行政院國家科學委員會補助專題研究計畫成果報告

※※※※※※※※※※※※※※※※※※※※※※※※
※ ※
※ 熱休克效應調節 NF-kappa B 在敗血性休克大白鼠之保護機制的探討 ※
※ ※
※※※※※※※※※※※※※※※※※※※※※※※※

計畫類別：☑ 個別型計畫 □整合型計畫

計畫編號：NSC 90-2320-B-041-016-

執行期間：90 年 08 月 01 日至 91 年 07 月 31 日

計畫主持人：陳香文

本成果報告包括以下應繳交之附件：

□赴國外出差或研習心得報告一份

□赴大陸地區出差或研習心得報告一份

□出席國際學術會議心得報告及發表之論文各一份

□國際合作研究計畫國外研究報告書一份

執行單位：嘉南藥理科技大學

中 華 民 國 91 年 07 月 31 日

熱休克效應調節 NF-kappa B 在敗血性休克大白鼠之保護機制的探討

Mechanism of heat shock response in regulating NF-κB activation in experimental septic rats

計畫編號：NSC 90-2320-B-041-016-
執行期限：90 年 08 月 01 日至 91 年 07 月 31 日

主持人：陳香文　嘉南藥理科技大學
計畫參與人員：楊瑞成、王淑容、呂宗禧　高雄醫學大學

中文摘要

從感染乃至敗血症及造成多器官衰竭的過程中，將激發宿主防禦反應，刺激免疫系統的啟動運作及免疫細胞的活化。這些作用將涉及了很多前發炎物質 或抗發炎物質之基因的誘發。若這些前發炎細胞激素的分泌沒有得到適當的調節，反而將從保護性的細胞防禦性反應物質，轉變成一種傷害性的物質，更進一步的促使後續之敗血性休克症狀的產生了。而 NF-κB 在此發炎物質的分泌中，參與了居中調節的角色，有助於回饋抑制路徑的完成及耐受性的產生。本計劃的目的在探討敗血症期間，肝臟組織中 NF-kappa B 活化調節的情形，及熱休克處置誘發熱休克蛋白的合成可能造成的效應。本實驗利用盲腸結紮與穿孔手術來誘發實驗動物敗血症的產生。以全身加熱的方式誘發熱休克反應。實驗結果顯示，在敗血症期間肝臟細胞質中 I-kappa B 及 NF-kappa B(p65)的表現有明顯的下降。同時亦可測得在敗血症期間將促使 NFκB 高度的活化，且在 iNOS 及 TNF-α之 mRNA 的表現上亦有明顯的增加。若實行盲腸結紮手術誘發敗血症之手術前，先進行熱休克處置，有助於提升敗血症期間 I-kappa B 及 p65 在細胞質中的表現。再者導致 NFκB 活性的抑制及 iNOS 及 TNF-α之 mRNA 表現的下降。吾等認為，熱休克處置誘發熱休克蛋白質的合成，可藉由維持 NF-kappa B 及 I-kappa B 複合體穩定的存在於細胞質中，而降低 NF-kappa B 的活化。

關鍵詞：敗血症，粒腺體，熱休克蛋白質，盲腸結紮與穿孔

Abstract

The present study was designed to investigate the role of NFκB in influencing the outcome of sepsis modulated by previous heat shock treatment. Rats were induced experimental sepsis by CLP method and heated by whole-bodily hyperthermia. They were sacrificed at 9 hr and 18 hr after CLP as early and late sepsis, respectively, and then the cytosol and nuclear of liver was collected. The expression of Hsp72, NF-κB (p65) and I-κB in cyotosol of liver was evaluated by Western blot analysis and NF-κB activity was detected by commercial ActivELISA™ kits. The interaction between Hsp72, p65 and I-κB was assessed by co-immunoprecipitation. The mRNA accumulation of iNOS and TNF-α were detected by RT-PCR. The results showed that the expression of I-kB and NF- kB in cytosol of liver is declined during sepsis. NF-kB activity in nucleus of liver is significant enhancement during sepsis, and then the target gene of NF-kB, such TNF-α and iNOS, is activated. Heat shock treatment, inducing heat shock protein synthesis, can prevent I-kB expression from decline in cytosol of liver and preserve the NF-kB in cytosol. Concomitantly, the increase of NF-kB activity induced by sepsis is inhibited, and then the expressions of TNF-αand iNOS mRNA were also down-regulation by heat shock treatment. The interaction of Hsp72 with NF-kB and I-kB was verified by co-immunoprecipitation with anti-NF-kB p65 and anti-I-kB antibodies. We suggest that the mechanisms of Hsp 72 in preventing NF-kB activation during sepsis may involve in stabilizing the complexes of NF-kB and I-kB and preserving the complex in cytoplasm.

Keywords: sepsis, heat shock protein, cecal ligation and puncture, NF-kappa B, I-kappa B

Introduction

In spite of advances in medical equipment and new drugs administration, the mortality rate of sepsis remains high. For decades, the pathogenesis of sepsis has been extensively investigated by animal model studies, leading to the clinical trial of anti-toxin or anti-cytokine therapy. However, the effectiveness is still limited. In the last decade, a novel self-protective phenomenon called heat shock response has been introduced to reduce the subsequent injury. In response to various stresses, series of high conversed protein named heat shock proteins (Hsps) can be induced in almost all living cells. These proteins protect cells or organisms through a mechanism called thermotolerance or cross-tolerance. Amazingly, no Hsps is induced by cecal ligation and puncture (CLP)-induced sepsis or endotoxin-induced shock, even during the dying late stage. On the other hand, we previously showed that heat shock pretreatment decreased significantly the mortality rate of CLP-induced sepsis in rats. Similar results were also obtained from studies that involved administration of sodium arsenite, a chemical capable of inducing the synthesis of Hsps. It is clear that over-expression of Hsps is beneficial in the outcome of sepsis, even though the molecular mechanism remain a mystery. Nuclear factor-kB (NF-kB) is an inducible eukaryotic transcription factor of the rel family and normally exists as an inactive cytoplasmic complex. Its predominant form is a heterodimer composed of p50

and p65 (Rel A) subunit. In the majority of cells, NF-kB exists in an inactive from in the cytoplasm, bound to the inhibitory I-kB protein and is activated in response to primary (viruses, bacteria, UV) or secondary (inflammatory cytokines) pathogenic stimuli. Following stimulation, IkB protein was found to be phosphorylated on two conserved serine residues, leading to protein degradation via the proteasome pathway. The released NF-kB/Rel dimers are then free to migrate into the nucleus to activate the target gene expression, such TNF α, IL- β or iNOS. During infection and its associated situations, sepsis or multiple organ dysfunction syndromes, lipopolysaccharide and endotoxin initiate the host response. Following the cascade of the disease, many inflammatory mediators, such as pro-inflammatory substances or cytokine, are released and induce NF-kB activity. Not only does NF-kB response to inflammatory mediators, it also participates in the regulation of cytoplasmic / nuclear signaling of immune and inflammatory response, and then activates a great variety of genes expression. The present study was designed to investigate the role of NF-kB activity in influencing the outcome of sepsis and the mechanisms of heat shock treatment in regulating the NF-kB activity during sepsis.

Result

Figure 1. Changes of NF-kB (p65) expression in cytosol of liver during various stages of sepsis and influenced by previous heat shock treatment.
Expression of NF-kB (p65) was detected by Western blot and immunochemical analysis (*upper panel)*. The results of statistical analysis of relative content of NF-kB (p65) (ratio of OD p65 / actin) was showed in lower panel. Equal amount of cytosolic extract from liver of rats were applied. The expressions of NF-kB(p65) were down-regulation in early and late stage of sepsis. Heat shock treatment contributed in preserving the NF-kB(p65) expression in cytosol of liver during sepsis.
S: sham operation, E: early stage of sepsis, L: late stage of sepsis, HE: early stage of sepsis with previous heat shock treatment. HL: late stage of sepsis with previous heat shock treatment.

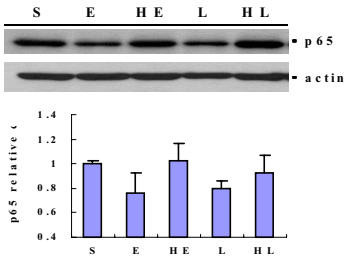


Figure 2. Changes of I-kB expression in cytosol of liver during various stages of sepsis and influenced by previous heat shock treatment.
Expression of I-kB was detected by Western blot and immunochemical analysis (*upper panel)*. The results of statistical analysis of relative content of I-kB (ratio of OD I-kB / actin) was showed in lower panel. Equal amount of cytosolic extract from liver of rats were applied. The expressions of I-kB were down-regulation in early and late stage of sepsis. Heat shock treatment contributed in preserving the I-kB expression in cytosol of liver during sepsis.
S: sham operation, E: early stage of CLP-induced sepsis without previous heat, L: late stage of CLP-induced sepsis without previous heat, HE: early stage of CLP-induced sepsis with previous heat shock treatment. HL: late stage of CLP-induced sepsis with previous heat shock treatment.

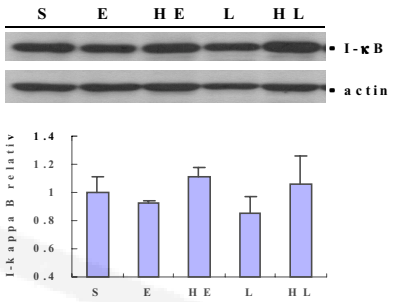


Figure 3. Regulation of NF-kB activity in nuclear of liver by heat shock pretreatment during sepsis.
NF-kB activity was detected by commercial TransAM™ NF-kB kits, one of the ELISA based kit. The NF-kB activity can be detected by spectrophotometer at 45nm. Equal amount of nuclear extract from liver of non-heated or heated group was applied. The activity of NF-kB significantly increase in early (E) and late (L) stage of sepsis compared with sham operation (S)($P < 0.05$). Heat shock treatment prevent the up-regulation of NF-kB activity induced by sepsis ($P < 0.05$).

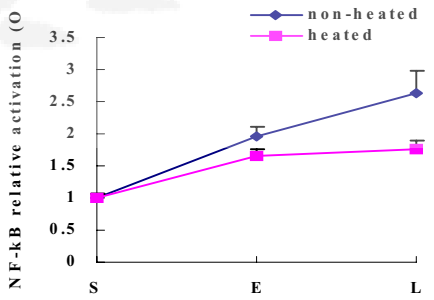


Figure 4. Heat shock treatment influences the mRNA expression of tumor necrosis factor- a and inducible nitric oxide synthase during sepsis Messenger RNA expression of tumor necrosis factor- a (TNF- a) (upper panel) and inducible nitric oxide synthase (iNOS)(lower panel) were evaluated by reverse transcription-polymerase chain reaction (RT-PCR). Total RNA from livers was subjected to RT-PCR using TNF- a (541 bp) and iNOS (264 bp) specific primers. while b-actin mRNA was amplified simultaneously as a reference marker (420 bp). The mRNA expression of TNF- a and iNOS were promoted by sepsis, while it were prevented by heat shock treatment.

S: sham operation, E: early stage of CLP-induced sepsis without previous heat, L: late stage of CLP-induced sepsis without previous heat, HE: early stage of CLP-induced sepsis with previous heat shock treatment. HL: late stage of CLP-induced sepsis with previous heat shock treatment.

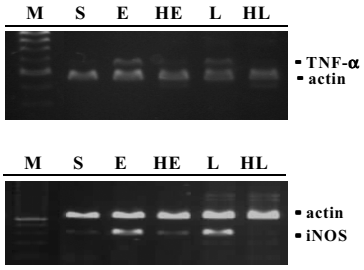


Figure 5. The changes of I-kB/ NF-kB relative content in the I-kB/ NF-kB complex in cytosol of liver during sepsis.
The changes of I-kB/ NF-kB relative content were investigated by co-immunoprecipitation with anti-NF-kB (p65), and then the immuno-precipitates were immunoblotted with anti-I-kB antibodies. Equal amount of cytosolic extract from liver of rats were applied. The results showed that I-kB/ NF-kB relative content is without significant changes during sepsis.
S: sham operation, E: early stage of CLP-induced sepsis without previous heat, L: late stage of CLP-induced sepsis without previous heat, HE: early stage of CLP-induced sepsis with previous heat shock treatment. HL: late stage of CLP-induced sepsis with previous heat shock treatment.

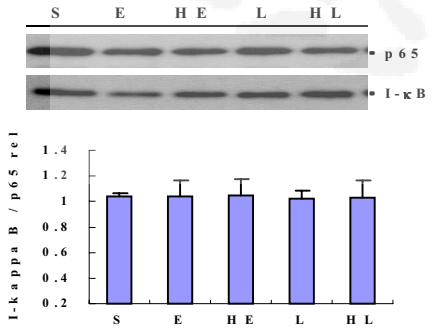


Figure 6. The interaction of Hsp72 with NF-kB and I-kB in cytosol of liver during sepsis
The interaction of Hsp72 with NF-kB and I-kB was verified by co-immunoprecipitation with anti-NF-kB p65 and anti-I-kB antibodies respectively , and then the immuno-precipitates were immunoblotted with anti-Hsp72 antibody. The results showed that Hsp72 can be co-immunoprecipitated with anti-NF-kB p65 and anti-I-kB antibodies.
Lane p65: immunoprecipitation with anti-NF-kB p65. Lane I-kB: immunoprecipitation with anti-I-kB. PC: positive control.

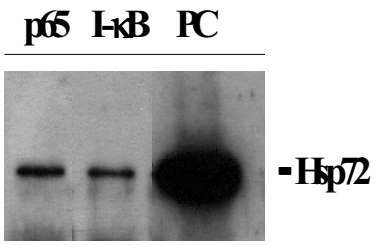
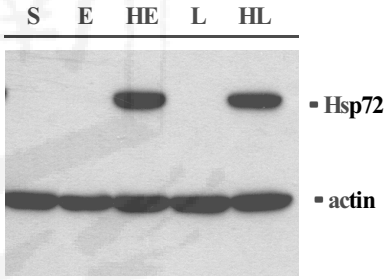


Figure 7. Hsp72 expression in cytosol of liver during sepsis Expression of Hsp72 was detected by Western blot and immunochemical analysis. Beta-actin was co-reacted as the internal standard. Equal amount of cellular extract from livers of rats were applied. The Hsp72 was undetectable in liver of sham control and can not be induced by sepsis. Heat shock treatment induced the Hsp72 over-expression.S: sham operation, E: early stage of CLP-induced sepsis without previous heat, L: late stage of CLP-induced sepsis without previous heat, HE: early stage of CLP-induced sepsis with previous heat shock treatment. HL: late stage of CLP-induced sepsis with previous heat shock treatment.



Summary
1. The expression of I-kB and NF- kB in cytosol of liver is declined during sepsis. According the result, we consider that the sepsis can lead to the I-kB degradation in cytosol . Following I-kB dissociation from NF- kB and degradation, the NF-kB was turn-activation and translocation from cytoplasm to nucleus.
2. During sepsis, NF-kB activity in nucleus of liver is signifincnt enhancement, and then the target gene of NF-kB, such TNF-a and iNOS, is activated.
3. Heat shock treatment can prevent I-kB expression from decline in cytosol of liver and preserve the NF-kB in cytosol. Concomitantly, the increase of NF-kB activity induced by sepsis is inhibited by heat shock treatment. We suggest that heat shock protein, induced by heat shock treatment, may contribute in regulation of NF-kB activity during sepsis.
4. The interaction of Hsp72 with NF-kB and I-kB was verified by co-immunoprecipitation with anti-NF-kB p65 and anti-I-kB antibodies.
5. In conclusion, We suggest that the mechanisms of Hsp 72 in preventing NF-kB activation during sepsis may involve in stabilizing the complexes of NF-kB and I-kB and preserving the complex in cytoplasm.