Selective inhibition of protein kinase C $\beta_2$ attenuates the adaptor P66$^{Shc}$-mediated intestinal ischemia–reperfusion injury

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Apoptosis is a major mode of cell death occurring during ischemia–reperfusion (I/R) induced injury. The p66$^{Shc}$ adaptor protein, which is mediated by PKC$\beta$, has an essential role in apoptosis under oxidative stress. This study aimed to investigate the role of PKC$\beta_2/p66^{Shc}$ pathway in intestinal I/R injury. In vivo, ischemia was induced by superior mesenteric artery occlusion in mice. Ruboxistaurin (PKC$\beta$ inhibitor) or normal saline was administered before ischemia. Then blood and gut tissues were collected after reperfusion for various measurements. In vitro, Caco-2 cells were challenged with hypoxia–reoxygenation (H/R) to simulate intestinal I/R. Translocation and activation of PKC$\beta_2$ were markedly induced in the I/R intestine. Ruboxistaurin significantly attenuated gut damage and decreased the serum levels of tumor necrosis factor-$\alpha$ (TNF-$\alpha$) and interleukin-6 (IL-6). Pharmacological blockade of PKC$\beta_2$ suppressed p66$^{Shc}$ overexpression and phosphorylation in the I/R intestine. Gene knockdown of PKC$\beta_2$ via small interfering RNA (siRNA) inhibited H/R-induced p66$^{Shc}$ overexpression and phosphorylation in Caco-2 cells. Phorbol 12-myristate 13-acetate (PMA), which stimulates PKCs, induced p66$^{Shc}$ phosphorylation and this was inhibited by ruboxistaurin and PKC$\beta_2$ siRNA. Ruboxistaurin attenuated gut oxidative stress after I/R by suppressing the decreased expression of manganese superoxide dismutase (MnSOD), the exhaustion of the glutathione (GSH) system, and the overproduction of malondialdehyde (MDA). As a consequence, ruboxistaurin inhibited intestinal mucosa apoptosis after I/R. Therefore, PKC$\beta_2$ inhibition protects mice from gut I/R injury by suppressing the adaptor p66$^{Shc}$-mediated oxidative stress and subsequent apoptosis. This may represent a novel therapeutic approach for the prevention of intestinal I/R injury.

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Critical massive intestinal ischemia occurs in response to conditions such as acute mesenteric thrombotic or embolic occlusion, which are associated with high mortality.$^1,2$ Other gut ischemia cases followed by hemorrhagic shock, volvulus, sepsis, and abdominal aortic aneurysm surgery have a more subtle but no less damaging injury. Although restoration of the blood supply to the ischemic gut is critical to salvage, the reperfusion may paradoxically aggravate ischemic tissue damage. Although restoration of the blood supply to the ischemic gut is critical to salvage, the reperfusion may paradoxically aggravate ischemic tissue damage and systemic inflammatory response.$^3$ During the reperfusion period, a vicious cascade occurs including massive reactive oxygen species (ROS) generation, the massive reactive oxygen species generation, and polymorphonuclear neutrophil infiltration.$^4,5$ It becomes recognized that oxidative stress-induced ischemia/reperfusion (I/R) damage involves multiple signaling pathways.

PKC, a family of serine/threonine protein kinases comprising at least 12 members, has a central role in signal transduction and intracellular crosstalk.$^6$ PKC$\beta_1$ and PKC$\beta_2$ isoforms are encoded by the same gene, PKC$\beta$, and are not expressed in homozygous PKC$\beta^{−/−}$ mice (NCBI Gene Database, identification number 18751). Multiple PKC isoforms are expressed in the intestine.$^7$ Gene deletion or pharmacological blockade of PKC$\beta$ protects ischemic myocardium, decreases infarct size, and enhances recovery of ventricular function.$^8$ Homozygous PKC$\beta$-null mice and WT mice fed with ruboxistaurin (LY333531, selective PKC$\beta$ inhibitor) and subjected to single-lung I/R display increased survival, indicating that PKC$\beta$ has a pivotal role in the I/R-induced apoptosis.$^9$ Despite these observations, the underlying mechanism by which PKC$\beta$ exerts deleterious effects in the intestinal I/R remains unclear.

The Shc adaptor protein family, consisting of the p66$^{Shc}$, p52$^{Shc}$, and p46$^{Shc}$ isoforms, is encoded by the ShcA locus.$^{10}$ Due to the presence of a unique N-terminal domain (CH2), which is required for redox activity, p66$^{Shc}$ is the only isoform
that acts as a redox enzyme implicated in mitochondrial ROS generation and the translation of oxidative signals into apoptosis.\textsuperscript{11} Phosphorylation at Ser36 of p66\textsuperscript{Shc} is required for conferring increased susceptibility to oxidative stress, and is critical for the cell apoptosis elicited by oxidative damage.\textsuperscript{12} Migliaccio \textit{et al}.\textsuperscript{11} reported the p66\textsuperscript{Shc}/C0/C0 mouse increased resistance to oxidative stress and extended lifespan by 30\%. Deletion of the p66\textsuperscript{Shc} gene in mice is shown to protect hind limb,\textsuperscript{13} brain,\textsuperscript{14} and ex vivo hearts\textsuperscript{15} from I/R injury. It suggests that p66\textsuperscript{Shc} would be a target to decrease the injury caused by intestinal I/R.

Hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) and hyperglycemic stress activate the PKC\textsubscript{b\textsuperscript{2}} isoform to induce p66\textsuperscript{Shc} phosphorylation at Ser-36, allowing transfer of the adaptor protein from the cytosol to the inner mitochondrial membrane, where it amplifies oxidative stress and catalyzes ROS production via cytochrome c oxidation.\textsuperscript{16–18} Therefore, we hypothesize that there may be a PKC\textsubscript{b\textsuperscript{2}}/p66\textsuperscript{Shc} signaling pathway in the pathogenesis of intestinal I/R.

**Results**

**Membrane translocation and phosphorylation of PKC\textsubscript{b\textsuperscript{2}} in response to intestinal I/R.** To test the hypothesis that PKC could be activated by I/R injury, we assessed cell membranous fraction of distinct PKC isoforms in the intestinal tissue subjected to 45 min ischemia followed by 45, 90, or 180 min reperfusion. A selective membrane translocation of PKC\textsubscript{b\textsuperscript{2}} was detected, whereas PKC\textsubscript{b\textsuperscript{1}}, PKC\textsubscript{d}, and PKC\textsubscript{e} showed no differences in membrane fraction after various reperfusion times (Figure 1a), indicating that PKC\textsubscript{b\textsuperscript{2}} is specifically activated by I/R. To support this notion, we detected that a 90-min reperfusion significantly increased PKC\textsubscript{b\textsuperscript{2}} phosphorylation at the thr-641 residue, leading to a markedly increased ratio of phosphorylated PKC\textsubscript{b\textsuperscript{2}}/total PKC\textsubscript{b\textsuperscript{2}} (Figure 1b). These results demonstrated that both membrane translocation and activation of PKC\textsubscript{b\textsuperscript{2}} occurred in the model of intestinal I/R.

Ruboxistaurin attenuates gut damage and the systemic inflammatory response after intestinal I/R. Next, ruboxistaurin (oral PKC\textsubscript{b} inhibitor) and normal saline were given as a pretreatment before the superior mesenteric artery was occluded for 45 min followed by 90 min reperfusion. On examination of the histological changes, ruboxistaurin preserved the integrity of morphological structure well, and reduced both hemorrhage and neutrophil infiltration in the I/R intestine (Figure 2a). Similarly, the gut histological injury scores were significantly increased following I/R injury versus sham, and was reduced by ruboxistaurin (Figure 2b). Additionally, intestinal I/R significantly increased the serum levels of tumor necrosis factor-\textgreek{a} (TNF-\textgreek{a}) and interleukin 6 (IL-6). Ruboxistaurin, however, almost abrogated the increase in TNF-\textgreek{a} and IL-6 concentrations (Figure 2c).

**Ruboxistaurin suppresses intestinal I/R-induced activation of PKC\textsubscript{b\textsuperscript{2}} and p66\textsuperscript{Shc}.** Figure 3a showed that ruboxistaurin greatly suppressed the translocation of PKC\textsubscript{b\textsuperscript{2}} in the I/R intestine over the same time period in which PKC\textsubscript{b\textsuperscript{1}} was not impacted. Meanwhile, ruboxistaurin prevented the intestinal I/R-induced increase in the phosphorylation of PKC\textsubscript{b\textsuperscript{2}} without affecting the expression of total PKC\textsubscript{b\textsuperscript{2}}, and suppressed the increased ratio of phosphorylated PKC\textsubscript{b\textsuperscript{2}}/total PKC\textsubscript{b\textsuperscript{2}} (Figure 3b). Intestinal I/R moderately increased the expression of p66\textsuperscript{Shc}, and greatly induced p66\textsuperscript{Shc} phosphorylation. However, ruboxistaurin significantly reduced I/R-induced p66\textsuperscript{Shc} overexpression and phosphorylation at ser36 (Figure 3c). Therefore, our study indicated that ruboxistaurin inhibited both PKC\textsubscript{b\textsuperscript{2}} activation and PKC\textsubscript{b\textsuperscript{2}}-mediated p66\textsuperscript{Shc} activation in the I/R intestine.

![Figure 1](image-url) **Intestinal I/R-mediated membrane translocation and phosphorylation of PKC\textsubscript{b\textsuperscript{2}}.** Mice were subjected to 45 min ischemia followed by 45, 90, or 180 min reperfusion. (a) Representative western blot demonstrating the expression of PKC\textsubscript{b\textsuperscript{1}}, PKC\textsubscript{b\textsuperscript{2}}, PKC\textsubscript{d}, and PKC\textsubscript{e} in membranous fractions with Na,K-ATPase as a loading control. (b) Representative western blot demonstrating p-PKC\textsubscript{b\textsuperscript{2}} (Thr 641) and total-PKC\textsubscript{b\textsuperscript{2}} expression from sham and 90 min reperfusion intestine. All results are expressed as means \(\pm\) S.E.M., \(n=3\) per group, \(*\) \(P<0.05\) versus sham.
Hypoxia/reoxygenation or phorbol 12-myristate 13-acetate-induced p66 Shc activation: involvement of PKCβ2.

Hypoxia/reoxygenation (H/R) of cells in vitro is a simple model of organ I/R, at least partly reflecting the pathophysiology in vivo. To simulate in vivo intestinal I/R, Caco-2 cells were exposed to H/R. To determine whether PKCβ2 is specifically required for the activation of p66Shc, we suppressed its expression using human-specific PKCβ2 small interfering RNA (siRNA) under normoxic and H/R conditions. Knockdown of PKCβ2 by siRNA reduced the expression of PKCβ2 and its phosphorylation in Caco-2 cells under normoxic and H/R conditions (Figure 4a). Our data showed that PKCβ2-siRNA had no effects upon p66Shc activation under normoxic conditions, but prevented p66Shc overexpression and phosphorylation under H/R conditions (Figure 4b). To further confirm whether p66Shc activation was activated by PKCβ2, we examined the effect of phorbol 12-myristate 13-acetate (PMA), a classical PKC activator, on the activation of p66Shc. The exposure of PMA markedly increased p66Shc phosphorylation in Caco-2 cells, which was inhibited significantly by PKCβ2 siRNA and ruboxistaurin (Figure 4c).

Inhibition of PKCβ2 activation by ruboxistaurin attenuates gut oxidative stress after intestinal I/R. To evaluate the oxidative state of the gut after I/R, we measured the levels of manganese superoxide dismutase (MnSOD), glutathione (GSH), glutathione peroxidase (GSH-PX), and malondialdehyde (MDA) in the intestinal tissues. Ruboxistaurin reversed intestinal I/R-induced anti-oxidant enzyme MnSOD downregulation (Figure 5a). ROS accumulation was increased in intestinal I/R tissue based on the assessment of MDA activity, which was reduced by ruboxistaurin (Figure 5b). In parallel, ruboxistaurin preserved intestinal I/R-induced GSH exhaustion and GSH-PX activity reduction (Figures 5c and d). Taken together, these data indicated that blockade of PKCβ2 decreased gut oxidative stress after intestinal I/R.

Inhibition of PKCβ2 activation by ruboxistaurin inhibits gut apoptosis after intestinal I/R. To determine the apoptosis state of the gut after I/R, a terminal deoxynucleotidyl transferase mediated deoxyuridinetriphosphate nick end labeling (TUNEL) assay was conducted. The apoptotic cells in the gut were elevated from non-detectable to well observed after intestinal I/R, whereas ruboxistaurin significantly reduced the number of apoptotic cells (Figure 6a). In addition, ruboxistaurin significantly suppressed the increased levels of cleaved caspase-3, another marker of cell apoptosis, in the I/R intestinal tissue (Figure 6b).

Discussion
In the present study, we have demonstrated that I/R-induced intestinal dysfunction involved the PKCβ2/p66Shc signaling pathway. PKCβ2 activation played an essential role in the
pathogenesis of intestinal I/R injury, and inhibition of excessive activation of PKCβ2 by ruboxistaurin reduced intestinal I/R injury at least partly via attenuation of the p66Shc activation. P66Shc acted as a redox enzyme implicated in mitochondrial ROS generation and the translation of oxidative signals into apoptosis. We provided evidence that...
pharmacological blockade or gene knockdown of PKCβ2 inhibited I/R-induced p66Shc activation, demonstrating that excessive p66Shc activation is associated with PKCβ2 activation. To the best of our knowledge, this is the first study examining the relationship between PKCβ2 and p66Shc in intestinal I/R.

Previous studies have reported activation of PKCβ2, PKCα, and PKCe in cardiac ischemia or I/R, 8,19,20 activation of PKCβ2 associated with the response to single-lung I/R, 8 and activation of PKCδ and PKCe related to cerebral I/R. 21 Our results demonstrated that the activated principal isoform of PKC in intestinal I/R was specifically PKCβ2, not PKCβ1, PKCα, or PKCe (Figures 1a and b). These data suggested that the activation of individual PKC isoforms in ischemia or I/R is tissue specific. Moreover, our results indicated that in intestinal I/R, ruboxistaurin did not change the translocation of PKCβ1 over the same time period in which PKCβ2 was greatly impacted (Figure 3a). Taken together, it is likely that the primary role of ruboxistaurin was to inhibit the activation of PKCβ2 in intestinal I/R.

H/R significantly induces the activation of p66Shc, and ablation of p66Shc is cytoprotective against oxidative stress.

**Figure 5** Inhibition of PKCβ2 activation by ruboxistaurin attenuates gut oxidative stress after intestinal I/R. (a) Representative western blot demonstrating MnSOD protein expression (n = 3). (b) The activity of MDA in the intestine was determined. (c) The GSH levels in the intestine. (d) The GSH-PX levels in the intestine (n = 8 per group for b, c, and d). All results are expressed as means ± S.E.M., **P < 0.01 versus sham; #P < 0.05 versus I/R.

**Figure 6** Inhibition of PKCβ2 activation by ruboxistaurin inhibits gut apoptosis after intestinal I/R. (a) TUNEL staining of paraffin-embedded intestinal tissue sections. Representative images for sham, I/R, sham ruboxistaurin pretreatment, and I/R ruboxistaurin pretreatment groups (n = 8). (b) Representative western blot demonstrating cleaved caspase-3 protein expression. All results are expressed as means ± S.E.M., n = 3 per group, **P < 0.01 versus sham; #P < 0.01 versus I/R.
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and apoptosis in hepatocytes. This may be clinically relevant as the mRNA level of p66Shc is increased in peripheral blood mononuclear cells of patients with acute myocardial infarction. In human aortic endothelial cells, selective inhibitor of PKCβ2 prevented p66Shc activation after exposure to hyperglycemic stress or oxidized low-density lipoprotein, respectively. Our data showed that inhibition of PKCβ2 activation by ruboxistaurin attenuated p66Shc overexpression and phosphorylation at ser36 in the I/R intestine (Figure 3c). In vitro studies, knocking down PKCβ2 via siRNA inhibited the activation of PKCβ2, and further prevented p66Shc overexpression and phosphorylation under H/R conditions (Figure 4). By using both pharmacological blockade and gene knockdown PKCβ2 in vivo and in vitro experiments, we tested the above hypothesis that there may be a PKCβ2/p66Shc signaling pathway in intestinal I/R.

Gut I/R produces excessive amounts of ROS, which is responsible for the intestinal mucosa damage. Given exposure to ROS, mitochondrial proteins, lipids, and DNA are believed to be primary targets of oxidative damage, leading to alteration or loss of cellular functions, and causing inhibition of proliferation and induction of apoptosis. A growing body of evidence links p66Shc to oxidative stress as the adaptor protein has a pivotal role in modulating the intracellular redox state, increasing susceptibility to oxidative stress, and resulting in apoptosis elicited by oxidative damage. Our data demonstrated ruboxistaurin increased the intestinal I/R-induced downregulation of MnSOD, a primary ROS scavenging enzyme, but suppressed the accumulation of MDA, an indicator of lipid peroxidation (Figures 5a and b). Meanwhile, ruboxistaurin preserved intestinal I/R-induced GSH exhaustion and GSH-PX activity reduction (Figures 5c and d). Furthermore, the apoptosis execution enzyme caspase-3 has a crucial role in cell apoptosis by resulting in DNA fragmentation, degradation of cytoskeleton, and formation of apoptotic bodies. Arany et al. showed that p66Shc was associated with cytochrome c, which is responsible for the activation of caspase-3 in the kidneys of mice with I/R injury. Our data showed that ruboxistaurin significantly attenuated intestinal caspase-3 activity and inhibited the apoptosis of the intestine subjected to I/R (Figures 6a and b). Therefore, it is conceivable that the inhibition of PKCβ2 activation by ruboxistaurin attenuates p66Shc-mediated oxidative stress and subsequent apoptosis in intestinal I/R.

During the reperfusion period, mucosal barrier integrity is destroyed and the systemic release of pro-inflammatory cytokines occurs, with concurrent leukocyte activation and bacterial translocation. In this study, intestinal I/R injury significantly increased the serum levels of TNF-α and IL-6, suggesting that a severe systemic inflammation response was induced during the reperfusion period. Ruboxistaurin administration almost abrogated the increase in TNF-α and IL-6 serum concentration (Figure 2c).

Ruboxistaurin, an oral PKCβ inhibitor, is currently undergoing phase 2 and phase 3 clinical testing for several cardiovascular diseases, such as diabetic retinopathy and diabetic kidney disease. Due to be administrated orally, ruboxistaurin was gavaged for 3 days before I/R, which would be a potential limitation in acute clinical cases. However, the focus of this study was to investigate the role of PKCβ2 in regulating p66Shc-mediated intestinal I/R injury.

In summary, our results demonstrate that the inhibition of PKCβ2 activation attenuated intestinal I/R injury and systemic inflammation response by inhibiting the adaptor p66Shc-mediated oxidative stress and subsequent apoptosis. Furthermore, the activated principal isoform of PKC in intestinal I/R was specifically PKCβ2, not PKCβ1, PKCδ, or PKCε. These may represent a novel therapeutic avenue for intestinal I/R injury.

Materials and Methods

Murine model of intestinal I/R. Male ICR mice (aged 4 weeks) weighing 20 ± 2 g were obtained from the Animal Center of Dalian Medical University (Dalian, China), and kept under standard laboratory conditions with standard laboratory chow and water. The mouse intestinal occlusion-and-reperfusion procedure was performed as described previously. Briefly, the superior mesenteric artery was occluded by a microvascular clamp for 45 min and then 45, 90, or 180 min reperfusion was performed. Normal saline and ruboxistaurin (LY 333531; ENZO, Lausen, Switzerland) were given by oral gavage before sham surgery. All procedures were conducted according to the Institutional Animal Care Guidelines, and were approved by the Institutional Ethics Committee.

Histological and TUNEL staining. For histological and TUNEL analysis, formalin-fixed sections were embedded in paraffin and sectioned. The 4-μm sections were stained by hematoxylin–eosin. Intestinal I/R-induced mucosal injury was evaluated according to Chiou’s score. TUNEL staining was performed using an apoptosis assay kit (Roche, Mannheim, Germany) according to the manufacturer’s instructions.

Measurement of cytokines. The levels of serum TNF-α and IL-6 were measured using Enzyme-linked immunosorbent assay (ELISA) kits (ENGTON Bio-engineering Limited Company, Shanghai, China) according to the manufacturer’s protocols.

Intestinal GSH, GSH-PX, and MDA activity assay. The GSH and GSH-PX activities were determined using an assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer’s recommendations. The level of MDA in the intestinal tissues was quantified by a lipid peroxidation MDA assay kit (Beyotime Institute of Biotechnology, Jiangsu, China) according to the manufacturer’s protocol.

Cell culture. Caco-2 cells were cultured at 37 °C in a humidified atmosphere of 5% CO2 in DMEM, supplemented with 10% fetal bovine serum, 1% non-essential amino acids, and 1% glutamine (Gibco, Carlsbad, CA, USA). To simulate physiologic conditions, Caco-2 cells were grown as monolayers on plates, providing both apical and basolateral areas, thereby allowing cells to become polarized. The culture medium was then replaced with serum-free DMEM before experimental treatment.

Transient transfection of siRNA. Caco-2 cells (1 × 104) were seeded on six-well plates and transfected at the time of 70–80% confluence with a PKCβ2 siRNA or non-binding control siRNA using Lipofectamine 2000 (Invitrogen, Karlsruhe, Germany), according to the manufacturer’s instructions. The siRNA which was used to target PKCβ2 had the sequences: 5′-GGCGACACUAGUUAUCACAUdTdT-3′ and 5′-AUGUGAUACAUUGAGGUGCCUdTdT-3′ (GenePharma, Shanghai, China). Scrambled siRNA which was used as a negative control had the sequences: 5′-ACGUAGACAGUUGGAGCAAGAdTdT-3′ and 5′-UUCUCCGUACGUGACAGUCGdTdT-3′. Commercial PKCβ2 siRNA was utilized for the inhibition of PKCβ2 expression as per manufacturer’s protocol.

H/R incubation and PMA exposure. To simulate in vivo intestinal ischemia, unless otherwise noted, cellular hypoxic conditions were created. For the hypoxic conditions, cells were incubated in a microaerophilic system (Thermo Fisher Scientific 8000, Marietta, GA, USA) at 5% CO2 and 1% O2, and balanced...
with 94% N₂ gas for 15h.35 The cells were then cultured in normoxic conditions for 6h of reoxygenation. After transfection with control or PKCβ2 siRNA, cells were incubated in either normoxic or H/R DMEM medium. Caco-2 cells were exposed to 100mM PMA (Sigma-Aldrich, St. Louis, MO, USA) for 30min in the absence or in the presence of PKCβ2 siRNA or roxubastin (20mM).

Western blot analysis. Equal protein amounts from isolated intestinal tissue and Caco-2 cell homogenate were removed using 10−15% SDS-PAGE (Bio-Rad, Hercules, CA, USA), and subsequently transferred onto PVDF membranes (Millipore, Bedford, MA, USA). Antibodies used for western blotting included those for PKCα, PKCβ1, and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA); PKCδ, PKCε, cleaved caspase-3, and Na⁺-K-ATPase (BioTime Technology, St. Louis Park, MN, USA); phospho-PKCβ2 (Cell Signaling Technology, Danvers, MA, USA); and p66Shc-phospho-p66Shc and MnSOD (Abcam Ltd., Cambridge, UK). Appropriate secondary antibodies were used to detect the primary antibody/antigen complexes. The membranes were exposed to enhanced chemiluminescence-plus reagents (Beyotime Institute of Biotechnology). Emitted light was documented using a multispectral imaging system (UVP, Upland, CA, USA), and the bands were analyzed using a Gel-Pro Analyzer, Version 4.0 (Media Cybernetics, Rockville, MD, USA).

Statistical analysis. Densityometry was obtained by the image analysis software (UVP). All values are presented as means ± S.E.M. The data were analyzed with a two-tailed Student's t-test when comparing means between two groups. One-way analysis of variance (ANOVA) followed by Student–Newman–Keuls (SNK) test was used when comparing multiple groups. The ordinal values of the gut injury scores were analyzed by the Kruskal–Wallis non-parametric test. Statistical significance was considered at P-values less than 0.05.

Conflict of Interest. The authors declare no conflict of interest.

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