZnSOD and MnSOD levels were also examined. Western immunoblot analysis of whole liver homogenates of diabetic rats (Fig. 2b, $n = 15$) showed a nearly twofold decrease ($P < 0.05$) in CAT protein expression and a twofold increase ($P < 0.05$) in MnSOD protein expression, while the level of CuZnSOD protein was unchanged when compared to the control group. Compared to untreated diabetic rats, the EP-treated diabetic rats displayed a 1.5-fold ($P < 0.05$) increase in liver CAT protein expression and a 30% ($P < 0.05$) decrease in liver MnSOD protein expression. No differences were observed between control rats and EP-treated control rats, as the treatment of control rats with EP did not elicit significant changes in activities nor in relative protein levels of the examined antioxidative enzymes.

Since the inflammation in STZ-induced diabetic rats is characterized by increased levels of pro-inflammatory cytokines and elevated hepatic production of Hp [2], we examined the effects of EP on the changes in the relative expression levels of TNF-α, IL-6, and Hp. Western analysis of whole liver homogenates showed that in diabetic rats, the levels of TNF-α and IL-6 were increased 6.7- and 3.3-folds, respectively (Fig. 3, $P < 0.05$, $n = 15$) as compared to control rats. EP treatment of diabetic rats led to three and twofold decreases in TNF-α and IL-6, respectively, when compared to the diabetic group (Fig. 3, $P < 0.05$, $n = 15$). However, their expression remained increased 2.3- and 1.7-folds, respectively ($P < 0.05$), when compared to control rats. The changes in TNF-α and IL-6 levels were accompanied by changes in hepatic production

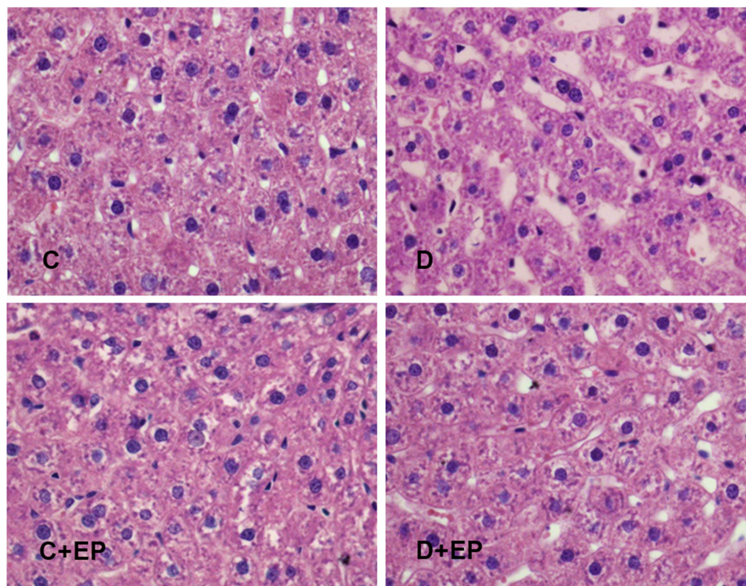

Fig. 1 EP ameliorates histopathological changes in the liver of diabetic rats. Photomicrographs of liver specimens showing normal architecture and cell structure in control rats (*C*) and control rats treated with EP (*C + EP*). In diabetic rats (*D*), photomicrographs show damaged architecture, prominent Kupffer cells, significant hydropic degeneration, and necrosis of hepatocytes. The diabetic group treated with EP (*D + EP*) exhibits almost normal architecture, minimal presence of necrosis and hepatocyte degeneration, and less prominent Kupffer cells. H&E stain, ×400

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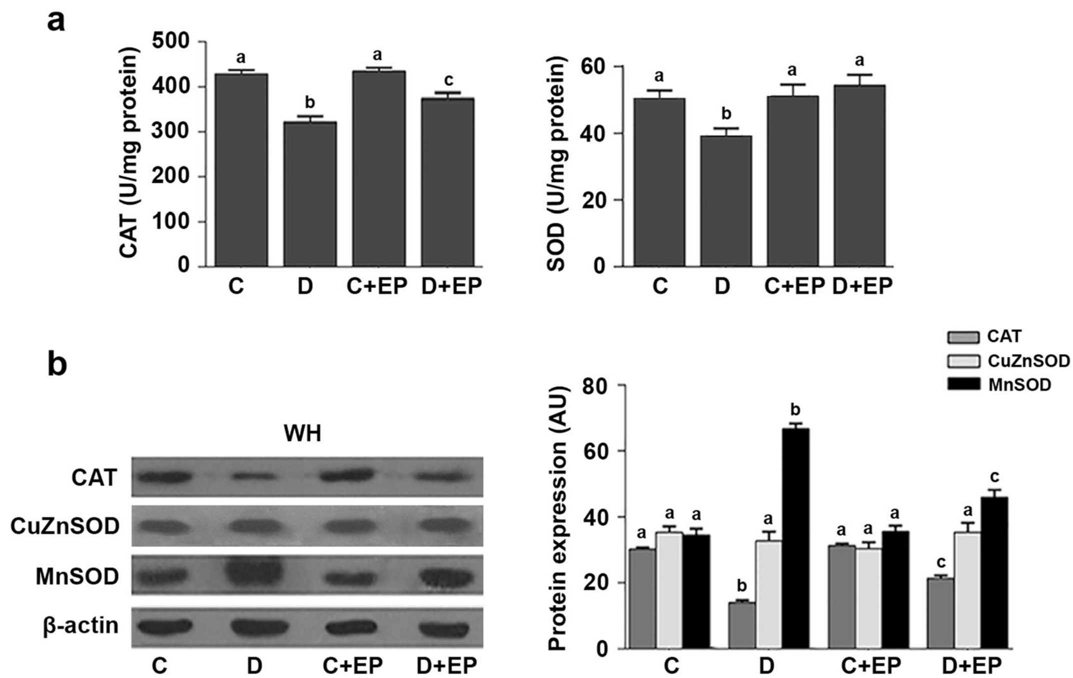

Fig. 2 Antioxidative status in diabetic rat liver after treatment with EP. (**a**) The activities of antioxidative enzymes, catalase (CAT), and total superoxide-dismutase (SOD) were determined spectrophotometrically. (**b**) Protein expression levels of CAT, copper-zinc superoxide-dismutase (CuZnSOD), and manganese superoxide-dismutase (MnSOD) in whole liver homogenates (WH) were determined by Western immunoblotting. β-actin was used as a loading control. *C* control rats, *D* diabetic rats, *C + EP* control rats treated with EP, *D + EP* diabetic rats treated with EP. Immunoblots obtained from at least three independent experiments were quantified by densitometric analysis and are presented in the graphs. The values in the graphs are means ± SE, $n = 15$. [a,b,c]Mean values with unlike letters were significantly different ($P < 0.05$)

of Hp. According to Western analysis of whole liver homogenates, treatment of diabetic rats with EP led to a 33% decrease in elevated Hp-β, which remained 1.3-fold higher than in the control group (Fig. 3, $P < 0.05$, $n = 15$). Again, no differences were observed between control rats and EP-treated control rats, as control rats that were treated with EP did not exhibit any significant changes in protein levels of the examined inflammatory markers.

Effect of EP on HMGB1 expression in diabetic rat serum and liver

According to numerous studies, EP inhibits HMGB1 secretion/release mostly at the level of nuclear-to-cytoplasmic translocation [37], suggesting that this contributes to its anti-inflammatory effect. To determine whether EP inhibits diabetes-induced subcellular translocation of

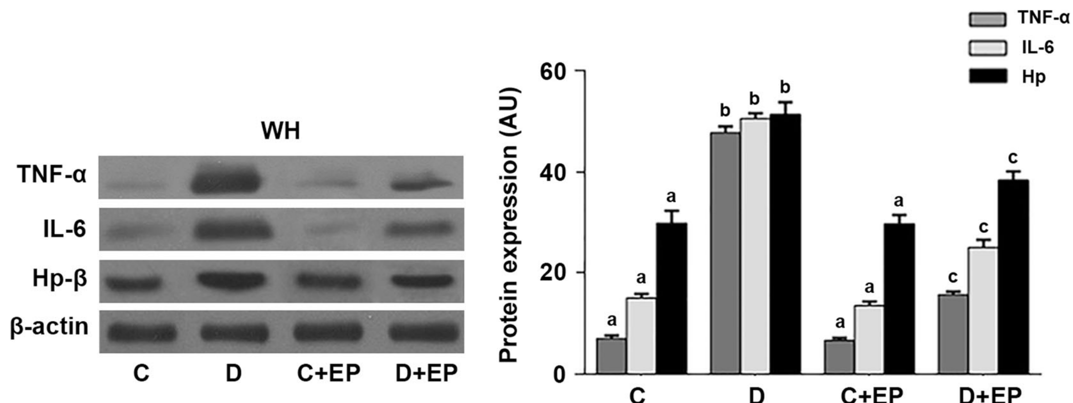

Fig. 3 EP reduces diabetes-induced expression of inflammatory markers TNF-α, IL-6, and haptoglobin (Hp). The protein expression levels of TNF-α, IL-6, and Hp in whole liver homogenates (WH) were determined by Western immunoblotting. β-actin was used as a loading control. *C* control rats, *D* diabetic rats, *C + EP* control rats treated with EP, *D + EP* diabetic rats treated with EP. Immunoblots obtained from at least three independent experiments were quantified by densitometric analysis and are presented in the graphs. The values in the graphs are means ± SE, $n = 15$. [a,b,c]Mean values with unlike letters were significantly different ($P < 0.05$)

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HMGB1, liver tissue sections were first analyzed by immunostaining. Liver sections of diabetic rats (Fig. 4a, $n = 9$) showed HMGB1 staining inside the cytoplasm, in contrast to control sections, where intra-nuclear HMGB1 staining was prominent. In contrast to diabetic rats, in the EP-treated diabetic group, aside from a faint cytoplasmic staining, HMGB1-positive staining was mostly intra-nuclear (Fig. 4a, $n = 9$). These changes were confirmed by Western analysis of HMGB1 expression in cytosolic and nuclear extracts, and in whole liver homogenates and serum (Fig. 4b, $n = 15$). In the liver of diabetic rats, the relative level of nuclear HMGB1 decreased 5.5-fold ($P < 0.05$) and increased 2.4-fold ($P < 0.05$) in the cytosolic extracts. These changes were accompanied by a 2.4-fold ($P < 0.05$) increase in HMGB1 expression in the whole liver homogenate and a 2.2-fold increase in the serum ($P < 0.05$). The EP treatment prevented the extracellular release of HMGB1, which is supported by the 1.7- and 1.2-fold ($P < 0.05$) increases in relative HMGB1 levels in both nuclear and cytoplasmic extracts, respectively, and its reduced expression in the liver homogenate and serum by about 41 and 31%, respectively ($P < 0.05$) when compared to the diabetic group. The EP treatment of control rats did not affect subcellular translocation of HMGB1.

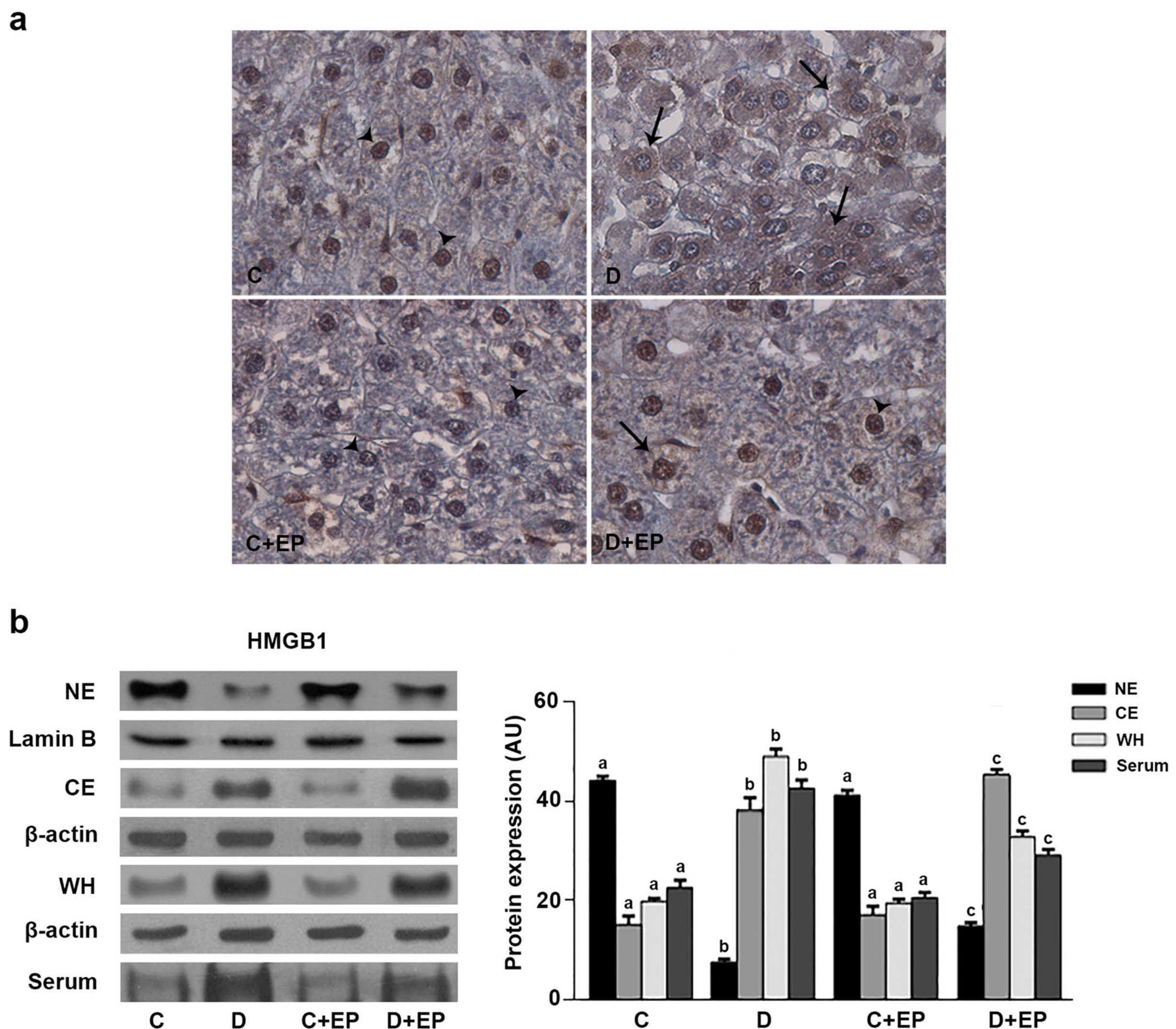

Fig. 4 EP reduces HMGB1 expression and its cytoplasmic translocation in diabetic liver. (**a**) Immunohistochemistry staining showing that in the control (*C*) and control group treated with EP (*C + EP*), HMGB1 was distributed mostly in the cell nucleus (indicated with arrowheads). Cytoplasmic translocation of HMGB1 was observed in the diabetic (*D*) group (indicated with arrows). EP treatment of diabetic rats (*D + EP*) significantly decreased cytoplasmic translocation of HMGB1. ×400. (**b**) HMGB1 protein expression levels in the liver nuclear extract (NE), cytosolic extract (CE), whole homogenate (WH), and serum were assessed by Western immunoblotting . β-actin and lamin B were used as a loading controls. Immunoblots obtained from at least three independent experiments were quantified by densitometric analysis and are presented in the graphs. The values in the graphs are means ± SE, $n = 15$. [a,b,c]Mean values with unlike letters were significantly different ($P < 0.05$)

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Effect of EP on the interaction of HMGB1 with TLR4 in diabetic rat livers

Since extracellular HMGB1 initiates inflammatory pathways through interaction with TLR4, we next performed co-immunoprecipitation analysis. Immunoprecipitation of HMGB1 from whole liver homogenates, followed by Western immunoblot analysis with TLR4 antibody, revealed a 1.7-fold increase (Fig. 5, $P < 0.05$, $n = 9$) in interaction between these two proteins in diabetes, as compared to the control. The interaction of HMGB1 and TLR4 decreased to the control level after the treatment with EP (Fig. 5, $P < 0.05$).

HMGB1/TLR4-mediated activation of MAPK signaling pathways in diabetic liver

MAPKs (p38, JNK, ERK1/2) and NF-κB p65 play pivotal roles in intracellular TLR4 signaling events. NF-κB p65 has been shown to be involved in the regulation of expression of genes that encode inflammatory cytokines TNF-α and IL-6, as well as Hp and the antioxidative enzymes CuZn- and MnSOD [31]. We therefore, investigated whether changes in the level of interaction between HMGB1 and TLR4 influence p38, JNK, ERK, and NF-κB p65 activation in the liver of diabetic rats. Western immunoblot analysis of the cytosol extracts did not reveal any changes in the total expression of these proteins in the liver of diabetic rats, irrespective of whether they were or were not treated with EP (Fig. 6a, $n = 15$). However, phosphorylation activates all three types of MAPKs. Western immunoblot analysis with antibodies against phosphorylated forms of p38, JNK, and ERK revealed that the increased level of interaction between HMGB1 and TLR4 was accompanied by 2- and 1.9-fold increases in activation of p38 and 46 kDa JNK in diabetic rat liver, respectively (Fig. 6a, $P < 0.05$, $n = 15$), as compared to the control. Increased phosphorylation of 42 kDa and 44 kDa ERK isoforms (about 2.4-fold and 1.7-folds, respectively, $P < 0.05$) was also detected. A decrease in the level of interaction between HMGB1 and TLR4 after the treatment with EP led to decreased activation of p38, 46 kDa JNK, and both 42 kDa and 44 kDa isoforms of ERK (1.7-, 1.3-, 1.2-, and 1.3-folds, respectively, $P < 0.05$), as compared to diabetic rats. However, the presence of activated MAPKs remained higher ($P < 0.05$) than in the control group ($P < 0.05$). Western analysis showed an increased nuclear presence of phosphorylated NF-κB p65 (1.6-fold, $P < 0.05$, $n = 15$) in diabetic rat liver. While the treatment with EP reduces its nuclear presence by about 30% ($P < 0.05$), compared to the diabetic group, it remained 24% ($P < 0.05$) above the control level. The EP treatment of control rats did not elicit significant changes in the levels of the examined MAPKs and NF-κB p65.

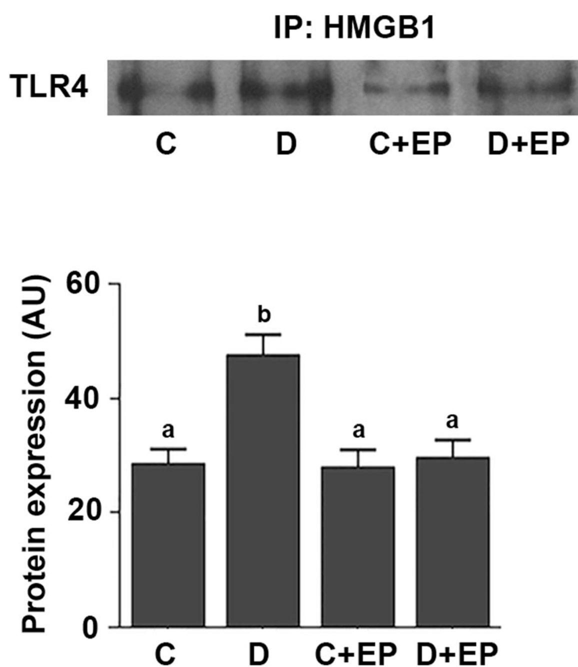

Fig. 5 EP reduces HMGB1 interactions with TLR4 in the liver of diabetic rats. Western immunoblotting of immunoprecipitated HMGB1 (*IP* HMGB1) from whole liver homogenates, with anti-TLR4 antibody. *C* control rats, *D* diabetic rats, *C + EP* control rats treated with EP, *D + EP* diabetic rats treated with EP. Immunoblots were quantified by densitometric analysis and are presented in the graphs. The values in the graphs are means ± SE, $n = 9$. [a,b]Mean values with unlike letters were significantly different ($P < 0.05$)

HMGB1/TLR4-mediated activation of JAK1/STAT3 signaling in diabetic liver

STAT3 is one of the main transcription factors involved in the upregulation of Hp gene transcription [41]. Studies have revealed a correlation between the HMGB1-induced macrophage release of TNF-α and IL-6 and the activation of the JAK/STAT signaling pathway [23] and crosstalk between JAK1/STAT3 signaling and TLR4 [42, 48]. Thus, we next investigated whether changes in the level of interaction between HMGB1 and TLR4 influence the activation of the JAK1/STAT3 signaling pathway in diabetic liver. Western analysis of the cytosol extract from the liver of diabetic rats showed that the increased level of HMGB1 and TLR4 interaction (Fig. 5) was associated with a 3.3-fold increase (Fig. 6b, $P < 0.05$, $n = 15$) in JAK1 activation by phosphorylation. JAK1 activation was accompanied by the increased presence of phosphorylated STAT3 isoforms on serine residues (3.3-fold for STAT3 p91 and 2.0-fold for STAT3 p86, $P < 0.05$, $n = 15$) in the nuclear extracts. The decrease in interaction between HMGB1 and TLR4 in the liver of diabetic rats treated with EP was accompanied by a 20% ($P < 0.05$) decrease in JAK1 phosphorylation and by significant decreases in phosphorylation of both 91 kDa and 86 kDa STAT3 (25 and 32%, respectively, $P < 0.05$, $n = 15$), levels that were 2.4-fold higher

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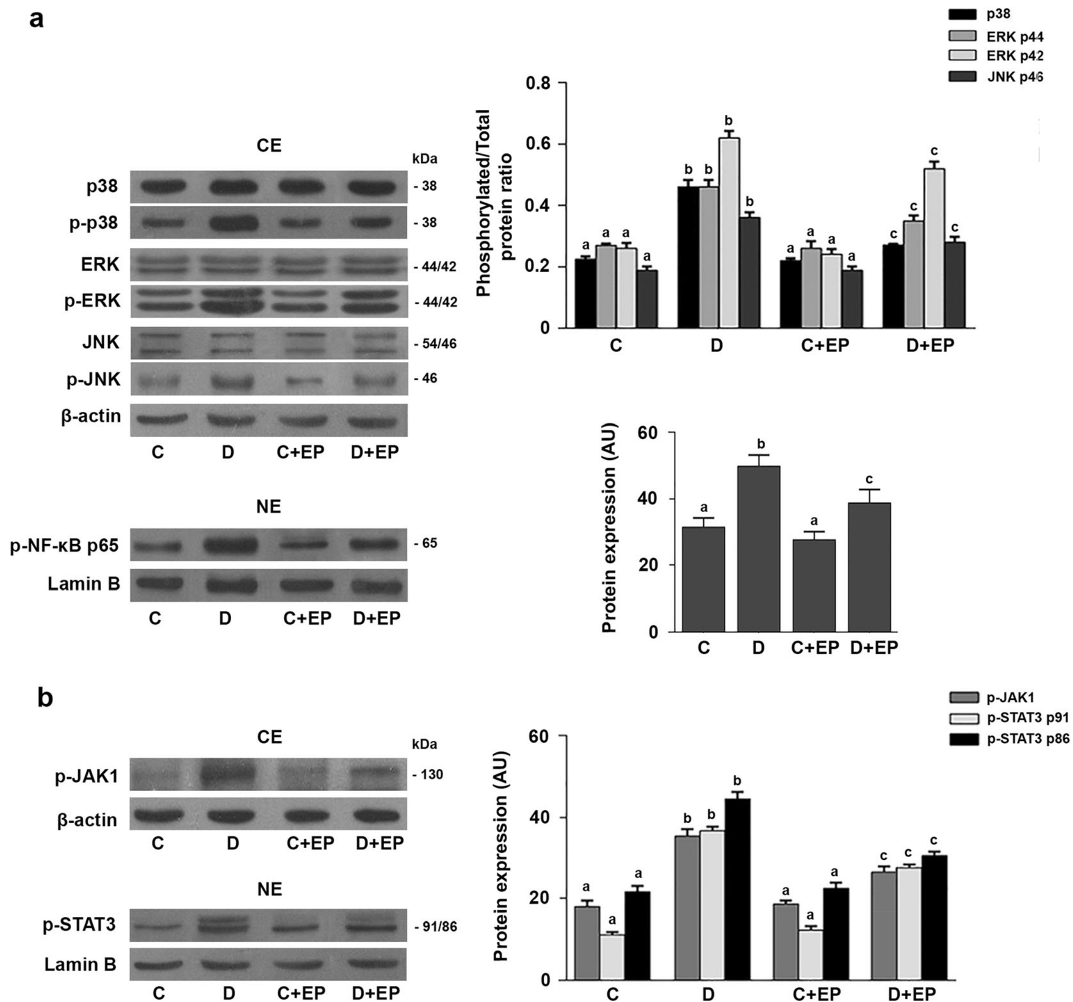

Fig. 6 EP reduces HMGB1/TLR4-induced activation of downstream (**a**) MAPKs/NF-κB p65 and (**b**) JAK1/STAT3 inflammatory pathways in diabetic liver. Protein expression levels of total and phosphorylated MAPKs (p38, ERK, JNK) and phosphorylated JAK1 in the cytosolic extracts (CEs) of rat liver and phosphorylated NF-kB p65 and STAT3 in the nuclear extracts (NE) were assessed by Western immunoblotting. β-actin and lamin B were used as a loading controls. *C* control rats, *D* diabetic rats, *C + EP* control rats treated with EP, *D + EP* diabetic rats treated with EP. Immunoblots obtained from at least three independent experiments were quantified by densitometric analysis (summarized in the graphs). The graph (a, upper) shows the results of quantification of the p-p38/p38, p-ERK/ERK, p-JNK p46/JNK p46 ratios determined at the protein level. The values in the graphs are means ± SE, $n = 15$. [a,b,c]Mean values with unlike letters are significantly different ($P < 0.05$)

for STAT3 p91 and 1.4-fold higher for STAT3 p86 when compared to the control ($P < 0.05$).

HMGB1/TLR4 interactions, Nrf2 signaling, and HO-1expression in diabetic liver

Persistent oxidative stress, which contributes to the progress of inflammatory response in diabetes, is the consequence of downregulation antioxidative enzyme genes and their decreased enzymatic activities. Nrf2 is a transcription factor that regulates the transcription of antioxidant genes, such as CAT and HO-1, and is an established inhibitor of NF-κB p65 activity [24]. Several lines of evidence indicate that induced expression of HO-1 attenuates cellular damage and inflammation [1] and that HO-1 negatively regulates HMGB1 in inflammatory conditions [32]. To examine whether diabetes-related increased expression of HMGB1 downregulates the cytoprotective response through induction of an inflammatory pathway (NF-κB p65), we analyzed the expression of Nrf2 in liver cytosol and nuclear extracts of diabetic rats, the Nrf2 to NF-κB p65 ratio, and HO-1 expression in the cytosol. Using Western blot analysis (Fig. 7), we observed that the level of Nrf2 in the cytosol fraction prepared from diabetic rats was increased 1.25-fold ($P < 0.05$, $n = 15$) when compared to the control group, while nuclear expression was decreased by 20% ($P < 0.05$, $n = 15$). Considering that NF-κB p65 negatively regulates Nrf2 signaling, we determined the ratio between Nrf2 and NF-κB p65 (Fig. 7). This ratio was not significantly changed in the cytosol fraction of diabetic liver despite the increase in the relative cytosol level of NF-κB p65 (1.4-fold, $P < 0.05$). In diabetic liver, nuclear expression of total NF-κB p65 increased 1.23-fold ($P < 0.05$, $n = 15$),

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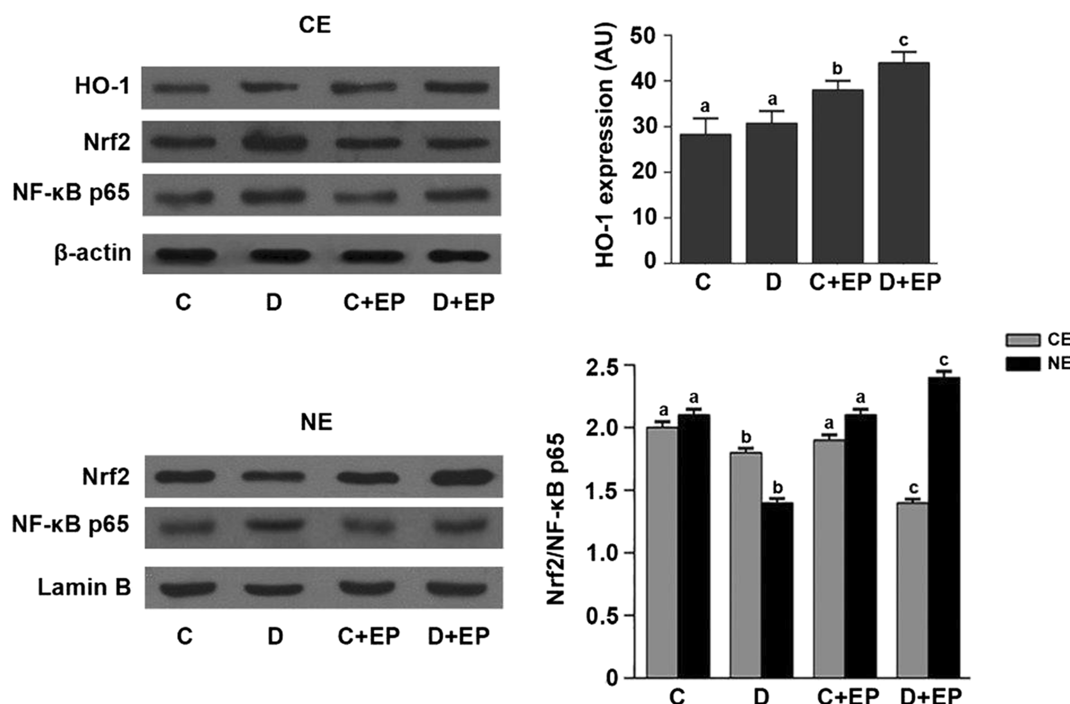

Fig. 7 EP reduces the HMGB1/TLR4-mediated decrease in Nrf2-antioxidant signaling in diabetic liver. Protein expression levels of HO-1 in the cytosolic fraction (CE) and Nrf-2 and NF-κB p65 in the cytosolic and nuclear fraction (NE) were assessed by Western immunoblotting and quantified by densitometric analysis. The upper graph shows the quantification of HO-1 protein levels. The lower graph shows the results of quantification of the Nrf2/NF-κB p65 ratios. *C* control rats, *D* diabetic rats, *C + EP* control rats treated with EP, *D + EP* diabetic rats treated with EP. The values in the graphs are means ± SE, $n = 15$. [a,b,c]Mean values with unlike letters are significantly different ($P < 0.05$)

resulting in a decrease in the Nrf2 to NF-κB p65 ratio by 34% ($P < 0.05$), when compared to the control. The expression of HO-1 in the cytosol from diabetic liver was not significantly different when compared to the control. Treatment of control rats with EP did not elicit significant changes in the cytosol and nuclear Nrf2/NF-κB p65 ratios, while the expression of HO-1 in the cytosol was increased 1.3-fold ($P < 0.05$, $n = 15$). In the liver of diabetic rats treated with EP, the relative level of Nrf2 was decreased by 29% ($P < 0.05$, $n = 15$) in the cytosol, when compared to the diabetic group, while its nuclear expression was increased by 50% ($P < 0.05$, $n = 15$) when compared to the diabetic group and 20% ($P < 0.05$), relative to the control. Accordingly, the Nrf2/NF-κB p65 ratio was decreased by 23% ($P < 0.05$) in the cytosol and increased 1.7-fold ($P < 0.05$) in the nuclear fraction, when compared to the diabetic group. The expression of HO-1 in the cytosol from the liver of diabetic rats treated with EP was increased 1.4-fold ($P < 0.05$, $n = 15$), when compared to the diabetic group.

Discussion

Adjustment of the inflammatory processes in diabetes is a matter of great interest in basic and clinical research, given that inflammatory pathways assume pivotal roles in the development and progression of many diabetic complications. The presented results highlight the contribution of HMGB1 protein in the development/progression of liver injury in experimentally induced diabetes because of its pro-inflammatory activities. In diabetic liver, HMGB1 is secreted from necrotic or damaged cells and immunologically activated innate immune cells [35]. Recently, we showed that diabetes upregulates HMGB1 expression [16] and that the increased expression of HMGB1 and the prevailing HMGB1/TLR4 signaling promote liver cell death, contributing to liver damage [33]. The results presented in this study further show that HMGB1 significantly furthered the inflammatory state in diabetic liver as a result of the HMGB1/TLR4-mediated increase in production of inflammatory markers TNFα, IL-6, and Hp. Increased interaction between HMGB1 and TLR4 leads to the activation of MAPK and JAK1 signaling cascades and subsequent to activation and nuclear translocation of NF-κB p65 and STAT3, the key transcriptional activators of the genes for TNF-α, IL-6, and Hp. TNF-α and IL-6 which are secreted after HMGB1 stimulation by promoting further HMGB1 release from liver cells amplify the inflammatory response in a positive feedback loop [28]. Additionally, we observed that activation of the HMGB1/TLR4 axis leads to decreased nuclear expression of Nrf2 and consequently Nrf2-related expression of antioxidative enzymes, such as CAT, thereby augmenting the oxidative stress. The ROS, by inducing the release of HMGB1, can also amplify inflammatory

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responses and liver damage. Although expression of HO-1 is predominantly Nrf2-regulated, it can be augmented in different cells and conditions by other mechanisms of transcriptional activation. In accordance with Liu et al. [25] who showed that HO-1 transcription can be positively regulated by activated STAT3, we propose that sustained HO-1 expression in diabetic liver was the consequence of STAT3 activity. Evankovich et al. [13] reported that the release of HMGB1 can be induced by TLR4 itself, since in ischemic liver tissue, TLR4-dependent ROS production was shown to be responsible for the release of HMGB1 into the extracellular milieu. The reduction in HMGB1 expression in the serum and liver of diabetic rats after chronic administration of EP reduced the cytokine activity of HMGB1 and had a protective effect on diabetic liver which was observed as improvements in liver histology and the antioxidant and inflammatory status. These results suggest that the extent of liver damage in diabetes depends on the expression level of HMGB1 and its pro-inflammatory activities, indicating that inhibition of HMGB1 secretion could be considered as a prime target for alleviating liver damage in diabetes.

Accumulating evidences indicate that EP is a multifunctional protective agent capable to mitigating tissue damage caused by various stressors [21]. The mechanisms underlying its anti-inflammatory and antioxidative effects are different and include suppression of the HMGB1 release [37], suppression of pro-inflammatory cytokine production via inhibition of ROS-dependent STAT signaling [18] or DNA-binding of NF-κB p65 [17], and induction of Nrf2 translocation from the cytosol to the nucleus and subsequent upregulation of HO-1 [19]. The results from this study show that all of the proposed mechanisms were associated with the observed protective effects of EP on diabetic rat liver. They also suggest that prevention of HMGB1 release is the primary event through which EP decreases STAT3 and NF-κB p65 activity and increases nuclear expression of Nrf2 and liver expression of HO-1.

The inflammatory process is complex since it is regulated by the intricate interplay between cytokines and factors with pro- and anti-inflammatory effects. HMGB1, as a cytokine-like protein, is capable of inducing the production of multiple pro-inflammatory mediators by a variety of cells and, simultaneously, it probably induces its own release from activated monocytes and macrophages. This implicates HMGB1 as the central player in pro-inflammatory reactions. However, some of the pro-inflammatory mediators produced in response to HMGB1, such as IL-6, produce anti-inflammatory effects thus revealing the dual role of HMGB1 as a pro- and anti-inflammatory molecule. Hp is a major AP protein principally produced in liver by hepatocytes, whose concentration in the circulation increases after establishment of the inflammatory reaction, and is generally viewed as an anti-inflammatory component [5]. Hp gene is primarily induced by IL-6 type cytokines whose receptors mediate the activation of transcription factor STAT3 and CCAAT/enhancer binding protein β [5, 41]. In the present study, we show that the increased expression of HMGB1 in diabetic liver was accompanied by increased production of Hp. Decreased HMGB1 release caused by EP administration attenuated Hp expression. The observed change in Hp expression correlated with the changes in the level of HMGB1/TLR4 interactions, the activation of downstream MAPK and JAK kinases, and with the activation of NF-κB p65 and STAT3 transcription factors. Recently, it was reported that secreted Hp, or its β subunit, acts as an active HMGB1 antagonist capable of binding and inhibiting the pro-inflammatory properties of HMGB1 that are mediated through the TLR4 signaling pathway [46]. Hp can bind to the fully reduced and disulfide form of HMGB1 which is generated in oxidative stress and is involved in cytokine production. This suggests that HMGB1, by stimulating Hp synthesis and secretion in diabetes, could form a negative feedback loop and thereby lower its own pro-inflammatory activities. However, the results from our study suggest that inhibition of HMGB1 activities with Hp was probably ineffective in diabetes. A possible explanation could be that glycation of Hp, which is markedly accelerated in diabetes, impaired protein binding and functions. Spiller et al. [38] reported that Hp in the bloodstream of diabetic patients underwent glycation on lysine-141. Also, Asleh et al. [3] established that in diabetic patients, glycated hemoglobin abrogated the Hp protection function against hemoglobin-driven oxidative injury.

Activated STAT3 also possesses pro-inflammatory and anti-inflammatory activities and is primarily activated by the IL-6-type receptor-stimulated JAK/STAT pathway. Phosphorylation of both JAK1 and JAK 2 is necessary for STAT3 phosphorylation on tyrosine residues, its dimerization, and translocation to the nucleus [42]. Activation of STAT3 from TLR4 has also been described [47]. Activated TLR4 could trigger the activation of ERK, JNK, and p38 MAPKs that phosphorylate STAT3 on serine 727 residues, which is required for maximum transactivation capacity of STAT3 [7]. In the present study, we observed that the HMGB1-mediated activation of TLR4 in diabetic liver was accompanied by increased activation of JAK 1 and STAT3 phosphorylation on serine residues. The activation of STAT3 by phosphorylation is essential for a normal inflammatory response; however, prolonged activation of STAT3 could contribute to chronic inflammation by supporting the sustained expression of a subset of proteins with roles in inflammation, including the STAT3 gene itself [42]. Also, STAT3 can stimulate the expression of a number of effector genes which in turn suppress the expression of pro-inflammatory genes. These include Bcl3, which impairs NF-κB binding to DNA and suppress TNF-α production [20], as well as the suppressors of cytokine signaling 3 (SOCS3) protein, which blocks the IL-6-mediated pro-inflammatory response by binding to the IL-6

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receptor [47]. According to Wang [42], prolonged activation of STAT3 requires repeated phosphorylation which is mediated by new synthesis and secretion of IL-6. Our results suggest that HMGB1 contributed to this process through TLR4 signaling. We observed that increased HMGB1 expression and its interactions with TLR4 were accompanied by increased phosphorylation of JAK1 and increased nuclear expression of phosphorylated STAT3 isoforms. The EP-associated decrease in HMGB1 expression in diabetic liver accompanied the decreases in JAK1 phosphorylation and STAT3 activation. According to Kim et al. [18], the mechanism of inhibition of STAT3 by EP is the result of JAK2 inhibition. This implies that EP, which decreased HMGB1 secretion and HMGB1/TLR4 interactions, reduced JAK1 activation and consequently STAT3 activation and the expression of STAT3-responsive inflammatory genes (e.g., IL-6, TNF-α, Hp).

All TLR signaling pathways culminate in the activation of MAPKs and downstream NF-κB transcription factors. Also, TNF-α signals via distinct cell surface receptors that activate all three types of MAPKs and NF-κB p65 [34]. In the present study, we observed that increased HMGB1/TLR4 interactions in diabetic liver were accompanied by increased activation of NF-κB p65 and its accumulation in the nucleus where it controls the expression of an array of inflammatory cytokine genes (IL-6, TNF-α) whose expression is involved in the AP reaction. This includes increased Hp production, as well as the response to oxidative stress and apoptosis induction [26]. Gene knockout studies suggest that NF-κB p50/p65 heterodimers possess anti-inflammatory roles due to direct inhibition of the expression of some pro-inflammatory genes and the effect on the expression or activity of anti-inflammatory cytokines [15]. We also show that the treatment of diabetic rats with EP significantly decreased the activation of MAPKs, the nuclear accumulation of activated NF-κB p65, and induced a decline in Hp expression in the liver although it remained significantly higher than in control rats. A possible explanation for this result is that EP induced the translocation of Nrf2 transcription factor to the nucleus where it transactivated the genes involved in oxidative stress and the inflammatory responses. According to Thimmulappa et al. [39], Hp gene could be one of the genes regulated by Nrf2; however, in what way Nrf2 contributes to Hp expression is still not fully understood. Zhao et al. [49] demonstrated that after intracerebral hemorrhage, the levels of Hp in blood plasma were strongly increased in animals treated with the activator of Nrf2, sulforaphane.

It is now clear that the ratio of nuclear Nrf2 and NF-κB p65 is an essential element of cellular reactions that determine the inflammatory response and oxidative status of the cell and tissue. Nrf2 and NF-κB pathways have been proposed to inhibit each other at the transcription level via protein-protein interactions or through secondary messenger effects [43]. By inhibiting NF-κB p65-mediated cytokine production, Nrf2 acts as an anti-inflammatory component. In diabetic liver, we observed a significant decrease in the nuclear presence of Nrf2 and increased production of IL-6 and TNF-α, which suggests that excessive HMGB1 release potentiates a decreasing Nrf2/NF-kB p65 ratio, thus attenuating the anti-inflammatory potential in diabetic liver. Also, this decrease correlates with attenuation of the antioxidative potential, as judged by the observed decrease in the activity and expression of CAT whose gene transcription is primarily regulated by Nrf2 [24]. We also assume that the decrease in the Nrf2/NF-κB p65 ratio probably lowered to some extent the efficacy of HO-1 gene expression, especially because increases in this ratio in the liver of EP-treated diabetic rats followed increased expression of HO-1 and CAT. Despite observed decrease in total SOD activity, the results presented herein show that relative expression level of CuZnSOD was unchanged in diabetic liver, while the level of MnSOD was increased. The rise could be interpreted as the result of increased NF-kB p65 expression. Induction of the MnSOD gene transcription in response to oxidative stress, TNF-α, and IL-6 has been well established, and studies have identified NF-κB p65 as the most crucial transcriptional factor regulating MnSOD induction [29]. CuZnSOD gene is often constitutively expressed and not as easily inducible as other SOD. In accordance with this, increased Nrf2/NF-kB p65 ratio in the liver of EP-treated diabetic rats follows decreased expression of MnSOD. The disagreement between the detected levels of SOD enzymes and activity of total SOD could be the consequence of post-translational modification(s) of CuZn- and MnSOD. Namely, according to Dinić et al. [9], both SOD enzymes, as well as CAT, are subjected to O-linked β-N-acetylglucosamine (O-GlcNAc) modification in the liver of STZ-diabetic rats. By modulating the local protein structure, O-GlcNAcylation of these enzymes could influence the effects of phosphorylation, which has been detected on SOD proteins and/or it could partially or completely block their catalytic domains and activity.

The presented data highlight the intricacy of HMGB1 action as an inflammatory mediator during diabetes and its complicated influence on the extent of liver damage. The expression level of exogenous HMGB1 and the functional interaction between HMGB1/TLR4, IL-6, and TNF-α signaling pathways probably determine the physiological outcome of HMGB1-generated responses. Despite the described anti-inflammatory roles of HMGB1 and participants in HMGB1/TLR4 signaling, inflammation persists in diabetic liver. In the state with a high presence of exogenous HMGB1, anti-inflammatory actions are overwhelmed by pro-inflammatory actions that ultimately prevail as the consequence of continual HMGB1/TLR4 signaling. Despite this, the prevention of HMGB1 release or blockade of the HMGB/TLR4 axis should be effective in ameliorating diabetes-induced inflammation and slowing down the advance in liver injury.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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