Intestinal ischemia-reperfusion of macaques triggers a strong innate immune response

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Abstract

AIM: To investigate inflammatory injury in the intestinal mucosa after intestinal ischemia-reperfusion (IIR) with Toll-like receptor (TLR)-mediated innate immunity.

METHODS: Ten macaques were randomized into control and IIR groups. The distribution and expression level of TLR2, TLR4, MD2, nuclear factor (NF)-κB p65 and interferon (IFN)-γ were measured by immunohistochemical stain and western blotting. The mRNA expression of TLR4, TLR2, MD2, interleukin (IL)-1β and tumor necrosis factor (TNF)-α were measured by reverse transcriptase-polymerase chain reaction. The cytokine levels in blood and intestinal tissues were measured by ELISA.

RESULTS: Obvious hemorrhage and erosion of mucosa were seen in the IIR group. Expression of TLR2, TLR4, MD2, NF-κB p65 and IFN-γ was significantly higher in the IIR group than in the control group (0.13 ± 0.04, 0.22 ± 0.04, 0.16 ± 0.06, 0.65 ± 0.12, 0.38 ± 0.10 vs 0.07 ± 0.04, 0.08 ± 0.03, 0.04 ± 0.02, 0.19 ± 0.06, 0.14 ± 0.05, P < 0.05). In addition, the expression of TLR2, TLR4, MD2, IL-1β and TNF-α mRNA in the IIR group were significantly higher than those of control group(1.52 ± 0.15, 1.39 ± 0.06, 1.94 ± 0.12, 1.48 ± 0.15, 0.66 ± 0.08 vs 0.31 ± 0.05, 0.5 ± 0.04, 0.77 ± 0.05, 0.35 ± 0.08, 0.18 ± 0.04, P < 0.05). Furthermore, IL-1β, IL-6 and TNF-α levels in the macaques ileum and plasma were significantly higher than in the control group (plasma: 86.3 ± 15.2, 1129 ± 248.3, 77.8 ± 16.2 vs 29.5 ± 7.3, 19.8 ± 8.2, 5.6 ± 1.7; ileum: 273.4. ± 44.7, 1636 ± 168.0, 205.5 ± 30.7 vs 76.8 ± 20.5, 663.4 ± 186.9, 49.0 ± 9.4; P < 0.05).

CONCLUSION: After IIR, general inflammatory injury in the intestinal mucosa is correlated with a strong innate immune response, mediated by activation of the TLR-NF-κB-cytokine pathway.

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Key words: Intestine ischemia reperfusion; Toll-like receptors; Nuclear factor-κB; Cytokine; Macaques

Core tip: After intestine ischemia-reperfusion, general inflammatory injury of the intestinal tract, and the ensuing multiple organ dysfunction syndrome, are correlated with a strong innate immune response, which is mediated by the extensive activation of the Toll-like receptor-nuclear factor-κB-cytokine pathway throughout the whole system.
INTRODUCTION

Multiple organ dysfunction syndrome (MODS) is the most severe complication of trauma, infection, or severe acute pancreatitis, and it has a high mortality rate. The intestinal tract is not only the target organ injured, but also the root of a systemic inflammatory reaction and a regulator of the internal environment under stress. For this reason, the gastrointestinal tract is thought to be an activating organ in the onset of MODS\textsuperscript{[1,2]}\textsuperscript{,}. Damage to the intestinal mucosa barrier and bacteria translocation remain the chief mechanisms of MODS theory\textsuperscript{[3,4]}\textsuperscript{.}

According to the theory of intestinal mucosa barrier injury and bacterial translocation, intestinal damage should occur in the colon. In a previous study we noticed general inflammatory damage to the intestine, but only slight damage to the right colon after occluding the superior mesenteric artery (SMA) during MODS caused by intestinal ischemia-reperfusion (IIR) of macaques\textsuperscript{[5]}. As the SMA supplies both the intestine and the right colon\textsuperscript{[5]}, the difference in the tissue damage cannot be explained by the theory of local oxygen metabolism disturbance, oxygen free radical damage, or bacteria toxins. The intestine is the biggest immune organ in the whole body, and the fact that general inflammatory damage to the mucosa occurred rapidly after IIR reminds us of the possibility that the innate immune response may play a role in this damage.

The innate immune response acts rapidly, which can protect the body from injury from microorganisms and harmful substances. Widespread Toll-like receptors (TLRs) are thought to be signal recognition receptors for the innate immune system, which activate nuclear factor (NF)-κB and induce transcription and expression of immune-response genes and inflammatory cytokines after combining with pathogen-associated molecular patterns (PAMPs), and then start an inflammatory reaction\textsuperscript{[6]}\textsuperscript{,}. Lipo polysaccharide (LPS) is one of the most widely recognized PAMPs. In previous studies, TLR4 and TLR2 were found to be the general receptors for LPS\textsuperscript{[6]}\textsuperscript{,}.

To investigate whether the general inflammatory damage following IIR and subsequent MODS is caused by an innate immune response mediated by TLRs in the intestine, we observed the change of TLR4 and TLR2 expression in the intestinal mucosa after inducing IIR in macaques, and analyzed the relationship between TLR4/TLR2 and NF-κB p65 and cytokines.

MATERIALS AND METHODS

Experimental animals and surgical procedures

Ten adult rhesus macaques (body weight, 6.92 ± 1.67 kg, aged 4-7 years) were provided by the Experimental Animal Center of Sichuan University, and the study was approved by the Animal Ethics Committee of West China Hospital of Sichuan University. The animals were randomly divided into the IIR group and control group (n = 5 each, male/female ratio 3:2). Before surgery, all groups of animals were fasted for 12 h with free access to water and then anesthetized through xylazine (0.2 mL/kg, intramuscularly) and maintained with carbrital [20 mg/kg, intravenously (iv)] and diazepam (0.1 mL/kg, iv) as needed. Upon satisfactory anesthesia, the animal was fixed on an operating table for skin preparation of the abdominal surgical area, followed by routine disinfection and draping.

IIR group: An incision was made right in the middle of the upper abdomen of rhesus macaques. The duodenum was exposed, lifted, and pulled. The SMA was isolated and then occluded with a microsurgical clip. One hour later, the clip was removed, and intestinal perfusion was reestablished.

Control group: The same procedure as for the IIR group except for occlusion of SMA with a microsurgical clip.

After surgery, animals in both groups received infusion of saline and glucose (0.2 mL/kg per minute, iv glucose tolerance test) for 24 h. Venous blood samples were taken, the macaques were killed 24 h after surgery, and the vital organs were removed for further examination.

Histopathological scoring of macaque intestine

Terminal ileum and the right colon specimens, which were fixed in 4% paraformaldehyde, were dehydrated, embedded, and sectioned following routine procedure. After HE staining, tissue sections were examined by microscopy. For semiquantitative evaluation of lesions, 10 randomly selected microscopic fields were observed carefully in each sample. The scoring system was based on the area of the lesion as follows: +, < 1/3 total area of field; ++, 1/3-2/3 total area of field; and ++++, > 2/3 total area of field. The remaining parts of the specimens were stored in a freezer at -70 °C.

Immunohistochemistry of TLR4, TLR2, MD2, NF-κB p65 and interferon (IFN)-γ

The terminal ileum and the right colon tissue sections of macaques were used for the immunohistochemical examinations. Frozen 10-μm terminal ileum tissue sections were cut in a cryostat and placed on a glass slide. The sections were fixed in 100% methanol for 5 min at -20 °C, treated with 0.1% goat serum for 20 min at 20 °C, and then treated for 8 h at 4 °C with PBS containing 0.1% goat serum and the following primary antibodies: rab-
Table 1  Sequences of primers and polymerase chain reaction products

| mRNA   | Sense            | Antisense                  | Size (bp) |
|--------|------------------|----------------------------|-----------|
| TLR4   | TGCAATGATCAAGGACCAGAGGC | GTGCTGGCACACCAACAATCACCC   | 449       |
| TLR2   | GGGCGCAAAATTACCTGTTG  | CTGAGCTTCGTCATGGCCACTCC    | 657       |
| MD2    | GAAGCTCAGAAGCATATTGGTCT | GGTGGTGTTAGAGTACAAACTCC    | 422       |
| IL-1β  | AAACCAGATGAGGGTCTCCAGG | TGGAGACACCACTTGTTGCTCCA    | 388       |
| TNF-α  | AGGGCTTACCGGGGTCTTGGT | TGTTAGAAGCAGCGGATCGGCC     | 418       |
| β-actin| CACCACACCTTACACATGAGC | GTGATCTCCTTCATGATGAGCC     | 695       |

TLR: Toll-like receptor; IL: Interleukin; TNF: Tumor necrosis factor.

Western blotting to detect TLR4, TLR2, MD2 and NF-κB
The quantities of TLR4, TLR2 and MD2 protein extracted from isolated ileum epithelial cells were measured by western blotting. Protein samples were separated by 12% SDS-PAGE. Samples were transferred to nitrocellulose membranes, which were then blocked for 2 h in 5% nonfat dry milk suspended in 0.1% Tween-20 Tris-buffered saline (TTBS, pH 7.4). Nitrocellulose membranes were incubated with rabbit polyclonal TLR4/TLR2 antibody (1:500), MD2 (1:500), NF-κB p65 (1:1000), IFN-γ (1:500), or β-actin (Cell Signaling Technology, Boston, MA, United States), at 4°C overnight. Membranes were washed in TTBS, incubated with horseradish-peroxidase-conjugated secondary antibody (Univ-bio, Shanghai, China), and developed using enhanced chemiluminescence reagents. The objective bands were analyzed by a UVIB (ultraviolet irradiation band) imaging system and were normalized to 1 IOD unit.

Reverse transcriptase-polymerase chain reaction of TLR4, TLR2, MD2, IL-1β, and TNF-α mRNA levels in terminal ileum
Total RNA was extracted from terminal ileum tissue specimens using TRIzol reagent (Roche, Burlington, NC, United States). The purity of RNA extract was tested by spectrophotometry analysis of optical density (OD, OD260/OD280 = 1.95-2.0). Reverse transcription polymerase chain reaction (RT-PCR) amplification were conducted with PTC-100 PCR (Bio-Rad Laboratories, Hercules, CA, United States), in accordance with the illustrations of the RT-PCR core kit (TaKaRa, Shiga, Japan). The sequences of primers and PCR products are listed in Table 1[10,11]. The obtained cDNA (2 μL each) was used as a template for PCR amplification with β-actin as an internal reference. The PCR products (2 μL each) were electrophoresed on 2% agarose gel, and quantified by scanning the gel in an imaging system (Bio-Rad Gel Doc 2000). The data were standardized as a ratio of gray scale (IOD) of objective band to β-actin.

ELISA of cytokines levels
The levels of IL-1β, IL-6, and TNF-α in plasma and intestinal were measured by ELISA (Senxiong Company, Shanghai, China), according to the manufacturer’s instructions. The plasma levels of cytokines were standardized as pg/mL. The ileal concentration of cytokines was standardized as pg/g protein.

Statistical analysis
All experimental data were processed in SPSS version 17.0 (SPSS, Chicago, IL, United States). Data were expressed as mean ± SD and subjected to tests for homogeneity of variance and normality (P > 0.05 for both). Intergroup comparisons were performed using one-way analysis of variance, and significant difference among means was identified by Fisher LSD test at the level of α = 0.05. P < 0.05 was considered statistically significant.

RESULTS
Diverse pathological changes in the small intestine after IIR
In the IIR group, rhesus macaques presented with obvious distension of the small intestine, pale mucous membrane, hemorrhage, and erosion in the small intestine, compared with the control group. No apparent changes were found in the colon of both groups. Marked mucosal inflammatory injury of the ileum, including increased macrophage and neutrophil infiltration into the mucosa, erosion or necrosis, and hemorrhage of the intestinal mucosa were observed under the microscope. In the IIR group, rhesus macaques showed significantly higher inflammatory lesion scores compared with the control group (P < 0.05) (Table 2). In contrast, inflammatory lesion scores of right colon were minor and no significant difference in both groups (Figure 1).
DISCUSSION

A balance is normally kept between the human body and the intestinal flora and microorganisms inside it. Few bacteria can survive in the small intestine because of the mechanical scouring due to bowel movements and the integrated bacteria-killing mechanisms of the upper gastrointestinal tract, especially bile [1]. Even though macaques eat more unclean food than humans, the germ-free rate measured in our previous study of healthy macaque ileum bacteria groups was as high as 40% [3]. TLR4, TLR2 and MD2 were found to be slightly expressed in macaque intestinal mucosal epithelia, but were almost negative in Peyer's nodules. This low expression was because, due to the mild stimulus that microorganisms provide to normal intestinal mucosae, the TLR-NF-κB cytokine signal transduction pathway in intestinal epithelia and antigen-presenting cells was in a mildly activated state, which was observed as a physiological inflammatory reaction.

We have seen from observation of bacterial counts and spectra in the small intestine during IIR that ileal bacteria increase dramatically, by a factor of $10^6$ after IIR. This increase is predominantly in aerobic bacteria, among which Escherichia coli (E. coli) increased by a factor of $10^6$ [19]. The abundant LPS of E. coli creates essential conditions for extensive activation of TLR4. We know that the recognition of LPS by TLR4 needs mediation of MD2 and CD14 on the cell surface and formation of an LPS recognition compound [18]. MD2 is one kind of secretary protein that exists on the surface of cell membranes, which helps TLR4 to recognize LPS/LBP/CD14 compounds and to lock LPS onto the combining site, so the presence of MD2 can increase the sensitivity of TLR4 to LPS [15,17]. Mutation of MD2 can induce non-response of TLR4-expressing cells to LPS [19], which is why MD2 plays an important role in the function of TLR4. We found in the present study that MD2 expression in the intestine following IIR increased dramatically, and assisted LPS in activating inactive TLR4. We thus draw the conclusion that the combined upregulation of TLR4-MD2 expression is one of the important factors in the innate immunity mediated by TLRs following IIR. This increase is predominantly in aerobic bacteria, by a factor of $10^6$ after IIR. This increase is predominantly in aerobic bacteria, among which Escherichia coli (E. coli) increased by a factor of $10^6$ [19]. The abundant LPS of E. coli creates essential conditions for extensive activation of TLR4. We know that the recognition of LPS by TLR4 needs mediation of MD2 and CD14 on the cell surface and formation of an LPS recognition compound [18]. 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increase of TLR4 and TLR2 expression in intestinal epithelia and lymph tissues, either at the gene transcription or protein level, while at the same time, the expression of NF-κB and proinflammatory cytokines IL-1β, IL-6 and TNF-α also increased, and these increases showed good correlation. IIR accelerated the whole process of mRNA transcription, protein molecular expression, and functioning of immunological factors in the TLR-NF-κB-cytokine pathway in mucosal epithelia, which on the one hand fully displayed the characteristics of a fast reaction of the innate immune system, and on the other hand, the positive feedback of releasing inflammatory mediators of TLR expression induced a stream of inflammatory mediators. The dramatic increase in peripheral inflammatory mediators by a factor of 20-100, induced by the rapid and severe innate immune reaction...
in intestinal mucosae, should be the basis of MODS theory.

Organs and tissues may be severely harmed by inflammatory mediators produced by an innate immune response. The inter-reaction between inflammatory mediators and inflammatory cells increases vascular permeability, and causes tissue edema and reduction of vascular quantity per unit volume, which aggravates cellular anoxia and induces tissue lesions; the large amount of enzymes and inflammatory products released by phagocytes directly injures tissue; also, increased cytokines tend to induce hypercoagulability, which is why extensive micro-thromboembolism can often be seen in the internal organs, especially the liver and lungs, of MODS patients. The inhibition of cardiac contractility by abundant TNF-α, IL-1β and IL-6 causes changes in hemodynamics such as blood pressure decrease, tachycardia, and left ventricular ejection fraction decrease, and these are clinically manifest as hypotension and low cardiac output. We observed that TLRI-mediated inflammation following IIR damaged the integrity of the small intestinal mucosal barrier, and that progression of propagative E. coli from the intestinal cavity into tissue or even the bloodstream led to bacterial translocation and bacteremia. In addition, the integrity of the small intestinal mucosal barrier prevents contact of TLRs on B cells in Peyer’s nodules with bacteria, and ex-

Figure 4   mRNA for Toll-like receptor 4, Toll-like receptor 2, MD2, TNF-α and IL-1β in ileal epithelia of macaques (RT-PCR). *P<0.05 vs control group. TLR: Toll-like receptor; TNF-α: Tumor necrosis factor-α; IL-1β: Interleukin-1β; IIR: Intestinal ischemia-reperfusion; RT-PCR: Reverse transcription polymerase chain reaction.

### Table 3 Quantitative comparison of TLR4, TLR2, MD2, and NF-κB p65 protein in intestinal mucosa (IOD value)

| Group   | TLR4 (pg/mL) | TLR2 (pg/mL) | MD2 (pg/mL) | NF-κB p65 (pg/mL) | IFN-γ (pg/mL) |
|---------|--------------|--------------|-------------|-------------------|---------------|
| Control | 0.80 ± 0.03  | 0.07 ± 0.04  | 0.04 ± 0.02  | 0.19 ± 0.06       | 0.14 ± 0.05   |
| IIR     | 0.22 ± 0.04  | 0.13 ± 0.04  | 0.16 ± 0.06  | 0.65 ± 0.12       | 0.36 ± 0.10   |

### Table 4 mRNA expression of TLRs, MD2 and inflammatory mediators in intestinal mucosa (IOD value)

| Group   | TLR4 | TLR2 | MD2 | TNF-α | IL-1β |
|---------|------|------|-----|-------|-------|
| Control | 0.50 ± 0.04 | 0.31 ± 0.05 | 0.77 ± 0.05 | 0.35 ± 0.08 | 0.18 ± 0.04 |
| IIR     | 1.39 ± 0.06* | 1.52 ± 0.15* | 1.94 ± 0.12* | 1.48 ± 0.15* | 0.66 ± 0.08* |

### Table 5 Inflammatory mediators in the small intestine mucosa and peripheral plasma

| Group | Plasma IL-1β (pg/mL) | Plasma IL-6 (pg/mL) | Plasma TNF-α (pg/mL) |
|-------|---------------------|---------------------|----------------------|
|       | Plasma IL-1β (pg/g protein) | Plasma IL-6 (pg/g protein) | Plasma TNF-α (pg/g protein) |
| Control | 29.5 ± 7.3 | 19.8 ± 8.2 | 5.6 ± 1.7 | 205.5 ± 30.7 |
| IIR | 86.3 ± 15.2* | 663.4 ± 186.9 | 49.0 ± 9.4 |

5 in each group; *P<0.05 vs control group.
pression of TLRs was low. Although this integrity is dam-
gaged because of a local innate immune response, an op-
opportunity emerges for TLRs on B cells in Peyer’s nodule
beneath the mucosal epithelium to come into contact with
a large amount of pathogenic microorganisms. So, during
IIR, not only the epithelium strongly expresses TLR4 and
TLR2. Rather, TLR4 and TLR2 on B cells in Peyer’s nodule
are also significantly activated. NF-κB shows a strong posi-
tive reaction, therefore, B cells are involved in the
innate immune response, which aggravates local mucosal
inflammatory reactions.

In conclusion, all macaques developed MODS fol-
lowing IIR, and this was related to the expansion of the
severe innate immune response, which was mediated by
the general activation of the TLR-NF-κB-cytokine path-
way in the small intestinal mucosa throughout the whole
system.

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