Pinocembrin ameliorates lipopolysaccharide-induced HK-2 cell apoptosis and inflammation by regulating endoplasmic reticulum stress

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Abstract. Pinocembrin (PINO) is a natural flavonoid drug that possesses a range of biological activities, including antimicrobial, antioxidant and anti-inflammatory activities. The specific aim of the present study was to examine the pharmacological role of PINO in sepsis-mediated acute kidney injury (AKI), as well as to investigate the potential underlying mechanism. Human renal tubular epithelial cells (of the HK-2 cell line) were stimulated with lipopolysaccharide (LPS) for 24 h to simulate septic AKI *in vitro*, after which the experiments were repeated and the cells were pretreated with increasing concentrations of PINO (0, 50, 100 and 200 μg/ml). Using an MTT cell viability assay, PINO was revealed to be non-toxic to HK-2 cells. In LPS-treated HK-2 cells, PINO alleviated the loss of cell viability. Western blotting was used to analyze the expression levels of pro-inflammatory cytokines, including IL-1β, IL-6 and TNF-α, and the results revealed that PINO decreased the expression levels of these cytokines in a concentration-dependent manner. Furthermore, malondialdehyde (MDA) and glutathione (GSH) activities were assessed using MDA and GSH assay kits and it was revealed that PINO decreased the significantly increased level of malondialdehyde, while it also decreased the reduction in the level of GSH in LPS-challenged HK-2 cells. In addition, a TUNEL assay and western blotting were performed to examine cell apoptosis, and PINO was identified to significantly inhibit the level of apoptosis in LPS-induced HK-2 cells. Subsequently, the expression levels of endoplasmic reticulum stress (ERS)-associated factors, including activating transcription factor 4, C/EBP homologous protein and phosphorylated/total eukaryotic translation initiation factor 2 subunit 1 were examined by western blotting and it was demonstrated that ERS was triggered in HK-2 cells exposed to LPS, although this was partly circumvented through PINO treatment in a concentration-dependent manner. Furthermore, after the addition of tunicamycin, which acts as an agonist of ERS, the aforementioned experiments were performed again. Tunicamycin led to partial abolition of the protective function of PINO against inflammation, oxidative stress and apoptosis in LPS-challenged HK-2 cells. Overall, the results of the present study demonstrated that PINO was able to ameliorate the injuries sustained by LPS-challenged HK-2 cells via modulating ERS to reduce inflammation, oxidative stress and apoptosis; therefore, PINO may be a novel candidate drug for treating septic AKI.

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Introduction

Sepsis, characterized by life-threatening organ dysfunction resulting from a maladaptive host response to infection, is the leading cause of death in intensive care units worldwide (1). As the kidney is one of the most vulnerable organs to sepsis, acute kidney injury (AKI) is generally observed to be the most commonly occurring and serious complication of sepsis. As reported previously (2,3), the incidence of septic AKI accounts for 45-70% of all cases of AKI, and the majority of the supportive therapies for septic AKI treatment that are currently available are largely ineffective, leading to an unsatisfactory clinical outcome. An emerging body of evidence has indicated that the occurrence of septic AKI portends an increased mortality rate and longer hospital stays compared with non-septic AKI (4,5). Therefore, there is an urgent need to develop novel effective strategies for the treatment of septic AKI.

Pinocembrin [(2S)-5,7-dihydroxy-2-phenyl-2,3-dihydro chromen-4-one; PINO] is a major bioactive flavonoid that is mainly isolated from honey, propolis, wild marjoram and the roots of ginger, and it is tolerated well without any obvious adverse reactions (6,7). At present, a great deal of attention is being focused on the study of PINO for its diverse pharmacological activities, including its anti-inflammatory, antioxidative,

antimicrobial and neuroprotective properties, and its ability to exert protective effects in multiple diseases (8). It is worth noting that PINO has been approved by China Food and Drug Administration as a novel drug for ischemic stroke, and it is currently in phase II clinical trials (8-10). In addition, as a potential anti-inflammatory drug, its ability to control inflammation has been demonstrated in situations of lung injury, liver injury and intestinal injury (11-13). A previous study has uncovered a role for PINO in attenuating gentamicin-induced nephrotoxicity in rats, suggesting a protective effect for PINO against kidney injury (14). In addition, PINO has been proposed as a new candidate drug for preventing the progression of septic shock, as PINO has been demonstrated to improve host survival against lipopolysaccharide (LPS)-induced lethal endotoxemia by lowering the overproduction of pro-inflammatory cytokines (15). Furthermore, PINO has been reported to alleviate septic cardiomyopathy, a complication of sepsis (16), suggesting that PINO may exert protective functions against both sepsis and its complications.

To the best of the authors' knowledge, with respect to septic AKI, the benefits of PINO have not yet been investigated. Based on the findings above, the present study aimed to investigate whether PINO may exert a protective role in septic AKI, and to elucidate the potential underlying mechanisms in an LPS-induced *in vitro* septic AKI model in HK-2 cells.

Materials and methods

Cell culture and treatment. Human renal tubular epithelial cells (HK-2 cells) were obtained from American Type Culture Collection and kept in culture in RPMI-1640 medium supplemented with 10% Invitrogen® fetal bovine serum (Thermo Fisher Scientific, Inc.) at 37˚C in an incubator in an atmosphere of 5% CO_2. To simulate sepsis-induced AKI, HK-2 cells were treated with 1 μg/ml LPS (Sigma-Aldrich; Merck KGaA) at 37˚C for 24 h. To explore the role of PINO in LPS-induced HK-2 cells, increasing concentrations of PINO (0, 50, 100 and 200 μg/ml; Merck KGaA) were applied for the various treatments 1 h prior to LPS induction (17). In addition, tunicamycin (TM; MedChemExpress), an agonist of endoplasmic reticulum stress (ERS), was introduced into HK-2 cells at a concentration of 1 μg/ml at 37˚C at 2 h prior to 200 μg/ml PINO treatment to explore the regulatory mechanism of PINO.

Cell viability assay. HK-2 cells were seeded into 96-well plates ($2x10^3$ cells/well) and incubated at 37˚C in an atmosphere of 5% CO_2. After adherence of the cells, 20 μl 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT; Merck KGaA) solution was added to each well, and the cells were cultured at 37˚C for a further 4 h. Subsequently, the culture medium was discarded, 100 μl dimethyl sulfoxide (Merck KGaA) was added to each well, and the cell culture was allowed to continue for a further 15 min at 37˚C to dissolve the crystalline substances. Finally, the absorbance at 490 nm was measured using a microplate reader.

Measurement of malondialdehyde (MDA) and glutathione (GSH). HK-2 cells were seeded into 96-well plates ($2x10^3$ cells/well) and incubated at 37˚C in an atmosphere of 5% CO_2. After LPS induction for 24 h, with or without pre-treatment of PINO as aforementioned, 100 μl supernatant of each well was collected. Subsequently, the concentrations of MDA and GSH in the supernatant were measured using the corresponding commercial detection kits for MDA (cat. no. A003-1 for MDA and cat. no. A006-2 for GSH; from Nanjing Jiancheng Bioengineering Institute), strictly following the manufacturer's instructions; the absorbances at 532 and 420 nm for MDA and GSH, respectively, were measured using a microplate reader.

TUNEL analysis. Apoptosis was assessed using a TUNEL Apoptosis Assay kit (Beyotime Institute of Biotechnology). In brief, after LPS induction for 24 h, with or without pre-treatment of PINO, HK-2 cells were subjected to fixation with 4% paraformaldehyde for 30 min at room temperature, and 5 min permeabilization with 0.3% Triton X-100 at room temperature. Subsequently, the TUNEL detection solution mixture was added to the HK-2 cells at 37˚C for 1 h in the dark, followed by an incubation with 4',6-diamidino-2-phenylinodole (50 μg/ml) for 10 min at room temperature and mounting in an anti-fade reagent (Beijing Solarbio Science & Technology Co., Ltd.). The TUNEL-positive cells from at least five random fields were then observed under an inverted fluorescence microscope (Olympus Corporation).

Western blotting. Total protein was extracted from HK-2 cells using ice-cold radio-immunoprecipitation assay (RIPA) lysis buffer (Beyotime Institute of Biotechnology). After the protein concentration had been determined using the bicinchoninic acid protein assay (Beyotime Institute of Biotechnology), the protein samples were adjusted to the same amount (30 μg/lane) and subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, before being transferred onto polyvinylidene difluoride membranes. After blocking with 5% skimmed milk for 1 h at 4˚C, the membranes were probed with primary antibodies against IL-1β (cat. no. ab9722; Abcam), IL-6 (cat. no. ab233706; Abcam), TNF-α (cat. no. ab215188; Abcam), Bcl-2 (cat. no. ab32124; Abcam), Bax (cat. no. ab32503; Abcam), cleaved caspase 9 (cat. no. ab2324; Abcam), caspase 9 (cat. no. 10380-1-AP; ProteinTech Group, Inc.), activating transcription factor 4 (ATF4; cat. no. ab184909; Abcam), C/EBP homologous protein (CHOP; cat. no. ab11419; Abcam), phosphorylated eukaryotic translation initiation factor 2 subunit 1 (p-eIF2α; cat. no. ab32157; Abcam), eIF2α (cat. no. ab157478; Abcam) (all 1:1,000), and GAPDH (1:2,500, cat. no. ab9485; Abcam) at 4˚C overnight. On the following day, the membranes were washed three times with TBS-0.1% Tween-20, and incubated with HRP-conjugated goat anti-rabbit IgG (cat. no. ab6721) or goat anti-mouse IgG (cat. no. ab6789) (both 1:2,000; Abcam) secondary antibodies at room temperature for 2 h. The bands were visualized using an ECL Western Blotting Detection kit (Beijing Solarbio Science & Technology Co., Ltd.) and quantified using ImageJ software (version 1.48; National Institutes of Health), with GAPDH as the internal control.

Statistical analysis. GraphPad Prism 8 software (version 8.0; GraphPad Software, Inc.) was used for the statistical analysis. The experimental data are presented as the mean ± standard deviation from at least three independent

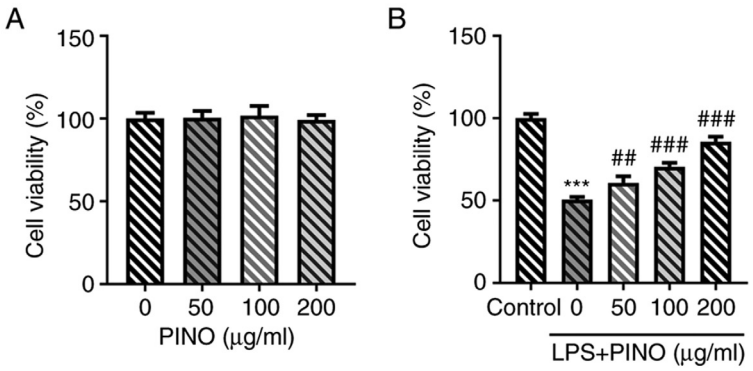

Figure 1. Effect of PINO on cell viability in HK-2 cells with or without LPS induction. (A) HK-2 cells were treated with increasing concentrations of PINO (0, 50, 100 and 200 μg/ml), and the cell viability was detected using MTT assay. (B) HK-2 cells were pretreated with various dosages of PINO (0, 50, 100 and 200 μg/ml) for 1 h, and subsequently treated with LPS for 24 h. The cell viability was detected using MTT assay. ***P<0.005 vs. control; $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. the LPS group. PINO, pinocembrin; LPS, lipopolysaccharide.

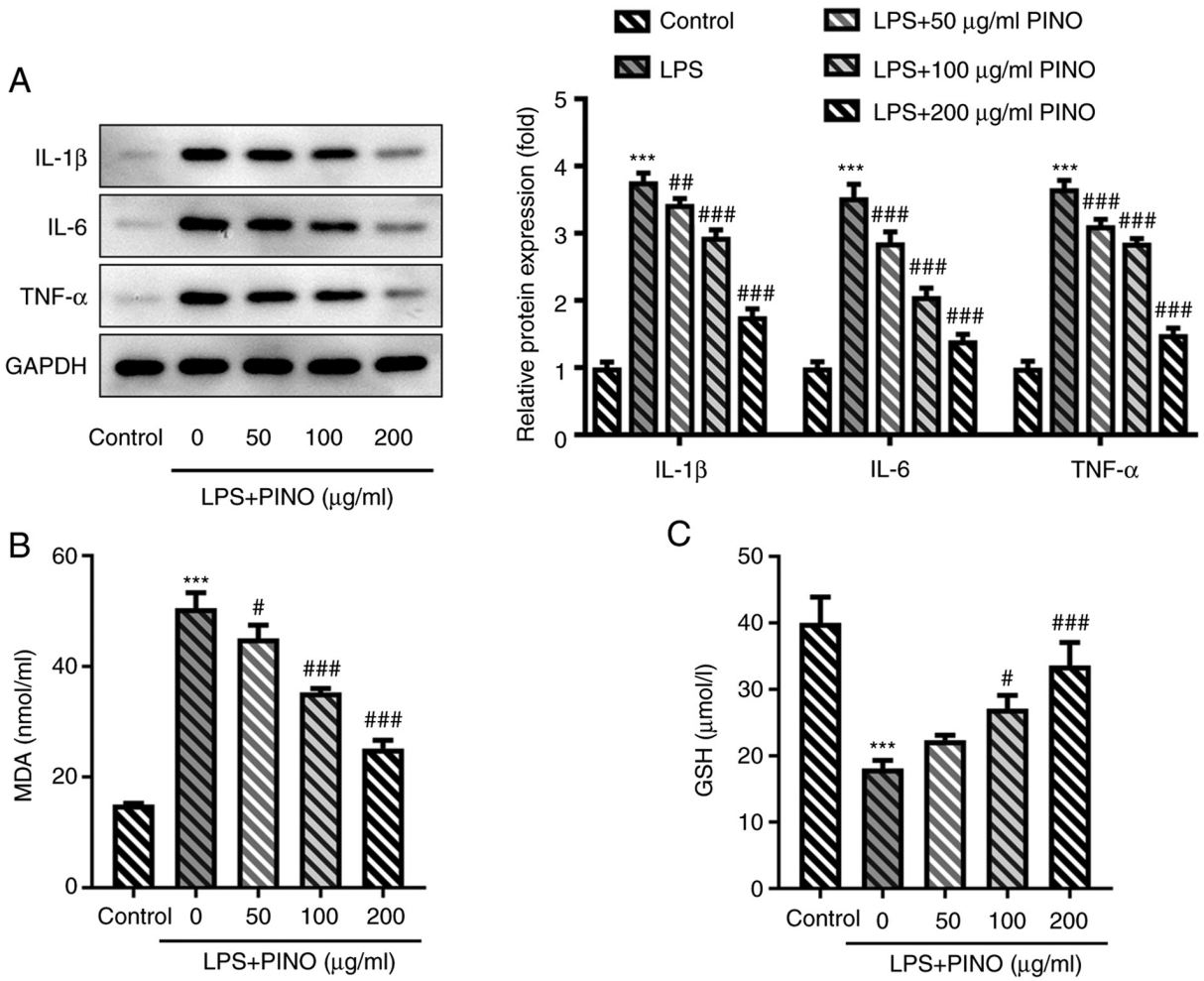

Figure 2. Effects of PINO on inflammation and oxidative stress in LPS-induced HK-2 cells. HK-2 cells were pre-treated with PINO (0, 50, 100 and 200 μg/ml) for 1 h, and then treated with LPS for 24 h. (A) Protein expression levels of TNF-α, IL-6 and IL-1β were measured using western blotting. The concentration of (B) MDA and (C) GSH in the supernatant of culture media was determined using the corresponding commercial kits. ***P<0.005 vs. control; $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. the LPS group. PINO, pinocembrin; LPS, lipopolysaccharide; IL, interleukin; TNF-α, tumor necrosis factor-α; GSH, glutathione; MDA, malondialdehyde.

experiments. Comparisons among groups were made using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of PINO on cell viability in HK-2 cells with or without LPS induction. To assess whether PINO was toxic to HK-2

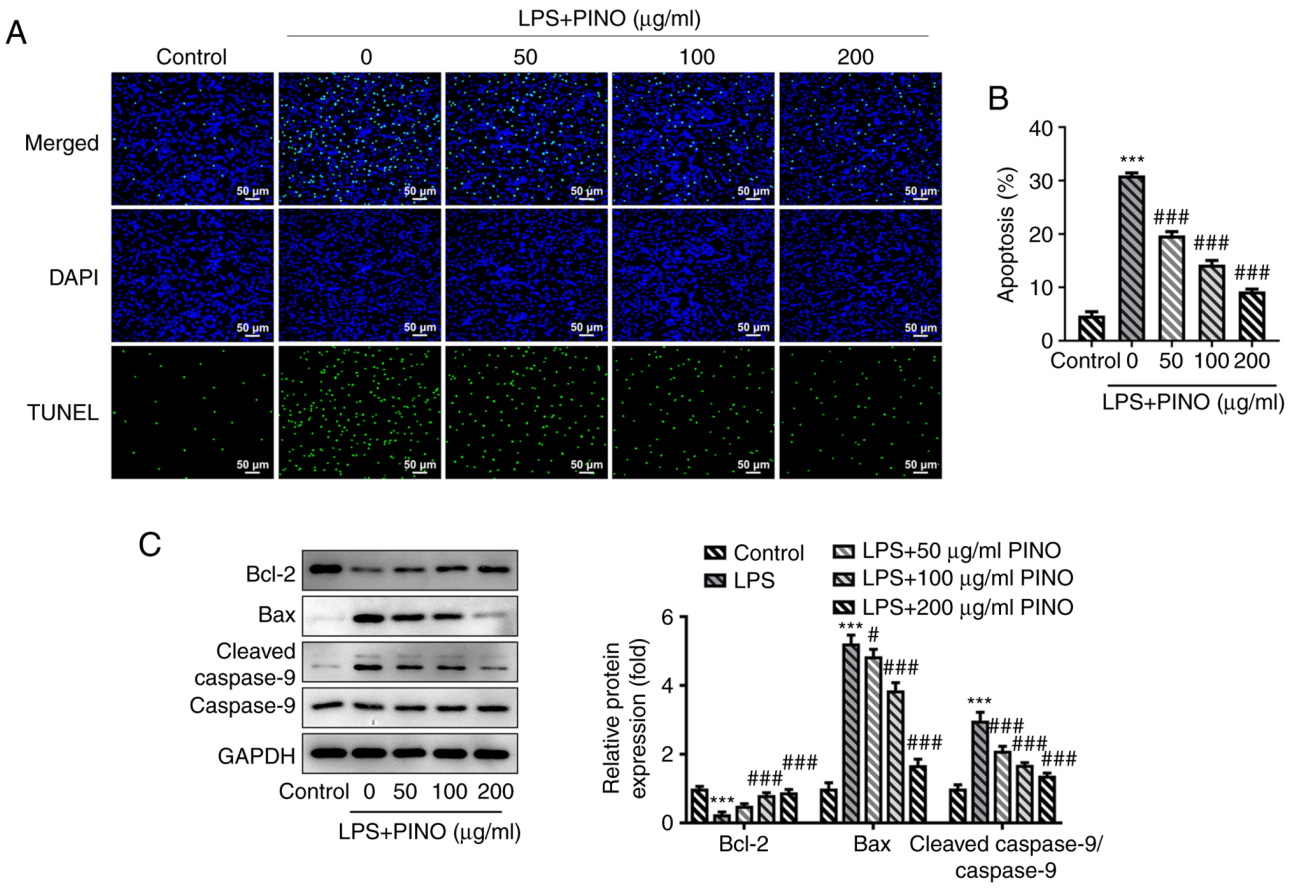

Figure 3. Effect of PINO on apoptosis in LPS-induced HK-2 cells. HK-2 cells were pretreated with PINO (0, 50, 100 and 200 μg/ml) for 1 h, and then treated with LPS for 24 h. (A) TUNEL assay was performed to assess the extent of cell apoptosis. (B) Quantification of the apoptosis rate. (C) Apoptosis-associated proteins were detected using western blotting. ***P<0.005 vs. control; $^{\#}$P<0.05, $^{\#\#\#}$P<0.001 vs. the LPS group. PINO, pinocembrin; LPS, lipopolysaccharide.

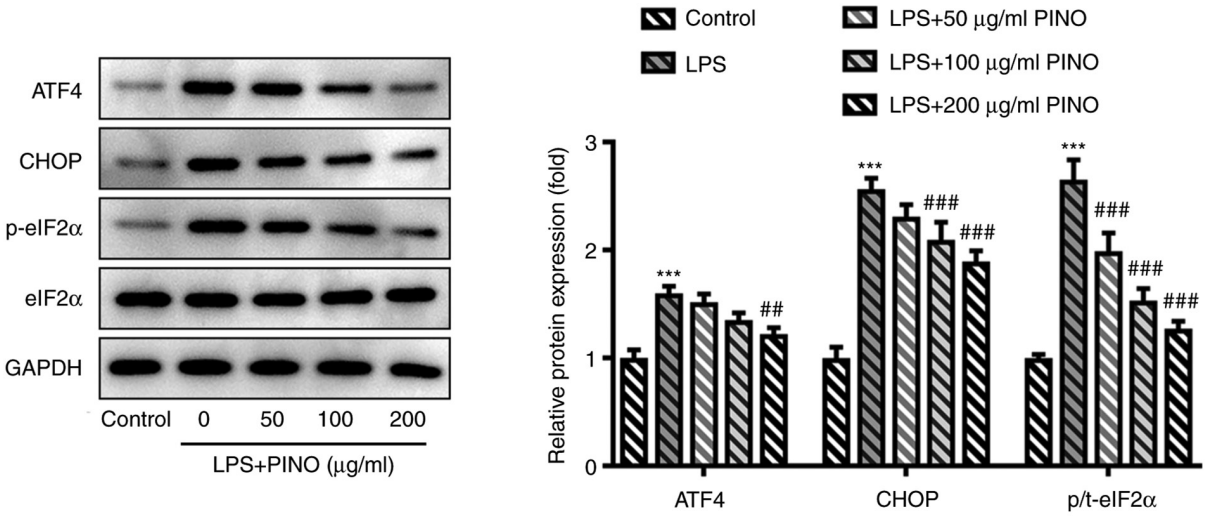

Figure 4. Effect of PINO on ERS in LPS-induced HK-2 cells. HK-2 cells were pretreated with PINO (0, 50, 100 and 200 μg/ml) for 1 h, and then treated with LPS for 24 h. The ERS-associated proteins were detected using western blotting. ***P<0.005 vs. control; $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. the LPS group. PINO, pinocembrin; LPS, lipopolysaccharide; ERS, endoplasmic reticulum stress; ATF4, activating transcription factor 4; CHOP, C/EBP homologous protein; p-, phosphorylated; t-, total; eIF2α, eukaryotic translation initiation factor 2 subunit 1.

cells, the effects of different concentrations of PINO (0, 50, 100 and 200 μg/ml) on cell viability were first examined. It was observed that no significant differences in cell viability occurred when HK-2 cells were treated with PINO at concentrations of 0-200 μg/ml (Fig. 1A). Subsequently, HK-2 cells were pretreated with various doses of PINO for 1 h prior to LPS treatment for 24 h. These experiments revealed that treatment with LPS led to a significant reduction in cell

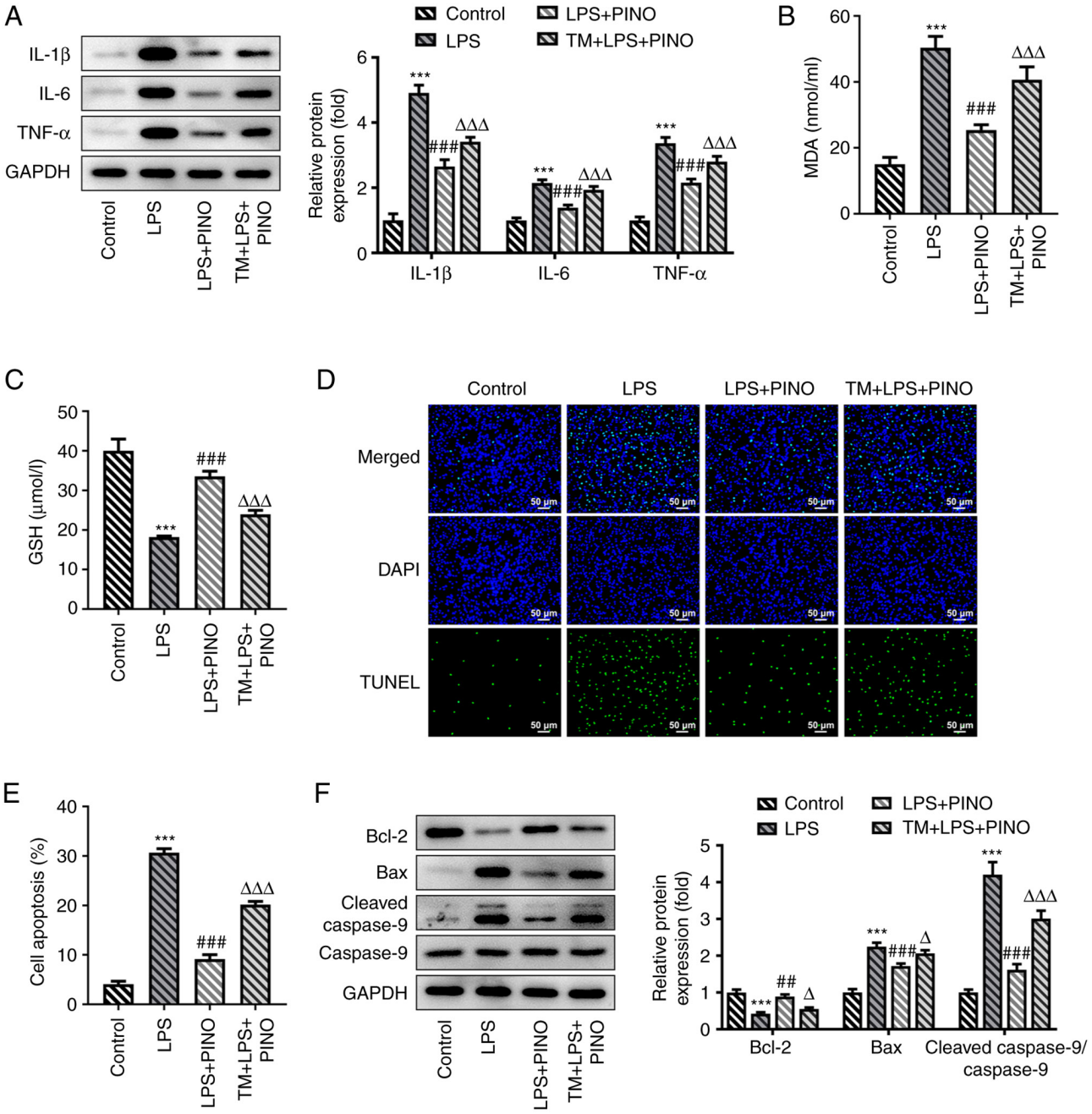

Figure 5. PINO exerts effects on inflammation, oxidative stress and apoptosis in LPS-induced HK-2 cells by regulating ERS. HK-2 cells were treated with TM (1 μg/ml) for 2 h prior to PINO treatment and LPS stimulation. (A) Protein expression levels of TNF-α, IL-6 and IL-1β were measured using western blotting. The concentrations of (B) MDA and (C) GSH in the supernatant of the culture media were determined using their corresponding commercial kits. (D) TUNEL assay was performed to assess apoptosis. (E) Quantification of the apoptosis rate. (F) Apoptosis-associated proteins were detected using western blotting. ***P<0.005 vs. control; $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. the LPS group; $^{\Delta}$P<0.05, $^{\Delta\Delta\Delta}$P<0.001 vs. the LPS + PINO group. PINO, pinocembrin; LPS, lipopolysaccharide; IL, interleukin; TNF-α, tumor necrosis factor-α; GSH, glutathione; MDA, malondialdehyde; ERS, endoplasmic reticulum stress; TM, tunicamycin.

viability compared with the control, whereas pretreatment with PINO led to significant improvements in the viability of LPS-challenged HK-2 cells in a concentration-dependent manner compared with the LPS group (Fig. 1B).

Effects of PINO on inflammation, oxidative stress and apoptosis in LPS-induced HK-2 cells. Subsequently, the biological activity of PINO in sepsis-induced AKI *in vitro* was investigated. As expected, LPS significantly promoted the secretion of pro-inflammatory cytokines compared with the control, including interleukin (IL)-6, IL-1β and tumor necrosis factor-α (TNF-α), in HK-2 cells, thereby simulating the inflammatory environment surrounding kidney cells exposed to sepsis. By contrast, PINO treatment exerted significant inhibitory effects on the levels of IL-6, IL-1β and TNF-α in LPS-induced HK-2 cells compared with the LPS group in a concentration-dependent manner (Fig. 2A). The level of MDA, a marker of oxidative stress, was significantly increased in LPS-induced HK-2 cells compared with the control, although this enhancement was inhibited by PINO in a concentration-dependent manner (Fig. 2B). Compared with the control, the concentration of GSH, one of the major cellular

antioxidants, was revealed to be significantly decreased in HK-2 cells upon exposure to LPS, although this decrease in the LPS group was attenuated upon PINO treatment in a concentration-dependent manner (Fig. 2C). Furthermore, the results of the TUNEL assay demonstrated that there was a significant increase in TUNEL-positive cells in LPS-challenged HK-2 cells compared with the numbers of cells in the control group, whereas the numbers of TUNEL-positive cells were gradually reduced following treatment with increasing concentrations of PINO (Fig. 3A and B). In addition, a series of apoptosis-associated proteins were detected using western blotting. The expression level of Bcl-2 was significantly downregulated upon LPS induction, whereas the protein expression levels of Bax and cleaved-caspase 9 exhibited a significantly upregulated trend, which suggested that LPS induced apoptosis of the HK-2 cells. However, these changes upon LPS induction were subsequently reversed by treatment with PINO in a concentration-dependent manner (Fig. 3C). Collectively, these findings suggested that PINO could exert anti-inflammatory, anti-oxidative and anti-apoptosis effects on the LPS-induced HK-2 cells.

Effect of PINO on ERS in LPS-induced HK-2 cells. To explore the potential regulatory mechanism of PINO, its possible influence on ERS, which is involved in multiple pathological processes and stress reactions, was explored. As presented in Fig. 4, the levels of ATF4, CHOP and p-eIF2α, which are considered important mediators and markers of ERS, were significantly enhanced in LPS-induced HK-2 cells compared with the control, demonstrating the occurrence of ERS in LPS-challenged HK-2 cells. Subsequently, the suppressive effects of PINO on the protein expression levels of ATF4, CHOP and p-eIF2α in HK-2 cells exposed to LPS implied that PINO could ameliorate ERS in the LPS-challenged HK-2 cells.

PINO exerts effects on inflammation, oxidative stress and apoptosis in LPS-induced HK-2 cells via regulating ERS. To establish whether ERS may have an important influence on the biological activity of PINO in LPS-induced HK-2 cells, TM, an agonist of ERS, was introduced into HK-2 cells at a concentration of 1 μg/ml at 2 h prior to PINO treatment (200 μg/ml). These experiments revealed that the expression levels of TNF-α, IL-6 and IL-1β in the TM + LPS + PINO group were significantly elevated in comparison with the LPS + PINO group (Fig. 5A). Furthermore, the reduced MDA level caused by PINO was significantly increased upon additional treatment of TM in the LPS-induced HK-2 cells; conversely, the upregulated level of GSH caused by PINO was significantly reduced upon the additional treatment with TM in the LPS-induced HK-2 cells (Fig. 5B and C). Furthermore, treatment with TM also led to a partial significant decrease in the inhibitory effect of PINO on apoptosis in LPS-induced HK-2 cells, as evidenced by increased numbers of TUNEL-positive cells, significantly upregulated protein expression levels of Bax and cleaved-caspase 9 and decreased protein expression of Bcl-2 in the TM + LPS + PINO group compared with the LPS + PINO group (Fig. 5D-F). Overall, these findings suggested that the activation of ERS could partly weaken the protective role of PINO against cell injuries sustained through exposure to LPS.

Discussion

Sepsis is a systemic inflammatory response caused by multiple infections, which results in damage to multiple organs, ultimately leading to a high level of mortality worldwide (18). Sepsis has long been regarded as the foremost precipitant of AKI, which is attributed to the high susceptibility of the kidney to sepsis (2,3). PINO, a bioactive flavonoid isolated from honey, propolis, wild marjoram and the roots of ginger, possesses multiple biological properties and is recognized as a promising natural small-molecule drug in inflammation-associated diseases (8,11,15). The present study was designed to determine the functional roles of PINO in septic AKI, and to investigate the underlying mechanism. The findings obtained have revealed that treatment with PINO clearly alleviated LPS-induced inflammation, oxidative stress and apoptosis in HK-2 cells. Furthermore, the aforementioned protective effects of PINO were weakened upon activating ERS. Hence, it was possible to surmise that PINO may act in a protective role in LPS-challenged HK-2 cells through regulating ERS.

The endotoxin LPS, which serves as an important component of Gram-negative bacteria, is involved in the pathogenesis of septic AKI, and has been widely applied to establish a septic AKI model (19). LPS stimulation usually results in a severe inflammatory response, accompanied by the excessive production of pro-inflammatory cytokines, including TNF-α and IL-6, leading to subsequent apoptosis and renal injury (20,21). The results of the present study also revealed increased expression levels of TNF-α, IL-6 and IL-1β in LPS-challenged HK-2 cells. Several previous studies have indicated that drug candidates with anti-inflammatory properties may protect the kidney against sepsis-triggered organ damage (20,22). In the present study, decreased expression levels of these cytokines were observed after PINO treatment, suggesting that PINO acted in a protective role in septic AKI.

An imbalance of the oxidant-antioxidant system leaning towards the dominance of oxidants during inflammation will lead to oxidative stress. Accumulating evidence has revealed that oxidative stress is as important as inflammation with respect to the pathophysiology of sepsis, as numerous drug candidates exert their protective effects on septic AKI via the suppression of inflammation as well as oxidative stress, and these drugs include etanercept, honokiol and glycyrrhizic acid (23-25). In the present study, the results also illustrated that PINO could significantly attenuate oxidative stress by reducing MDA production and increasing the level of GSH in LPS-challenged HK-2 cells, further affirming the protective role of PINO in septic AKI. In addition, a previous study has revealed that aberrant inflammation and oxidative stress in the kidney initiated apoptosis, which subsequently led to kidney epithelial cell apoptosis and promoted kidney cell viability loss (26). Likewise, abundant apoptotic cells were observed in LPS-challenged HK-2 cells in the present study, demonstrating that severe injury of kidney cells was manifested upon LPS stimulation; however, the aforementioned injuries were ameliorated by PINO owing to its inhibitory action on apoptosis. Therefore, overall, the data in the present study suggested

that PINO exerted a protective role in LPS-stimulated HK-2 cells by reducing inflammation, oxidative stress and apoptosis.

ER serves an important role in maintaining protein homeostasis, and is sensitive to various stimuli, including infection and trauma, which further leads to the accumulation of misfolded or unfolded proteins and ERS (27). During ERS, the unfolded protein response (URP) is activated to maintain cellular homeostasis (28); however, a prolonged URP can lead to excessive ERS and cause CHOP-mediated apoptosis (29). An increasing body of evidence suggests that sepsis is associated with the activation of ERS, which has been identified as a contributor to kidney injury (30,31). Therefore, focusing on the modulation of ERS may be a potential approach to elucidating the underlying mechanism of the pathogenesis of sepsis-induced AKI. The study performed by Jia *et al* (32) revealed that methane-rich saline solution can exert its anti-inflammatory, antioxidative and antiapoptotic properties in terms of ameliorating sepsis-induced AKI by suppressing the ERS-associated GRP78/ATF4/CHOP/caspase-12 apoptotic signaling pathway. Wang *et al* (33) considered resveratrol as a promising drug for protecting against sepsis-induced kidney injury, and demonstrated that resveratrol inhibits the renal inflammatory response by regulating the ERS-mediated NF-κB pathway. Notably, PINO has been demonstrated to reduce the expression levels of CHOP and caspase-12 in ischemia/reperfusion-induced brain injury, suggesting that PINO may prevent brain injury by attenuating ERS-induced apoptosis (34).

Therefore, to elucidate the mechanism contributing to the protective effects of PINO on LPS-induced kidney injury, the present study also evaluated the expression levels of ERS-associated proteins in LPS-induced kidney injury. Likewise, the results obtained revealed that ERS was triggered upon LPS stimulation, and that this was then inhibited by PINO treatment, whereas additional treatment with tunicamycin, an agonist of ERS, served to weaken the protective function of PINO against inflammation, oxidative stress and apoptosis in LPS-challenged HK-2 cells, suggesting that PINO may protect the kidney against sepsis-triggered inflammation, oxidative stress and apoptosis partly through inhibiting ERS.

In conclusion, the present study demonstrated that PINO protected against LPS-induced septic AKI by reducing the levels of inflammation, oxidative stress and apoptosis, at least in part through regulating ERS. Consequently, PINO is a potential drug candidate for the prevention and treatment of sepsis and its complications, including AKI.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

YF designed the study. YZ, CY and YF collected, analyzed and interpreted the data. YZ and CY drafted the manuscript and YF revised the manuscript. YF and YZ confirm the authenticity of all the raw data. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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