

Blockade of PKC β protects against remote organ injury induced by intestinal ischemia and reperfusion via a p66shc-mediated mitochondrial apoptotic pathway

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Published online: 15 June 2014
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Abstract Intestinal ischemia–reperfusion (I/R) is a serious clinical dilemma with high morbidity and mortality. Remote organ damage, especially acute lung injury and liver injury are common complications that contribute to the high mortality rate. We previously demonstrated that activation of PKC β II is specifically involved in the primary injury of intestinal I/R. Considering the tissue-specific features of PKC activation, we hypothesized that some kind of PKC isoform may play important roles in the progression of secondary injury in the remote organ. Mice were studied in in vivo model of intestinal I/R. The activation of PKC isoforms were screened in the lung and liver. Interestingly, we found that PKC β II was also activated exclusively in the lung and liver after intestinal I/R. PKC β II suppression by a specific inhibitor, LY333531, significantly attenuated I/R-induced histologic damage, inflammatory cell infiltration, oxidative stress, and apoptosis in these organs, and also alleviated systemic inflammation. In addition, LY333531 markedly restrained p66shc activation, mitochondrial translocation, and binding to cytochrome-c. These resulted in the decrease of cytochrome-c release and caspase-3 cleavage, and an increase

in glutathione and glutathione peroxidase. These data indicated that activated PKC isoform in the remote organ, specifically PKC β II, is the same as that in the intestine after intestinal I/R. PKC β II suppression protects against remote organ injury, which may be partially attributed to the p66shc-cytochrome-c axis. Combined with our previous study, the development of a specific inhibitor for prophylaxis against intestinal I/R is promising, to prevent multiple organ injury.

Keywords Intestinal ischemia reperfusion · Remote organ injury · Protein kinase C β · Adaptor protein p66shc

Introduction

Intestinal ischemia–reperfusion (I/R) is a lethal complication that occurs in severe burns, hemorrhagic shock, and trauma [1–3]. Critically ill patients who undergo these severe clinical situations have usually experienced occult intestinal ischemia, given the preferential shunting of blood flow to the heart [4] and brain [5]. After fluid resuscitation, intestinal reperfusion immediately resumes. Although close monitoring of these patients had improved outcomes, morbidity and mortality remains high [6]. Intestinal I/R results in gut barrier dysfunction and bacterial translocation, which leads to inflammatory cytokine release, reactive oxygen species (ROS) overproduction, and even apoptosis. Ultimately, systemic inflammation and multiple organ failure results [7–9]. Remote organs, especially the lung [7] and liver [8], are very susceptible to intestinal I/R injury. Increasingly reports demonstrated that the secondary remote organ injury (including acute respiratory distress syndrome and acute liver failure, etc.) is much more severe than the primary damage and is proved to be the leading

Electronic supplementary material The online version of this article (doi:10.1007/s10495-014-1008-x) contains supplementary material, which is available to authorized users.

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cause of death in intestinal I/R patients [7–9]. However, multiple signaling pathways are involved in this pathogenesis and the specific mechanism is extremely complicated.

The protein kinase C (PKC) family comprises a group of multi-functional protein kinases that play important roles as signal transducers of cellular stress by phosphorylation of the serine and threonine residues [10]. Activation of PKC, indicated by translocation of the protein from the cytoplasm to the membrane with subsequent phosphorylation, occurs in response to many conditions, such as I/R injury [11] and hemorrhagic shock [12]. Of the various PKC isoforms, PKC β II is exclusively activated during the primary injury of intestinal I/R [13]. We have demonstrated that inhibition of PKC β II protects intestine from the I/R injury [13]. However, other researchers have reported that the activation of individual PKC isoforms in particular pathogenesis is tissue-specific [10]. Thus, it is essential to further determine which PKC isoform contributes to the remote organ injury induced by intestinal I/R and whether it has a critical pathological role during disease progression.

Adaptor protein p66shc (p66shc) is a member of the ShcA protein family, and is comprised of two other proteins, p46shc and p52shc. Phosphorylation of the tyrosine residues of p46shc and p52shc plays an important role in their interaction with the epidermal growth factor receptor. In contrast to p46shc and p52shc, p66shc has a unique N-terminal proline-rich domain with a serine phosphorylation site (serine 36). Endogenous or exogenous stress, such as free radicals attack, results in serine 36 phosphorylation of the p66shc, contributing to cell oxidative stress and apoptosis [14, 15]. Prior studies have demonstrated that p66shc knockout mice showed strong resistance to severe tissue injury after heart or hind limb ischemia and secondary ROS attack induced by reperfusion [16, 17]. Likewise, p66shc-deficient cells were protected against oxidative stress and apoptosis when exposed to hypoxia and reoxygenation [18]. Although our previous study has demonstrated that p66shc phosphorylation was associated with acute lung injury (ALI) after intestinal I/R [19], the detailed mechanism by which p66shc exerts its deleterious effect in I/R induced remote organ injury requires further investigation.

Considering the tissue-specific features of PKC activation, we generated a mouse model of intestinal I/R and screened the activated isoform of PKC in the remote organ after intestinal I/R. Further, we explored whether the inhibition of PKC activation shows protective effects and elucidated the potential p66shc-mediated mechanisms. Our work aims to highlight a new target for preventing intestinal I/R induced remote organ injury.

Materials and methods

Experimental model

Adult male ICR mice (18–22 grams, SPF Animal Centre of Dalian Medical University, Dalian, China) were housed at a temperature of 22 ± 2 °C, kept on a 12:12-h photoperiod, and provided with food and water ad libitum. Animals were premedicated by intragastric administration of the PKC β inhibitor LY333531 (10 mg/kg daily; Enzo Life Sciences, NY, USA), or vehicle, 3 days prior to I/R surgery. The murine intestinal I/R model was used, according to previously established standards [20]. Briefly, a midline laparotomy was performed, and the superior mesenteric artery (SMA) was isolated at its origin, and occluded with an atraumatic microvascular clamp. After 45 min of ischemia, the clamp was removed and mice were administered 0.5 ml of warm sterile saline intraperitoneally to improve hydration. Reperfusion was then performed at 45, 90, or 180 min intervals. Sham animals underwent the same protocol without occlusion of the SMA. After reperfusion, animals were sacrificed by exsanguination via the abdominal aorta. Intestinal, lung and liver tissues and blood samples were collected for analysis.

All experiments were performed in accordance with the institutional guidelines for the care and use of laboratory animals and were approved by the Committee on the Ethics of Animal Experiments of the Dalian Medical University (Dalian, China).

Histopathologic examination

Intestinal, lung, and liver tissues were fixed in 10 % buffered formalin phosphate and paraffin-embedded. Tissue blocks were sectioned at a thickness of 4 μ m and hematoxylin-eosin stained. Histopathologic examination of these samples was performed under light microscopy in a blinded manner. The intestinal injury score was graded on a six-tiered scale modified from Chiu [21]. The severity of lung injury was scored based on Mikawa's report [22]. Liver pathologic scores were evaluated according to Eckhoff's report [23].

Measurement of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), TNF- α , and interleukin (IL)-6

Serum ALT and AST levels were determined with a commercial assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and presented as international units per liter (IU/L). Serum IL-6 and TNF- α levels were measured by a enzyme-linked immunosorbent assay kit

(BOSTER, Wuhan, China) according to the manufacturer's instructions.

Lung and liver H₂O₂, malondialdehyde (MDA), reduced glutathione (GSH), glutathione peroxidase (GSH-PX), and myeloperoxidase (MPO) assay

H₂O₂, MDA and GSH levels, and GSH-PX and MPO activity were analyzed using commercial assay kits, according to the manufacturer's instructions (Nanjing Ji-an Cheng Bioengineering Institute, Nanjing, China), and were expressed as mmol/g protein, nmol/mg protein, mg/g protein, U/mg protein, and U/g, respectively.

Western blot

Membranous, cytosolic, and mitochondrial protein fractions or total protein were prepared using a commercial protein isolation kit (KeyGEN Biotech, Nanjing, China). Equal amounts of total protein (50 µg/sample) were subject to sodium dodecyl sulfate polyacrylamide gel electrophoresis (10–15 %, Bio-Rad, Hercules, USA) and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, USA). Western blot analysis was performed with antibodies specific for PKCβII, PKCγ, PKCδ, PKCε, cleaved-caspase-3, and ATPase (Bioworld Technology, Inc., MN, USA), PKCβI (Santa Cruz Biotechnology, CA, USA), phospho-PKCβII (Cell Signaling Technology, MA, USA), manganese superoxide dismutase (MnSOD), phospho-p66shc, total-p66shc (Abcam Ltd., Cambridge, UK), cytochrome-c (Beyotime Institute of Biotechnology, Shanghai, China), voltage-dependent anion channels (VDAC, Proteintech Group, Wuhan, China), and β-actin (ZSGB-BIO, Beijing, China). Nonspecific binding was blocked by incubation of membranes with 5 % skim milk for 2 h at 37 °C. Membranes were exposed to enhanced chemiluminescence plus reagents (Beyotime Institute of Biotechnology, Shanghai, China). Spectrophotometric analysis was performed with a BioSpectrum-410 multi-spectral imaging system and analyzed with a Gel-Pro Analyzer Version 4.0 (Media Cybernetics, MD, USA).

Co-immunoprecipitation

Mitochondrial fractions were extracted as described above. An equal amount of cytochrome-c antibody was added into 500 µg proteins and gently rotated at 4 °C overnight. Immunocomplexes were captured by adding 40 µl protein A + G agarose beads (Beyotime Institute of Biotechnology, Shanghai, China) and gently rotating at 4 °C for 4 h. The mixture was then centrifuged at 1,000g for 5 min at 4 °C and the supernatant was discarded. The precipitate was washed for 5 times with ice-cold phosphate buffer

saline. After washing, the immunocomplex was boiled in sodium dodecyl sulfate sample buffer for 5 min to dissociate the immunocomplex from the beads. Samples were then subjected to western blot with anti-p66shc and anti-cytochrome-c antibody, according to the manufacturer's instructions.

Terminal deoxynucleotidyl transferase dUTP nick end-labeling assay

Lung or liver histopathologic slides were dewaxed and treated with 20 µg/ml of proteinase K. Slides were stained using a terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) kit (Roche Diagnostics, Indianapolis, IN). All images were captured under fluorescent microscopy. Apoptotic cells appeared fluorescent green and were counted per 10 visual fields.

Caspase-3 activity assay

The caspase-3 activity was determined using a commercial assay kit (Beyotime Institute of Biotechnology, Shanghai, China), according to the manufacturer's protocols.

Statistical analysis

Data were expressed as mean ± standard deviation (SD). The statistical analysis was carried out using the SPSS16.0 statistical software package (SPSS Inc., Chicago, IL, USA). Statistical comparisons were analyzed by Kruskal–Wallis test for non-normal distributions followed by Wilcoxon Rank Sum test with Bonferroni adjustments for multiple comparisons, or a one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls (SNK) test for normal distributions. A *p* value less than 0.05 was considered statistically significant.

Results

PKCβ is activated in response to intestinal I/R in the lung and liver

When mice were subjected to intestinal I/R, a significant translocation of PKCβII to the cell membrane in the lung and liver was observed, implying activation of PKCβII. The increase of PKCβII in the membrane fraction reached an apparent maximum after 90 min reperfusion (Fig. 1a). In contrast, the PKCβI isoform showed no significant change (Fig. 1b). Meanwhile, membranous PKCγ, PKCδ, and PKCε showed no significant change after any period of reperfusion (Fig. 1b). Additionally, we detected phosphorylated PKCβII, recognized to be a crucial element in

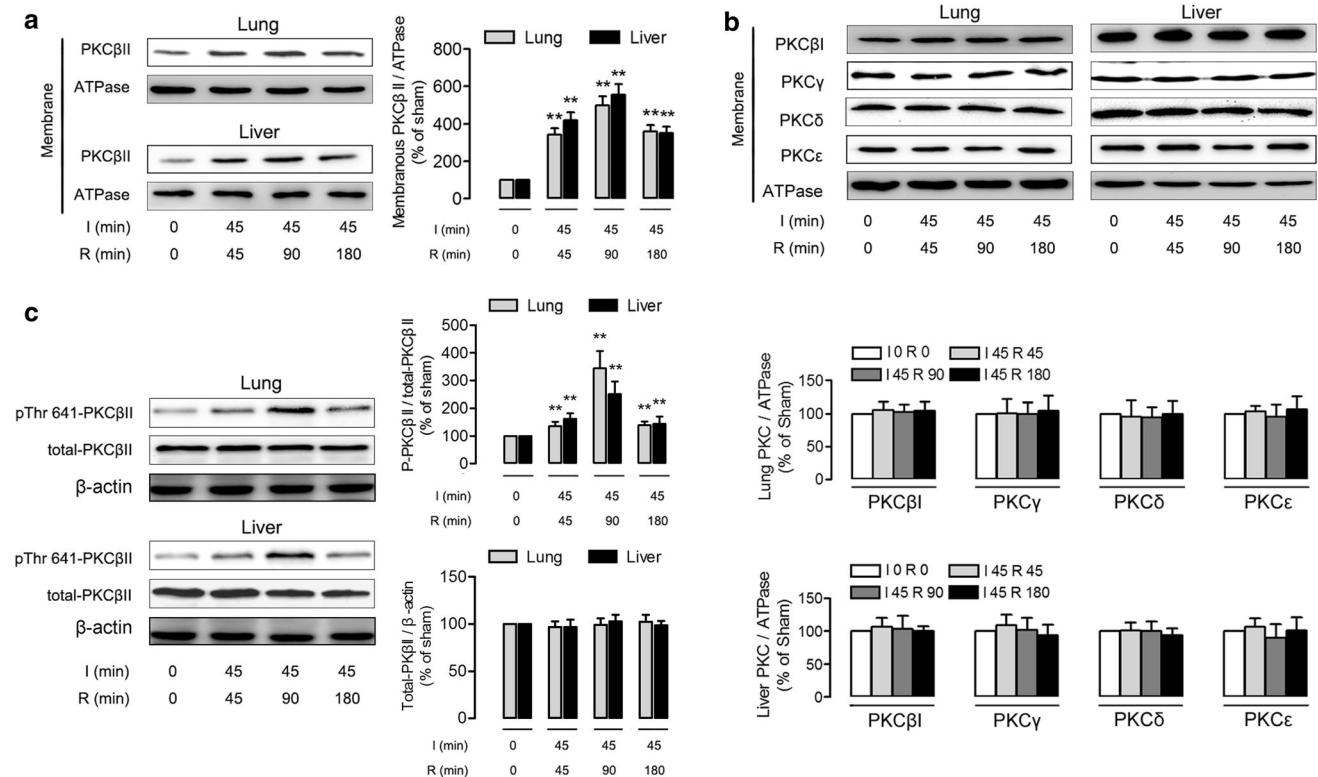


Fig. 1 PKC β is activated in response to intestinal I/R in the lung and liver. **a** Protein expression of membranous PKC β II in the lung and liver after intestinal I/R. ATPase was used to normalize membranous protein levels. **b** Protein expression of membranous PKC β I, PKC γ , PKC δ , and PKC ϵ in the lung and liver after intestinal I/R. ATPase

was used to normalize membranous protein levels. **c** Relative expression of phosphorylated PKC β II and total-PKC β II in the lung and liver after intestinal I/R. Results are presented as the mean \pm SD, $n = 3$. $^{***}p < 0.01$ versus sham. *I* indicates ischemia, *R* reperfusion, *Thr* Threonine

the catalytic function of PKC β II. As shown in Fig. 1c, 90 min of reperfusion of the intestine resulted in significantly increased level of Thr641-phosphorylated PKC β II without affecting the total level of PKC β II. Thus, we confirmed that the activated principal isoform of PKC relevant to intestinal I/R in the lung and liver was the PKC β II isoform, and not the β I, γ , δ or ϵ isoforms.

LY333531 attenuates lung and liver injury induced by intestinal I/R

To determine the role of PKC β in intestinal I/R induced remote organ injury, we used LY333531, a specific inhibitor of PKC β , to suppress PKC β II activation. As shown in Fig. 2a, LY333531 suppressed the membranous translocation of PKC β II after intestinal I/R, but without impact on PKC β I translocation. These findings were consistent with previous reports [24]. Furthermore, intestinal I/R led to marked phosphorylation of PKC β II, which was abolished by administration of LY333531 (Fig. 2b).

Next, we assessed whether PKC β inhibition protected against intestinal I/R injury and secondary remote organ injury. As shown in Fig. 3, after intestinal I/R, small

intestinal tissues were apparently damaged with severe irregularities of the villi, disintegrated lamina propria, and ulceration. Lung tissue was also markedly damaged, and was characterized by infiltration of inflammatory cells, thickened alveolar walls, and hemorrhage. Liver tissue was damaged with extensive nuclear pyknosis, cytoplasmic hypereosinophilia, and disintegration of hepatic cords. By contrast, in the LY333531 treated group, significantly decreased injury was shown on histopathology, in all three organ systems. Serum ALT and AST levels also showed the same trend, accordingly (Table 1).

To verify the effect of PKC β suppression on neutrophil infiltration in the lung and liver, tissue MPO activity was measured. As shown in Table 1, intestinal I/R caused a significant increase of MPO activity compared to sham animals, which was remarkably reduced with administration of LY333531.

To determine whether intestinal I/R affected systemic inflammation, we measured serum TNF- α and IL-6 concentrations. Compared with the sham group, TNF- α and IL-6 concentrations were significantly elevated in the I/R group. In contrast, PKC β inhibition by LY333531 administration prevented TNF- α and IL-6 overproduction (Table 1).

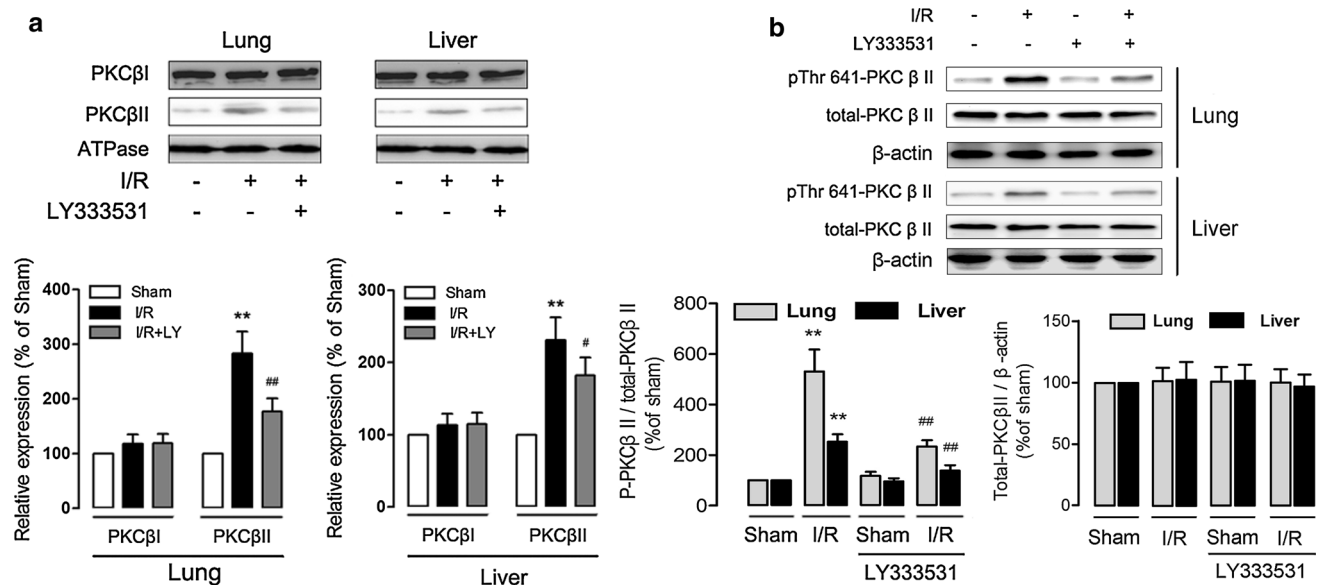
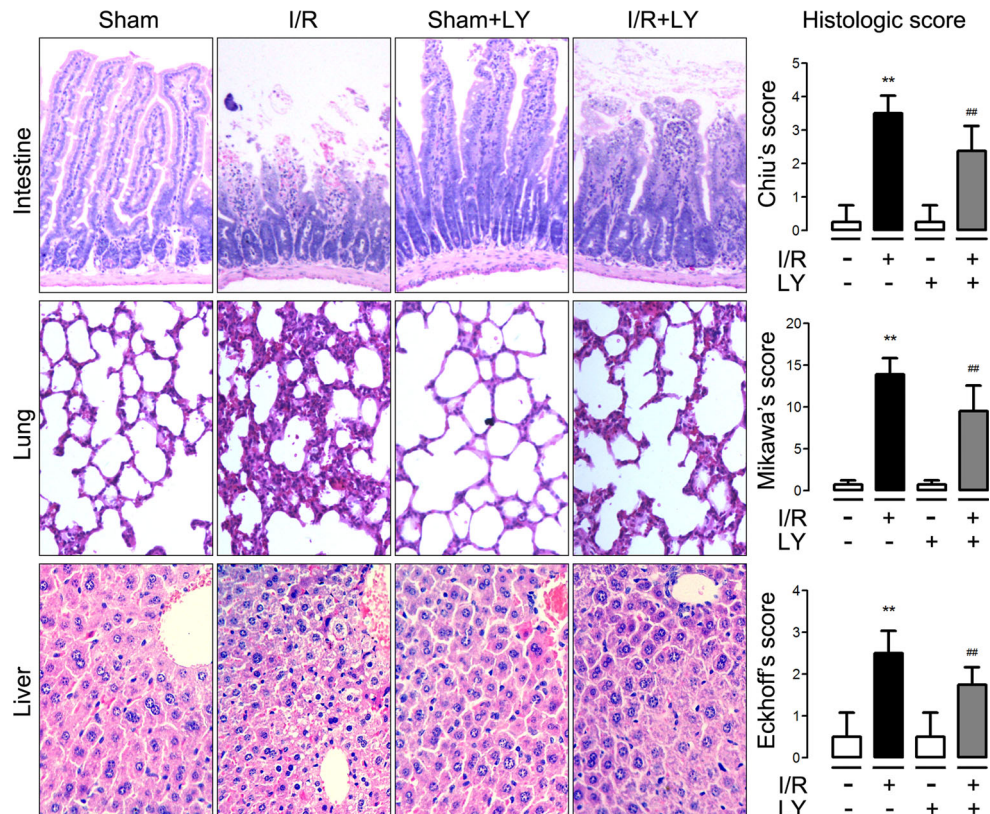


Fig. 2 LY333531 inhibits PKCβII activation induced by intestinal I/R in the lung and liver. Mice were subjected to 45 min of intestinal ischemia followed by 90 min of reperfusion. **a** Membranous expression of PKCβI and PKCβII in the lung and liver. ATPase was used to

normalize membranous protein levels. **b** Relative expression of phosphorylated PKCβII and total-PKCβII in the lung and liver from each group. Results are presented as the mean ± SD, *n* = 3. ***p* < 0.01 versus sham; #*p* < 0.05 versus I/R; ###*p* < 0.01 versus I/R

Fig. 3 PKCβ suppression improves intestinal I/R-induced intestine, lung and liver histopathologic injury. Mice were subjected to 45 min of intestinal ischemia followed by 90 min of reperfusion. Tissues were harvested at the end of reperfusion and were stained with hematoxylin-eosin. Slides were examined under light microscopy at ×100 (intestine), ×200 (lung), or ×400 (liver) magnification. Histologic injury scores in groups were quantified. Results are presented as the mean ± SD, *n* = 10. ***p* < 0.01 versus sham; ##*p* < 0.01 versus I/R group. LY indicates LY333531



LY333531 ameliorates oxidative stress and apoptosis in the lung and liver after intestinal I/R

Oxidative stress is certainly involved in the lung and liver injury after intestinal I/R [19, 25]. As a major component

of ROS, the content of H₂O₂ were significantly increased after intestinal I/R, while LY333531 administration reduced the content of H₂O₂ markedly (Fig. 4a). MDA is an end-product of lipid peroxidation, which is regarded as a major marker of oxidative stress [26]. As shown in Fig. 4b,

Table 1 Concentration of ALT, AST, TNF- α , and IL-6 in the serum and MPO in the lung and liver

Group	Serum ALT (IU/L)	Serum AST (IU/L)	Serum TNF- α (pg/ml)	Serum IL-6 (pg/ml)	Lung MPO (U/g)	Liver MPO (U/g)
Sham	53.49 \pm 7.92	38.74 \pm 6.51	159.19 \pm 15.42	53.78 \pm 11.02	0.39 \pm 0.21	2.10 \pm 0.64
I/R	159.31 \pm 17.70**	108.84 \pm 21.11**	285.82 \pm 83.53**	129.59 \pm 22.14**	2.38 \pm 0.65**	6.17 \pm 2.15**
Sham + LY333531	54.30 \pm 5.78	42.90 \pm 15.45	136.37 \pm 23.87	65.08 \pm 11.26	0.50 \pm 0.30	2.11 \pm 0.63
I/R + LY333531	122.82 \pm 16.46##	89.58 \pm 13.63##	219.67 \pm 51.51#	96.24 \pm 15.62##	1.80 \pm 0.24##	4.38 \pm 1.18#

Results are presented as the mean \pm SD, $n = 10$. ** $p < 0.01$ versus sham; # $p < 0.05$ versus I/R; ## $p < 0.01$ versus I/R

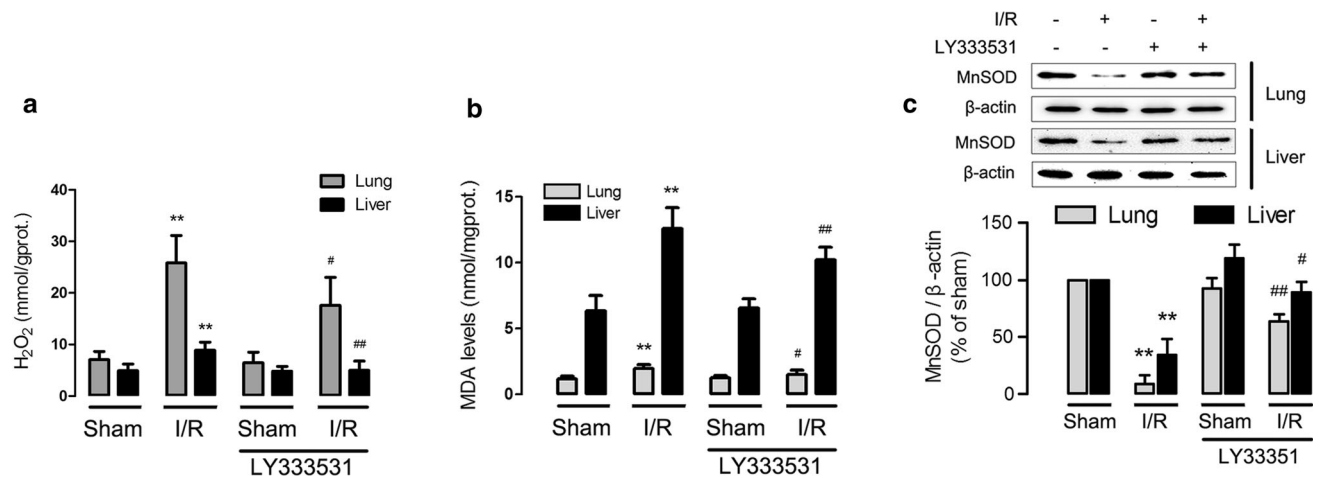


Fig. 4 PKC β suppression ameliorates lung and liver oxidative stress after intestinal I/R. Mice were subjected to 45 min of intestinal ischemia followed by 90 min reperfusion. **a** Lung and liver H₂O₂ levels. Results are presented as the mean \pm SD, $n = 10$. **b** Lung and liver MDA levels. Results are presented as the mean \pm SD, $n = 10$.

c Protein expression of MnSOD in the lung and liver; β -actin was used to normalized protein levels. Results are presented as the mean \pm SD, $n = 3$. ** $p < 0.01$ versus sham; # $p < 0.05$ versus I/R; ## $p < 0.01$ versus I/R. Prot. indicates protein

MDA content was markedly increased after intestinal I/R. However, LY333531 administration decreased MDA levels in comparison with the I/R group. Anti-oxidant enzyme MnSOD (a downstream molecule of p66shc-foxo3a axis) expression was also assayed, and found to be significantly suppressed after intestinal I/R. LY333531 administration prevented suppression of MnSOD expression, implying a potential antioxidative effect of PKC β suppression (Fig. 4c).

To determine the effect of PKC β suppression on apoptosis, TUNEL staining was performed. As shown in Fig. 5a, apoptotic cells in the lung and liver were significantly increased after intestinal I/R, whereas LY333531 administration significantly reduced the number of apoptotic cells compared with the I/R group. To further confirm these results, we detected the expression of cleaved caspase-3 and the caspase-3 activity. As expected, intestinal I/R enhanced lung and liver cleaved-caspase-3 expression and the caspase-3 activity, compared with the sham group. However, these effects were significantly attenuated by LY333531 administration (Fig. 5b, c).

LY333531 inhibits p66shc activation in the lung and liver after intestinal I/R

P66shc is involved in modulating oxidative stress and apoptosis [15, 27, 28]. P66shc silenced by small interfering RNA (siRNA) showed resistance to H₂O₂-induced caspase-3 activation and apoptosis in human alveolar epithelial cell line A549 and human normal liver cell line L02 (Suppl Fig. 1). To evaluate the activation of p66shc in the lung and liver after intestinal I/R, p66shc phosphorylation was measured. As shown in Fig. 6a, time-dependent phosphorylation of p66shc was observed after 45–180 min reperfusion. Next, we evaluated whether I/R-induced p66shc activation was PKC β dependent. As a specific inhibitor of PKC β , LY333531 showed a significant restraining effect on p66shc phosphorylation in the lung and liver after intestinal I/R, without impact on the sham or the sham + LY333531 groups (Fig. 6b). To further determine whether PKC β , especially PKC β II, is specifically required for the activation of p66shc, we suppressed its expression using siRNA in A549 cells and L02 cells (Suppl Fig. 2a). The exposure of

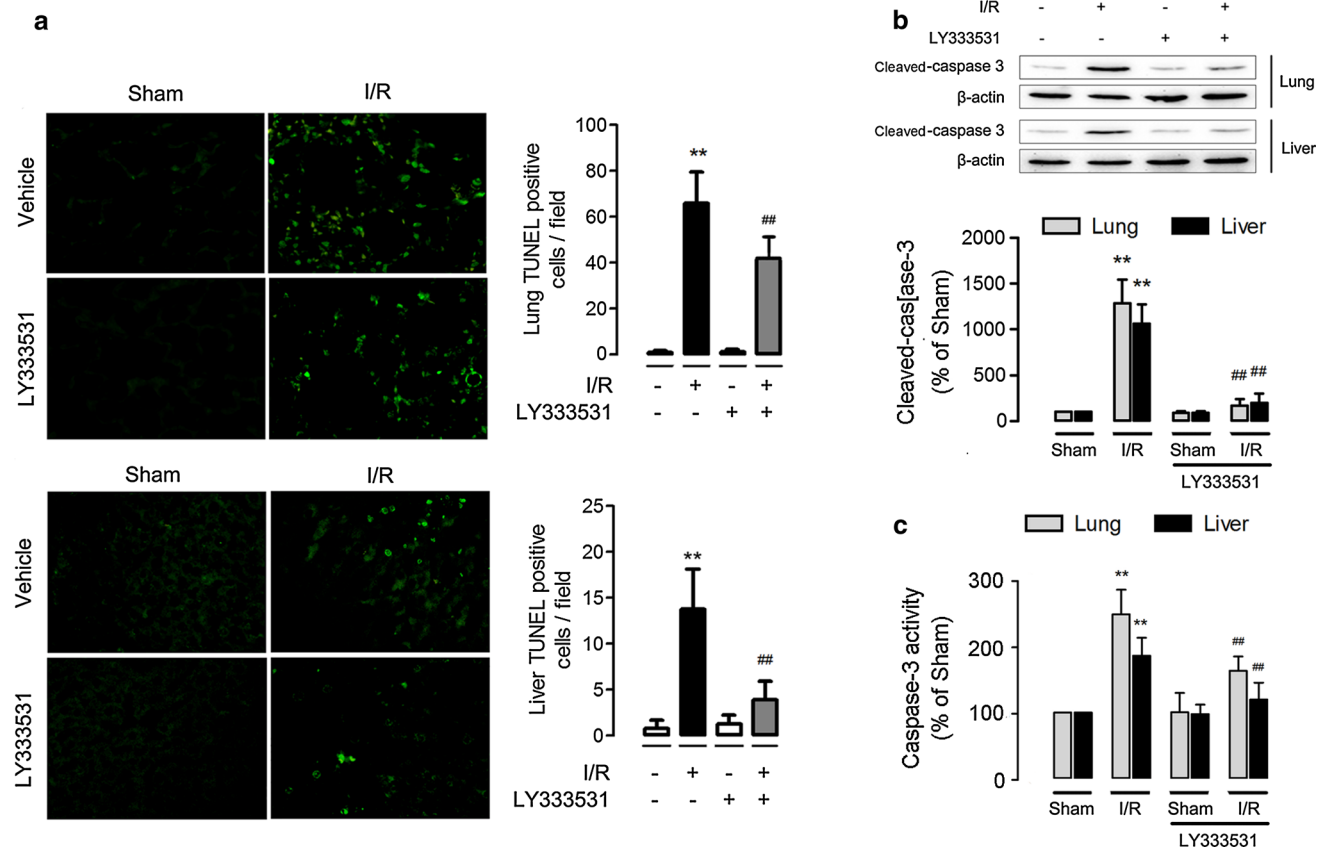
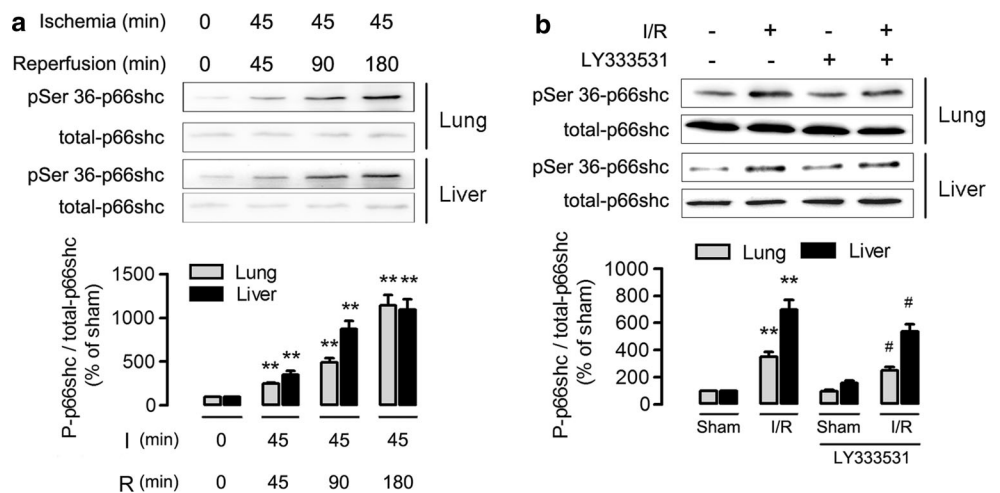


Fig. 5 PKC β suppression ameliorates lung and liver apoptosis after intestinal I/R. Mice were subjected to 45 min of intestinal ischemia followed by 90 min reperfusion. **a** Representative TUNEL staining of lung and liver after intestinal I/R. Quantification of TUNEL staining. Results are presented as the mean \pm SD of 10 frames per group from

three animals per group. **b** Protein expression of cleaved-caspase-3 in the lung and liver. β -actin was used to normalize protein levels. Results are presented as the mean \pm SD, $n = 3$. **c** Lung and liver caspase-3 activities. Results are presented as the mean \pm SD, $n = 10$. ** $p < 0.01$ versus sham; ## $p < 0.01$ versus I/R

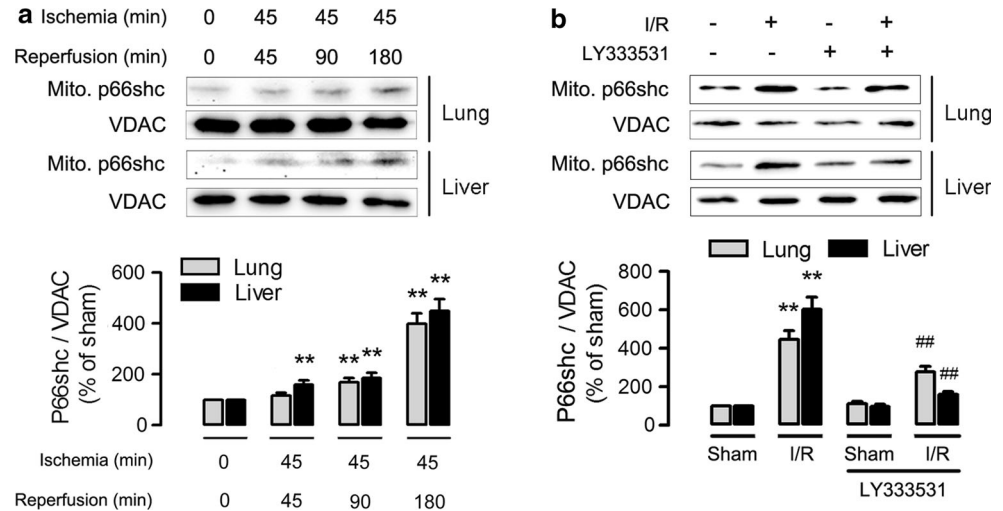
Fig. 6 P66shc is phosphorylated after intestinal I/R, which is restrained by PKC β suppression. **a** Relative expression of phosphorylated p66shc in the total protein in the lung and liver. **b** Mice were subjected to 45 min of intestinal ischemia followed by 90 min reperfusion. Relative expression of phosphorylated p66shc in the total protein in the lung and liver. Results are presented as the mean \pm SD, $n = 3$. ** $p < 0.01$ versus sham; # $p < 0.05$ versus I/R. Ser indicates serine



PMA (a classical PKC activator) markedly increased p66shc phosphorylation in A549 cells and L02 cells, which were inhibited significantly by PKC β II siRNA or LY333531

(Suppl Fig. 2b). As a whole, these findings suggest that intestinal I/R induced p66shc activation in the lung and liver may partially depend on PKC β activation.

Fig. 7 P66shc is translocated into mitochondria after intestinal I/R, which is restrained by PKC β suppression. **a** Mitochondrial expression of p66shc in the lung and liver. **b** Mice were subjected to 45 min of intestinal ischemia followed by 90 min reperfusion. Mitochondrial expression of p66shc in the lung and liver. VDAC was used to normalize mitochondrial protein levels. Results are presented as the mean \pm SD, $n = 3$. ** $p < 0.01$ versus sham; ## $p < 0.01$ versus I/R



LY333531 inhibits p66shc mitochondrial translocation and binding to cytochrome-c

As verified by previous studies, the pro-oxidative and proapoptotic properties of p66shc depend on mitochondrial translocation and binding to cytochrome-c [27]. To assess whether intestinal I/R increases mitochondrial translocation of p66shc, Western blotting of p66shc in the mitochondrial lysates was performed. As shown in Fig. 7a, after 45–180 min reperfusion, mitochondrial p66shc expression significantly increased in a time-dependent manner. Additionally, LY333531 administration suppressed p66shc levels in the mitochondria after intestinal I/R (Fig. 7b). To further investigate the relationship between mitochondrial p66shc and cytochrome-c, co-immunoprecipitation analysis was performed. Cytochrome-c co-precipitated with p66shc significantly in the I/R group compared with sham animals. However, this effect was significantly attenuated by LY333531 administration (Fig. 8a). Taken together, these results suggest that LY333531 suppresses mitochondrial translocation and cytochrome-c binding of p66shc in the lung and liver after intestinal I/R.

LY333531 ameliorates cytochrome-c releasing and GSH system dysfunction

In the mitochondria, p66shc oxidizes cytochrome-c to generate ROS (H_2O_2). This process induces opening of mitochondrial permeability transition pore, with a subsequent release of cytochrome-c and H_2O_2 into the cytosol [16, 27]. Then, cytosolic cytochrome-c activates caspase-3 and results in apoptosis [29]. To confirm this process, we investigated mitochondrial and cytoplasmic cytochrome-c levels in each group. As shown in Fig. 8b, intestinal I/R caused a significant release of cytochrome-c from the mitochondria into the cytosol in the lung and liver, while

control mice did not demonstrate this event. In contrast, PKC β inhibition markedly restored mitochondrial localization of cytochrome-c.

P66shc induced H_2O_2 releasing also plays important role in promoting apoptosis [27]. In physiological conditions, cytosolic H_2O_2 released from the mitochondria will interact with GSH and ultimately convert into H_2O by GSH-PX catalysis, thereby maintaining the homeostasis [30]. Once the GSH system is getting out-of-balance, apoptosis is more prone to occur. As shown in Table 2, in accordance with cytochrome-c release in the I/R group, lung and liver GSH content and GSH-PX activity dramatically decreased. In contrast, LY333531 administration showed an efficient recovery of GSH content and GSH-PX activity, compared with the I/R group.

Discussion

Intestinal I/R is a serious clinical dilemma with high morbidity and mortality [1, 2, 6, 31]. Multiple organ damage, including ALI and liver injury, are common complications of intestinal I/R and contribute to the high mortality rate [7, 8]. Only a limited number of pharmacologic treatment options provide benefit in intestinal I/R and remote organ injury. In this study, we provided the first evidence that (1) PKC β (especially PKC β II) is selectively activated in the lung and liver after intestinal I/R; (2) PKC β suppression by a specific inhibitor (LY333531) markedly reduced I/R-induced remote organ injury, as indicated by improved morphological damage, attenuated systemic inflammation, and decreased oxidative stress and apoptosis; (3) the protective effect of LY333531 is partially attributed to PKC β dependent phospho-p66shc suppression and alleviation of p66shc-mediated oxidative stress and apoptosis.

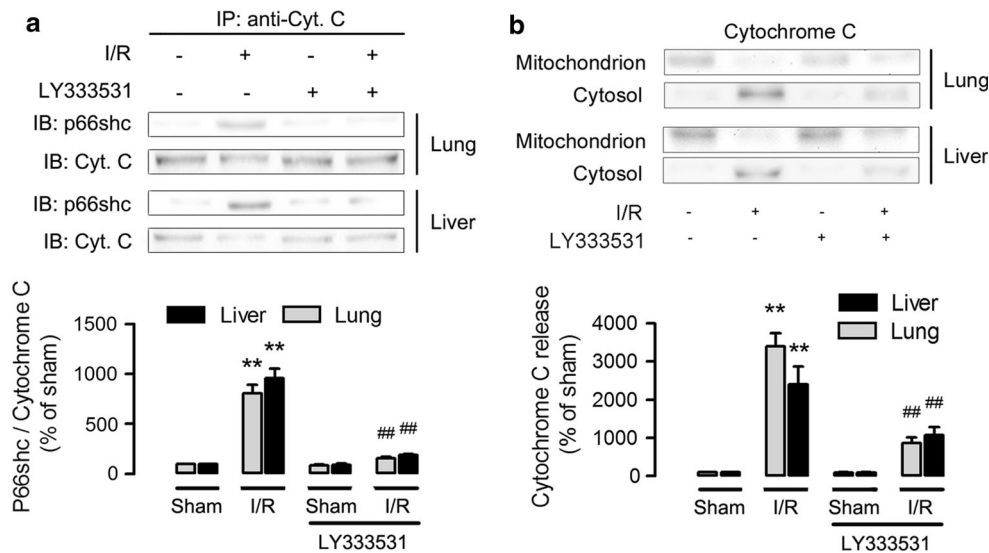


Fig. 8 PKC β suppression inhibits p66shc binding to cytochrome-c and attenuates the subsequent cytochrome-c release. Mice were subjected to 45 min of intestinal ischemia followed by 90 min reperfusion. **a** Mitochondrial lysate was immunoprecipitated with anti-cytochrome-c antibody and immunoblotted with anti-p66shc

antibody. The same blot was re-hybridized with anti-cytochrome-c antibody. Cyt. C indicates cytochrome-c. **b** Protein expression of mitochondrial cytochrome-c and cytosolic cytochrome-c in the lung and liver. Results are presented as the mean \pm SD, $n = 3$. ** $p < 0.01$ versus sham; ## $p < 0.01$ versus I/R

Table 2 LY333531 administration ameliorated intestinal I/R induced GSH system dysfunction in the lung and liver

Group	Lung GSH (mg/g protein)	Lung GSH-PX (U/g protein)	Liver GSH (mg/g protein)	Liver GSH-PX (U/g protein)
Sham	1.26 \pm 0.10	184.01 \pm 14.91	2.68 \pm 0.30	411.56 \pm 21.51
I/R	0.87 \pm 0.16**	138.97 \pm 11.28**	1.77 \pm 0.16**	350.67 \pm 32.02**
Sham + LY333531	1.23 \pm 0.18	206.92 \pm 17.12	2.55 \pm 0.29	413.52 \pm 16.26
I/R + LY333531	1.07 \pm 0.18 [#]	157.14 \pm 17.95 [#]	2.02 \pm 0.22 [#]	390.89 \pm 20.00 ^{##}

Results are presented as the mean \pm SD, $n = 10$. ** $p < 0.01$ versus sham; [#] $p < 0.05$ versus I/R; ^{##} $p < 0.01$ versus I/R

Intestinal I/R is associated with severe damage to the mucosal structure and reduced epithelial function, eventually leading to the loss of barrier integrity and bacterial translocation [32]. Previous studies suggest that migratory bacteria result in the release of cytokines or chemokines, such as IL-6 and TNF- α , which exert a significantly harmful effect via direct inflammatory stimuli on remote organs. Therefore, patients who underwent intestinal I/R develop systemic inflammation in multiple organs [33]. We found that PKC β suppression showed a less obvious inflammatory response compared with the I/R group. PKC β suppression enhanced the mucosal integrity of the intestine (Fig. 3), thus reducing bacterial translocation and cytokines release. Our data indicates that PKC β may play an important role in modulating inflammatory pathways. Consistent with our results, a recent study revealed that PKC β suppression by LY333531 or hispidin (specific inhibitors of PKC β) prevented nuclear factor κ B activation in H₂O₂-treated cells, thus reducing the production of inflammatory cytokines and oxidative stress. In contrast,

phorbol ester (an activator of PKC) treatment promoted PKC β -associated inflammation [34].

Previous studies demonstrated that the oxidative stress response induced by intestinal I/R plays a pivotal role in the pathogenesis of remote organ damage [9, 19]. After ROS exposure, cells undergo organelle disintegration, membrane lipid breakdown, and DNA damage [35, 36]. P66shc is an essential regulator of mitochondrial and cytoplasmic oxidative stress, since p66shc knockout mice had decreased ROS generation and had anti-oxidative features [14, 15]. Previous study identified that oxidative stress induces the phosphorylation of p66shc, which allowing it to be recognized by prolyl isomerase Pin1 and isomerized. After dephosphorylated by protein serine/threonine phosphatase, p66shc translocates into mitochondria and promotes the ROS generation [14]. In this study, we found that significant oxidative stress was induced in the lung and liver after intestinal I/R, which accompanied p66shc phosphorylation. However, LY333531 administration attenuated p66shc phosphorylation, while also alleviating oxidative stress in

these organs. Moreover, MnSOD is a mitochondrial anti-oxidative enzyme regulated by foxo3a [28]. It has been known that phosphorylation of p66shc promotes phosphorylation of foxo3a, with foxo3a translocation into the cytosol in an inactive form. Indeed, p66shc suppression blocks foxo3a phosphorylation and leads to a resistance to oxidative stress, in accordance with MnSOD upregulation [28]. In the current work, MnSOD expression was actually attenuated in the I/R group. In contrast, phospho-p66shc suppression by LY333531 enhanced MnSOD expression, thereby strengthening the anti-oxidative capacity. Furthermore, we showed that p66shc phosphorylation facilitated mitochondrial translocation and binding to cytochrome-c, recognized as the trigger event of H₂O₂ release [27]. Although cytoplasmic GSH could turn the H₂O₂ into H₂O with the catalysis of GSH-PX and maintain the balance of intracellular redox state, we observed H₂O₂ over production, GSH exhaustion and GSH-PX inactivation in this study. However, the PKC β suppressed mice displayed restraining effects on p66shc translocation as well as binding to cytochrome-c. As a result, GSH and GSH-PX were recovered and oxidative stress was relieved. Taken together, PKC β suppression by LY333531 may restrain p66shc-associated oxidative stress in remote organs after intestinal I/R.

Apoptosis and oxidative stress are inseparable parts during various pathophysiological processes. Given exposure to ROS, mitochondrial proteins, lipids, and DNA are believed to be primary targets of oxidative stress damage, leading to alteration or loss of cellular functions, and causing inhibition of proliferation and induction of apoptosis [35, 36]. It has been shown that apoptosis plays a critical role in multiple organ dysfunction in critically ill patients [37]. P66shc acts as a pro-apoptotic protein which plays a pivotal role in modulating the intracellular redox state, increasing susceptibility to oxidative stress, and resulting in apoptosis elicited by oxidative damage eventually. Several researchers have delineated a functional link between p66shc and caspase-3 [38], which plays a crucial role in cell apoptosis by resulting in DNA fragmentation, cytoskeleton degradation, and the formation of apoptotic bodies. P66shc is an indispensable protein that regulates mitochondrial-mediated apoptosis induction in mammals. The realization of its pro-apoptotic function mainly depends on binding of cytochrome-c and its oxidation in the mitochondria. During oxidization, cytochrome-c is released into the cytosol and triggers caspase-9 dependent caspase-3 activation, thus inducing apoptosis [27, 29, 39]. Conversely, mutations in p66shc impair its ability to mediate apoptosis by restraining the interaction between p66shc and mitochondrial cytochrome-c [29]. Consistent with these observations, our data showed that expression of cleaved-caspase-3 was upregulated after intestinal I/R, and accompanied by cytochrome-c release. Meanwhile,

TUNEL data showed significant apoptosis in the lung and liver. In contrast, phospho-p66shc suppression by PKC β inhibition was associated with dysfunction of the cytochrome-c-caspase-3 network and improved apoptosis. Taken together, intestinal I/R activated the cascade from PKC β dependent p66shc phosphorylation, cytochrome-c release, and caspase-3 activation. However, LY333531 administration inhibited PKC β activation, while also suppressing p66shc phosphorylation, translocation, and further interaction with cytochrome-c. Thus, cytochrome-c release was reduced and apoptosis was alleviated.

PKC has become an attractive target in the pathogenesis of various diseases [10]. In the case of ischemic or I/R injury, previous studies have reported activation of PKC ϵ , PKC δ , and PKC β II in cardiac ischemia or I/R [40–42], activation of PKC β II associated with the response to single-lung I/R [11], and activation of PKC α related to hepatic I/R [43]. Thus, the activation of individual PKC isoforms in particular disease is tissue-specific. Despite this feature of PKC activation, we found firstly that the activated PKC isoform in the remote organ, specifically PKC β II and not the other isoforms, is the same as that in the intestine after intestinal I/R. This finding is interesting and makes PKC β II/p66shc pathway as a specific therapeutic target not only in attenuating the primary damage of intestinal I/R, but also in improving the secondary injury in the remote organs.

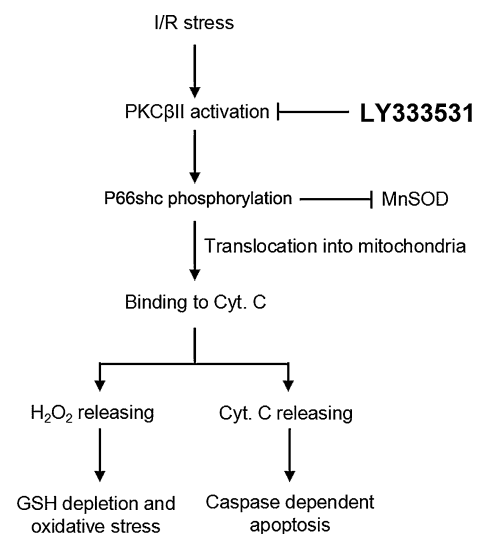


Fig. 9 Proposed mechanism of LY333531 for preventing remote organ injury induced by intestinal I/R. I/R stress activates PKC β II to induce phosphorylation of p66shc, allowing transfer of the protein from the cytosol to the mitochondria. In the mitochondria, p66shc binds to cytochrome-c and oxidizes it, and catalyzes the reduction of H₂O₂. This latter induces the release of cytochrome-c and H₂O₂ into the cytosol. Besides, phosphorylated p66shc inhibits expression of MnSOD. LY333531 inhibits activation of PKC β II, thereby suppressing the above mentioned process. Cyt. C indicates cytochrome-c

In recent years, specific regulators of PKC (agonist or inhibitor) have been used in many animal models [10]. As a highly selective inhibitor of PKC β , LY333531 protected against several ROS and apoptosis associated pathological processes [11, 44]. Moreover, LY333531 is now the focus of testing in human clinical trials [45–47]. We propose that the activation of p66shc-mediated pro-oxidative and pro-apoptosis signaling pathway is particularly dependent on PKC β ; hence, administration of LY333531 to antagonize PKC β may be a novel strategy to prevent intestinal I/R-induced remote organ injury via attenuation of the PKC β -p66shc pathway.

In summary, these findings highlight a novel signaling pathway regulating the remote organ injury induced by intestinal I/R. As shown in Fig. 9, PKC β II is strongly activated in remote organs after intestinal I/R. PKC β suppression by LY333531 protects against lung and liver injury induced by intestinal I/R, partially through the p66shc-cytochrome-c axis. Our results have identified a novel therapeutic target in the management of remote organ injury induced by intestinal I/R.

Acknowledgments This work was supported by grants from the Chinese National Natural Science Foundation (No. 81171850, 81372037).

Conflict of interests The authors have no competing interests to declare.

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