PICK1 mediates reactive oxygen species release and apoptosis in endotoxin-induced acute lung injury through interacting with PKCα and Ca 2+ /Calmodulin-activated Kinase II (CaMKII)

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Research Article

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Abstract

Background: The pathophysiological mechanism of sepsis-related acute lung injury, which leads to a major cause of mortality in critically ill patients remains ambiguous. Our previous study demonstrated that PICK1 played a pivotal role in regulating antioxidants (Glutathione) synthesis via affecting xCT, however, its effects on oxidants was still unknown. This study aimed to explore whether PICK1 mediates reactive oxygen species release and apoptosis in endotoxin-induced acute lung injury, thereby funding effective treatments for ALI.

Methods: In this study, male C57BL/6 mice or PICK1 knock out mice and mouse macrophage cell line, RAW264.7 were treated with LPS to establish endotoxin-induced acute lung injury model in vivo and in vitro. FSC-231 were used to disturb the interaction between PICK1 and PKCα. KN-93 were used to inhibit the activity of CaMKII. The alterations of protein PICK1, PKCα, CaMKII, p-CaMKII, BAX, Bcl-2, Cleaved-Caspase 3 were observed. Meanwhile, PICK1-PKCα complexes, PICK1-p-CaMKII complexes were detected. Cell Viability, ROS release, and cell apoptosis were examed as well.

Results: We found that in endotoxin-related ALI, the content of PICK1 and p-CaMKII were augmented, while, PKCα were activated and declined. LPS stimulated the formation of PICK1-PKCα complexes, PICK1-p-CaMKII complexes. Moreover, PICK1 knock out exacerbate ROS release and apoptosis. Either PDZ or BAR domain disruption could mimic the effects of PICK1 knock out.

Conclusions: Our results suggest that PICK1 mediates ROS release and apoptosis via interaction with PKCα and CaMKII.

Introduction

Despite intensive research, sepsis-associated acute lung injury (ALI) remains one of the top reasons of mortality in intensive care unit yearly, imposing immense health and economic burden(1, 2). The lung is the most vulnerable critical organ when organ dysfunction developed induced by dysregulation of host defense against infection, resulting in ALI and its final devastating result acute respiratory distress syndrome (ARDS)(3).

Recently, mounting evidence have demonstrated that the redox cascade during sepsis resulting in oxidative stress amplified inflammation and apoptosis, leading to severe noxious effect on organs(4).While, existed clinical antibiotic treatment cannot directly affect detrimental redox cycles, thus, oxidative stress-targeted antioxidant treatments could represent an interesting line of research in the treatment of sepsis (5).

Previously, our laboratory has shown that Protein interaction with C-kinase 1 (PICK1) played a pivotal role in sepsis-induced ALI by regulating GSH synthesis via affecting the substrate-specific subunit of lung cystine/glutamate transporter, xCT(6). In the present study, we applied lipopolysaccharide (LPS) administration to establish an endotoxin -associated acute lung injury model, meanwhile, we treated

RAW264.7 cells with LPS to mimic vitro septic condition. We aim to investigate whether PICK1 mediates radical oxygen species (ROS) release and apoptosis during the pathophysiological process of sepsis-induced ALI/ARDS and to explore the possibility of its underlying mechanism.

Materials And Methods

Animals

Male C57BL/6 mice and PICK1 KO mice (aged 8–10 weeks) were used in this study. The original breeding pairs of PICK1$^{-/-}$ mice were obtained from Ying Shen (Zhejiang University School of Medicine, Hangzhou) and maintained at the Experimental Animal Center of Wenzhou Medical University. Animals were approved by the Animal Experimentation Ethics Committee of Wenzhou Medical University (Approval No. SYXK 2010 – 0150).

Sepsis-associated acute lung injury model

C57BL/6J mice and PICK1 KO mice were intraperitoneally injected with LPS (10 mg/kg) or saline(7).After all treatment for 12h, the mice were euthanized and the lung tissues were collected and used for subsequent staining and tests.

Cell culture

The mouse monocyte/macrophage cell line RAW264.7 was incubated in DMEM with 10% FBS. PDZ domain inhibitor FSC-231 was purchased from sigma (Ref: 529531-10mg) .CaMKII inhibitor KN93 and its inactive analog KN92 was purchased from GLPBIO (Ref:GC12501-10mM, GC15988-10mM)

Cell apoptosis detection

1×10^6 cells were seeded in 6-well plates and treated with LPS for 18h, and then cells were suspended for further study. Apoptosis was determined using an Annexin-V fluorescein isothiocyanate and propidium iodide (FITC/PI) apoptosis detection kit (Life Technologies, Waltham, MA) with flow cytometry in accordance with the manufacturer's instruction. Annexin-V (+)/PI (−) and Annexin-V (+)/PI (−) represented the RAW264.7 cells in early apoptosis and late apoptosis/necrosis, respectively. Flow cytometry data was collected on a FACScan (Becton Dickinson) using CellQuest software, then analyzed using FlowJo software (version 9). Each sample was assayed in triplicate to ensure the authenticity of the experiments.

Cell Viability Assay

Cell viability was determined by MTT assay. RAW264.7 cells were cultured in 96-well plates at a density of $10^{\wedge 5}$ cells/mL and exposed to different concentrations of LPS (500 ng/mL) or 0.1% DMSO as control for 48 h and 10 μL of MTT (5 mg/mL) was added to each well and incubated for 4 h. The supernatant was discarded and the formation was resolved with 150 μL/well of DMSO. The absorbance at 570nm was measured on a microplate reader (BioTek Epoch, Winooski, VT, USA). Concentrations were determined for three wells of each sample, and each experiment was performed in triplicate.

Western blotting

The protein concentration of the supernatant was determined by the BCA protein assay. Then, equal amounts (20μg) of proteins were denatured by heating at 100°C for 5min in 1×nuPAGE loading buffer (Life Technologies, Carlsbad, NM, USA), fractionated by 10% SDS-polyacrylamide gel electrophoresis (Life Technologies), and blotted to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% nonfat milk in Tris-buffered saline with 0.05% Tween-20 (TBST) and cut into strips, which were incubated with PICK1 (Neurolab, L20/8, 1:1000), PKCα (Abcam, Ab32376, 1:1000), GAPDH (Bio-world, AP0063, 1:1000), Bcl-2 (CST, 3498s, 1:1000), BAX (Abcam, Ab32503, 1:1000), cleaved-Caspase 3(CST, 9663s, 1:1000), respectively, at 4°C overnight. After three washes with TBST for 10min, the strips were incubated with HRP-linked anti-mouse (DC-02L) or anti-rabbit IgG (DC-03L) (Calbiochem), and then followed by ECL (Amersham, RPN2106). The strips were detected using an ECL detector (Image Lab Software, Bio-Rad). The signals were scanned and quantified using Image Lab software (Bio-Rad).

Quantitative polymerase chain reaction

Total RNA was extracted from lung tissues of mice with TRIzol reagent (Invitrogen, Carlsbad, CA).The RNA concentration was measured with absorbance at a wavelength of 260 nm.Then, cDNA was synthesized by reverse transcriptase reaction with cDNA Synthesis kit (TaKaRa, Dalian, China). The resulting cDNA was subsequently amplified with the following IL-6 primers: forward 5’-ACAGGGAGAGGGAGCGATA-3’ and reverse 5’- CCAGTCCTCTTTGTTGGGGAT-3’.

IL-1β: forward 5’-TGATGGCTTATTACAGTGGCA-3’ and reverse 5’-CGGAGATTCGTAGCTGGATG-3’.

TNF-α: forward 5’-CACAGTGAAGTGCTGGCAAC-3’ and reverse 5’-GATCAAAGCTGTAGGCCCCA-3’

GAPDH : forward 5'-AAGAAGGTGGTGAAGCAG-3' and reverse 5'-AGGTGGAAGAGTGGGAGT-3'. (8)

Lung wet/dry weight ratio

Fresh lung samples were dissected and weighed immediately to obtain the wet weight. Then, lungs were placed in a drying oven at 65°C for 72 h until a constant weight was obtained. The wet to dry weight ratio was calculated to represent the degree of lung edema.(6)

Coimmunoprecipitation

Homogenized tissues and cells were collected and lysed using lysis buffer (50mM Tris, 150mM NaCl, 0.5% Nonidet-40, 5mM EDTA, 1mM PMSF (pH 8.0)) and centrifuged. The decimus supernatant was used as the input, and the remainder was used for coimmunoprecipitation (co-IP). Add 10 μL Mouse anti-PICK1 antibody and 20 μL of resuspended volume of Protein A/G PLUS-Agarose incubate at 4°C on a rocker platform or rotating device for 1 hour to overnight. Collect immunoprecipitates by centrifugation and wash pellet 4 times with 1.0 mL RIPA buffer, each time repeating centrifugation step above. After final wash, aspirate and discard supernatant and resuspend pellet in 40 μL of 2×nuPAGE loading buffer (Life

Technologies, Carlsbad, NM, USA) and boiled for 5 minutes at 100°C.The samples were then run on SDSPAGE gels and transferred to PVDF membrane for Western blotting with the indicated antibodies.

Flow cytometry assay for ROS

RAW264.7 cells were seeded at a density of 2 ×10^5/well in 12-well cell culture plates. Following FSC-231 (50 μM) pre-treatment at 37°C, the cells were stimulated with LPS (500 ng/mL) for 18 hours, and intracellular total ROS was detected using a Reactive Oxygen Species Assay kit (Shanghai Beyotime Bio-Tech Co., Ltd.) according to the manufacturer's protocol. Briefly, following treatment, the cell culture medium was removed, and dichloro-dihydro-fluorescein diacetate (DCFH-DA) was added to a final concentration of 10 μM. Then, the cells were incubated in a CO_2 incubator for 20 min at 37°C and washed 3 times with PBS to completely remove the DCFH-DA from the cells. RAW264.7 cells were collected by centrifugation, aspirating and discarding supernatant and resuspending with PBS. DCF fluorescence intensities were detected by flow cytometry or a multi-detection reader at excitation and emission wavelengths of 485nm and 535 nm, respectively. Each sample was assayed in triplicate to ensure the authenticity of the experiments.

Lung superoxide measurement

Superoxide production was evaluated by lucigenin chemiluminescence. Briefly, lungs were homogenized in Krebs-Hepes buffer and centrifuged at 4500 g for 15 min. The supernatants were transferred to scintillation vials containing 5 mM lucigenin (Sigma) in the same buffer, and incubated in the dark for 10 min at 37°C. The chemiluminescence was measured by a luminometer (AB-2200 LuminescencerPSN, ATTO Co., Nagoya, Japan) at 1-min intervals over a 5-min period.Data are expressed as relative light units (RLU) per minute per milligram protein.(6)

Statistical analysis

All measured data in this experiment were expressed as Mean ± SEM.GraphPad Pro Prism 7.00 software was used for statistical analysis. The comparison between the two groups was performed by Student's t test, and the differences between the groups were compared by one-way ANOVA and corrected by Bonferroni.$P < 0.05$ was considered statistically significant.

Results

Elevated PICK1 levels was accompanied by consumption of PKCα and phosphorylation of CaMKII in endotoxin-induced lung injury model in vivo and in vitro

In previous study, we have identified that PICK1 played a pivotal role in lung oxidative stress defense in sepsis-associated ALI by maintaining glutathione homeostasis via xCT(6). In mice cecal ligation and puncture model, we previously observed that the PICK1 expression levels in septic lung tissue were significantly increased, which was accompanied with the depletion of PKCα protein, however, whether there is an interaction between PICK1 and PKCα has not been deeply explored. In this study, male

C57BL/6 mice were subjected to LPS injection intraperitoneally, and then, lung tissues were collected for further study after 12 hours. Twelve hours after LPS treatment, the level of PICK1 in LPS group was found to have increment in contrast to that in the sham group (Fig. 1E), while, the PKCα was declined at 12 hours after LPS stimulation (Fig. 1F). In addition, we explored the phosphorylation of CaMKII and the levels of CaMKII. A significant increment of CaMKII and p-CaMKII were found in mice subjected to LPS (Fig. 1G&H). Collectively, RAW264.7 cells were treated with LPS to mimic the septic shock condition in vitro. Next, the alteration of levels of PICK1, PKCα, CaMKII and phosphorylation of CaMKII were detected at 9 (early septic), and 18 (late septic) hours after LPS treatment. LPS stimulation increased the expression of PICK1 (Fig. 1A) and the phosphorylation of CaMKII (Fig. 1C). Meanwhile, the depletion of PKCα, which representing the activation of PKCα pathway was observed in RAW264.7 after LPS assault (Fig. 1B).

LPS stimulation increased the formation of PICK1-PKCα complexes and PICK1-pCaMKII complexes

PICK1 forms heteromeric PDZ domain complexes with PKCα, which was initially identified on the basis of the yeast two-hybrid system (9, 10), while, it also interacts with CaMKII via BAR domain involving in Ca^{2+} releasing to stimulate GluA2(11)and in regulating inflammatory pain (12). To explore the underlying mechanism of the alterations on the levels of PICK1, PKCα and CaMKII, we examined whether endogenous PICK1-PKCα complexes (abbreviated as P-P complexes) and PICK1-pCaMKII complexes (abbreviated as P-pC complexes) were regulated by LPS. Through co-IP experiments, we observed a significant increase in the content of P-P complexes and P-pC complexes in the LPS treatment group compared with the control group in vitro(P<0.05)(Fig. 2A&B). When the phosphorylation activity of CaMKII was inhibited by KN-93, LPS stimulation could not induce the increment in pCaMKII level that co-immunoprecipitated with PICK1 anymore. While, such a phenomenon could not be detected in KN-92, an inactive analog of KN-93, treated macrophages (Fig. 2B). Thus, we hypnotized that PICK1 was involved in the pathogenesis of endotoxin-induced sepsis through interacting with PKCα and pCaMKII.

PICK1 KO mice have aggravated apoptosis and more reactive oxygen species released leading to severe lung injury when subjected to endotoxin

To corroborate the role of PICK1 in the endotoxin-induced acute lung injury, PICK1 knock out mice were used to figure out the underlying mechanism. To begin with, we found that the reactive oxygen species release of $PICK1^{-/-}$ mice that underwent LPS challenge was significantly higher than that of wild type (WT) mice that underwent LPS challenge (Fig. 3D). Next, Tunel staining results showed that $PICK1^{-/-}$ mice had more

apoptosis cells than wild-type mice after LPS treatment (Fig. 3F&G). As a result, lung wet/dry weight ratio showed that deficiency of PICK1 led to more severe lung damage in the $PICK1^{-/-}$ mice after LPS injection, compared with WT mice with LPS injection (Fig. 3E). Meanwhile, elevated mRNA levels of TNFα, IL-1β, IL-6 also indicated more severe inflammatory storm in LPS treated $PICK1^{-/-}$ mice than LPS treated WT mice

(Fig. 3A-C). Collectively, we found that depletion of PKCα was abolished by PICK1 knock out when subjected to LPS (Fig. 1F), the phosphorylation level of CaMKII was much reduced in PICK1 KO mice after LPS challenge (Fig. 1G), implying that deficiency of PICK1 might affect the activation of PKCα and the phosphorylation of CaMKII to regulate apoptosis and ROS release.

PDZ domain interference could mimic the effects of PICK1 deficiency

In order to further validate this hypothesis, FSC-231, a PDZ domain inhibitor of PICK1 was used to provide experimental basis for it. In vitro, we observed that when PDZ-domain intervention was conducted, the abolish of PKCα in response to LPS was vanished (Fig. 4B). Meanwhile, Western blotting results showed that the expression of Bcl-2 decreased quantitatively, BAX and cleaved-Casepase3 increased even further when treated with FSC-231 plus LPS than those cells only treated with LPS indicating the aggravation of apoptosis (Fig. 4C-E). Flow cytometry results revealed that apoptotic cell population and ROS release were increased quantitatively when PDZ-domain was disrupted (Fig. 4G&H), and poor cell viability was detected via MTT as well (Fig. 4F). Above, we noticed that FSC-231 aggravated LPS-treated RAW264.7 cell apoptosis and superoxide formation, demonstrating that when interfered the interaction of PICK1 and PKCα (equaling to blockade PKCα pathways), cell apoptosis and oxidative stress were exacerbated.

KN-93 exacerbated apoptosis and oxidative stress in LPS treated RAW264.7 macrophages

As we know, CaMKII interacts with PICK1 via BAR domain, which need the activation of CaMKII (11). Thus, KN-93, a selective CaMKII inhibitor, was used to disrupt the complexes formation of PICK1 and CaMKII. In RAW264.7 cells, KN93 and its inactive derivative, KN-92 were pretreated 1 hour before LPS stimulation. As determined in Fig. 5B, KN-93, but not KN-92 significantly reduced p-CaMKII levels after LPS stimulation, which indicating the interference of the interaction of PICK1 and p-CaMKII. Then, apoptosis indicators and ROS were examined. Compared with the LPS group, the LPS + KN-93 group aggravated cell apoptosis and ROS release (Fig. 5D-G). On the contrast, the LPS + KN92 group had no significant changes in the p-CaMKII, apoptosis and ROS levels when compared with LPS group. Comparing with LPS group, Cell viability was observed much declined in LPS + KN-93 group as well (Fig. 5H). Thus, interferent the interaction between PICK1 and pCaMKII also can simulated the apoptosis and ROS effects of PICK1 knock out.

Discussion

The main finding of the present study is the involvement of PICK1 in mediating reactive oxygen species release and apoptosis through interacting with PKCα and CaMKII in the pathogenesis of endotoxin-induced acute lung injury, which, to our knowledge, has never been reported before. Our previous investigation showed that when subjected to CLP surgery, PKCα protein exhibited a downward trend in the lungs. Meanwhile, the increased pulmonary PICK1 level during polymicrobial sepsis seemed to be

related with the alteration of PKCα levels, on account of that when PICK1 gene knocked down, the decline effect of PKCα during sepsis was abolished. Similar results were obtained in this endotoxin-induced septic shock model: interference of PICK1 function using FSC-231 blocked the assumption of PKCα and its downstream apoptosis and oxidative stress pathways, resulting in exacerbated apoptosis and oxidative burst. Furthermore, disrupting the interaction between PICK1 and CaMKII via CaMKII activity inhibitor, KN-93, also received similar effect to PICK1 disfunction. Above, we implied that the noxious effect attributed to the disfunction of PICK1 in a septic condition may owing to the imbalance of the Ca^{2+} flux, leading to the inactivation of PKCα and its downstream anti-apoptotic and anti-oxidant pathways.

Owing to its normal physiological function and its direct exposure to external atmospheric pollutants, lung became the most common organ to be adversely affected when systemic inflammatory onset response to polymicrobial infectio(13).Oxidative stress as one of the well-appreciated underlying mechanisms of the development of sepsis-associated ALI/ARDS has been well documented(14). It is well known that excessive production of ROS and massive oxidative stress resulting in cell apoptosis and lung inflammation, which are two of the most principal causes of sepsis-associated acute lung injury. Therein, ROS mediates intracellular signaling cascades to induce apoptosis via disintegration of mitochondrial membranes. (15, 16)von Knethen et al. have suggested that the depletion of PKCα was correlated with the inhibition of the oxidative burst in septic patients, pointing out that PKCα is a critical transmitter in provoking the oxidative burst in monocytes/macrophages response to LPS. In contrast, restraining the activation-induced depletion of PKCα led to aggravated oxidative burst (17). As we known, PKC family is divided into three groups (classical, novel and atypical) based on their structure and cofactor requirements. Among these related enzymes, PKCα, which was known as the activator of the signaling cascade causing superoxide generation, belongs to classical PKC members owing to it has DAG and Ca^{2+} binding sites and requires Ca^{2+} for its optimal activity(18, 19). Mounting evidence suggests that PKCα regulated the phosphorylation of the key anti-apoptotic protein Bcl-2, resulting in PKCα involving in the anti-apoptosis signal for cell survival(18, 20). In this study, similar results were obtained that the inactivation of PKCα induced burst of apoptosis of RAW264.7 cells when treated with LPS. Beyond its anti-apoptosis function, PKCα is also known to have an anti-oxidative stress function which is linked to the GSH regulation function of Bcl-2 (21, 22) According to our experiments, the production of reactive oxygen species of LPS treated cells was extremely augmented when PKCα activity was blocked.

PICK1, known as the only protein that possesses both the PSD-95/ DlgA/ZO-1 (PDZ) domain and the Bin-Amphiphysin-Rvs (BAR) domain (23), was initially named as a protein kinase C alpha (PKCα)-binding protein on the basis of the yeast two-hybrid system(9).It interacts with numerous neurotransmitters receptors, transporters, ion channels, and enzymes, and controls their trafficking through its PDZ and BAR domains, consequently participates in various diseases including neurobiological disorders and non-neurological disorders. Our previous experimental and molecular modeling study demonstrated that PICK1 deficiency exacerbated sepsis-associated acute lung injury and impairs glutathione synthesis via reduction of xCT. In the meantime, our CO-IP results showed that the content of PICK1-PKCα complex

increased during polymicrobial sepsis, which led us to hypothesized that the participation of PICK1 in septic-induced oxidative stress might not only through its regulation of GSH but also its interaction with PKCα to modulate the balance of ROS versus GSH. Owing to the intimate interaction within PICK1 and Ca^{2+}/calmodulin activated kinase II (CaMKII), whose activation regulated the release of Ca^{2+} from the ER via the IP3 and ryanodine, intracellular Ca^{2+} blockage was found to mimic the disfunction effect of PICK1. This further highlights the pivotal role of PICK1 in the mechanism of oxidative stress in sepsis.

In this study, the increased Ca^{2+} flux was observed in RAW264.7 cells when subjected to LPS stimulation, and blocking intracellular Ca^{2+} flux led to the inactivation of PKCα, which was similar to the effect of PDZ domain inhibition of PICK1. Consider this, we suggested that the interaction between CaMKII and PICK1 should be further explored and whether supplement of Ca^{2+} could rescue the detrimental effect of PICK1 deficiency, to verified the certain mechanism of how PICK1 regulated PKCα pathway via affecting Ca^{2+} during polymicrobial sepsis.

Declarations

Acknowledgements

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Figures

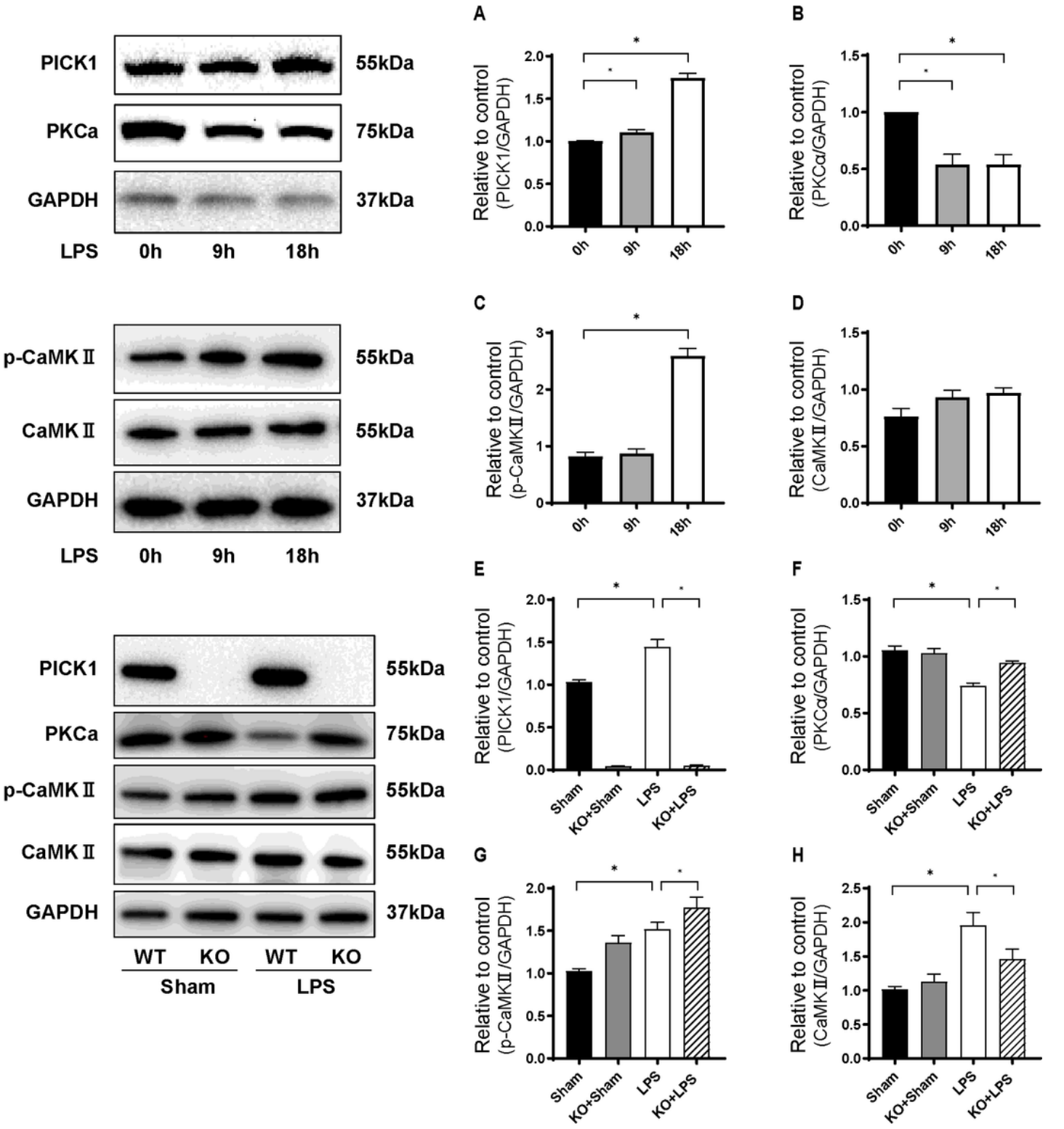

Figure 1

Elevated PICK1 levels was accompanied by consumption of PKCα and phosphorylation of CaMKII in endotoxin-induced lung injury model in vivo and in vitro.

The protein levels of PICK1,PKCα,p-CaMKII and CaMKII were determined by Western blot 0h,9h and 18h after LPS administration in RAW 264.7. *indicates P□0.05

1A-D:The intensities of each specific band were quantitated, analyzed with one-way ANOVA, and expressed as means±SD of three individual experiments.

The protein levels of PICK1,p-CaMKII,CaMKII and PKCα were determined by Western blot 12h after LPS administration in C57BL/6 and PICK1 KO mice. n = 5 per group. *indicates P0.05

1E-H,The intensities of each specific band were quantitated, analyzed with one-way ANOVA, and expressed as means±SD of three individual experiments. LPS indicates lipopolysaccharide;KO,PICK1 knock out;PICK1,Protein interacting with C kinase-1;SD, standard deviation;

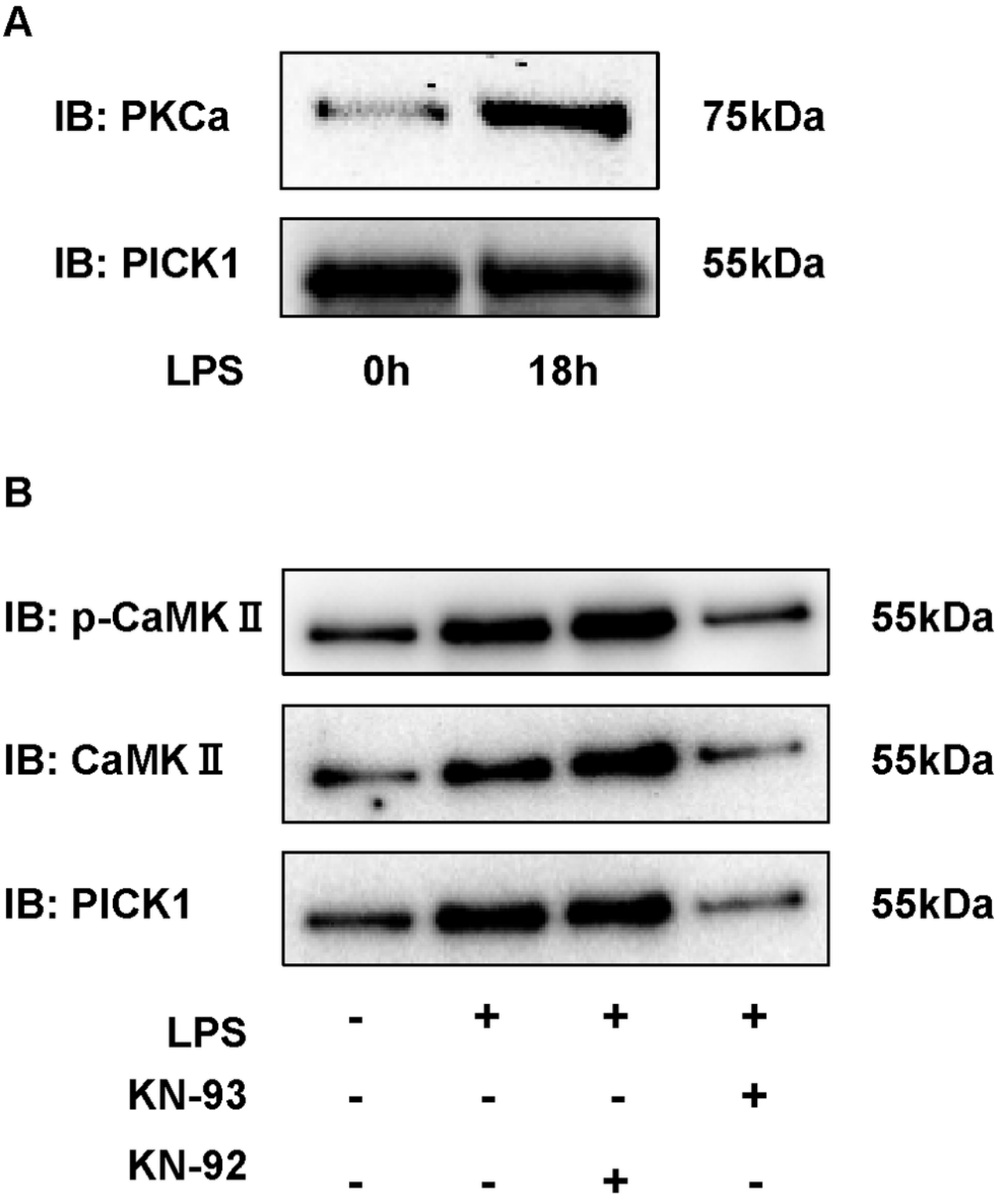

Figure 2

LPS stimulation increased the formation of PICK1-PKCα complexes and PICK1-pCaMKII complexes.

2A.Representative images of IP of PICK1 and PKCα,*indicates P<0.05

2B.Representative images of IP of PICK1,p-CaMKII and CaMKII,*indicates P<0.05

LPS indicates lipopolysaccharide;KN-93,CaMKII inhibitor;KN-92,inactive analog of KN-93;PICK1,Protein interacting with C kinase-1;

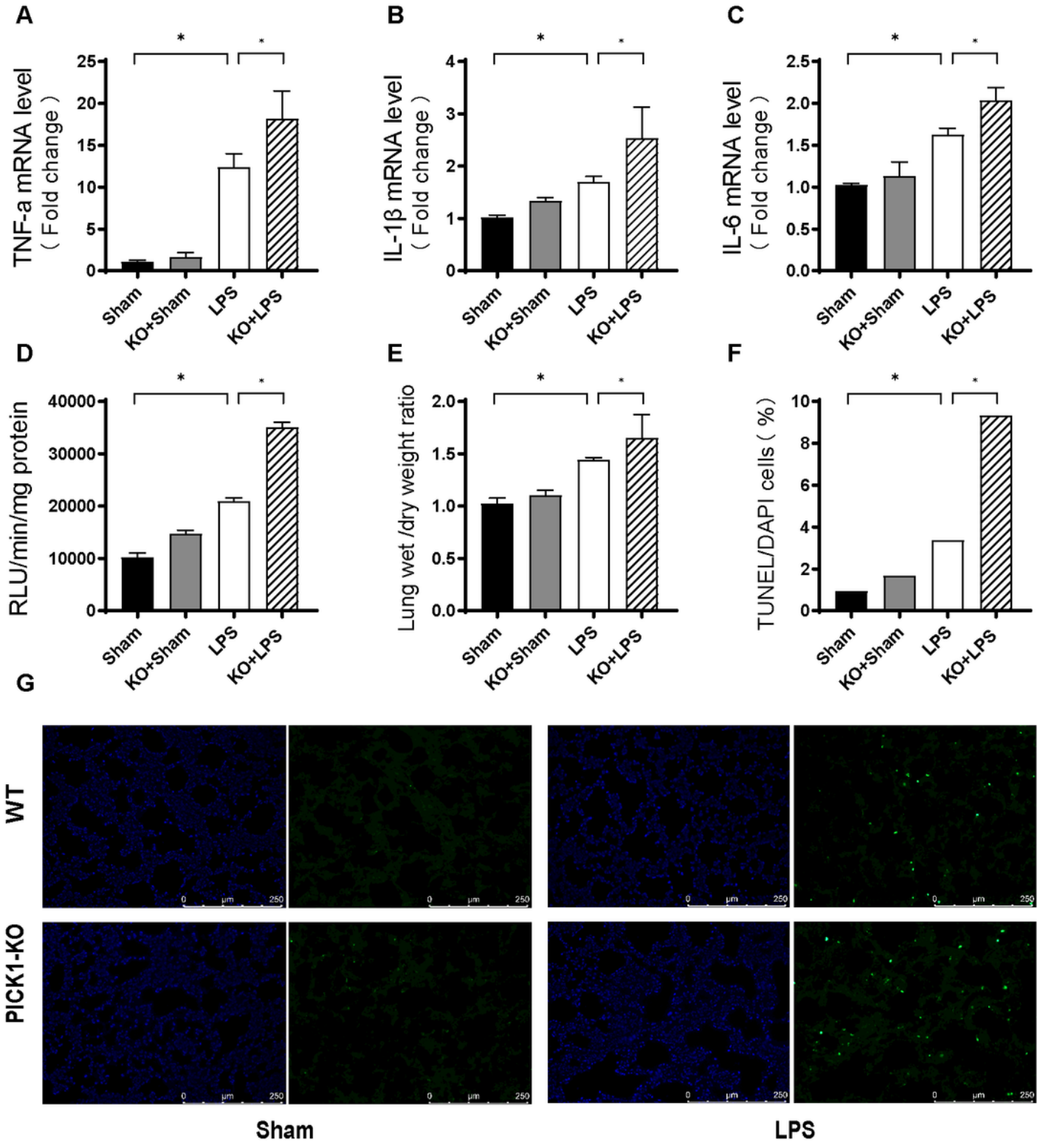

Figure 3

PICK1 KO mice have aggravated apoptosis and more reactive oxygen species released leading to severe lung injury when subjected to endotoxin

3A-C.Increasment of TNF-α,IL-1and IL-6β expression in lung after LPS challenge.The level of mRNA was determined 12h after LPS challenge by real-time PCR. Data are normalized with the GAPDH and expressed as the means±SD (n=5 per group).*indicates P0.05

3D.Superoxide production after PICK1 knock out and/or LPS challenge was analyzed by luminometer using the lucigenin chemiluminescence. Data are expressed as relative light units (RLU) per minute per milligram protein and corresponding histogram are shown. n = 5 per group*indicates P0.05

3E. Lung wet/dry weight ratio. The data are presented as mean±SD, n=5, *indicates P0.05

3F.Corresponding histogram of TUNEL staining. Data were presented as the ratios of TUNEL-positive/DAPI-positive cells. *indicates P0.05

3G.Cell apoptosis was identified by TUNEL staining in lung from WT and KO mice. Representative staining with DAPI and TUNEL in lungs from WT and KO mice underwent LPS or sham manipulation. n = 5 per group. Scale bars, 50 μm.LPS indicates lipopolysaccharide;KO,PICK1 knock out;PICK1,Protein interacting with C kinase-1;IL, interleukin;TNF,tumor necrosis factor;SD, standard deviation;

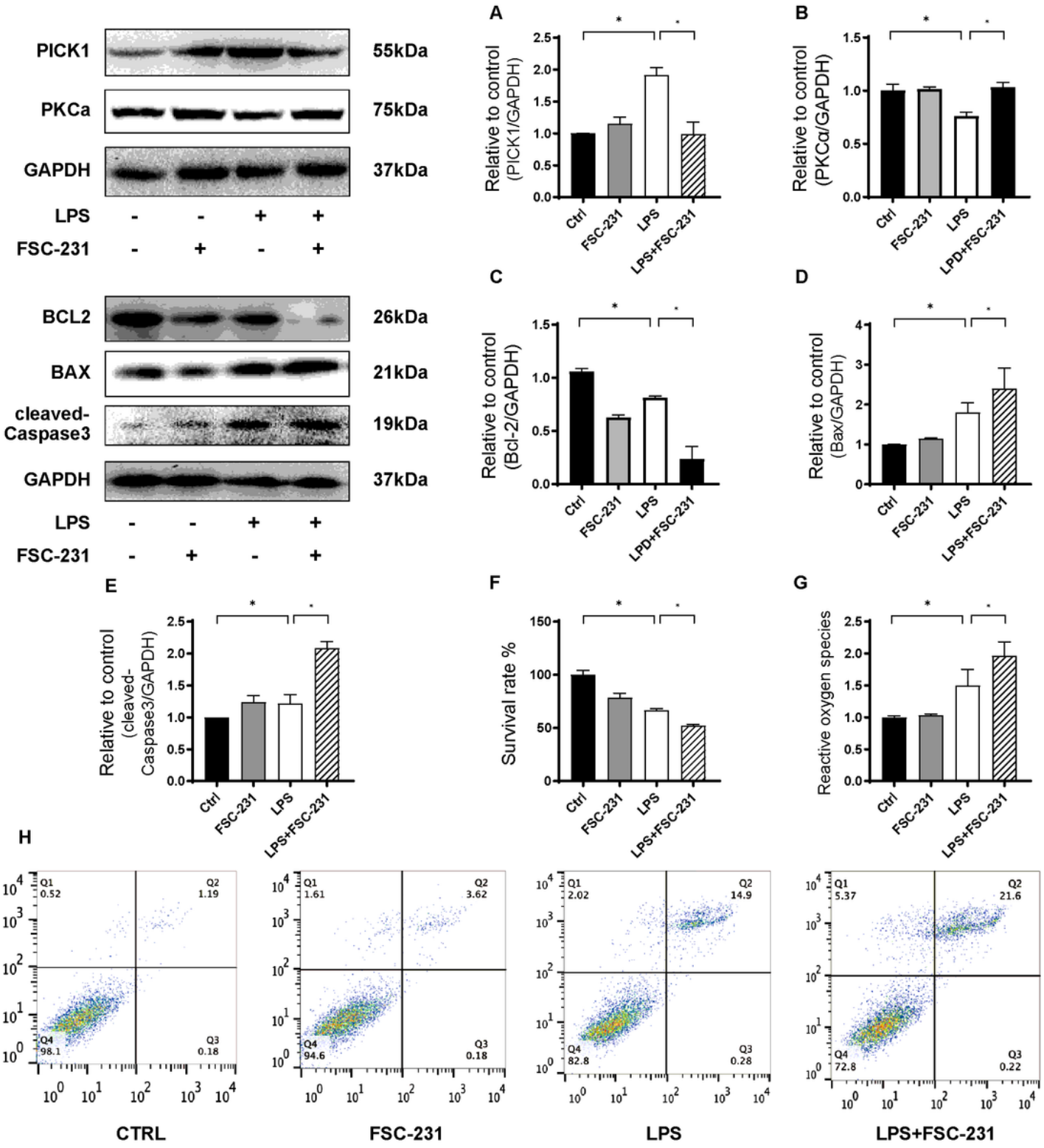

Figure 4

PDZ domain interference could mimic the effects of PICK1 deficiency.

The protein levels of PICK1、PKCα、Bax、Bcl-2 and cleaved-Caspase3 in RAW264.7 cells 18h after LPS challenge.*indicates P<0.05

4A-E The intensities of each specific band were quantitated, analyzed with one-way ANOVA, and expressed as means±SD of three individual experiments.

4F.Effect of FCS-231 on the viability of RAW264.7 cells 18h after LPS stimulation, measured by MTT assay.

4G.The values shown are mean±SD , *indicates P ≤0.05

4H.Superoxide was measured following 18h LPS treatment and FCS-231 was pretreated 1 hour before LPS stimulation.50uMLPS indicates lipopolysaccharide;SD, standard deviation;Ctrl,control group;PICK1,Protein interacting with C kinase-1;

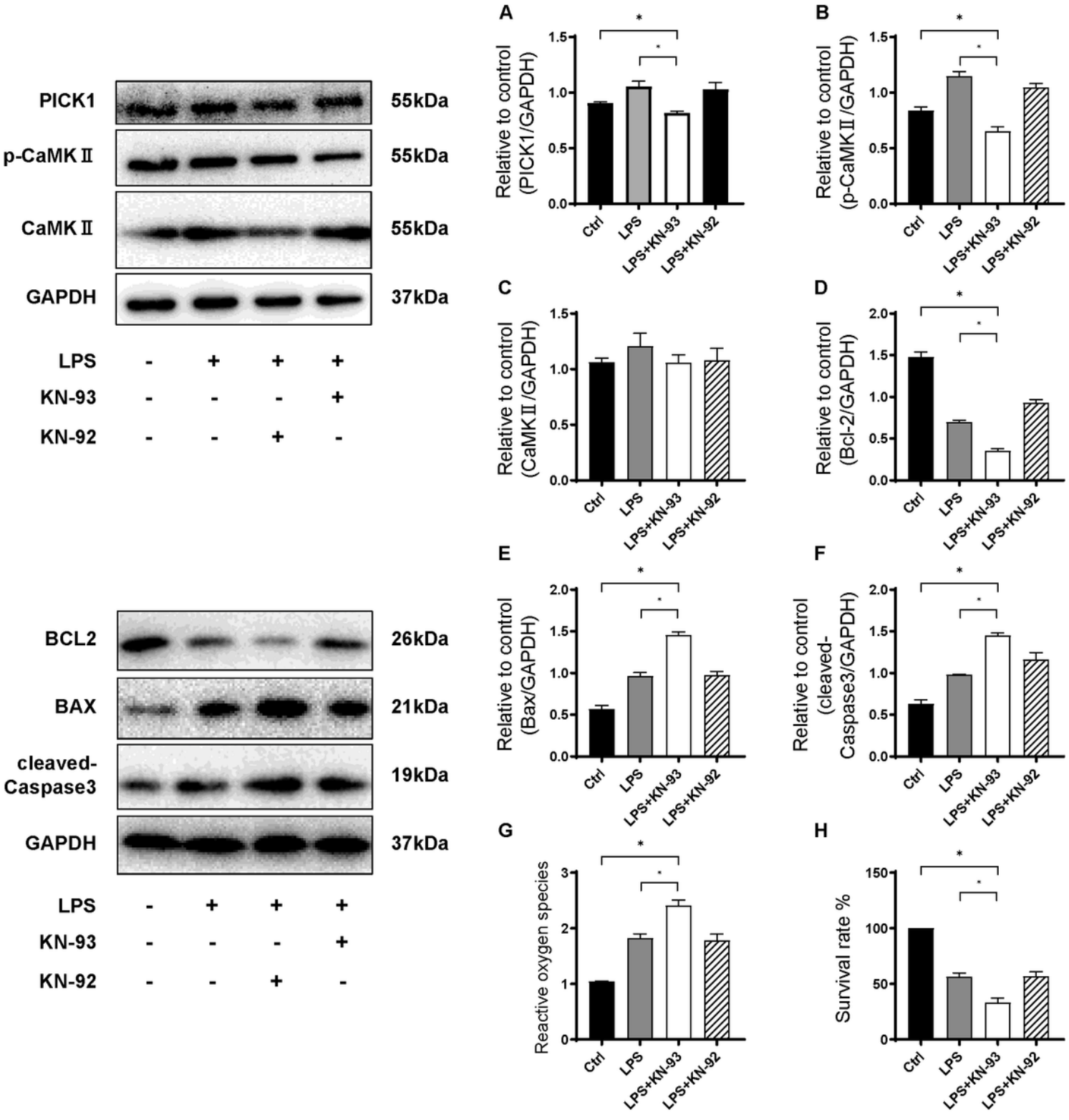

Figure 5

KN-93 exacerbated apoptosis and oxidative stress in RAW264.7 macrophages.

The protein levels of PICK1、p-CaMKII、CaMKII、Bax、Bcl-2 and cleaved-Caspase3 18h after LPS treatment and KN-92,KN-93 were pretreated 1 hour before LPS stimulation in RAW264.7 cells.

5A-F.The intensities of each specific band were quantitated, analyzed with one-way ANOVA, and expressed as means±SD of three individual experiments.

5G.Superoxide was measured following 18h LPS treatment and KN-92,KN-93 were pretreated 1 hour before LPS stimulation. The values shown are mean±SD of three replicate wells per experiment. * indicates $P < 0.05$

5H.Effect of KN-92,KN-93 on the viability of RAW264.7 after LPS stimulation, measured by MTT assay.

LPS indicates lipopolysaccharide;SD, standard deviation;Ctrl,control group;PICK1,Protein interacting with C kinase-1;