2023

Modulation of TLR4/NF- Modulation of TLR4/NF-κB, Nrf2/HO-1 and PI3K/Akt signaling by cilostazol mitigates lipopolysaccharide-induced septic acute kidney injury

Ahmed F. Mohamed
Faculty of Pharmacy Cairo University, ahmed.fathi@pharma.cu.edu.eg

Helmy M. Sayed
Faculty of Pharmacy Cairo University, helmy.said@pharma.cu.edu.eg

Hala F. Zaki
Faculty of Pharmacy Cairo University, hala.fahmy@pharma.cu.edu.eg

Marwa Safar
marwa.safar@bue.edu.eg

Modulation of TLR4/NF-κB, Nrf2/HO-1 and PI3K/Akt signaling by cilostazol mitigates lipopolysaccharide-induced septic acute kidney injury

Ahmed F. Mohamed

Helmy M. Sayed

Hala F. Zaki

See next page for additional authors

Modulation of TLR4/NF-κB, Nrf2/HO-1 and PI3K/Akt signaling by cilostazol mitigates lipopolysaccharide-induced septic acute kidney injury

Authors

Ahmed F. Mohamed, Helmy M. Sayed, Hala F. Zaki, and Marwa M. Safar

ORIGINAL ARTICLE-PHARMACOLOGY AND CLINICAL PHARMACY

Modulation of Toll-Like Receptor 4/Nuclear Factor-Kappa B, Nuclear Factor Erythroid 2-Related Factor 2/Hemeoxygenase-1, and Phosphoinositide 3-Kinase/Akt Signaling by Cilostazol Mitigates Lipopolysaccharide-Induced Septic Acute Kidney Injury

Ahmed F. Mohamed, PhD [a,*], Helmy M. Sayed [a], Hala F. Zaki [a], Marwa M. Safar [a,b]

[a] Department of Pharmacology and Toxicology, Faculty of Pharmacy, Cairo University, Egypt
[b] Department of Pharmacology and Biochemistry, Faculty of Pharmacy, The British University in Egypt, Cairo, Egypt

Abstract

Aim: **Cilostazol was investigated as a protective agent against lipopolysaccharide (LPS)-associated acute kidney damage in mice.**

Methods: **Cilostazol (50 mg/kg/day; p.o.) administered for 7 consecutive days before a single LPS dose (2 mg/kg; i.p.).**

Results: **Cilostazol hampered serum creatinine, cystatin C, and renal kidney injury molecule-1 and neutrophil gelatinase-associated lipocalin; repressed toll-like receptor 4 and MyD88 transcription, as well as nuclear factor-kappa B p65, interleukin-1β, and malondialdehyde content; and boosted Nrf2 mRNA expression, hemeoxygenase-1 activity, and reduced glutathione content. This was synchronous with an upregulation of p-phosphoinositide 3-kinase and p-Akt expressions.**

Conclusion: **Collectively, cilostazol prevented LPS renal injury, which might correspond to modulation of toll-like receptor 4/nuclear factor-kappa B, nuclear factor erythroid 2-related factor 2/hemeoxygenase-1, and phosphoinositide 3-kinase/Akt pathways.**

Keywords: Cystatin C, Kidney injury molecule-1, Neutrophil gelatinase-associated lipocalin

1. Introduction

Sepsis is a grave clinical problem that is linked to multiple organ dysfunction, including acute kidney injury (AKI), which contributes to overall mortality of nearly 26% [1]. Lipopolysaccharide (LPS), a constituent of outer membrane of gram-negative bacteria, is commonly used for septic AKI induction in experimental models [2,3]. Indeed, a wide array of events underlie LPS-associated renal dysfunction, including glomerular ischemia, peritubular microcirculatory dysfunction, local and systemic inflammatory reactions, as well as oxidative tubular damage [4,5].

The noxious renal effects of LPS are probably mediated by toll-like receptor 4 (TLR4) pathway [6], which eventually upregulates pro-inflammatory cytokine synthesis, such as interleukin-1 (IL-1) [7]. These cytokines mediate renal tubular injury via enhanced inflammatory cell accumulation and reactive oxygen species (ROS) upleveling [8].

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a pleiotropic transcriptional regulator that counteracts oxidative tissue insults [9]. Under oxidative stress conditions, Nrf2 nuclear translocation promotes the production of antioxidant enzymes [10].

A number of studies have delineated phosphoinositide 3-kinase (PI3K)/Akt pathway as a central

Received 14 September 2022; accepted 18 December 2022.
Available online 15 February 2023

* Corresponding author at: Faculty of Pharmacy, Kasr El-Aini Street, Cairo, 11562, Egypt. Tel: +201220069121; Fax: +20 223 628 246.
E-mail address: ahmed.fathi@pharma.cu.edu.eg (A.F. Mohamed).

https://doi.org/10.54634/2090-9101.1034

moderator of various intracellular signals secondary to inflammatory and apoptotic cues [11,12]. Additionally, several reports highlighted a potential cross-talk between TLR4 and PI3K/Akt signaling [13,14].

Cilostazol, an antiplatelet drug commonly used for the treatment of peripheral arterial disease-associated claudication [15], was shown to alleviate inflammatory and oxidative manifestations in various experimental settings, possibly via suppression of vascular adhesion molecules and nuclear factor-kappa B (NF-κB) translocation to the nucleus [16,17]. Furthermore, cilostazol demonstrated profound nephroprotective effects against renal I/R, diabetic nephropathy, and cyclosporine-induced nephrotoxicity [18–20]. Nevertheless, its potential value against septic kidney damage is yet to be determined.

Therefore, the current study aimed to examine the nephroprotective potential of cilostazol against endotoxemic AKI. Additionally, modulation of TLR4/MyD88/NF-κB, Nrf2/hemeoxygenase-1 (HO-1), and PI3K/Akt pathways was targeted to explore some of the molecular machinery underlying cilostazol's potential anti-inflammatory and redox-modulating properties in this model.

2. Materials and methods

2.1. Animals

Male Swiss mice (25–30 g) were used in this work with an acclimatization period of 1 week before testing. Housing was done at a temperature of 24–27 °C, humidity of 60 ± 10%, and 12 : 12-h light/dark cycles. Standard pellet chew and water were freely provided. The study procedures adhered to the ARRIVE guidelines as well as the US National Institutes of Health's Guide for Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised Applied Biosystems 1996) and in conformance with the Ethics Committee of Faculty of Pharmacy, Cairo University (PT 1510).

2.2. Chemicals

LPS *Escherichia coli* serotype 0111:B4 (Sigma-Aldrich, St. Louis, Missouri, USA) and cilostazol (Otsuka Pharmaceutical Co., Tokushima, Japan) were used in this investigation. Other chemicals used were of analytical quality.

2.3. Experimental design

Mice were randomly distributed into three groups of 19 mice each. Groups I and II received the vehicle (1% Tween 80, p.o.). Cilostazol (50 mg/kg; p.o.) in 1% Tween 80 was given for 7 consecutive days to group III [18]. LPS (2 mg/kg; i.p.) was freshly prepared in endotoxin-free 0.9% saline and was administered as a single dose to mice of groups II and III [21] 1 h following the final dose of the vehicle or cilostazol, respectively. Fig. 1 depicts a diagrammatic representation of the experimental design.

2.4. Preparation of samples

At 24 h after the LPS challenge, mice were killed by decapitation under thiopental (20 mg/kg; i.p.) anesthesia, and blood and kidney specimens were obtained for biochemical and serological estimations. Kidneys were washed in PBS, and each group was further divided into three subsets. In the first set ($n = 8$), kidneys were stored at −80 °C, where one kidney was employed for real-time PCR or Western blot analyses, whereas the other kidney was used for mitochondrial isolation and heme oxygenase-1 assay. The second set ($n = 8$) was used to prepare a 10% (w/v) homogenate in PBS, which was used for colorimetric reduced glutathione (GSH) or malondialdehyde (MDA) determination and ELISA assessments. The last subset ($n = 3$) was used for renal histopathological examination.

2.5. Biochemical examination

2.5.1. Serum creatinine and renal redox biomarkers

Serum creatinine was determined colorimetrically using a test reagent kit (QCA, Spain) as per the manufacturer's directions. GSH in kidney was quantified using Ellman's reagent as previously described [22]. Measurement of lipid peroxidation was done based on tissue MDA reaction with thiobarbituric acid according to the method of Uchiyama and Mihara [23].

2.5.2. Mitochondrial isolation and hemeoxygenase-1 activity assay

HO-1 activity was measured as elaborated previously [24]. In brief, to obtain the mitochondrial pellet, kidneys were homogenized in Tris-HCl buffer (pH 7.6) with 0.4-mM PMSF and 250-mM sucrose and then centrifuged. The supernatant was incubated in PBS with heme (50 μM), rat liver cytosol (5 mg/ml), G6PDH (1 U), G6P (2 mM), $MgCl_2$ (2 mM), and NADPH (0.8 mM) at 37 °C for an hour. Absorbance was determined at 463 and 520 nm for chloroform extract of the generated bilirubin. Results were represented as pmol bilirubin/mg protein.

2.5.3. Quantitative RT-PCR analysis

Renal total RNA was extracted using RNeasy Kit (Qiagen, Germantown, MD, USA). Superscript Choice system (Life Technologies, Cleveland, OH, USA) was used for RNA/cDNA reverse transcription.

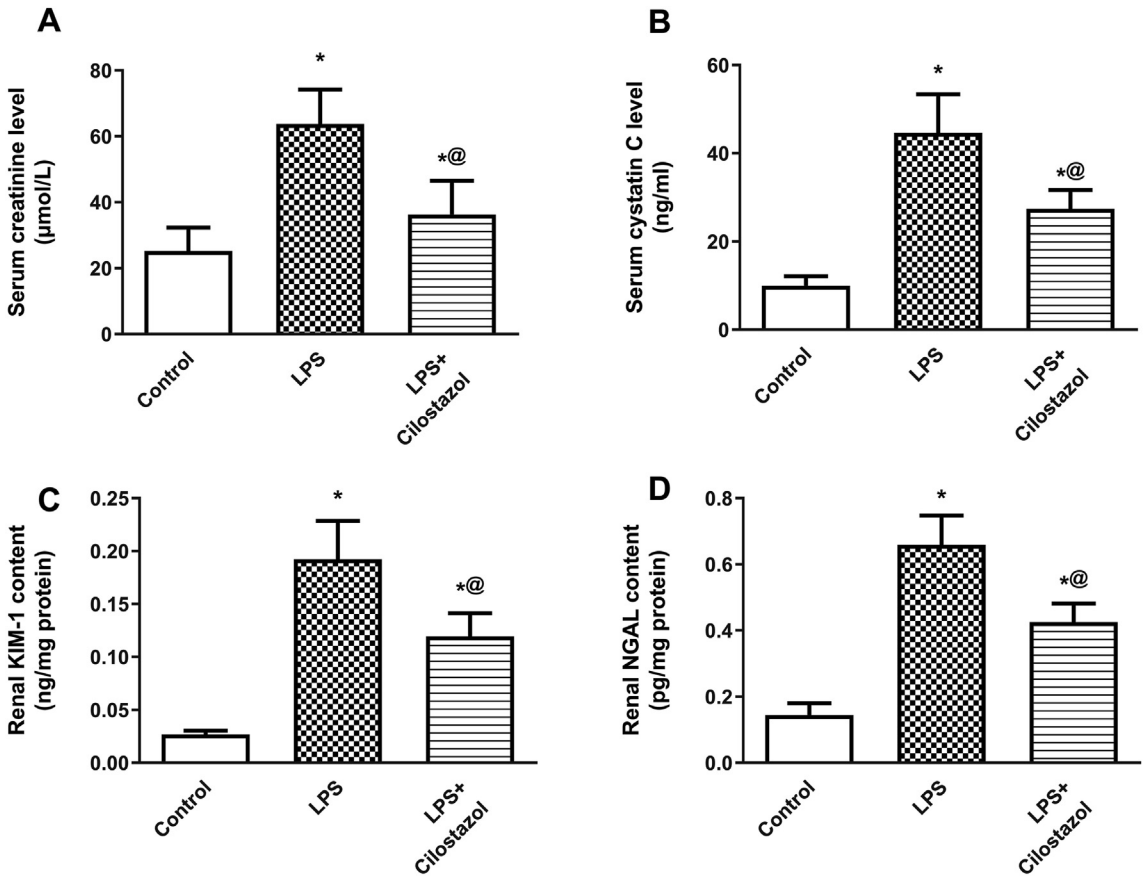

Fig. 1. Diagrammatic representation of the experimental study.

SYBR Green PCR Master Mix (Applied Biosystems, Cleveland, OH, USA) was used for quantitative RT-PCR. In brief, 5 µl of cDNA, 12.5 µl of 2 × SYBR Green Master Mix, and 200 ng of TLR4, MyD88, and Nrf2 gene primers were mixed. Table 1 describes the used primer sequences. PCR reactions included 40 cycles for 15 s at 95 °C (denaturing), 60 °C for 60 s (annealing), and 72 °C for 60 s (extension). The $2^{-\Delta\Delta CT}$ formula was employed to assess relative gene expression using GAPDH as a housekeeping gene [25].

2.5.4. Enzyme-linked immunosorbent assays

Mouse ELISA kits (Cusabio, Wuhan, China) were used for quantification of renal interleukin-1β (IL-1β) (cat#: CSB-E08054 m), kidney injury molecule-1 (KIM-1) (cat#: CSB-E08809 m), NF-κB p65 (cat#: CSB-E08789 m), and neutrophil gelatinase-associated lipocalin (NGAL) (cat#: CSB-E09410 m) levels. Serum cystatin C was determined with a mouse ELISA kit (cat#: MSCTC0; R&D Systems Inc., Minneapolis, Minnesota, USA) as per the manufacturer's directions. Results were represented as ng/mg protein (NF-κB p65 as well as KIM-1), pg/mg protein (NGAL and IL-1β), and ng/ml (cystatin C).

Table 1. Primer sequences used for RT-PCR analysis.

Gene	Primer sequence
TLR4	F: 5′-AGGCAGCAGGTGGAATTGTATC-3′
	R: 5′-TCGAGGCTTTTCCATCCAATAG-3′
MyD88	F: 5′- GTCCATTGCCAGCGAGCTAA-3′
	R: 5′- GGAGACAGGCTGAGTGCAAA-3′
Nrf2	F: 5′- TCTCCTCGCTGGAAAAAGAA -3′
	R: 5′- AATGTGCTGGCTGTGCTTTA -3′
GAPDH	F: 5′- ACCCCAGCAAGGACACTGAGCAAG-3′
	R: 5′- GGCCCCTCCTGTTATTATGGGGGT-3′

TLR4, toll-like receptor 4.

2.5.5. Western blot analysis of p85/p55 (pY458/199)-phosphoinositide 3-kinase and pS473-Akt

Renal tissue was homogenized with RIPA buffer containing phosphatase inhibitor cocktail. Protein concentration was quantified with a BCA assay kit (Thermo Fisher, Waltham, MA, USA). SDS-PAGE was used to separate equal quantities of protein, which was then transferred to a polyvinylidene fluoride membrane before being blocked with 5% bovine serum albumin. The membrane was incubated at 4 °C overnight with rabbit anti-p85/p55 (*p*Y458/199)-PI3K polyclonal antibody (1 : 1000; Thermo Fisher, Cat# PA5-17387, RRID: AB_10985894) or a rabbit anti-*p*S473-Akt monoclonal antibody (1 :

500; Thermo Fisher, Cat# OMA1-03061, RRID: AB_557533). As a secondary antibody, we employed HRP-conjugated donkey anti-rabbit immunoglobulin (Amersham, Life Science Inc., Marlborough, MA, USA). A scanning laser densitometer was used to perform immunoblot analysis (GS-800 system; Bio-Rad, California, Marlborough, MA, USA). After normalization with β-actin protein expression, the results are expressed in arbitrary units.

2.6. Histopathology

The kidneys were detached, cleaned with ice cold saline, and preserved for 24 h in 10% formalin. Samples were fixed in paraffin, and 4-μm slices were cut and stained with hematoxylin and eosin before being studied under a light microscope (Leica Microsystems GmbH, Wetzlar, Germany).

2.7. Statistical analysis

The data were reported as mean ± SD and compared using a one-way analysis of variance test and Tukey's multiple comparisons test. GraphPad Prism software was used for statistical analysis (version 9). For all tests, the level of significance was set at *P* value less than 0.05.

3. Results

3.1. Effects of cilostazol on kidney dysfunction following lipopolysaccharide challenge

Following LPS treatment, a significant decrease of kidney function was seen, as evidenced by increases in serum creatinine and cystatin C, as shown in Fig. 2, mounting to 253.17 and 446.48%, respectively, in comparison with control mice. Such a rise was largely blunted by pretreatment with cilostazol (56.93 and 61.39%, respectively) when compared with the LPS group. A similar pattern was also noted in renal KIM-1 and NGAL contents, reaching 7.16 = and 4.57-fold in the LPS group, respectively, relative to normal control mice. Cilostazol pretreatment effectively counteracted these effects for both KIM-1 (62.09%) and NGAL (64.46%).

3.2. Effects of cilostazol on toll-like receptor 4 signaling and inflammatory markers following lipopolysaccharide challenge

Fig. 3 depicts a conceivable transcriptional stimulation of TLR4 and its downstream adaptor MyD88 in the LPS group versus the normal control mice (9.06-fold and 12.38-fold, respectively). Such a spike was mitigated by cilostazol pretreatment for both TLR4 (28.83%) and MyD88 (32.35%) mRNA expressions. Likewise, NF-κB p65 and IL-1β, two downstream mediators of TLR4, were increased in the LPS group compared with control mice by 8.83-fold and 4.06-fold, respectively. Pretreatment with cilostazol substantially suppressed the levels of both mediators (47.77 and 61.44%, respectively).

3.3. Effects of cilostazol on redox biomarkers and Nrf2 signaling following lipopolysaccharide challenge

As presented in Fig. 4, a state of oxidative stress was evoked after LPS insult. A notable escalation in renal MDA content (231.90%) coupled with a drastic GSH depletion (52.77%) was observed in the LPS untreated group. Cilostazol pretreatment reversed these outcomes, which was reflected by the reduced renal MDA (65.98%) and the replenishment of GSH (128.26%). Upregulation of Nrf2 mRNA (3.51 fold) and HO-1 activity (135.81%) in LPS mice marked the adaptive renal response to oxidative insult; such effects were further magnified by cilostazol pretreatment, reaching 1.88-fold and 1.33-fold (Nrf2 and HO-1, respectively) as compared with the LPS group.

3.4. Effects of cilostazol on phosphoinositide 3-kinase/Akt signaling following lipopolysaccharide challenge

Fig. 5 shows that LPS administration elicited a rise of *p*-PI3K and *p*-Akt protein expressions (347.70 and 382.24%, respectively), which was distinctly augmented in mice that were administered cilostazol, reaching 2.54 fold (*p*-PI3K) and 2.08 fold (*p*-Akt).

3.5. Effects of cilostazol on renal histopathology following lipopolysaccharide challenge

Histopathological findings presented in Fig. 6 confirm cilostazol-mediated renal protection. Normal structure of the renal glomeruli and tubules was portrayed in the control mice. Conversely, mice challenged with LPS showed focal periglomerular inflammatory cell infiltration together with vacuolar degeneration of tubular lining epithelium. Cilostazol pretreatment afforded a marked amendment of the deleterious renal effects of LPS, which was verified by the normal histological renal appearance in cilostazol-pretreated mice.

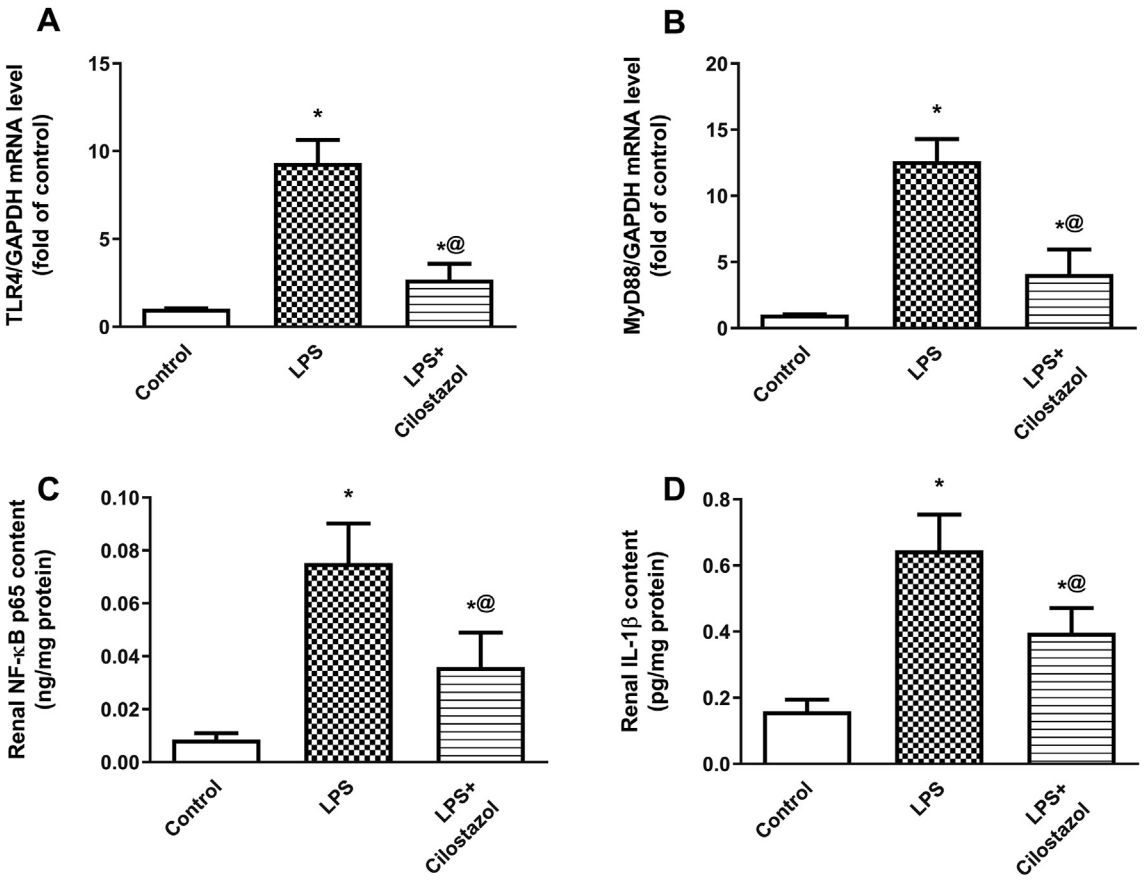

*Fig. 2. Cilostazol's effect on serum creatinine (a) and cystatin C (b) levels, renal KIM-1 (c) and NGAL (d) contents. n = 6–8 (mean ± SD). *versus control, @ versus LPS (one-way analysis of variance, Tukey's multiple comparisons test, P < 0.05). KIM-1, kidney injury molecule-1; LPS, lipopolysaccharide; NGAL, neutrophil gelatinase-associated lipocalin.*

4. Discussion

To the best of the authors' knowledge, the current investigation provides the first direct evidence that cilostazol, an antiplatelet with documented anti-inflammatory properties, exhibits perceivable nephroprotective effects in a murine model of LPS-associated AKI that mimics sepsis-related endotoxemic acute renal damage in humans. This is supported by (a) improved biochemical as well as histopathological renal perturbations instigated by LPS, (b) amendment of acute tubular injury biomarkers KIM-1 and NGAL, (c) declined TLR4 gene expression along with downstream MyD88/NF-κB/IL-1β inflammatory cascade, (d) mitigation of mitochondrial dysfunction and renal oxidative stress via Nrf2/HO-1 signaling, and (e) stimulation of the survival pathway PI3K/Akt.

A marked improvement of biochemical indicators of kidney function, namely creatinine and cystatin C, validated the nephroprotection attained by cilostazol pretreatment. The maintained glomerular and tubular architecture following cilostazol administration reiterated these findings. This is supported by findings of Hafez et al. [26] and Ragab et al. [18] among others [27–29] in rat models of thioacetamide-induced renal toxicity, renal ischemia/reperfusion, Bothrops alternatus envenomation, diclofenac-induced nephrotoxicity, and amikacin-induced nephrotoxicity.

Serum creatinine is considered the primary indicator for kidney function evaluation. However, its use as a sole indicator of kidney dysfunction is limited owing to being influenced by a number of non-renal factors [30]. More recently, other biomarkers have arisen as more reliable tools for kidney function and renal tubular injury evaluation, namely, cystatin C, KIM-1, and NGAL [31–33].

KIM-1, a prominent biomarker of renal tubular damage [34], is a phosphatidylserine receptor that facilitates the uptake of necrotic cell debris, thus restraining epithelial shedding and tubular obstruction [35]. NGAL is another molecule that signifies renal tubular injury [36]. It was initially detected as a component of neutrophil granules. NGAL expression was found to be unequivocally enhanced following renal injury, where it regulates

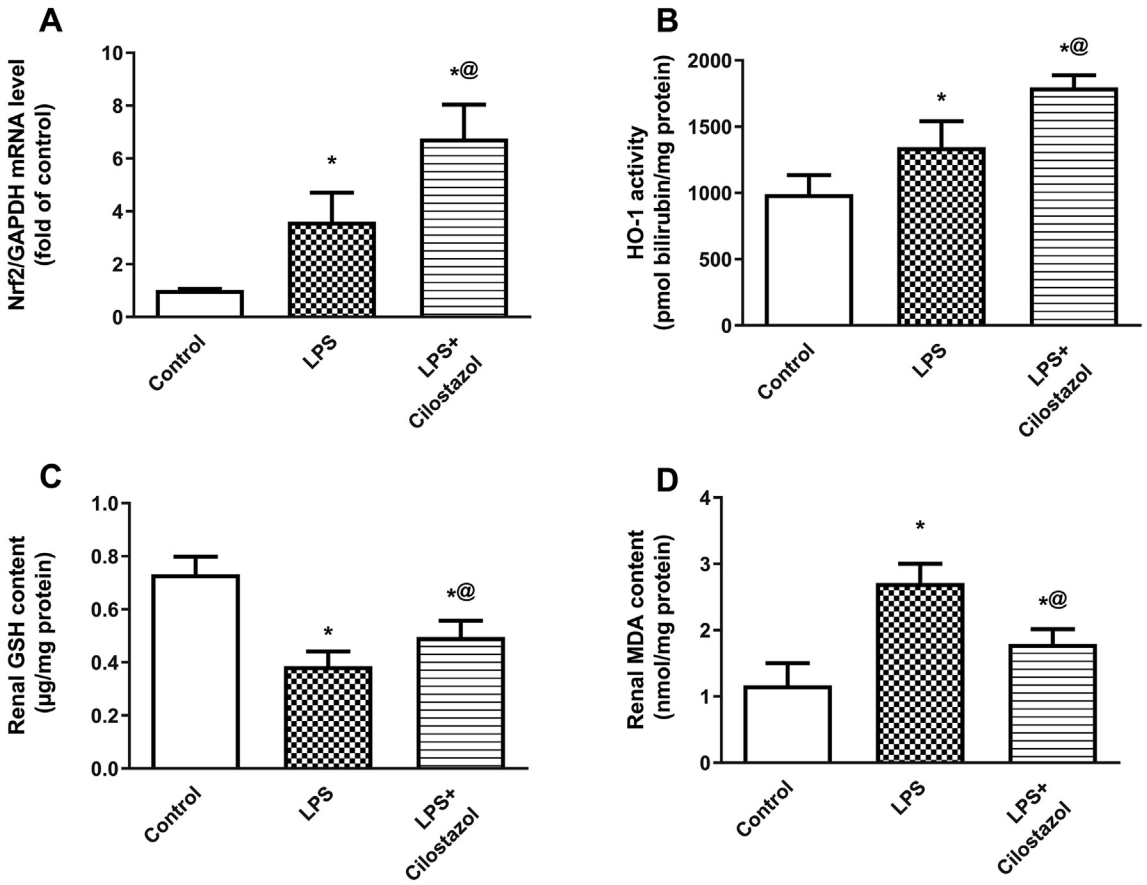

*Fig. 3. Cilostazol's effect on renal TLR4 (a) and MyD88 (b) mRNA expression, NF-κB p65 (c) and IL-1β (d) contents. n = 6−8 (mean ± SD). *versus control, @versus LPS (one-way analysis of variance, Tukey's multiple comparisons test, P < 0.05). LPS, lipopolysaccharide; TLR4, toll-like receptor 4.*

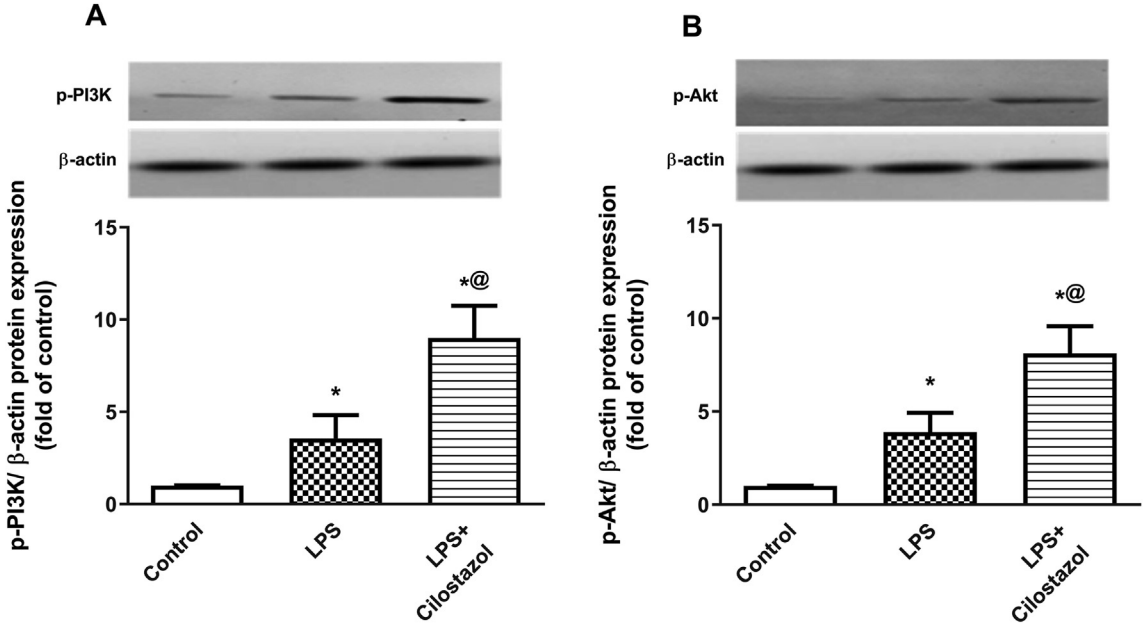

*Fig. 4. Cilostazol's effect on renal Nrf2 mRNA expression (a), HO-1 activity (b), GSH (c) and MDA contents (d). n = 6−8 (mean ± SD). *versus control, @versus LPS (one-way analysis of variance, Tukey's multiple comparisons test, P < 0.05). GSH, reduced glutathione; HO-1, hemeoxygenase-1; LPS, lipopolysaccharide; MDA, malondialdehyde.*

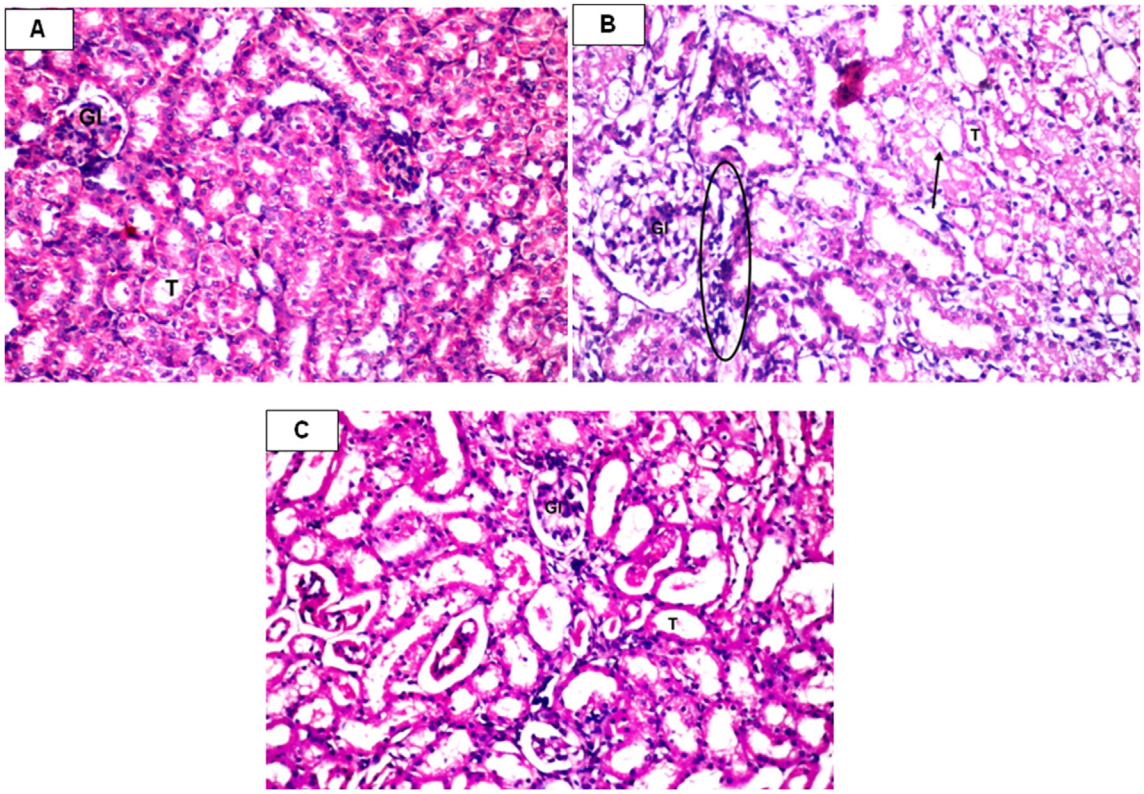

*Fig. 5. Cilostazol's effect on renal p-PI3K (a) and p-Akt (b) protein expression. n = 6–8 (mean ± SD). *versus control, @versus LPS (one-way analysis of variance, Tukey's multiple comparisons test, P < 0.05). LPS, lipopolysaccharide; PI3K, phosphoinositide 3-kinase.*

tubular cell differentiation, apoptosis, and re-epithelialization [33]. Consistent with this evidence, both KIM-1 and NGAL levels were amplified in endotoxemic mice kidneys, which upholds the findings of previous studies [37,38]. Cilostazol affirmed its renal protective endowment by the prohibition of both biomarkers. This is in conformance with Ragab et al. [18], who reported a comparable decline of KIM-1 and NGAL contents following kidney ischemia/reperfusion injury in rats pretreated with cilostazol.

The interplay between the pro-inflammatory cytokine storm elicited in response to the endotoxemic malady and the development of AKI has been stated by many [2,39]. The overproduction of these cytokines is probably instigated by enhanced TLR4 signaling on tissue macrophages and circulating monocytes by LPS [8]. As demonstrated herein, LPS triggers transcriptional activation of TLR4 and its adaptor MyD88, initiating a signaling cascade that eventually enhances the phosphorylation of the inhibitor of kappa B-alpha (IκB-α). NF-κB is normally sequestered in the cytoplasm by IκB. Once IκB is phosphorylated, NF-κB detaches and is transported to nucleus where it orchestrates pro-inflammatory repertoires production such as IL-1β [7], prompting systemic inflammation, redox perturbations, and multiple organ injury [40]. Therefore, inhibition of TLR4 signaling might provide a key strategy to alleviate LPS renal injury. To this end, this study delineated that cilostazol pretreatment conspicuously revoked TLR4 signaling as depicted by the transcriptional downregulation of TLR4 and MyD88, along with diminished renal NF-κB p65 and IL-1β levels. Cilostazol's anti-inflammatory capabilities were largely ascribed to the hindrance of neutrophils' vascular adhesion [16] and NF-κB activation [17]. The present results coincide with those of Park et al. [41] in LPS-stimulated RAW macrophages, where cilostazol reverted NF-κB nuclear transfer as well as DNA binding with a subsequent restriction of NF-κB-dependent cytokine production. In another study, cilostazol downregulated PU.1, a transcription factor linked to augmented TLR4 expression and TLR4/MyD88/NF-κB signaling, in LPS-stimulated synovial macrophages from patients with rheumatoid arthritis [42], thus explaining cilostazol-mediated halting of TLR4 mRNA expression documented herein. In a similar context, olprinone, a selective PDE3 inhibitor, was recently found to hamper inflammatory burst in experimental acute respiratory distress

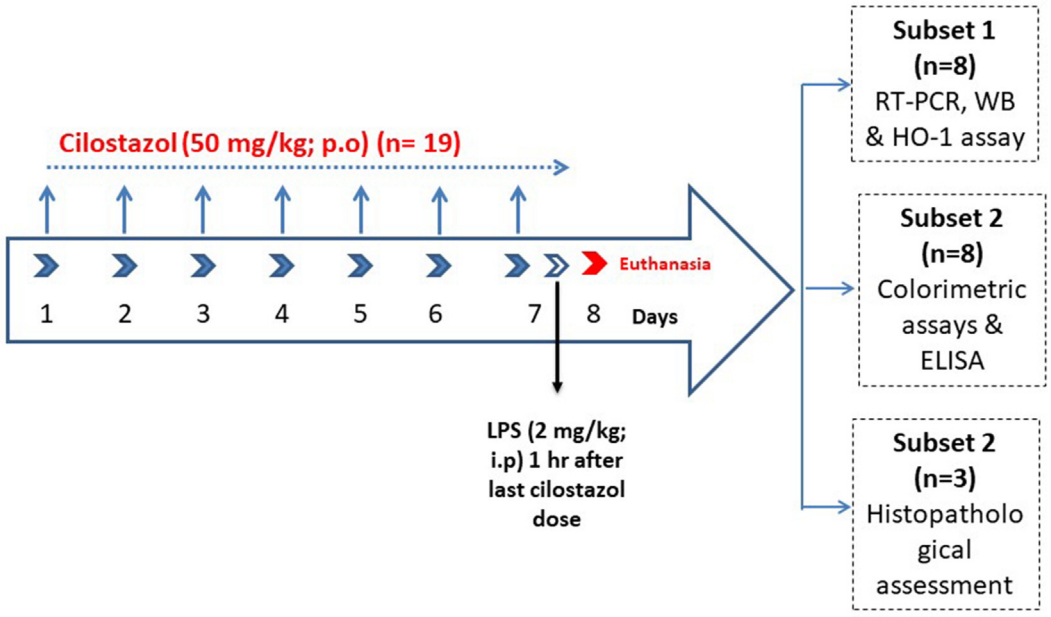

Fig. 6. Cilostazol's effect on renal histopathology following LPS challenge. Characteristic hematoxylin and eosin renal photomicrographs from control mice (a) depicting normal structure of the glomeruli (Gl) and tubules (T), LPS mice (b) showing inflammatory cell infiltration in the periglomerular tissue (circle) together with tubular epithelial degeneration (arrow), and mice pretreated with cilostazol (c) revealing no histopathological alterations of renal structures (hematoxylin and eosin × 40). LPS, lipopolysaccharide.

model [43]. PDE inhibition induces the synthesis of anti-inflammatory cytokines via upregulating CREB and, subsequently, activating transcription factor-1 [44].

Increasing evidence exists regarding the importance of ROS and cellular oxidative status upheaval in LPS-mediated nephrotoxicity [45]. Besides the damage inflicted to cellular macromolecules, ROS can stimulate redox-sensitive transcription factors like NF-κB, leading to magnified inflammatory response as well as tissue damage [46]. One of the compensatory mechanisms to guard against oxidative injury is the up-leveling of endogenous antioxidant enzymes [10], a fact that fits in with our findings where LPS injury caused a subtle, yet significant enhancement of Nrf2/HO-1 signaling, which denotes the cellular adaptation to the overwhelming ROS production secondary to LPS insult. Previously, Yin and Cao [47] reported that LPS enhanced Nrf2 activation by virtue of inhibiting keap 1, a major Nrf2 sequestrant, via promoting its incorporation in autophagosomes. The upregulation of Nrf2 and the magnified downstream HO-1 activity were even more vivid in cilostazol-pretreated mice, thus establishing this pathway as a cardinal weapon in cilostazol's antioxidant effects. Zuo et al. [48] affiliated this outcome to induction of cAMP-dependent PKA/CREB pathway by cilostazol, which leads to boosted expression of PGC-1α along with its downstream targets like Nrf2. Additionally, the arrested NF-κB levels, which negatively regulate the Nrf2 pathway via promoted binding of the co-repressor histone deacetylase 3 (HDAC3) to ARE [49], by cilostazol provides another validation for the reinforced downstream Nrf2 signaling interceded by the drug. Increased HO-1 activity, regulated by Nrf2, impedes cellular oxidative insults, which may be attributed to the breakdown of heme by HO-1 into biliverdin [50] and subsequently bilirubin, a powerful inhibitor of lipid peroxidation [51]. Furthermore, the anti-inflammatory effects of carbon monoxide can be anticipated as a contributor to the overall repression of the inflammatory process by cilostazol [52].

Cilostazol extended its antioxidant effect to entail the perturbed redox status where it partly preserved renal GSH, a crucial endogenous antioxidant that acts as a superoxide scavenger and a suppressor of hydrogen peroxide production [53]. Meanwhile, it counteracted LPS-triggered surplus levels of lipid peroxides, possibly reflecting the free radical quenching properties of cilostazol. Such effects are in close agreement with earlier observations on cilostazol in renal damage induced by ischemia/reperfusion [18] and diabetic nephropathy [17]. Cilostazol's ability to modulate tissue thiols and MDA might be related to the induction of Nrf2 pathway where ɣ-glutamylcysteine ligase enzyme, the rate-limiting step in GSH production, is positively affected by Nrf2 [54]. Moreover, aldo-keto

reductase 1C, a key player in neutralization of toxic aldehydes [55], is another downstream target of Nrf2 that might clarify the abrogated production of renal MDA following cilostazol administration [56]. As the build-up of ROS contributes to the sustained inflammatory response and the undermined renal integrity by LPS, their suppression by cilostazol identifies another mechanism for the nephroprotective effects of the drug.

A number of studies pinned down PI3K/Akt signaling as an influential regulator of pro-inflammatory responses [12,57]. In particular, a negative effect on TLR4 signaling and TLR-mediated NF-κB induction is supported by various reports [14,58]. We, among others [13,59], have elucidated increased *p*-PI3K and *p*-Akt protein levels in LPS mice, a finding that designates one of protective cellular machinery against endotoxemic tissue injury. The expression of both proteins in cilostazol-treated mice exceeded that of the LPS group, suggesting that the rescinded TLR downstream NF-κB level by cilostazol might arise from its capacity to incite PI3K/Akt signaling. Contrariwise, evidence exists that PI3K/Akt signaling might enhance NF-κB activation, which is potentially accredited to direct phosphorylation of IκB-α by Akt [60]. In context, Laird et al. [13] suggested that transformation of membrane phosphatidylinositol (4,5)-bisphosphate (PIP2) to phosphatidylinositol (3,4,5)-trisphosphate (PIP3) by PI3K/Akt stimulation impedes the former's binding to Toll-IL-1 resistance domain-containing adapter protein (TIRAP), which is a decisive factor for MyD88 recruitment and subsequent TLR4 downstream signaling, a fact that supports the current findings. Although cilostazol-associated PI3K/Akt pathway upregulation was formerly attested in human neuroblastoma cells [61], the current investigation offers the first evidence for a PI3K/Akt-facilitated nephroprotection exerted by cilostazol in the LPS-induced AKI model. Cilostazol's effect on PI3K/Akt signaling was previously attributed to retardation of tumor necrosis factor-α-provoked increased phosphatase and tensin homolog, an endogenous repressor of PI3K/Akt signaling [61]. It is noteworthy that a positive link exists between PI3K/Akt signaling and the Nrf2 pathway [62], representing another prospective mechanism for cilostazol's antioxidant quality.

4.1. Conclusion

In summary, these findings verified that cilostazol averted LPS-induced renal injury as evidenced by amendment of proximal tubular dysfunction, redox disturbance, and pro-inflammatory cytokine release. The underlying mechanisms may include modification of TLR4/MyD88/NF-κB and Nrf2/HO-1 signaling, which might be related to stimulation of PI3K/Akt pathway. Therefore, this study provides a strong impetus for cilostazol to be further evaluated as a valuable therapeutic option for the management of endotoxemic renal complications.

Conflict of interest

None declared.

Acknowledgements

The authors thank Prof. Adel M. Bakeer (Department of Pathology, Faculty of Veterinary Medicine, Cairo University, Egypt) for conducting histopathological examinations.

References

[1] Lemon JB, Bohn Q, Sloan SNB, Stahl G, Johnson K, Goade S, et al. Sepsis and acute kidney failure outcomes investigated in a rural, Midwestern population. J Emerg Crit Care Med 2022;6:13.
[2] Mårtensson J, Bellomo R. Pathophysiology of septic acute kidney injury. Contrib Nephrol 2016;187:36–46.
[3] Meyer-Schwesinger C, Dehde S, von Ruffer C, Gatzemeier S, Klug P, Wenzel UO, et al. Rho kinase inhibition attenuates LPS-induced renal failure in mice in part by attenuation of NF-kappaB p65 signaling. Am J Physiol Ren Physiol 2009;296: F1088–99.
[4] Schrier RW, Wang W. Acute renal failure and sepsis. N Engl J Med 2004;351:159–69.
[5] Wu L, Gokden N, Mayeux PR. Evidence for the role of reactive nitrogen species in polymicrobial sepsis-induced renal peritubular capillary dysfunction and tubular injury. J Am Soc Nephrol 2007;18:1807–15.
[6] Miyake K. Innate immune sensing of pathogens and danger signals by cell surface Toll-like receptors. Semin Immunol 2007;19:3–10.
[7] Akira S, Takeda K. Toll-like receptor signalling. Nat Rev Immunol 2004;4:499–511.
[8] Zarjou A, Agarwal A. Sepsis and acute kidney injury. J Am Soc Nephrol 2011;22:999–1006.
[9] Villeneuve NF, Lau A, Zhang DD. Regulation of the Nrf2-Keap1 antioxidant response by the ubiquitin proteasome system: an insight into cullin-ring ubiquitin ligases. Antioxidants Redox Signal 2010;13:1699–712.
[10] Surh YJ, Kundu JK, Na HK. Nrf2 as a master redox switch in turning on the cellular signaling involved in the induction of cytoprotective genes by some chemopreventive phytochemicals. Planta Med 2008;74:1526–39.
[11] Cao Z, Ren D, Ha T, Liu L, Wang X, Kalbfleisch J, et al. CpG-ODN, the TLR9 agonist, attenuates myocardial ischemia/reperfusion injury: involving activation of PI3K/Akt signaling. Biochim Biophys Acta, Mol Basis Dis 2013;1832:96–104.
[12] Williams DL, Ozment-Skelton T, Li C. Modulation of the phosphoinositide 3-kinase signaling pathway alters host response to sepsis, inflammation, and ischemia/reperfusion injury. Shock 2006;25:432–9.
[13] Laird MHW, Rhee SH, Perkins DJ, Medvedev AE, Piao W, Fenton MJ, et al. TLR4/MyD88/PI3K interactions regulate TLR4 signaling. J Leukoc Biol 2009;85:966–77.
[14] Guha M, Mackman N. The phosphatidylinositol 3-kinase-Akt pathway limits lipopolysaccharide activation of signaling

pathways and expression of inflammatory mediators in human monocytic cells. J Biol Chem 2002;277:32124–32.
[15] Lugnier C. Cyclic nucleotide phosphodiesterase (PDE) superfamily: a new target for the development of specific therapeutic agents. Pharmacol Ther 2006;109:366–98.
[16] Iwama D, Miyamoto K, Miyahara S, Tamura H, Tsujikawa A, Yamashiro K, et al. Neuroprotective effect of cilostazol against retinal ischemic damage via inhibition of leukocyte-endothelial cell interactions. J Thromb Haemostasis 2007;5:818–25.
[17] Lee W-C, Chen H-C, Wang C-Y, Lin P-Y, Ou T-T, Chen C-C, et al. Cilostazol ameliorates nephropathy in type 1 diabetic rats involving improvement in oxidative stress and regulation of TGF-β and NF-κB. Biosci Biotechnol Biochem 2010;74:1355–61.
[18] Ragab D, Abdallah DM, El-Abhar HS. Cilostazol renoprotective effect: modulation of PPAR-γ, NGAL, KIM-1 and IL-18 underlies its novel effect in a model of ischemia-reperfusion. PLoS One 2014;9:e95313.
[19] Gokce M, Yuzbasioglu MFF, Bulbuloglu E, Oksuz H, Yormaz S, Altnoren O, et al. Cilostazol and diltiazem attenuate cyclosporine-induced nephrotoxicity in rats. Transplant Proc 2012;44:1738–42.
[20] Wang F, Li M, Cheng L, Zhang T, Hu J, Cao M, et al. Intervention with cilostazol attenuates renal inflammation in streptozotocin-induced diabetic rats. Life Sci 2008;83:828–35.
[21] Pawlinski R, Pedersen B, Kehrle B, Aird WC, Frank RD, Guha M, et al. Regulation of tissue factor and inflammatory mediators by Egr-1 in a mouse endotoxemia model. Blood 2003;101:3940–7.
[22] Beutler E, Duron O, Kelly BM. Improved method for the determination of blood glutathione. J Lab Clin Med 1963;61:882–8.
[23] Uchiyama M, Mihara M. Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. Anal Biochem 1978;86:271–8.
[24] Abraham NG, Lutton JD, Levere RD. Heme metabolism and erythropoiesis in abnormal iron states: role of delta-aminolevulinic acid synthase and heme oxygenase. Exp Hematol 1985;13:838–43.
[25] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR. Methods 2001;25:402–8.
[26] Hafez HM, Ibrahim MA, Zedan MZ, Hassan M, Hassanein H. Nephroprotective effect of cilostazol and verapamil against thioacetamide-induced toxicity in rats may involve Nrf2/HO-1/NQO-1 signaling pathway. Toxicol Mech Methods 2019;29:146–52.
[27] Saeed ZM, Khattab MI, Khorshid NE, Salem AE. Ellagic acid and cilostazol ameliorate amikacin-induced nephrotoxicity in rats by downregulating oxidative stress, inflammation, and apoptosis. PLoS One 2022;17:e0271591.
[28] Marinho AD, Coelho Jorge AR, Nogueira Junior FA, Alison de Moraes Silveira J, Rocha DG, Negreiros Nunes Alves AP, et al. Effects of cilostazol, a Phosphodiesterase-3 inhibitor, on kidney function and redox imbalance in acute kidney injury caused by Bothrops alternatus venom. Toxicon 2022;220:106922.
[29] Wadie W, Abdel-Razek NS, Salem HA. Phosphodiesterase (1, 3 & 5) inhibitors attenuate diclofenac-induced acute kidney toxicity in rats. Life Sci 2021;277:119506.
[30] Mehta RL, Chertow GM. Acute renal failure definitions and classification: time for change? J Am Soc Nephrol 2003;14:2178–87.
[31] Herget-Rosenthal S, Marggraf G, Hüsing J, Göring F, Pietruck F, Janssen O, et al. Early detection of acute renal failure by serum cystatin C. Kidney Int 2004;66:1115–22.
[32] Miyanishi M, Tada K, Koike M, Uchiyama Y, Kitamura T, Nagata S. Identification of Tim4 as a phosphatidylserine receptor. Nature 2007;450:435–9.
[33] Mori K, Lee HT, Rapoport D, Drexler IR, Foster K, Yang J, et al. Endocytic delivery of lipocalin-siderophore-iron complex rescues the kidney from ischemia-reperfusion injury. J Clin Invest 2005;115:610–21.
[34] Bonventre JV. Kidney injury molecule-1 (KIM-1): a urinary biomarker and much more. Nephrol Dial Transplant 2009;24:3265–8.
[35] Ichimura T, Asseldonk EJPV, Humphreys BD, Gunaratnam L, Duffield JS, Bonventre JV. Kidney injury molecule-1 is a phosphatidylserine receptor that confers a phagocytic phenotype on epithelial cells. J Clin Invest 2008;118:1657–68.
[36] Devarajan P. Neutrophil gelatinase-associated lipocalin (NGAL): a new marker of kidney disease. Scand J Clin Lab Invest 2008;241:89–94.
[37] Nair AR, Masson GS, Ebenezer PJ, Del Piero F, Francis J. Role of TLR4 in lipopolysaccharide-induced acute kidney injury: protection by blueberry. Free Radic Biol Med 2014;71:16–25.
[38] Han M, Li Y, Liu M, Li Y, Cong B. Renal neutrophil gelatinase associated lipocalin expression in lipopolysaccharide-induced acute kidney injury in the rat. BMC Nephrol 2012;13:25.
[39] Okusa MD. The inflammatory cascade in acute ischemic renal failure. Nephron 2002;90:133–8.
[40] Van Der Poll T, Meijers JCM. Systemic inflammatory response syndrome and compensatory anti-inflammatory response syndrome in sepsis. J Innate Immunol 2010;2:379–80.
[41] Park WS, Jung WK, Lee DY, Moon C, Yea SS, Park SG, et al. Cilostazol protects mice against endotoxin shock and attenuates LPS-induced cytokine expression in RAW 264.7 macrophages via MAPK inhibition and NF-kB inactivation: not involved in cAMP mechanisms. Int Immunopharm 2010;10:1077–85.
[42] Park SY, Lee SW, Baek SH, Lee CW, Lee WS. Rhim BY, et al. Suppression of PU.1-linked TLR4 expression by cilostazol with decrease of cytokine production in macrophages from patients with rheumatoid arthritis. Br J Pharmacol 2013;168:1401–11.
[43] Kosutova P, Mikolka P, Balentova S, Adamkov M, Calkovska A, Mokra D. Effects of PDE3 inhibitor olprinone on the respiratory parameters, inflammation, and apoptosis in an experimental model of acute respiratory distress syndrome. Int J Mol Sci 2020;21:3382.
[44] AY W, KM S, LS M. The role of the transcription factor CREB in immune function. J Immunol 2010;185:6413–9.
[45] Sebai H, Ben-Attia M, Sani M, Aouani E, Ghanem-Boughanmi N. Protective effect of resveratrol on acute endotoxemia-induced nephrotoxicity in rat through nitric oxide independent mechanism. Free Radic Res 2008;42:913–20.
[46] Bhattacharyya J, Biswas S, Datta AG. Mode of action of endotoxin: role of free radicals and antioxidants. Curr Med Chem 2004;11:359–68.
[47] Yin S, Cao W. Toll-like receptor signaling induces Nrf2 pathway activation through p62-triggered keap1 degradation. Mol Cell Biol 2015;35:2673–83.
[48] Zuo L, Li Q, Sun B, Xu Z, Ge Z. Cilostazol promotes mitochondrial biogenesis in human umbilical vein endothelial cells through activating the expression of PGC-1? Biochem Biophys Res Commun 2013;433:52–7.
[49] Liu GH, Qu J, Shen X. NF-κB/p65 antagonizes Nrf2-ARE pathway by depriving CBP from Nrf2 and facilitating recruitment of HDAC3 to MafK. Biochim Biophys Acta Mol Cell Res 2008;1783:713–27.
[50] Takahashi T, Shimizu H, Morimatsu H, Maeshima K, Inoue K, Akagi R, et al. Heme oxygenase-1 is an essential cytoprotective component in oxidative tissue injury induced by hemorrhagic shock. J Clin Biochem Nutr 2009;44:28–40.
[51] Stocker R, Yamamoto Y, McDonagh AF, Glazer AN, Ames BN. Bilirubin is an antioxidant of possible physiological importance. Science 1987;235:1043–6.
[52] Otterbein LE, Bach FH, Alam J, Soares M, Tao Lu H, Wysk M, et al. Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway. Nat Med 2000;6:422–8.

[53] Davis W, Ronai Z, Tew KD. Cellular thiols and reactive oxygen species in drug-induced apoptosis. J Pharmacol Exp Therapeut 2001;296:1−6.
[54] Kaspar JW, Niture SK, Jaiswal AK. Nrf2:INrf2 (Keap1) signaling in oxidative stress. Free Radic Biol Med 2009;47: 1304−9.
[55] Rittner HL, Hafner V, Klimiuk PA, Szweda LI, Goronzy JJ, Weyand CM. Aldose reductase functions as a detoxification system for lipid peroxidation products in vasculitis. J Clin Invest 1999;103:1007−13.
[56] Kang ES, Woo IS, Kim HJ, Eun SY, Paek KS, Kim HJ, et al. Up-regulation of aldose reductase expression mediated by phosphatidylinositol 3-kinase/Akt and Nrf2 is involved in the protective effect of curcumin against oxidative damage. Free Radic Biol Med 2007;43:535−45.
[57] Martin M, Rehani K, Jope RS, Michalek SM. Toll-like receptor-mediated cytokine production is differentially regulated by glycogen synthase kinase 3. Nat Immunol 2005;6: 777−84.
[58] Díaz-Guerra MJ, Castrillo A, Martín-Sanz P, Boscá L. Negative regulation by phosphatidylinositol 3-kinase of inducible nitric oxide synthase expression in macrophages. J Immunol 1999;162:6184−90.
[59] Monick MM, Carter AB, Robeff PK, Flaherty DM, Peterson MW, Hunninghake GW. Lipopolysaccharide activates Akt in human alveolar macrophages resulting in nuclear accumulation and transcriptional activity of beta-catenin. J Immunol 2001;166:4713−20.
[60] Ozes ON, Mayo LD, Gustin JA, Pfeffer SR, Pfeffer LM, Donner DB. NF-κB activation by tumour necrosis factor requires tie Akt serine- threonine kinase. Nature 1999;401:82−5.
[61] Kim KY, Shin HK, Lee JH, Kim CD, Lee WS, BY Rhim, et al. Cilostazol enhances casein kinase 2 phosphorylation and suppresses tumor necrosis factor-α-induced increased phosphatase and tensin homolog deleted from chromosome 10 phosphorylation and apoptotic cell death in SK-N-SH Cells. J Pharmacol Exp Therapeut 2004;308:97−104.
[62] Hamdulay SS, Wang B, Birdsey GM, Ali F, Dumont O, Evans PC, et al. Celecoxib activates PI-3K/Akt and mitochondrial redox signaling to enhance heme oxygenase-1-mediated anti-inflammatory activity in vascular endothelium. Free Radic Biol Med 2010;48:1013−23.