Anti-inflammatory effects of aqueous extract from *Dichroa febrifuga* root in rat liver[1]

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ABSTRACT

AIM: To study the anti-inflammatory effects of aqueous extract from *Dichroa febrifuga* root (AEDF) for suppression in the process of lipopolysaccharide (LPS)-induced sepsis in the rat liver. **METHODS**: The inhibitory effect of AEDF on the alteration of inflammatory proteins was investigated by Western blot and immunohistochemical analysis. **RESULTS**: Western blot analysis showed that the level of nuclear factor (NF)-κBp65 was markedly up-regulated and (I)-κBα was down-regulated by LPS (8 mg/kg) challenge. However, AEDF 100 mg/kg inhibited induction of NF-κBp65 and degradation of I-κBα in the liver of LPS-challenged rats. Immunohistochemical analysis showed that while the expression of the NF-κBp65, tumor necrosis factor (TNF)-α, and inducible nitric oxide synthase (iNOS) tended to increase, that of I-κBα was decreased in the hepatocytes of rats challenged with LPS. A slight decline of NF-κBp65, TNF-α, and iNOS, but an increase of I-κBα were observed in the hepatocytes of the rats pretreated with AEDF. **CONCLUSION**: AEDF may act as a therapeutic agent for inflammatory disease through a regulation of inflammation-related proteins.

INTRODUCTION

Traditionally, the root of *Dichroa febrifuga* has clinically been used as an anti-malarial drug and also used in the treatment of productive cough in China and Korea. This plant has a wide application as a complementary therapeutic agent in Korea for the treatment of unstable fever caused by infection. According to our previous study, the aqueous extract from *D febrifuga* root (AEDF) suppressed the lipopolysaccharide (LPS)-induced inflammatory response through inhibiting the production of nitric oxide (NO) and tumor necrosis factor (TNF)-α in the peritoneal macrophage of mice[1].

Recently, nuclear factor κB (NF-κB) is known to mediate multiple LPS-induced inflammatory responses. The NF-κB is bound to inhibitory κB (I-κB), which is phosphorylated by I-κB kinase (IKK) and degraded by ubiquitin-mediated proteolysis under the activated condition. Activated NF-κB is translocated into the

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nucleus where it induces transcriptional up-regulation of various proinflammatory mediators that contribute to the systemic inflammatory response, such as iNOS, TNF-α, and interleukin (IL)-8[2,3]

Therefore, blocking NF-κB activation may be an effective strategy in the treatment of sepsis-induced multiple organ injury. In this study, we investigated the inhibitory effect of AEDF on the alteration of inflammatory proteins such as NF-κBp65, I-κBα, iNOS, and TNF-α in the process of LPS-induced sepsis by Western blot and immunohistochemical analysis.

MATERIALS AND METHODS

Preparation of aqueous extract The roots of *D febrifuga* Lour were purchased from a local herb store, Kwang Myung Dang (Pusan, Korea) in September 1999. The *D febrifuga* was confirmed and authenticated by Prof KO Woo-Shin, College of Oriental Medicine, Dong-Eui University. A voucher specimen (number: DF-98-2) has been deposited at the College of Oriental Medicine, Dong-Eui University, Busan, Korea. The *D febrifuga* was extracted by the method of Kim *et al*[1].

Reagents Anti-iNOS rabbit polyclonal antibody was obtained from CALBIOCHEM (San Diego, CA). Rabbit polyclonal antibodies raised against NF-κBp65, I-κBα, and TNF-α and horse radish peroxidase-conjugated anti-rabbit antibody and ECL kit were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Avidin-biotin-peroxidase complex kit and substrate kit for peroxidase were purchased from Vector Lab (Burlingame, CA) and LPS (phenol extracted *Salmonella enteritidis*) and other all reagents from Sigma (St Louis, MO).

LPS and AEDF administration and rat treatment Male Sprague-Dawley rats, weighing about 120 g on average, were obtained from Taconic & SamYuk Co in Korea. Rats of the LPS 8 mg/kg alone and LPS plus AEDF 100 mg/kg group were administrated intraperitoneally 3 times, 24, 8, and 3 h before the LPS challenge, with either phosphate buffered saline (PBS) or AEDF at a concentration of 100 mg/kg. After pretreatment, rats were challenged intravenously with 8 mg/kg of LPS and control one with the same volume of PBS. Rats were sacrificed at interval 4, 8, and 24 h after the LPS challenge.

Western blot analysis Rat livers were obtained from the control, LPS alone, and LPS plus AEDF groups and homogenized in 9 volumes of potassium HEPES buffer (pH 7.4) 20 mmol/L, containing 0.5 % Triton X-100, DTT 1 mmol/L, and protease inhibitor cocktail solution. The homogenates were centrifuged at 25 000 ×*g* for 30 min at 4 °C, and the supernatants served as liver protein extracts. The 30 μg of protein extracts were subjected to SDS-PAGE, and proteins were transferred to nitrocellulose membranes. For immunodetection, we used the ECL kit.

Histopathology The livers were fixed in 4 % paraformaldehyde in PBS for 18 h and dehydrated in a graded ethanol series. After embedment in paraffin, serial 5-μm thick sections were prepared. For histopathological examinations, hematoxylin-eosin stain and periodic acid Schiff's reaction were used.

Immunohistochemistry After deparaffinized in 58 °C xylene, the sections were exposed to 0.3 % methanolic hydrogen peroxide for 30 min, and washed with PBS. Tissues were then treated with goat normal serum at room temperature for 30 min followed by treatment with anti-NF-κBp65, IκBα, TNF-α, and iNOS diluted for 1:500 in moisture chamber at 4 °C for 16 h. After being washed by PBS, tissues were incubated with the secondary antisera, biotinylated anti-rabbit IgG for 30 min and washed with PBS. These sections were further incubated in avidin-biotin-peroxidase complex kit at room temperature for 60 min. Diaminoben-zidine substrate kit for peroxidase was applied. For the controls, treatment with primary and secondary antibodies was omitted.

RESULTS

Effect of AEDF on NF-kBp65 induction NF-κBp65 increased about 6-11 fold in the livers of LPS-treated rats compared to the controls (Fig 1A). However, in the rats pretreated with AEDF, the level of NF-κBp65 was reduced at 8 and 24 h after the LPS challenge compared to the LPS-treated rats. This result indicated that AEDF inhibited the induction of NF-κBp65 in the liver of LPS-treated rat.

Inhibitory effect of AEDF on the degradation of I-kBa Since LPS-induced activation of NF-κB is accompanied by the rapid degradation of the inhibitory protein I-κBα, we examined whether AEDF would inhibit the degradation of I-κBα in the livers of LPS-treated rats. While I-κBα rapidly degraded at 4 h in the LPS-treated rats, the degradation was inhibited at 4 h in the AEDF-pretreated rats. However AEDF showed partial inhibition on degradation of I-kBa at 8 and 24 h after the

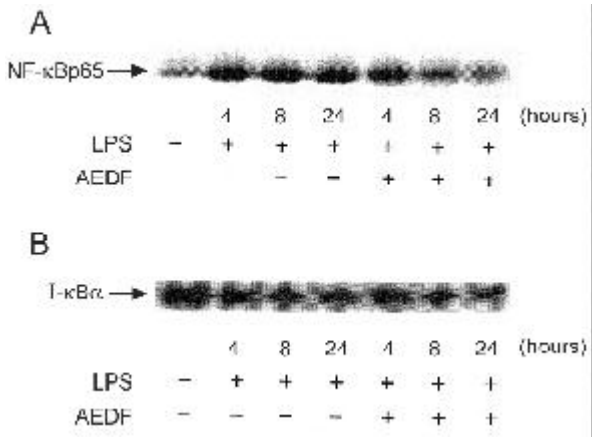

Fig 1. Inhibitory effect of aqueous extract from *Dichroa febrifuga* root (AEDF) on the induction of NF-k Bp65 (A) and the degradation of I-k Ba (B) in rat livers. Rats were pretreated with AEDF (100 mg/kg) or PBS for 3 times at 24, 8, and 3 h before LPS challenge, and then administered with LPS (8 mg/kg). Liver samples were taken from the rats at indicated times after the LPS challenge. Samples were subjected to SDS-PAGE followed by the Western blot analysis using an NF-k Bp65 and I-k Ba antibody. Results represent two independent experiments.

LPS challenge (Fig 1B).

Histopathological analysis Very severe epithelial changes such as cloudy swelling, hydropic degeneration, and Kupffer cell reaction were observed in the rats challenged with LPS. Stromatic changes including passive congestion and inflammatory cell infiltration were also demonstrated and the score of inflammation loci was markedly increased. Even though epithelial changes in rats pretreated with AEDF showed a similar histopathological pattern, slight differences such as fewer scores of inflammation loci were observed (Tab 1).

Immunohistochemical analysis In the hepatocytes of rats challenged with LPS, the immunoreaction of the NF-κBp65, TNF-α, and iNOS tended to increase compared with normal rats. Much more intensive expression of NF-κBp65 and iNOS was detected in the hepatocytes of centrolobular zone and TNF-α in the surface of hepatocytes. The number of nucleus of hepatocytes showing iNOS immunoreaction also increased in the LPS-treated rats. However, a slight decline of NF-κBp65, TNF-α, and iNOS expression was detectable in the AEDF-pretreated rats. In contrast, I-κBα expression decreased, especially in the hepatocytes located around necrotic loci, in rats challenged with LPS. But the expression of I-κBα partly reincreased in the AEDF pretreated rats. Especially, the number of hepatocytes showing immunoreaction in the nucleus increased in the AEDF-pretreated rats (Tab 2, Fig 2).

DISCUSSION

Endotoxin induces a release of various cytokines from macrophages and T-cells, which induce expression of iNOS mRNA or TNF-α through the activation of transcriptional factor NF-κB[4]. LPS stimulation elicits an increase of NF-κB activation with a corresponding degradation of its inhibitor I-κBα[3,5]. The prevention of NF-κB activation may be useful in the therapy of sepsis-induced disorders associated with local or systemic inflammation[6].

LPS-induced NF-κB activation is closely related to I-κB degradation. In the recent reports, the components of a specific signal transduction cascade activated in response to the proinflammatory cytokines, TNF-α or IL-1β, were elucidated. These cytokines initiate a

Tab 1. Effect of aqueous extract from *Dichroa febrifuga* root (AEDF) 100 mg/kg on the histological findings in the liver of rats challenged with LPS 8 mg/kg. *n*=5.

HC	Control	LPS-treated group			+AEDF-pretreated group		
		4 h	8 h	24 h	4 h	8 h	24 h
CS	+-++	++++	++++	+++	+++	+++	+++
HD	0	++	++	++	++	++	++
KC	0-+	+++	+++	+++	+++	+++	+++
FN	0	++	++-+++	++++	++	++	+++

HC, histological changes; CS, cloudy swelling; HD, hydropic degeneration; KC, Kupffer cell reaction; FN, focal necrosis.
0-++++ indicates the relative changes of the histological finding: 0, faint and negligible; +, weak; ++, moderate; +++, severe; ++++, very severe.

Tab 2. Immunohistochemistry for inflammation-related proteins in the liver of rats. *n*=5.

Stain	Region	Control	LPS treated group			+AEDF pretreated group		
			4 h	8 h	24 h	4 h	8 h	24 h
I-κBα	HC[1)]	+/+-++	0-+/+	0-+/+	0-+/+	0-+/0	+-+/++	++/++
	KC	0	0	0	0	0	0	0
	IFC	0	++	++-+++	++-+++	++	++	++
NF-κBp65	HC[2)]	0-+/+	0-+/++	0-+/++	0-+/++	0-+/+	0-+/++	0-+/+
	KC	0	0	0	0	0	0	0
	IFC	0	+	+	+	+	+	+
TNF-α	HC[3)]	0	0/+	0/++	0/+++	0/+	0/+	0/+
	KC	0	0	0	0	0	0	0
	IFC	0	++	++	++	++	++	++
iNOS	HC[2)]	0-+	+/++	++/+++	++/++	+/+	++/++	++/++
	KC	0-+	+	++	+-++	+	+-++	+-++
	IFC	0	++	++	++	++	++	++

HC, hepatocyte; KC, Kupffer cell; IFC, inflammatory cell in necrosis loci; /, two distinct reaction are observed in the same cell; 1) cytoplasm/nucleus; 2) most hepatocyte/hepatocytes located around the centrolobular vein; 3) cytoplasm/cell surface. 0-+++ indicate the relative intensity of the reaction : 0, faint and negligible; +, weak; ++, moderate; +++, intense.

signaling cascade leading to the activation of I-κB kinase (IKK). Phosphorylated I-κB is then selectively ubiquitinated by an E3 ubiquitin ligase[7,8]. In the last step of this signaling cascade, phosphorylated and ubiquitinated I-κB is selectively degraded by the 26S proteasome[9].

Inhibition of NF-κB activation with an inhibitor of I-κB degradation eliminated TNF-α synthesis and the expression of iNOS in the LPS-stimulated hepatocytes[10,11]. Up-regulation of iNOS mRNA level by LPS can be explained by observed activation of NF-κB and this shows an important degradation of I-κB in the presence of LPS which results in NF-κB activation and iNOS transcription[12].

In this report, AEDF inhibited the induction of NF-κB and the degradation of I-κBα in the liver of LPS-treated rats. Negative regulation of NF-κB activity is very complex in that several mechanisms are involved in the termination of NF-κB activation or its down-regulation in response to specific signals. The critical inhibitory step is thought to be the binding of newly synthesized I-κBα to NF-κB in the nucleus[13]. As shown in the results, up-regulation of I-κBα was observed in the AEDF-pretreated rats as compared with LPS-treated rats through the experiment. This result suggests two possibilities: 1)AEDF may affect regulatory machinery of IKK involving ubiquitin or proteasome system, 2) AEDF may enhance the transcriptional activation system of I-κBα.

As for the immunohistochemical studies, a more intensive immunoreaction of NF-κBp65, TNF-α, and iNOS and a weaker one of I-κBα were merely observed in the hepatocytes of rats challenged with LPS. Some differences between AEDF-pretreated rats and LPS-treated ones, such as a decline of NF-κBp65, TNF-α, and iNOS and an increase of I-κBα, were detected in the hepatocytes. There was no exact evidence for the translocation of NF-κBp65 into the nucleus by immunohistochemical analysis. However, the above results obtained from immunohistochemistry showed a different induction of inflammation-related protein in the hepatocytes.

In the present study, prominent immunoreaction of I-κBα was detected in the nucleus of the AEDF-pretreated rats. This may be due to the positive regulation of the I-κBα gene by NF-κB. The rapid reappearance of I-κBα following its degradation is the result of the activation of I-κBα gene by NF-κB and this reaccumulation correlates with the inhibition of NF-κB activity in the nucleus because I-κBα can enter the nucleus as previously observed[13]. As stated above, AEDF may act as an inhibitor of NF-kB activation and I-κBα degradation and then have inhibitory effects on the expression of iNOS and TNF-α production. Therefore it may be concluded that AEDF would be useful as a therapeutic agent for inflammatory disease.

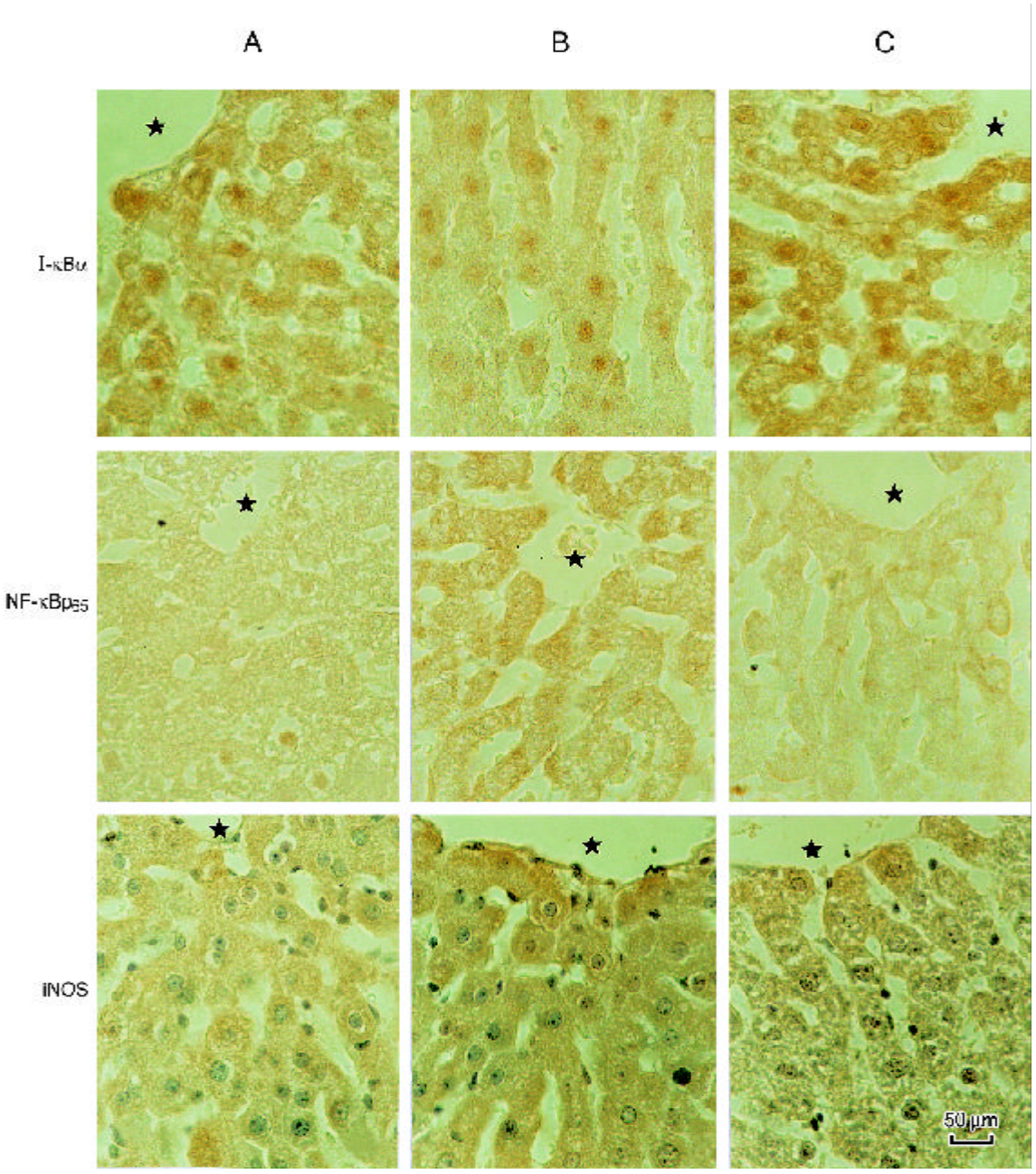

Fig 2. Immunohistochemical localization of I-k Ba , NF-k Bp65, and iNOS in the liver of normal (A), LPS (8 mg/kg alone)-treated (B), and AEDF (100 mg/kg)-pretreated rats (C) at 8 h after LPS challenge. Normal liver showed moderate immunoreaction of I-k Ba in the cytoplasm and nucleus of hepatocytes, but these decreased in the LPS-treated rats. Note immunoreaction of I-k Ba in the nucleus of the AEDF-pretreated rats. Although the NF-k Bp65 translocation into the nucleus was not detected in the LPS-administered rats, an increase of immunoreaction of NF-k Bp65 with iNOS was observed. A slight decline of NF-k Bp65 and iNOS was observed in the AEDF pretreated rats. ★, central vein.

REFERENCES

1 Kim YH, Ko WS, Ha MS, Lee CH, Choi BT, Kang HS, *etal.* The production of nitric oxide and TNF-α in peritoneal macrophages is inhibited by *Dichroa febrifuga* Lour. J Ethnopharmacol 2000; 69: 35-43.

2 Baeuerle PA, Baichwal VR. NF-kappa B as a frequent target for immunosuppressive and anti-inflammatory molecules. Adv Immunol 1997; 65: 111-37.

3 Kramer AA, Salhab KF, Shafii AE, Norman J, Carey LC, Mendez C. Induction of tolerance to hemorrhagic or endotoxic shock involves activation of NF-κB. J Surg Res 1999; 83: 89-94.

4 Ando N, Kono T, Iwamoto J, Kikuchi-Utsumi K, Yoneda M,

Karasaki H, *et al*. Nitric oxide release from the liver surface to the intraabdominal cavity during acute endotoxemia in rats. Nitric Oxide 1998; 2: 481-8.

5 Yamakawa T, Eguchi S, Matsumoto T, Yamakawa Y, Numaguchi K, Miyata I, *et al*. Intracellular signaling in rat cultured vascular smooth muscle cells: roles of nuclear factor-κB and p38 mitogen-activated protein kinase on tumor necrosis factor-α production. Endocrinology 1999; 140: 3562-73.

6 Ruetten H, Thiemermann C. Effect of calpain inhibitor I, an inhibitor of the proteolysis of IκB, on the circulatory failure and multiple organ dysfunction caused by endotoxin in the rat. Br J Pharmacol 1997; 121: 695-704.

7 Alkalay I, Yaron A, Hatzubai A, Orian A, Ciechanover A, Ben-Neriah Y. Stimulation-dependent I kappa B alpha phosphorylation marks the NF-kappa B inhibitor for degradation via the ubiquitin-proteasome pathway. Proc Natl Acad Sci USA 1995; 92: 10599-603.

8 Yaron A, Gonen H, Alkalay I, Hatzubai A, Jung S, Beyth S, *et al*. Inhibition of NF-kappa-B cellular function via specific targeting of the I-kappa-B-ubiquitin ligase. EMBO J 1997; 16: 6486-94.

9 Chen Z, Hagler J, Palombella VJ, Melandri F, Scherer D, Ballard D, *et al*. Signal-induced site-specific phosphorylation targets I kappa B alpha to the ubiquitin-proteasome pathway. Genes Dev 1995; 9: 1586-97.

10 Dí az-Guerra MJM, Velasco M, Martí n-Sanz P, Bosca L. Evidence for common mechanisms in the transcriptional control of type II nitric oxide synthase in isolated hepatocytes: requirement of NF-κB activation after stimulation with bacterial cell wall products and phorbol esters. J Biol Chem 1996; 271: 30114-20.

11 Iimuro Y, Bradford BU, Yamashina S, Rusyn I, Nakagami M, Enomoto N, *et al*. The glutathione precursor *L*-2-oxothiazolidine-4-carboxylic acid protects against liver injury due to chronic enternal ethanol exposure in the rat. Hepatology 2000; 31: 391-8.

12 Liu SF, Ye X, Malik AB. *In vivo* inhibition of nuclear factor-kappaB activation prevents inducible nitric oxide synthase expression and systemic hypotension in a rat model of septic shock. J Immunol 1997; 159: 3976-83.

13 Arenzana-Seisdedos F, Turpin P, Rodriguez M, Thomas D, Hay RT, Virelizier JL, *et al*. Nuclear localization of I kappa B alpha promotes active transport of NF-kappa B from the nucleus to the cytoplasm. J Cell Sci 1997; 110: 369-78.