

Acute carbon monoxide poisoning induces renal inflammation and apoptosis via CCL4 upregulation and activation of the PI3K/Akt pathway in mice

YU NI^{1,2*}, XUHENG JIANG^{3,4*}, SHUHONG WANG^{3,4},
TAO PU², TIAN TIAN YAN^{3,4}, ANYONG YU^{3,4} and QI LIU^{1,2}

¹Department of Nephrology, Clinical Medical College of Suzhou Medical College, Soochow University, Suzhou, Jiangsu 215123, P.R. China; ²Department of Nephrology, Affiliated Hospital of Zunyi Medical University, Zunyi, Guizhou 563003, P.R. China; ³Emergency Department, Affiliated Hospital of Zunyi Medical University, Zunyi, Guizhou 563003, P.R. China; ⁴Emergency Department, Basic and Clinical Innovation Research Center for Acute and Critical Care, Affiliated Hospital of Zunyi Medical University, Zunyi, Guizhou 563003, P.R. China

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Abstract. Acute carbon monoxide poisoning (ACOP) is one of the primary causes of poisoning worldwide, leading to multi-organ dysfunction, particularly affecting the kidneys. However, the molecular mechanisms underlying the effects of carbon monoxide-induced acute kidney injury (AKI) remain yet to be fully elucidated. Therefore, the present study aimed to identify key genes and signaling pathways involved in AKI following ACOP in a mouse model using the RNA sequencing (RNA-seq) technology. Renal function was assessed via measuring serum creatinine (SCr) and blood urea nitrogen (BUN) levels. Additionally, histopathological alterations, cell apoptosis and inflammation were evaluated by immunohistochemistry, TUNEL assay, ELISA and western blot analysis. The results revealed significant renal insufficiency and tubular injury, alongside a heightened inflammatory state and apoptosis in ACOP mice. RNA-seq and mechanistic studies identified the upregulation of C-C motif chemokine ligand 4 (CCL4) and activation of the PI3K/Akt pathway as key underlying events. Inhibition of PI3K with LY294002

attenuated these pathological changes and alleviated kidney injury. In conclusion, the present study demonstrated that ACOP induced renal inflammation and apoptosis through CCL4 upregulation and activation of the PI3K/Akt pathway, thus conveying a potential therapeutic target for mitigating ACOP-induced AKI (ACOP-AKI). Overall, the present study could provide important insights into the molecular mechanisms of ACOP-AKI.

Introduction

Acute carbon monoxide poisoning (ACOP) is one of the most common acute poisoning incidents globally. In the United States, ACOP is associated with 50,000-100,000 emergency visits and 1,500-2,000 fatalities each year (1). The pathophysiological mechanism of ACOP is complex, and can eventually lead to the dysfunction of multiple organs, which is occasionally life-threatening in severe cases. The kidneys are a highly metabolic and oxygen-dependent organ, rendering them sensitive to hypoxia and oxidative stress. Therefore, acute renal injury (AKI) is a notable complication of ACOP (2). It has been reported that ~18% of patients with ACOP experience AKI, which not only markedly increases short-term mortality, but is also associated with the development of chronic renal disease (CKD) (3). Therefore, investigating the causes of ACOP could promote the development of targeted therapies to prevent or alleviate AKI, thus improving patient outcomes and reducing the risk of progression to CKD.

Evidence has suggested that the mechanisms of ACOP-induced AKI (ACOP-AKI) involve several interrelated factors, such as decreased oxygen transport, mitochondrial respiratory inhibition, reperfusion injury, oxidative stress and the activation of abnormal inflammatory signal pathways (4-7). CO exposure may also trigger autophagy by inducing the formation of mitochondrial reactive oxygen species (8). These findings underscore the complexity of the molecular pathways involved in ACOP-AKI and emphasize the need for further studies to uncover these mechanisms.

Correspondence to: Professor Anyong Yu, Emergency Department, Affiliated Hospital of Zunyi Medical University, 149 Dalian Road, Zunyi, Guizhou 563003, P.R. China
E-mail: anyongyu@163.com

Professor Qi Liu, Department of Nephrology, Clinical Medical College of Suzhou Medical College, Soochow University, 199 Ren'ai Road, Industrial Park, Suzhou, Jiangsu 215123, P.R. China
E-mail: 2454640289@qq.com

*Contributed equally

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Previous advances in RNA sequencing (RNA-seq) technology have enabled comprehensive analysis of transcriptional alterations in complex tissues (9-12). This technique is applied to identify changes in gene expression and their functional significance, thus offering a novel approach for understanding the molecular mechanisms underlying the development of complicated diseases. However, to date, transcriptional modifications associated with ACOP-AKI remain yet to be fully elucidated.

The present study aimed to assess the molecular mechanisms underlying ACOP-AKI using RNA-seq technology. The present study hypothesized that ACOP-AKI was associated with the abnormal activation of important factors and signaling pathways. To assess this hypothesis, an ACOP mouse model was established, and RNA-seq was employed to comprehensively analyze gene expression profiles in renal tissues following exposure to CO, mainly focusing on important components and signaling pathways associated with inflammation and apoptosis. The primary objective of the present study was to elucidate the molecular mechanisms underlying ACOP-AKI and establish a theoretical basis for the development of targeted therapeutic interventions.

Materials and methods

Animals. A total of 60 adult C57BL/6 mice (male; age, 8-12 weeks; weight, 22-26 g) were obtained from the Animal Experimental Center of Zunyi Medical University. All experimental protocols were approved by the Animal Care Committee of Zunyi Medical University (approval no. zyfy-an-2024-0710/0358) and supervised by the Ethics Committee of the Affiliated Hospital of Zunyi Medical University. Mice were maintained under controlled environmental conditions (temperature, 22-25°C; humidity, 55-60%; 12-h light/dark cycle) with free access to food and water. All procedures adhered to China's guidelines for animal research, with efforts made to minimize animal use and alleviate potential distress, thus complying with ethical standards.

Animal models. The ACOP mouse model was established as previously described (13). Briefly, mice were placed in a transparent plastic chamber at 22-24°C. To monitor CO levels, two CO detectors were positioned, one at the center and another near the edge of the chamber. The chamber was then gradually filled with CO gas. The exposure protocol involved an initial phase of 1,000 ppm CO for 40 min, followed by a higher concentration of 3,000 ppm CO for 20 min, or until the mice displayed signs of unconsciousness. Following exposure to CO, the animals were promptly transferred to fresh ambient air and observed until they fully regained consciousness. Mice were anesthetized with 2% isoflurane in air (2-3 l/min) on days 1, 3 and 7. Subsequently, ~0.5-1 ml of whole blood was collected via the retro-orbital venous plexus equally divided into anticoagulant and plain tubes for the preparation of plasma and serum. Both plasma and serum were isolated following centrifugation at 1,000 x g for 10 min at 4°C. Following blood collection, deep anesthesia was maintained with 2% isoflurane, and the mice were sacrificed directly. Different euthanasia methods were selected according to different experimental purposes: In the experiments exploring

the time course of ACOP-induced renal injury and conducting transcriptome analysis, to minimize animal suffering and preserve the overall morphological structure of tissues, the experimental animals were sacrificed by cervical dislocation under isoflurane anesthesia. The collected renal tissues were mainly used for phenotypic observation studies. In the inhibitor intervention experiments, under the same isoflurane anesthesia, sacrifice was performed by transcardiac perfusion with ice-cold 0.1 M phosphate-buffered saline (PBS) to harvest renal tissues, which was mainly to meet the higher tissue quality requirements for mechanism research.

Experimental groups and process design. To explore the temporal changes in kidney injury subsequent to ACOP, a total of 24 mice were randomly divided into four groups (n=6): The control, ACOP 1-day, ACOP 3-day and ACOP 7-day groups. The degree of kidney injury was evaluated at each time point by histopathological and biochemical analyses. Follow-up experiments were performed at the time point exhibiting the most severe injury (determined by the highest tubular injury score). Total RNA was extracted from the renal tissue of both the control and ACOP model groups for RNA sequencing (RNA-seq) analysis. Raw reads were quality-checked and adapter-trimmed using Fastp (v0.23.2, github.com/OpenGene/fastp). The resulting high-quality clean reads were then aligned to the reference genome using HISAT2 (v2.2.1, <https://daehwankimlab.github.io/hisat2/>). Gene-level read counts were generated from the alignment files using FeatureCounts (v2.0.3, <https://sourceforge.net/>). Finally, differentially expressed genes (DEGs) between the control and model groups were identified using the DESeq2 (v1.40.2, <https://bioconductor.org/packages/release/bioc/html/DESeq2.html>) package, with a significance threshold set at an absolute fold change ≥ 2 and an adjusted P-value < 0.05 .

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was then performed to identify key signaling pathways involved in kidney injury. Based on the KEGG results, representative upregulated genes were selected as candidate targets and validated using western blotting. An *in vivo* intervention utilizing the PI3K inhibitor LY294002 was performed to assess the functional role of specific signaling pathways in ACOP-AKI. A total of 36 mice were randomly allocated into four groups (n=9) in the inhibitor experiments. The control group consisted of mice housed in identical transparent plastic cages as those in the experimental groups without CO treatment or injection. The ACOP group consisted of mice exposed to CO to establish an ACOP model.

Subsequently, mice were subject to intraperitoneal injections with varying components depending on their group. For the ACOP + LY294002 group, LY294002 was initially dissolved in DMSO at a concentration of $< 0.1\%$ and was subsequently diluted in 0.9% NaCl, resulting in a final injection dose of 10 mg/kg body weight. Mice in the ACOP group were treated with an intraperitoneal injection of an equivalent volume of 0.9% NaCl solution without DMSO, matching the injection volume of LY294002 in the ACOP + LY294002 group. In the ACOP + vehicle group, mice were also exposed to CO and then received an intraperitoneal injection of a 0.9% NaCl solution containing 0.1% DMSO. The first injection

was administered within 2 h post-modeling via CO exposure, followed by daily injections until the designated experimental endpoint. Mice were then sacrificed for subsequent analysis.

Measurement of blood CO concentration. The concentration of CO in blood was measured using CO assay kit (cat. no. A101-3-1; Nanjing Jiancheng Bioengineering Institute), according to the manufacturer's instructions. Total hemoglobin (Hb) concentration was determined. The optical density (OD) was measured at a wavelength of 540 nm (A540) using a microplate reader (Varioskan Flash; Thermo Fisher Scientific, Inc.). The Hb concentration was calculated as follows: $Hb = A540 \times 73.54$. Subsequently, the percentage of carboxyhemoglobin (COHb) was determined. The ODs of test and control samples were measured at 568 nm (A568) and 581 nm (A581). The ΔOD value was calculated using the following formula: $\Delta OD = \Delta OD \text{ of test sample (A568-A581)} - \Delta OD \text{ of control sample (A568-A581)}$. The COHb was then calculated using the following equation: $COHb (\%) = (0.822 \times \Delta OD + 0.001) \times 100$. The final blood CO concentration was calculated as follows: $CO \text{ concentration} = [COHb (\%) \times Hb (g/l) \times 10^6 \times 4] / 64456$.

Kidney function assessment. Blood urea nitrogen (BUN) and serum creatinine (SCr) were measured with commercially available assay kits (C013-2-1 and C011-2-1; both Nanjing Jiancheng Bioengineer Institute, China), according to the manufacturer's protocols. The OD was measured at a wavelength of 450 nm, and the concentrations of BUN and SCr were calculated based on a standard curve.

Histopathological analysis. Renal tissue samples were fixed in 4% phosphate-buffered formaldehyde for 24–48 h at room temperature and subsequently embedded in paraffin. The embedded tissues were sliced into 5 μm -thick sections and were then subjected to hematoxylin and eosin (H&E) as well as periodic acid-Schiff (PAS) staining. Specifically, hematoxylin staining was performed for 5–8 min at room temperature, followed by eosin staining for 1–2 min under the same conditions. For the PAS staining, sections were oxidized in periodic acid solution for 5–10 min at room temperature and then stained with Schiff's reagent for 15–30 min at room temperature. After staining, the sections were incubated with diaminobenzidine for 5 min at room temperature, counterstained with hematoxylin for 1–2 min at room temperature, and observed under a light microscope. Tubular injury was assessed according to a five-tier grading system that evaluated the extent of brush border loss, tubular dilation, cast formation, and cellular necrosis, as referenced in (14). The specific grading scheme was defined as: 0, no pathological changes; 1, less than 10% involvement; 2, 10–25% involvement; 3, 26–50% involvement; 4, 51–75% involvement; and 5, >75% involvement.

Immunohistochemical staining. Renal tissue samples were fixed in 4% phosphate-buffered formaldehyde for 24–48 h at room temperature. Paraffin-embedded tissue sections (thickness, 5 μm) were deparaffinized and rehydrated through a descending series of ethanol to water. Antigen retrieval was performed using citrate buffer (pH 6.0) at 95°C for 20 min. Endogenous peroxidase activity was blocked following tissue incubation with 3% hydrogen peroxide for 10 min at

room temperature. Subsequently, sections were blocked with 10% normal goat serum (cat. no. AR0009, Boster Biological Technology) for 1 h at room temperature. The sections were then incubated at 4°C overnight with a primary antibody against neutrophil gelatinase-associated lipocalin (NGAL; rabbit; dilution, 1:200; cat. no. ab125075; Abcam). Sections were washed three times with phosphate-buffered saline (PBS; pH 7.4) and incubated with an HRP-conjugated goat anti-rabbit IgG secondary antibody (dilution 1:500; cat. no. ab6721; Abcam) for 30 min at room temperature. Following secondary antibody incubation, the sections were washed again with PBS. The tissues were then counterstained with hematoxylin for 1–2 min at room temperature. Images were captured under an optical microscope and analyzed with ImageJ software (v1.8, National Institutes of Health).

TUNEL assay. The kidney tissue samples were fixed in 4% paraformaldehyde at room temperature for 24–48 h prior to paraffin embedding. Paraffin-embedded sections (5 μm) were deparaffinized and rehydrated as aforementioned, followed by antigen retrieval with citrate buffer (pH 6.0) at 95°C for 20 min. The tissue sections were then incubated with a TUNEL reaction mixture (One Step TUNEL Apoptosis Assay Kit, Beyotime, C1089) for 1 h at 37°C. Subsequently, the sections were counterstaining with hematoxylin (0.1% w/v, Sigma-Aldrich, H9627) for 1–2 min at room temperature, and mounted with antifade mounting medium (Beyotime, P0126). Finally, to assess cell apoptosis, images from five randomly selected fields of view/section were captured under a light microscope.

RNA-seq analysis. Total RNA was extracted using a TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc., cat. no. 15596026). RNA integrity and concentration were determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.). Subsequently, for library preparation, high-quality RNA samples (RNA integrity number ≥ 7.0) were further analyzed using the NEBNext Ultra RNA Library Prep Kit (New England BioLabs, Inc., cat. no. E7530L). The final libraries were quantified using the Qubit™ dsDNA HS Assay kit (Invitrogen; Thermo Fisher Scientific, Inc., cat. no. Q32851) and diluted to a loading concentration of 1.8 nM. Paired-end sequencing (2x150 bp) was performed on the Illumina NovaSeq 6000 platform with the NovaSeq 6000 S4 Reagent kit (300 cycles; Illumina, Inc., cat. no. 20028312). Then the data were processed and analyzed using Fastp (v0.23.2, github.com/OpenGene/fastp). High-quality reads were then aligned to the reference genome using HISAT2 (v2.2.1, <https://daehwan-kimlab.github.io/hisat2/>), followed by gene read counting using FeatureCounts (v2.0.3, <https://subread.sourceforge.net/>). Differential expression analysis was ultimately performed using the DESeq2 (v1.40.2, <https://bioconductor.org/packages/release/bioc/html/DESeq2.html>) software.

Candidate gene selection. A panel of candidate genes, including cytokine signaling 6 (SOCS6), mesothelin (MSLN), glutathione-specific γ -glutamylcyclotransferase 1 (CHAC1) and C-C motif chemokine ligand 4 (CCL4), was preselected for initial screening based on their previously reported associations with drug-induced organ injury, cell stress response,

inflammation or immune regulation pathways (14–17). CCL4 was prioritized for detailed reporting in the present study because it demonstrated the most consistent and significant dysregulation trends in our preliminary assessments (data not shown).

ELISA. The serum levels of TNF- α , IL-6 and IL-1 β were quantified according to the manufacturer's instructions using Mouse TNF- α ELISA Kit (Order NO. D721217, Sangon Biotech, Shanghai, China), Mouse IL-6 ELISA Kit (Order NO. D721022, Sangon Biotech, Shanghai), and Mouse IL-1 β ELISA kit (Order NO. D721017, Sangon Biotech, Shanghai, China).

Western blot analysis. Total proteins were extracted from kidney tissue using RIPA lysis buffer (Beyotime Institute of Biotechnology), supplemented with protease and phosphatase inhibitors. Protein concentration was measured using a BCA protein assay kit (Thermo Fisher Scientific, Inc.). Equal amounts of protein extracts (50 μ g per lane) were separated by 10% SDS-PAGE, depending on the molecular weight of each target protein, and were then transferred onto PVDF membranes (MilliporeSigma). Following blocking with 5% non-fat milk in TBS-Tween-20 for 1 h at room temperature, the membranes were incubated at 4°C overnight with primary antibodies against NGAL (rabbit; dilution, 1:5,000; cat. no. ab125075; Abcam), C-C motif chemokine ligand 4 (CCL4; rabbit; dilution, 1:800; cat. no. 26614-1-AP; Proteintech Group, Inc.), phosphorylated (p)-protein kinase B (p-Akt; rabbit; dilution, 1:3,000; cat. no. 28731-1-AP; Proteintech Group, Inc.), Akt (rabbit; dilution, 1:5,000; cat. no. 10176-2-AP; Proteintech Group, Inc.), NF- κ B p65 (Ser536) (rabbit; dilution, 1:5,000; cat. no. 80379-2-RR; Proteintech Group, Inc.), NF- κ B p65 (rabbit; dilution, 1:3,000; cat. no. 10745-1-AP; Proteintech Group, Inc.), IL-6 (mouse; dilution, 1:3,000; cat. no. 66146-1-Ig; Proteintech Group, Inc.), IL-1 β (rabbit; dilution, 1:1,000; cat. no. 26048-1-AP; Proteintech Group, Inc.), TNF- α (rabbit; dilution, 1:1,000; cat. no. 17590-1-AP; Proteintech Group, Inc.), Bax (rabbit; dilution, 1:8,000; cat. no. 50599-2-Ig; Proteintech Group, Inc.) and Bcl-2 (mouse; dilution, 1:1,000; cat. no. 26593-1-AP; Proteintech Group, Inc.). Membranes were washed three times with TBST (0.05% Tween-20), and incubated with HRP-conjugated goat anti-rabbit (cat. no. SA00001-2) or goat anti-mouse (1:5,000; cat. no. SA00001-1; Proteintech) secondary antibodies (dilution, 1:5,000; Proteintech Group, Inc.) for 1 h at room temperature. GAPDH (rabbit; dilution 1:20,000; cat. no. 10494-1-AP; Proteintech Group, Inc.) and β -actin (rabbit; dilution, 1:20,000; cat. no. 66009-1-Ig; Proteintech Group, Inc.) served as internal loading controls. The proteins were visualized using the WesternBright® ECL detection kit (Advansta Inc.) combined with the ChemiDoc XRS+ imaging system (Bio-Rad Laboratories, Inc.). The signal intensity of each band was subsequently quantified using ImageLab software v6.1 (Bio-Rad Laboratories, Inc.).

Statistical analysis. All data are expressed as the mean $\pm$ SD. All statistical analyses were performed using SPSS 22.0 (IBM Corp.). The differences between two groups were compared by unpaired Student's t-test, while those among multiple groups were compared by one-way ANOVA. For multiple comparisons

following ANOVA, Bonferroni correction was applied. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

ACOP induces AKI in mice. To evaluate whether ACOP treatment induces kidney injury in mice, a mouse model of ACOP exposure was established, and blood samples were collected at 1, 3 and 7 days following CO exposure to assess blood CO levels, renal function parameters and renal histopathology (Fig. 1A). The results demonstrated that blood CO levels were markedly elevated in ACOP-treated mice compared with control mice, thus verifying the successful establishment of the mouse model (Fig. 1B). In addition, SCR and BUN levels were markedly increased at all time points following ACOP treatment compared with the control group, thus indicating substantial renal dysfunction (Fig. 1C and D). Histological analysis, using H&E and PAS staining, revealed that the glomerular structure remained intact, although mild mesangial matrix expansion and prominent tubular injury were observed in the ACOP group (Fig. 1E). On day 1 post-ACOP exposure, renal tubular epithelial cells displayed marked swelling, peritubular capillary congestion, cytoplasmic dissolution and nuclear loss (red arrows). On day 3, severe vacuolar degeneration, tubular dilation, brush border disruption and extensive nuclear detachment were evident (yellow arrows). However, on day 7, partial tubular regeneration was observed, although vacuolar changes, tubular dilation and brush border damage persisted (blue arrows). To assess tubular injury, the tubular injury scoring method was applied (18), which showed a significant increase in injury scores in ACOP treated mice compared with control mice, with the highest injury scores observed on day 3. This finding was consistent with the morphological one (Fig. 1F). In addition, the expression levels of NGAL, an early biomarker of AKI, were detected. Immunohistochemical staining demonstrated markedly widespread NGAL expression in renal tubules in mice in the ACOP group compared with the control group (Fig. 1E and G). Western blot analysis further verified that NGAL was markedly upregulated in the ACOP group compared with the control group; this was most noticeable on days 1 and 3, followed by a decrease on day 7 (Fig. 1H and I). Collectively, the aforementioned results suggested that exposure of mice to ACOP could successfully induce AKI.

DEGs and signaling pathways in ACOP-AKI. To further explore the molecular mechanism of ACOP-AKI, RNA-seq analysis was carried out on the kidney tissue samples from the ACOP model and control groups on day 3, identified as the peak of kidney injury. Based on the analysis of differential gene expression, the top 20 DEGs with the highest average expression levels were presented in Table S1, including $\log_2$ (fold change) and P-value. The volcano plot in Fig. 2A depicts the distribution of DEGs, revealing significant up- and downregulation trends, whereas the heatmap in Fig. 2B focuses on genes functionally linked to the PI3K/Akt pathway. A KEGG pathway enrichment analysis of DEGs revealed that the 'PI3K/Akt signaling pathway' was markedly enriched in the ACOP group (Fig. 2C).

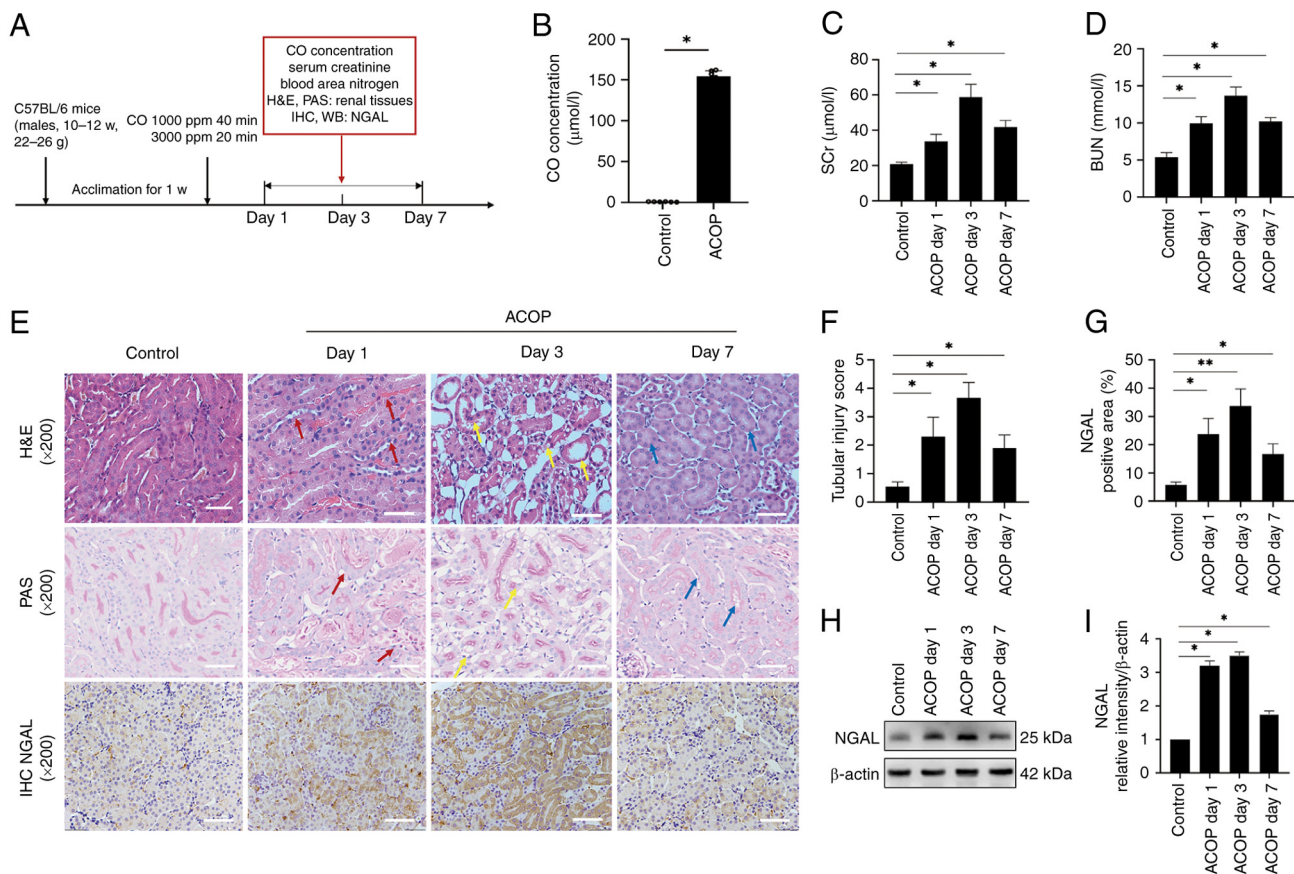


Figure 1. ACOP induces acute kidney injury in mice. (A) Schematic illustration of the ACOP mouse model and experimental design. (B) Bar graph showing CO concentrations in the blood of mice in the control and ACOP groups. (C) SCr and (D) BUN levels at different time points after ACOP exposure are shown. (E) Representative H&E, PAS and NGAL immunohistochemical staining of kidney tissues at different time points after mice exposure to ACOP exposure. On day 1, tubular epithelial cells displayed marked swelling, peritubular capillary congestion and cytoplasmic dissolution with nuclear loss, as indicated by the red arrows. On day 3, extensive vacuolar degeneration, tubular dilation, brush border disruption and nuclear shedding were observed (yellow arrows). By day 7, signs of tubular regeneration, with persisted vacuolar degeneration, tubular dilation and brush border damage (blue arrows), were observed. Scale bar, 100 μm. (F) Quantification of tubular injury scoring. (G) Quantification of NGAL protein expression in renal tissue detected by immunohistochemistry. (H) Expression of NGAL based on immunoblot bands. (I) Semi-quantitation of NGAL expression. Data are expressed as the mean ± SD (n=6). *P<0.05 and **P<0.01 vs. control. ACOP, acute carbon monoxide poisoning; NGAL, neutrophil gelatinase-associated lipocalin; SCr, serum creatinine; BUN, blood urea nitrogen; CO, carbon monoxide; w, week; ppm, parts per million; H&E, Hematoxylin and Eosin; PAS, periodic acid-Schiff; IHC, immunohistochemistry; WB, western blot analysis.

ACOP upregulates *CCL4* expression in renal tissue, activates the *PI3K/Akt* pathway and elevates renal cell apoptosis and systemic inflammatory cytokine levels. Through candidate gene screening and validation, *CCL4* exhibited consistent patterns at both the transcriptional and protein levels in the present model (data not shown), indicating its potential role in the mechanisms underlying ACOP-AKI. In addition, the present study observed that in ACOP-exposed mice, the p-Akt/Akt ratio and Bax protein levels were markedly increased, whereas Bcl-2 expression was markedly decreased when compared with control mice (Fig. 3A). TUNEL staining assays further revealed that the number of TUNEL-positive cells was notably increased in renal tissues of mice in the ACOP group compared with the control group. Merged images demonstrated co-localization of TUNEL signals with DAPI-stained nuclei, confirming the induction of cellular apoptosis in the renal tissue following ACOP injury. Furthermore, the apoptosis appeared to be predominantly localized within the renal tubule (Fig. 3B). Furthermore, the ELISA results demonstrated markedly elevated serum levels of the systemic pro-inflammatory cytokines *TNF-α*, *IL-6* and *IL-1β* in the

ACOP group compared with the control group (Fig. 3C). The aforementioned data indicated that ACOP may have upregulated the expression of *CCL4*, accompanied by activation of the *PI3K/Akt* signaling pathway. Furthermore, ACOP induced significant inflammatory responses and notable apoptosis.

LY294002 attenuates ACOP-AKI. To investigate the role of the *PI3K/Akt* signaling pathway in ACOP-AKI, mice were treated with the *PI3K* inhibitor LY294002, and renal function and histopathological changes were evaluated on day 3 (Fig. 4A). Biochemical analysis showed that mice in the ACOP group exhibited markedly elevated serum levels of SCr and BUN compared with the control group. In addition, LY294002 treatment partially yet markedly ameliorated these increased serum levels (Fig. 4B and C). Morphological assessment revealed pronounced tubular damage in the kidneys of ACOP-treated mice, which was markedly alleviated in the ACOP + LY294002 group (Fig. 4D). Consistently, the tubular injury score was markedly lower in the ACOP + LY294002 group compared with the ACOP group (Fig. 4E). Immunohistochemical analysis demonstrated a significant increase in NGAL expression in the ACOP

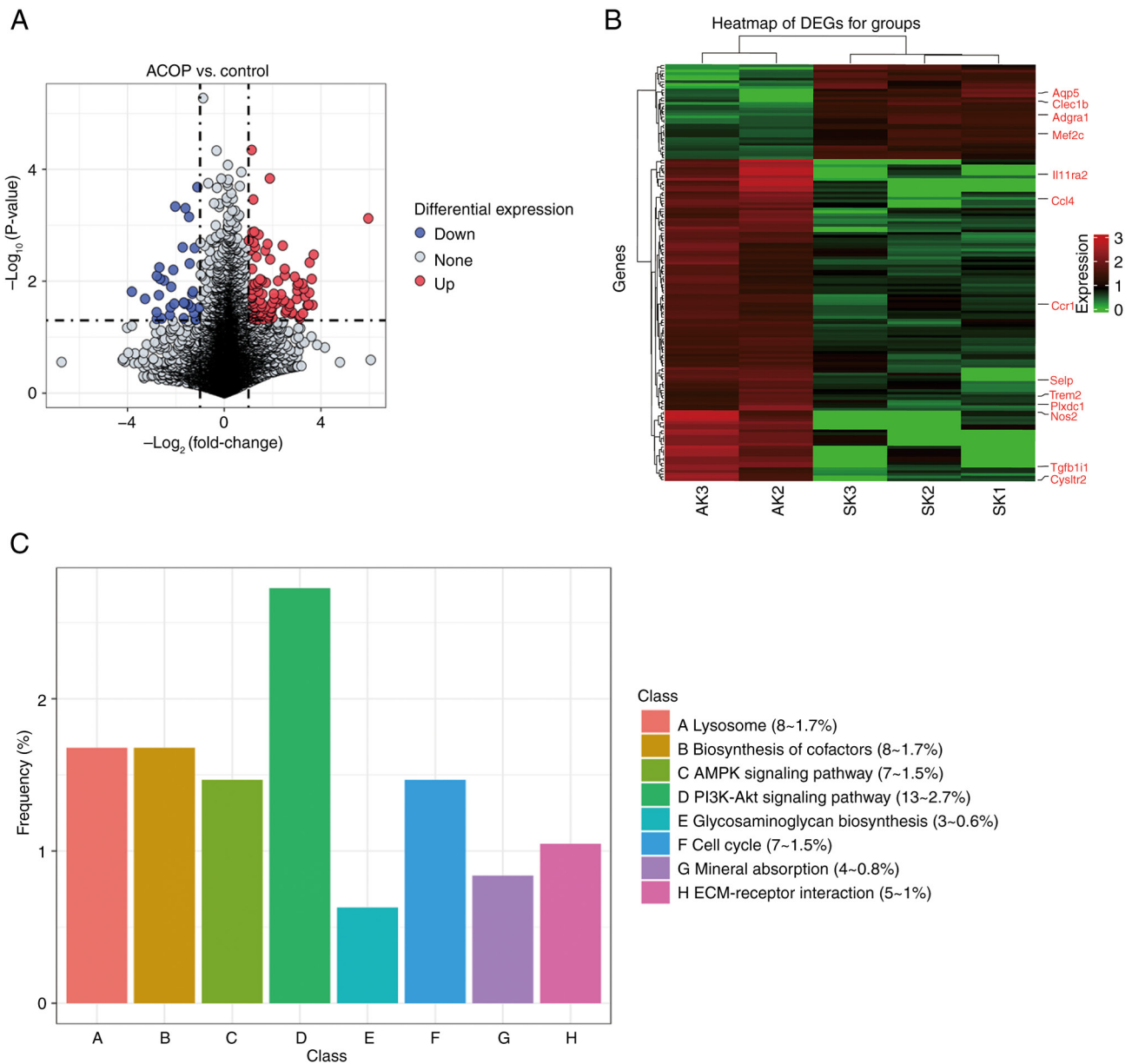


Figure 2. C-C motif chemokine ligand 4 is upregulated in ACOP-treated mice via the PI3K/Akt signaling pathway. (A) Volcano plots showing identified DEGs. Red and blue dots indicate markedly upregulated and downregulated genes, respectively, while gray dots represent genes that do not meet the differential expression criteria. (B) Heatmap of DEGs. Red and green indicate the upregulated and downregulated genes, respectively. (C) Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis of DEGs. DEG, differentially expressed genes; ACOP, acute carbon monoxide poisoning; AMPK, AMP-activated protein kinase; ECM, extracellular matrix; AK, acute kidney; SK, sham kidney Aqp5, aquaporin 5; Clec1b, C-type lectin domain family 1 member B; Adgra1, adhesion G protein-coupled receptor A1; Mef2c, myocyte enhancer factor 2C; Il11ra2, interleukin 11 receptor subunit $\alpha 2$; Ccl4, C-C motif chemokine ligand 4; Ccr1, C-C motif chemokine receptor 1; Selp, selectin P; Trem2, triggering receptor expressed on myeloid cells 2; Plxdc1, plexin domain containing 1; Nos2, nitric oxide synthase 2; Tgfb11, transforming growth factor $\beta 1$ induced transcript 1; Cyslr2, cysteinyl leukotriene receptor 2.

group compared with the control group, which was markedly reduced following treatment with LY294002 (Fig. 4F). Finally, western blot analysis was performed to assess the NGAL protein expression levels (Fig. 4G), and densitometric quantification verified that LY294002 markedly suppressed NGAL expression in the kidneys of ACOP-treated mice (Fig. 4H). These findings suggested that LY294002 could effectively mitigate ACOP-AKI.

LY294002 inhibits inflammation and apoptosis in the kidneys of ACOP mice. To further investigate the molecular mechanisms underlying the effects of LY294002 on ACOP-AKI, western blot analysis was employed to detect the expression

levels of inflammatory factors and apoptosis-related proteins. The results showed that the ratio of p-Akt/Akt was markedly elevated in the ACOP-treated group, compared with the control, accompanied by a significant upregulation of inflammatory factors such as IL-6, TNF- α and IL-1 β . By contrast, treatment with LY294002 markedly reduced the p-Akt/Akt ratio and the protein expression levels of IL-6, IL-1 β and TNF- α compared with the ACOP group, suggesting an inhibitory effect of LY294002 on the expression of pro-inflammatory cytokines.

Considering that NF- κ B is a key mediator of pro-inflammatory cytokine expression, the present study further assessed the ratio of NF- κ B p65 (Ser536)/NF- κ B p65. The

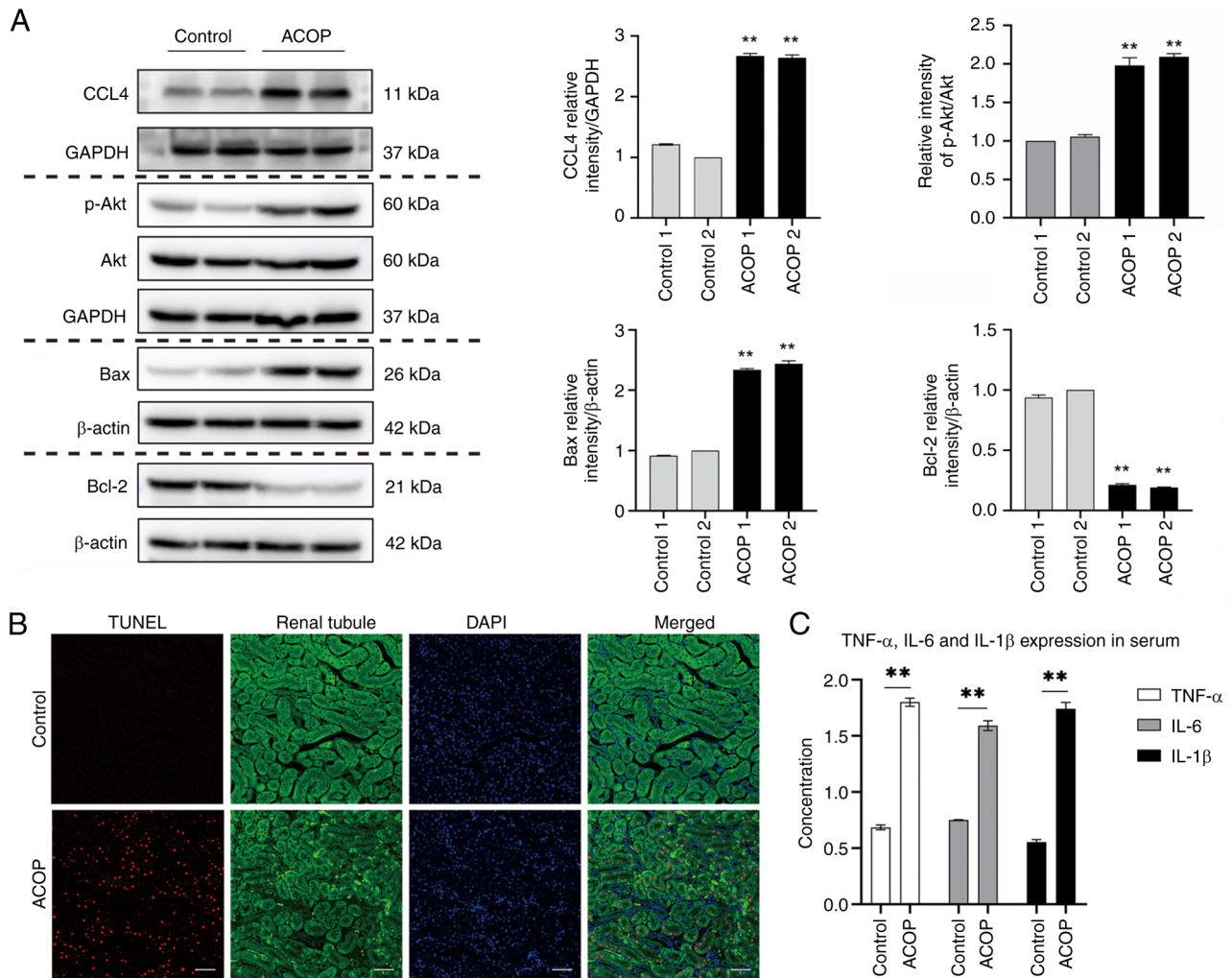


Figure 3. ACOP induces apoptosis in kidney tissues and triggers systemic inflammatory responses in mice. (A) Western blot analysis was performed to determine the expression levels of CCL4, p-Akt/Akt, Bax and Bcl-2 in renal tissues from the control and model groups. Statistical evaluation was also performed. (B) Apoptosis in kidney tissues from mice in the control and model groups was assessed by TUNEL staining. (C) Serum levels of the inflammatory cytokines TNF- α , IL-6 and IL-1 β in mice were measured using ELISA. Data are expressed as the mean $\pm$ SD (n=3). **P<0.01 vs. control. CCL4, C-C motif chemokine ligand 4; ACOP, acute carbon monoxide poisoning; p-, phosphorylated.

results revealed that treatment with LY294002 also markedly inhibited the elevation of this ratio in the kidneys of ACOP-treated mice. Furthermore, ACOP treatment induced a significant increase in the expression of the pro-apoptotic protein Bax and a significant decrease in the expression of the anti-apoptotic protein Bcl-2 when compared with the control group. However, LY294002 treatment markedly suppressed ACOP-induced Bax expression and restored Bcl-2 levels (Fig. 5). In conclusion, LY294002 effectively suppressed inflammation and apoptosis in the kidneys of ACOP-treated mice by modulating key signaling pathways such as PI3K/Akt and NF- κ B, providing a potential pharmacological target for the treatment of ACOP-AKI.

Discussion

ACOP is one of the most common causes of poisoning globally and can induce AKI, potentially leading to CKD in severe cases (19,20). In the present study, the successful establishment of the ACOP mouse model was verified via

measuring the serum levels of CO. Renal dysfunction was observed on the first day after exposure of mice to ACOP, which was characterized by elevated SCr and BUN levels, accompanied by hallmark histopathological alterations, including tubular dilatation, epithelial cell detachment, inflammatory infiltration and aberrant NGAL expression. A clinical study reported that the median time to the first diagnosis of ACOP-AKI is one day after Emergency Department (ED) admission, with the majority of patients being diagnosed at their highest KDIGO stage within three days of ED admission (3). Notably, renal injury reached its peak on day 3 and exhibited partial recovery by day 7, which was consistent with clinical observations (3). While individuals with stage I and II AKI typically achieved a full recovery, up to 55.6% of those with stage III AKI progressed to CKD (3,21). The underlying mechanisms of ACOP-AKI to CKD may involve a burst of reactive oxygen species (ROS) production and the activation of downstream signal cascades. ROS not only directly damage cellular components but also trigger sustained tissue injury via promoting inflammatory

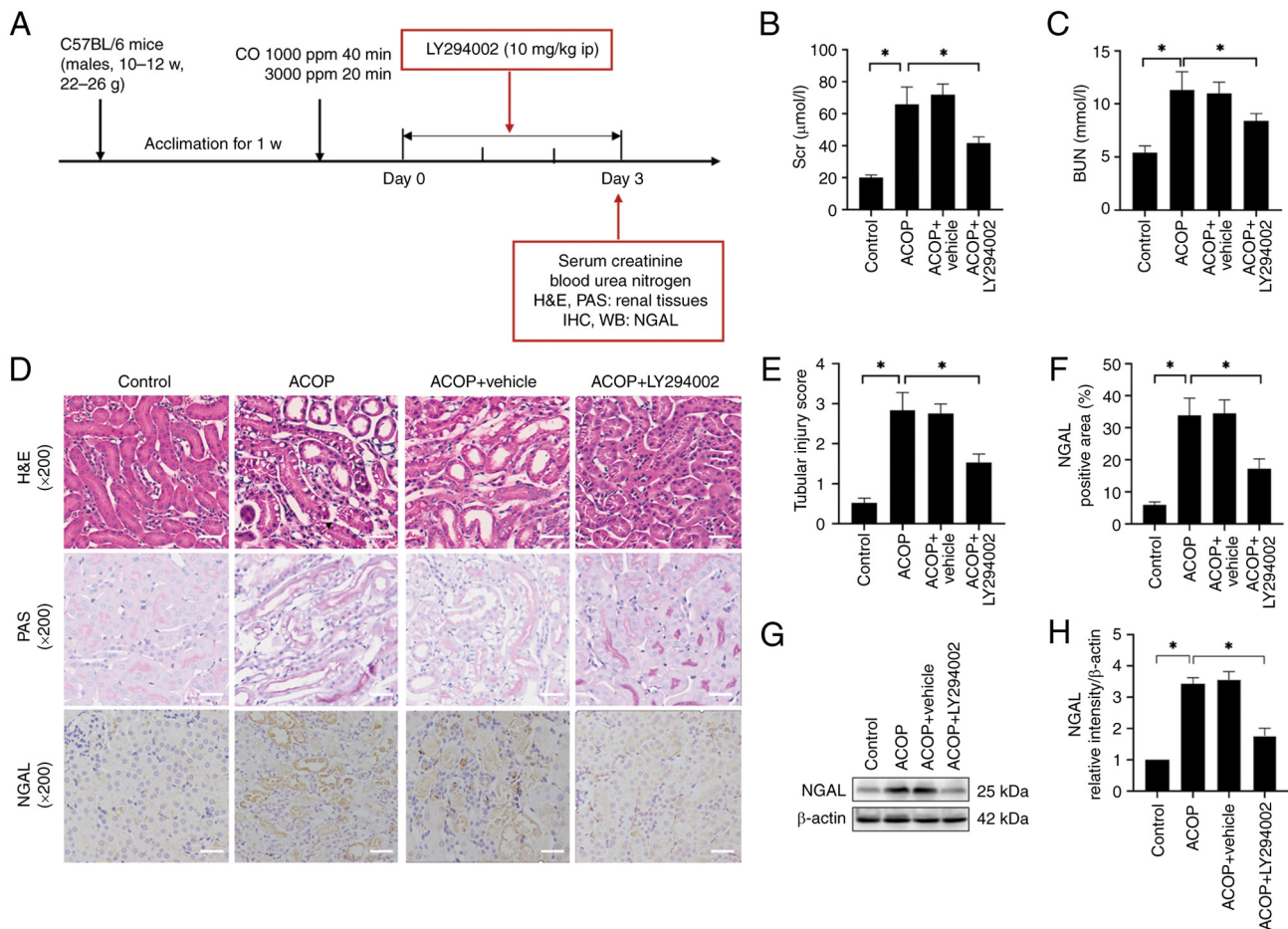


Figure 4. Effects of LY294002 on kidney injury in mice exposed to ACOP. (A) Schematic representation of the establishment of the ACOP + LY294002 mouse model and experimental design. The experiment was conducted on day 3 after ACOP exposure, when the injury was most severe. Blood and tissue samples were subsequently collected for serological, morphological and protein expression analyses. (B) SCr and (C) BUN levels in control, ACOP, vehicle-treated ACOP control and LY294002-treated ACOP groups are shown. (D) Representative H&E, PAS and NGAL immunohistochemical staining images of kidney tissues in different groups are shown. Scale bar, 100 μm. (E) Tubular injury scoring is presented. (F) Quantification of NGAL protein expression in renal tissue detected by immunohistochemistry. (G) Expression of NGAL based on immunoblot bands. (H) Semi-quantitation of NGAL expression. Data are expressed as the mean ± SD (n=3). *P<0.05 vs. control. ACOP, acute carbon monoxide poisoning; NGAL, neutrophil gelatinase-associated lipocalin; SCr, serum creatinine; BUN, blood urea nitrogen; CO, carbon monoxide; w, week; ppm, parts per million; PAS, periodic acid-Schiff; IHC, immunohistochemistry; WB, western blot analysis; ip, intraperitoneal.

responses and apoptotic signaling, potentially forming the pathological basis for long-term sequelae of ACOP (22). Histological analyses further revealed that metabolically active renal tubular epithelial cells were the primary targets of injury, whereas glomerular structures remained relatively intact. This may be attributed to the higher oxygen demand and greater susceptibility to oxidative stress of tubular epithelial cells (23,24). This observation was consistent with the clinical manifestations of patients with ACOP-AKI, who primarily present with oliguria, proteinuria, urinary casts, and disturbances in water and electrolyte balance (25).

The mechanisms underlying ACOP-AKI are complex, involving CO-mediated tissue hypoxia and ischemia, mitochondrial dysfunction, oxidative stress and inflammation. However, the underlying molecular mechanisms remain yet to be fully elucidated (2,23). Among the aforementioned mechanisms, inflammation was considered to be closely related to ACOP-AKI. In the present study, RNA-seq analysis of renal tissues from mice with ACOP-AKI revealed a significant upregulation of multiple inflammation-related genes. During

the initial phase of the present study, candidate genes were preselected, such as SOCS6, MSLN, CHAC1 and CCL4, for further investigation based on prior research. Notably, CCL4 exhibited consistent trends at both the transcriptional and protein levels in the present model, suggesting its potential role in the mechanisms underlying ACOP-AKI. While we are actively exploring the functional roles of other key candidate genes, further evidence is required to substantiate these findings. Given the novelty of CCL4 in ACOP-AKI, we prioritized reporting these results. Furthermore, KEGG pathway enrichment analysis indicated that the PI3K/Akt signaling pathway was markedly activated, thus suggesting that this signaling axis could serve an important role in the pathogenesis of ACOP-AKI.

In the present study, the RNA-seq results were further verified at the protein and functional levels by western blot analysis. The results demonstrated that in ACOP-treated mice, the expression levels of CCL4 and the p-Akt/Akt ratio were markedly increased compared with the control group. As a chemotactic factor, CCL4 is important for immune

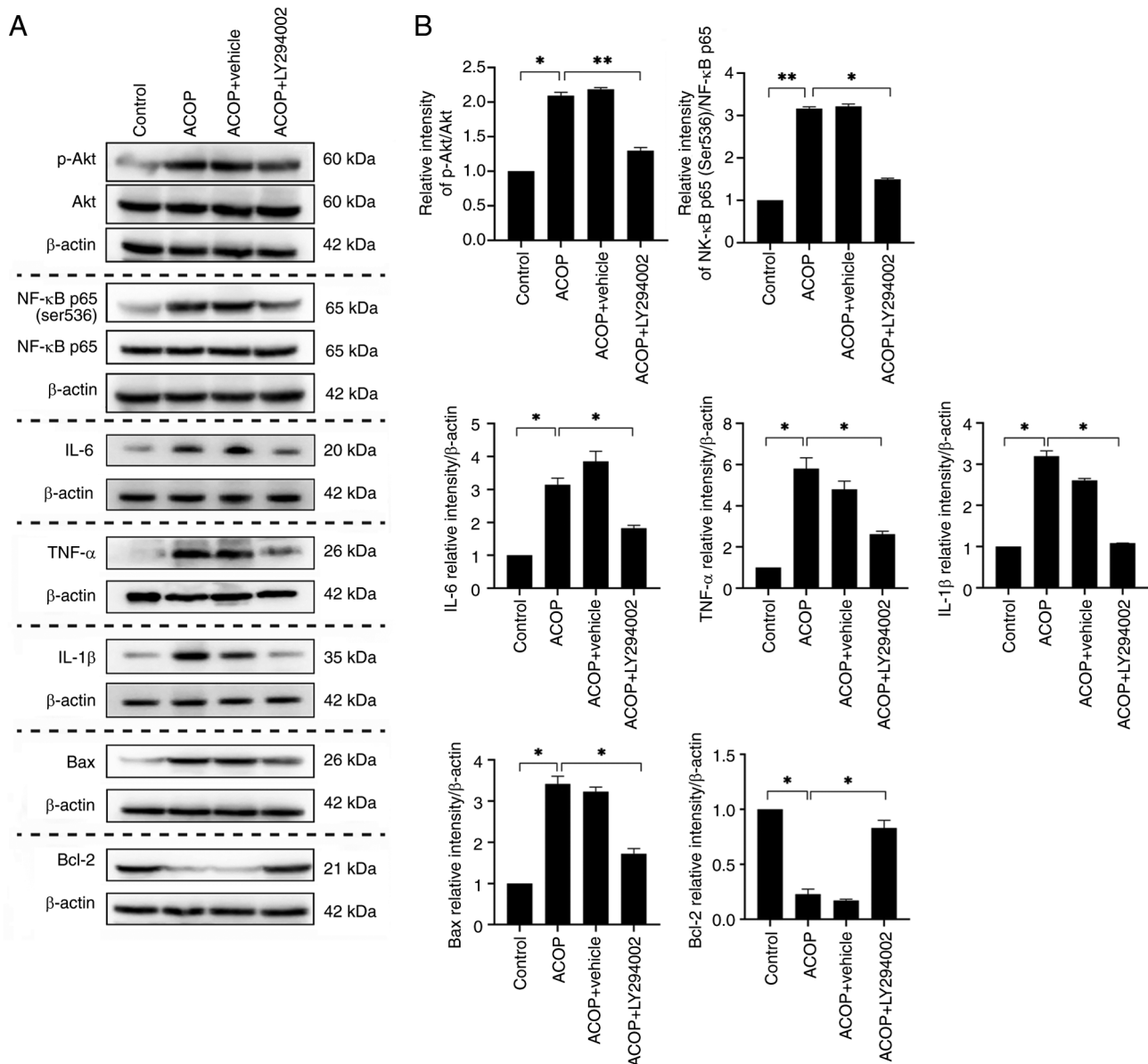


Figure 5. Treatment with LY294002 inhibits inflammation and apoptosis in the kidneys of ACOP mice. (A) The protein expression levels of p-Akt/Akt, NF- κ B p65 (Ser536)/NF- κ B p65, IL-6, TNF- α , IL-1 β , Bax and Bcl-2 were detected in kidney tissues of mice in the control, ACOP, vehicle-treated ACOP control and LY294002-treated ACOP groups by western blot analysis. (B) The quantified protein expression levels of (A) are shown. Data are expressed as the mean $\pm$ SD (n=3). *P<0.05 and **P<0.01 vs. control. ACOP, acute carbon monoxide poisoning; p-, phosphorylated.

cell recruitment and initiates local immune responses, thus promoting inflammation (26). The PI3K/Akt signaling pathway is a notable axis in cell growth, survival and stress responses, and its activation can enhance cell survival signals and inhibit apoptosis. However, chronic or excessive PI3K/Akt signaling activation can result in an amplified inflammatory response and promote apoptosis (27). The present study found that renal tubular cell apoptosis was accelerated in ACOP-treated mice, accompanied by a significant increase in Bax and decrease in Bcl-2 expression. In addition, the serum levels of TNF- α , IL-6 and IL-1 β were also markedly elevated, thus indicating that a systemic inflammatory response was activated. These findings supported the involvement of CCL4 upregulation and activation of the PI3K/Akt pathway in the process of ACOP-AKI, which may play a role in promoting the inflammatory response and apoptosis.

To further verify the functional involvement of the PI3K/Akt pathway, mice in the ACOP group were treated with the LY294002 PI3K inhibitor (28). The results demonstrated that LY294002 markedly improved renal dysfunction, as evidenced by the reduced SCr and BUN levels, and improved the morphological integrity of the renal tubular structures. Immunohistochemical and western blot analyses further revealed a significant decrease in the expression levels of NGAL in the LY294002-treated group compared with the ACOP model group, thus providing additional evidence for the nephroprotective effects of PI3K inhibition.

As a key regulatory factor in inflammatory responses, PI3K has emerged as a potential treatment target for inflammatory diseases (29). Previous studies showed that LY294002 could inhibit the PI3K/Akt pathway, eventually downregulating NF- κ B and pro-inflammatory factors, such as TNF- α and

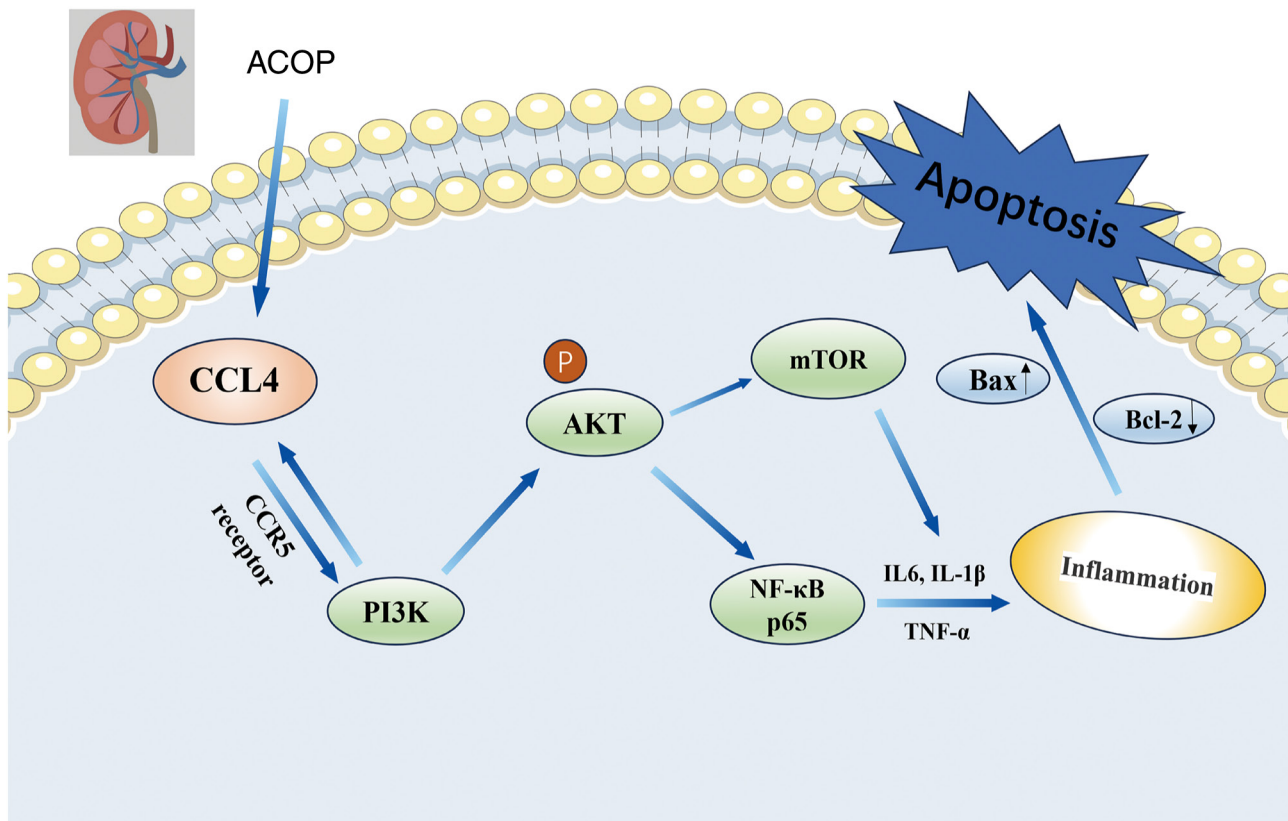


Figure 6. Schematic illustration depicting how the inflammatory response and apoptosis collectively contribute to kidney injury, and the underlying mechanisms following exposure to ACOP. ACOP, acute carbon monoxide poisoning; CCL4, C-C motif chemokine ligand 4; CCR5, C-C chemokine receptor type 5.

IL-1 β , in the spinal cords of bone cancer pain-model rats (30). NF- κ B is a key transcription factor, which is notably involved in several inflammatory reactions. The NF- κ B-induced release of downstream inflammatory mediators not only enhances the recruitment of immune cells but can also lead to cell damage and apoptosis through various mechanisms, such as generating excessive oxidative stress or directly activating apoptotic pathways (31,32). In particular, TNF- α and IL-1 β are considered significant pro-apoptotic factors.

In the present study, the activation of the PI3K/Akt pathway was associated with the phosphorylation of NF- κ B p65 (Ser 536), which may enhance its transcriptional activity and promote the expression of pro-inflammatory mediators, including TNF- α , IL-6 and IL-1 β . These inflammatory mediators further promoted the expression of the pro-apoptotic protein Bax, while reducing the expression levels of the anti-apoptotic protein Bcl-2, eventually aggravating renal cell apoptosis. This mechanism was reversed by LY294002 treatment. The aforementioned mechanism was consistent with that reported in a previous study, suggesting that NF- κ B-mediated inflammation could also be involved in CO poisoning-induced neurological injury (33). However, PI3K/Akt may also have affected apoptosis via additional downstream targets, such as mammalian target of rapamycin or forkhead box O. Nevertheless, further studies are needed to verify the involvement of these pathways.

However, the present study had some limitations. Previous studies have demonstrated that CCL4 can activate a variety of downstream signaling pathways, including the

PI3K/Akt pathway (34), and induce the phosphorylation of NF- κ B p65 via its receptor C-C chemokine receptor type (CCR)5 (35). This process promotes cell senescence and dysfunction via increasing ROS production and activating inflammatory responses (17). Although the results of RNA-seq showed that CCL4 was markedly upregulated at the transcription level and was closely associated with the activation of the PI3K/Akt pathway, the particular role of CCL4 in ACOP-AKI and its causal association with the PI3K/Akt pathway should be further verified. Functional experiments, such as gene knockout or antibody-mediated neutralization are necessary to validate this association. In addition, other differentially expressed inflammation-related genes identified through RNA-seq analysis, such as MSLN, SOCS6, CHAC1, CCR1, nitric oxide synthase 2 and P-selectin, could also interact with the PI3K/Akt axis, warranting further exploration into their molecular interaction mechanisms. Furthermore, clinical data correlating the CCL4/Akt axis with the severity of AKI in patients with ACOP remains lacking. Therefore, future studies exploring this potential association should be performed for improved understanding of its clinical relevance.

In conclusion, the present study demonstrated that during ACOP, CCL4 expression and the PI3K/Akt pathway were upregulated, resulting in renal dysfunction and promoting inflammatory responses and apoptosis. Additionally, LY294002-induced inhibition of the PI3K/Akt pathway attenuated NF- κ B-mediated inflammatory signaling, thus alleviating AKI in ACOP mice via suppressing

inflammatory responses and cell apoptosis (Fig. 6). To the best of our knowledge, the present study was the first study to utilize RNA-seq technology to identify abnormal gene expression and changes in signaling pathways in the renal tissues of ACOP-exposed mice. These findings could provide important experimental evidence and theoretical insights for further elucidating the molecular mechanisms underlying ACOP-AKI.

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Availability of data and materials

The data generated in the present study may be found in the Sequence Read Archive under accession number PRJNA1234475 or at the following URL: <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1234475>.

Authors' contributions

YYY and QL conceived and designed the study. YN, XHJ, SHW, TP and TTY analyzed and interpreted data. YN, XHJ and SHW improved the quality of the figures. XHJ, SHW, TTY and YN drafted the manuscript. TP, AYY and QL revised the manuscript. YN and QL confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

All experimental protocols were approved by the Animal Care Committee of Zunyi Medical University (approval no. zyfy-an-2024-0710/0358) and supervised by the Ethics Committee of the Affiliated Hospital of Zunyi Medical University. All procedures adhered to China's guidelines for animal research, ensuring ethical standards were met by minimizing animal usage and alleviating potential distress.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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