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Full Length Research Paper

Effect of seawater immersion on the NF-κB, IκB and TLR4 expression in small intestinal tissues in rats with abdominal open injury

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The aim of this study was to observe the impact of seawater immersion on the dynamic expressions of NF-κB and IκB mRNA in small intestinal tissues in rats with abdominal open injury. Wistar rats were randomly divided into three groups: the control group (n = 7), the abdominal open injury (AOI) group and abdominal open injury plus seawater immersion (AOI+SI) group. The dynamic expressions of NF-κB and IκB mRNA in small intestinal tissues were detected in each group/subgroup by real-time PCR method. After 72 h, NF-κB mRNA level of AOI+SI group was increased obviously compared to that of AOI group (P<0.01). The IκB mRNA expression of both AOI+SI and AOI groups were increased obviously after 48 h compared to that of the control, but the difference between AOI+SI group and AOI group showed that the increase of AOI+SI group was more significant (P<0.01). In the long time of seawater immersion (>72 h), the NF-κB mRNA expression was significantly up-regulated, which promoted the synthesis of NF-κB protein and further magnified NF-κB signal pathways to lead to the development of continued inflammatory course ultimately; meanwhile, the obviously up-regulated IκB-α mRNA level increased the expression of IκB-α protein to down-regulate the activity of NF-κb as much as possible.

Key words: Abdominal open injury, seawater immersion, real-time PCR, NF-κB, IκB.

INTRODUCTION

Abdominal trauma is one of the most common injuries in modern sea battles with an incidence rate of 3 to 4%. Seawater immersion after abdominal trauma can lead to severe disorders in metabolism and hemodynamic, it can activate and cause the release of inflammatory cytokines in a large quantity to induce inflammatory reactions. And during the process, the barrier function of intestines is destroyed and a large amount of bacteria and endotoxin (ETX) invade into organic circulation systems to induce more serious immune imbalance and induce the initiation and development of MODS (multiple organ dysfunction syndrome), which might also serve as an important reason for the aggravation of injury (Sen and Baltimore, 1986; Ghosh et al., 1998).

Inflammatory reactions constitute an essential part in a secondary injury, and also, they are the major cause for the progressive aggravation of injury or even MODS after trauma. During the process, inflammatory mediators and cytokines play important roles. Among different transcription factors regulating inflammatory genes, nuclear factor kappa B (NF-κB) occupies the most dominant position, which is also the necessary cytokine for transcriptional activation of many genes regulating inflammatory reactions in cells.

Meanwhile, Toll-like receptor 4 (TLR), as the upstream receptor of NF-κB, could activate NF-κB-centered signal transduction pathways, induce inflammatory reactions and promote the activation of antigen presenting cells

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during the inflammatory reaction process (Abbasi et al., 2010; Ghosh et al., 1998; Shishodia et al., 2003). However, the detail research of these inflammatory factors in seawater-immersed abdominal injury has not found. In this study, the expressions of NF-κB, IκB (inhibitor protein of nuclear factor κB) and TLR4 in small intestinal tissues in rats with seawater-immersed abdominal injury were detected by SYBR Green real-time PCR technique, and the role of NF-κB-centered inflammatory systems in seawater-immersed abdominal injury was explored.

MATERIALS AND METHODS

Animal grouping

A total of 63 male Wistar rats were randomly divided into three groups. The control group: rats were normally fed only for index observations (n = 7); abdominal open (AOI) group: 28 rats underwent abdominal open injury for model building, and they were subdivided according to different detection time points (12, 24, 48 and 72 h subgroups, n = 7); and abdominal open plus seawater immersion (AOI+SI) group: 28 rats underwent seawater immersion after abdominal open injury, and they were subdivided (12, 24, 48 and 72 h subgroups, n = 7).

Animal model

In order to wipe out the influences of biorhythm on experimental results, all experiments were started at 8 a.m. After being fed at experimental animal centre for about one-week, rats were experimented. And before model building, they were fasted without food for 24 h and without water for 1h.

AOI group: rats were anesthetized with 3% pentobarbital sodium, and then fixed with supine position on a self-made plate; to create a surgical abdominal open injury, a midline lower abdominal incision of 3 cm was performed by eye scissors, and a self-made iron mesh was used to prop open the incision and then fixed in case of evisceration; and rats were erectly exposed to 22℃ for 1 h.

AOI+SI group: animal models with abdominal open injury were established following the same procedures as those in AOI model building; and then, rats were immersed in seawater at 22℃ for 1 h, and the seawater surface was even with the xyphoid. Intestinal tissues at the distance of 15 cm upward away from ileocecal junction were harvested for sample detections after rats were killed.

Primers and cDNA synthesis

Primer sequences were designed by Primer premier 5 software as follows: actin: 5'-CCC ATC TAT GAG GGT TAC GC-3' (upstream) and 5'-TTT AAT GTC ACG CAC GAT TTC-3' (downstream), and the amplified fragment was 150 bp; TLR: 5'-TGC TCA GAC ATG GCA GTT TC-3' (upstream) and 5'-TCA AGG CTT TTC CAT CCA AC-3' (downstream) , and the amplified fragment was 206 bp; NF-κB: 5'-AAC ACT GCC GAG CTC AAG AT-3' (upstream) and 5'-CAT CGG CTT GAG AAA AGG AG-3' (downstream) , and the amplified fragment was 163bp; and IκB: 5'-CCT CAC CCT TCC CCA ATA AT-3' (upstream) and 5'-GTG TGA ATG GTG CCT GTG AC-3' (downstream) with an amplified fragment of 199 bp.

RNA extraction was carried out according to the instructions of RNA extraction kit (Tiangen Biotech Co., Ltd, China) and the first strand of cDNA was synthesized also according to the instructions of kit (Promega M170A, USA).

Real-time PCR

The real-time PCR reaction system (50 μl) for NF-κB and IκB-α contained 25 μl 2×SYBR mixture (4 mM Mg^{2+}), PCR primers(10μM) each 1μl, 0.3μl Taq enzyme and 2 μl cDNA. The amplification conditions: pre-denaturation of 95℃ for 2 min followed by 45 cycles of 95℃ 20 s, 58℃ 25 s and 72℃ 30 s, and one cycle of 65℃ 10 s, 95℃ and 20℃ (0.5℃/s).

ΔCT = CT value in each group/subgroup-actin CT value in the same group. And $2^{-\Delta CT}$ was used for comparisons of copy numbers of TLR_4 mRNA by RT PCR among different groups/subgroups.

Western blot analysis

Total protein samples from intestinal tissues were extracted and determined by BCA method. The proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the protein bands were then transferred to nitrocellulose membranes. Membranes were blocked in 5% skim milk for 2 h and incubated with a primary antibody in a 1:1000 dilution rabbit anti-human VEGF antibody overnight at 4℃. After TBST washing 3 times (10m for each), the membranes were incubated with 1:2500 dilution peroxidase-conjugated goat anti-rabbit IgG for 1 h at room temperature. After TBST washing 3 times (10 min for each), the membranes were stained by ECL and then exposed by autoradiography. The optical density of the film was scanned using a gel-scanner, and β-actin was used as the internal reference. The 10 times ratio of the integral optical density was calculated to represent the relative amount. The experiment was repeated three times, and the average was calculated.

Statistical analysis

Data were presented as $\dot{x} \pm s$ and analyzed by SPSS 16.0 statistical software. Repeated measure ANOVA was carried out for PCR $2^{-\Delta CT}$ value, and SNK-q method was used for pairwise comparison when a difference emerged. One-factor analysis of variance was used for differences among groups/subgroups, and SNK-q for pairwise comparisons between groups/subgroups. $P<0.05$ was considered statistically significant.

RESULTS

Amplification and melting curves of TLR, NF-κB and IκB

Amplification curves of TLR, NF-κB and IκB displayed that there were linear relationship between fluorescence intensities and initial copy numbers, and melting curves displayed that single peaks appeared, indicating RT-PCR products were at high purity and the reactions in our study were specific.

Changes of TLR, NF-κB and IκB mRNA expression

The expression of NF-κB mRNA in AOI+SI group was significantly increased at 72 h compared to that in the control or AOI group at the same time point ($P<0.01$); though the expressions of IκB-$_\alpha$ mRNA in both AOI and AOI+SI groups were obviously increased compared to

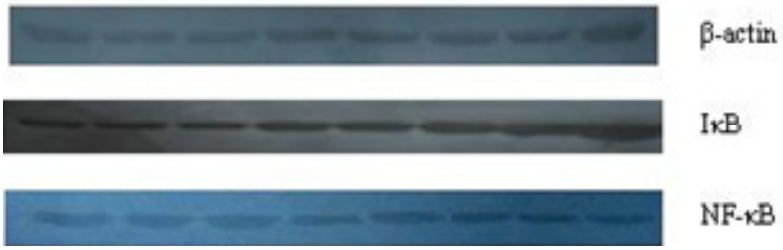

Figure 1. Electrophoresis results of the expressions of IκB and NF-κB in intestinal mucous membrane tissues 3 h later after injury. Lane 1-4: S+W group; Lane 5-7: W group; Lane 8: C group.

Figure 2. Electrophoresis results of the expressions of IκB and NF-κB in intestinal mucous membrane tissues 24 h later after injury. Lane 1 to 4: S+W group; Lane 5 to 7: W group; Lane 8: C group.

Figure 3. Electrophoresis results of the expressions of IκB and NF-κB in intestinal mucous membrane tissues 72 h later after injury. Lane S+W group; Lane 5 to 7: W group; Lane 8: C group.

that in the control at 48 h ($P<0.01$), the expression of IκB-$_{\alpha}$ mRNA in AOI+SI group was more significantly increased at 72 h compared to that in AOI group ($P<0.01$). And the expression of TLR_4 mRNA in AOI+SI group was also significantly increased compared to that in AOI or the blank group at 72 h ($P<0.01$).

Changes of NF-κB and IκB protein expression

Western blotting results showed that the expression of IκB in AOI+SI group was decreased at 3 h compared to that in the blank or AOI group, and such decrease was continued up to 72 h ($P<0.05$) while the difference of the expressions of IκB between AOI and the blank groups had no statistical significance though the expression in AOI group was a little lower than that in the blank ($P>0.05$); in addition, the expressions of NF-κB in different groups told totally different stories in contrast with those of IκB (Figures 1 to 3).

DISCUSSION

Sea battle injury belongs to a type of acute injury, which is far more complicated than injury in a matched field battle (Ghosh et al., 1998). Seawater immersion after abdominal trauma could lead to severe disorders in metabolism and hemodynamics (Shishodia et al., 2003). During this process, due to the devastating damage to the barrier function of intestines, a large amount of bacteria and endotoxin invade into organic circulations to

activate and induce the release of a large quantity of inflammatory cytokines, which in turn, causes inflammatory reactions. And in the process of inflammatory reactions, inflammatory mediator NF-κB promptly participated and played a central and unremitting role, which induced the increase of inflammatory cytokines such as TNF-α, blood vessel endothelial adhesion factors etc, leading to the aggravation of inflammatory reactions and even worse tissue injury (Schmitz et al., 2001; Ramakrishnan et al., 2004; Shenkar et al., 1996). But the mechanism underlying excessive inflammatory reactions and activation of NF-kB synthesis caused by seawater immersion is not known.

In our study, the expressions of TLR_4 mRNA, NF-κB mRNA and IκB-α mRNA in small intestinal tissues of rats immersed in seawater after abdominal injury were successfully detected by SYBR Green real-time PCR (Ethridge et al., 2002; Yan et al., 2006; Kuk et al., 2001). And our results showed that in AOI+SI group, the expression of TLR_4 mRNA was increased at 48 h and significantly increased at 72 h; the expression of NF-κB mRNA was significantly increased at 72 h; and the expression of IκB-α mRNA was more significantly increased compared to that in AOI group, though both groups exhibited significant increase in this respect compared to the blank.

TLR_4 (Toll-like receptror-4) is widely expressed in all cells, especially in monocytes. TLR_4 plays an important role in lipopolysaccharide (LPS) signal transduction pathways, which is the main component of bacteria in the natural immune system, like gram-negative bacteria. LPS, released by gram-negative bacteria, integrated with LPS binding proteins (LBP) in blood flow to form complexes, and these complexes interacted with mCD14s (LBP receptor) on the surfaces of monocytes and macrophagocytes to activate TLR_4 signal transduction pathways, which in turn, activated the increase of NF-κB activity and regulated related genes, leading to a series of pathological changes (Chen et al., 2008; Clemens, 2000; Medzhitov, 2001; Lin et al., 2004; Genovese et al., 2008).

Our study demonstrated that seawater immersion after injury, led to a damage of intestinal barrier function and translocation of bacteria and endotoxin through multiple pathways, causing bacterial translocation (BT) and gut original endotoxaemia. And due to this, LPS was released and the released LPS further activated TLR_4 signal transduction pathways (Rota et al., 2002; Bini et al., 2008; Wheeler and Bernard, 1999).

TLR_4 transducted signals and further activated a series of downstream responses via TIR domains: the structural domain of TIR interacted with that of MyD88 carboxyl terminus to activate MyD88, and through the interaction with the death domain (DD) of carboxyl terminus in serine/threonine protein kinase 4 ($IRAK_4$), the activated MyD88 recruited $IRAK_4$ into TLR signaling complex to cause the autophosphorylation of IRAK; the activated IRAK interacted with tumor necrosis factor receptor associated factor-6 (TRAF6), and the activated TRAF6 integrated with TGF-βassociated kinase (TAK1) and TAK binding protein (TAB) to form complex and to activate NF-κB induced kinase (NIK); the activated NIK further phosphorylate IκB multi-enzyme complex, and the activated IκB multi-enzyme complex exerted its effect on the inhibitor of NF-κB to phosphorylate two serine sites of IκB and lead to the degradation of IκB, by which transcription factor NF-κB was set free from IκB/ NF-κB complex. It immigrated into the nucleus and consequently, the immigrant led to the transcription of NF-κB mRNA, and induces the gradual increase of NF-κB synthesis, causing systemic inflammatory reactions, organ functional defects and multiple organ dysfunction syndromes (MODS) via the release of inflammatory factors (West et al., 2006; Abraham, 1999).

NF-κB is a eukaryotic nuclear transcription factor. After immigrating into the nucleus, it exerts its function of being a transcription factor by priming genetic transcription. Our results also indicated that the lasting existence of severe injury caused by seawater immersion after abdominal injury plus positive feedback stimulating factors synthetized and released by the promotion of NF-κB (such as inflammatory mediators TNF-α, IL-2β, etc.) lead to the increase of the expression of NF-κB mRNA, which further promotes the increase of NF-κB protein synthesis and magnify NF-κB-centered inflammatory signal transduction pathways. Meanwhile, 48 h later after seawater immersion, the intracellular negative feedback regulatory channel of NF-κB inflammatory signal transduction pathway had been initiated, and the manifest increase of $I\kappa B\text{-}_\alpha$ mRNA promoted the increase of $I\kappa B\text{-}_\alpha$ protein to make the dynamic balance of NF-κB between the nucleus and the cytoplasm tilt towards the side of cytoplasm, and in so doing, to down-regulate the activity of NF-κB in the nucleus thereby, terminating the transcription and generation of inflammatory mediators (Pegu et al., 2008; Hotchkiss et al., 2003).

In acute injuries, NF-κB participates in the activation of macrophages and leucocytes, and manipulates the genetic expressions of many pro-inflammatory factors. Thus, the loss of manipulation will cause the magnification of inflammatory reactions and even tissue injuries. From the perspective of molecular biology, the causes for the activation of NF-κB in abdominal injury with seawater immersion also include the translocation of microbial populations, apart from factors such as trauma, stress, etc. With seawater immersion, after LPS activates TLR signal transduction pathways, TLR may participate in the process of the activation of NF-κB inflammatory reactions, which may also promptly and everlastingly participate in the process of subsequent inflammatory reactions after abdominal injury with seawater immersion. The activation of NF-κB in injury with seawater immersion was more prompt and everlasting, and the excessive expression of NF-κB in injury with seawater immersion

could lead to the excessive release of cytokines such as TNFα, etc, leading to an water fall effect (Suk et al., 2001), which may further lead to systemic inflammatory reactions, organ functional defects and multiple organ dysfunction syndrome (MODS) (Hoffmann et al., 1999; Stone, 1994).

In this study, the expressions of NF-κB and IκB in intestinal mucous membrane tissues were detected by western blotting. Our results showed that the expression of NF-κB was increased and that of IκB was decreased at 3 h in AOI+SI group, and such effects lasted until 72 h while the expressions of NF-κB and IκB in AOI group didn't display the same changes, indicating NF-κB promptly and lastingly participates in the process of subsequent inflammatory reactions in abdominal injury with seawater immersion. The continuous down-regulation of IκB indicated that the mechanism of NF-κB negative feedback was not built for a rather long period, and the severity of injury with seawater immersion was more serious than that of injury alone and factors causing injury continuously existed, during which NF-κB might play a leading role (Akira and Hemmi, 2003).

In the abdominal seawater-immersion injury process, inflammatory reactions constitute an important component of a secondary injury, which are also the major cause for the aggravation of injury and MODS. During the whole process, inflammatory mediators and inflammatory cytokines play important roles in the pathological reactions after injury. The severity of inflammatory reaction is correlated with the expressions of genes coding inflammatory mediators, and the expressions of these genes are regulated by transcription factors. Studies have shown that seawater immersion after injury can activate inflammatory cells and release a variety of cells as well as humoral factors, leading to an excessive stress and inflammatory reactions. And our results indicate that during the above-mentioned process, NF-κB instantly and lastingly takes part, whose mechanism might be associated with its participation in the activation of macrophages and leucocytes and its control on the genetic expressions of pro-inflammatory factors, which may ultimately cause the magnification of inflammatory reactions or even tissue injuries.

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