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Original Article

Inhibitory Effect of Carbamylated Staphylococcal Enterotoxins B on Inflammatory Response in HL-60 Cells

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Staphylococcal enterotoxin B (SEB) is bacterial toxin that induces the activation of immune cells. Because the inhibition of pro-inflammatory effect of SEB can resolve the inflammation, I determined the influence of functional or structural change of SEB on immune cells. The post translational modification of protein occurs through carbamylation. Carbamylation can change the structure of proteins and can modify the biological activity of protein. In the present study, I investigated the effect of carbamylated SEB (CSEB) on the inflammatory response mediated by LPS in HL-60 cells. To determine the anti-inflammatory effect of CSEB, I produced carbamylated SEB using potassium cyanate (KCN) and then examined whether CSEB involved in cytokine releases and apoptosis of LPS-stimulated HL-60 cells. Although CSEB had not any effect on the LPS-stimulated HL-60 cells, the protein levels of IL-8, TNF-α and IL-1β were significantly decreased by CSEB without cytotoxicity. CSEB also blocked Akt and NF-κB activation. These results indicate that the suppressive effect of CSEB in LPS-stimulated cytokine releases is occurred by inhibition of Akt and NF-κB activity. Through further studies, CSEB may be used as anti-inflammatory molecule that makes the immune system more efficient.

Key Words: Carbamylation, Staphylococcal enterotoxin B, Anti-inflammatory molecule, HL-60 cells

INTRODUCTION

In infectious diseases, *Staphylococcus aureus* (*S. aureus*) can frequently colonize in the site of infection, and can contribute to persistent inflammation in the lesions by secreting various toxins (Baker, 2006). *S. aureus* secretes staphylococcal superantigens (SsAgs), including staphylococcal enterotoxin A (SEA), staphylococcal enterotoxin B (SEB) and toxic shock syndrome toxin-1 (TSST-1) (Pinchuk et al., 2010). In these SsAgs, SEB binds to the major histocompatibility complex (MHC) class II on antigen-presenting cells (APC) and the T-cell receptor (TCR) on T cells, and then induces the marked activation of T cells and other immune cells (Kotzin et al., 1993; Skov et al., 2000). Activated T cells produce various cytokines and modulate the cytokines function as important factors in these disease processes (Baker, 2006). A number of cytokines typically increase in inflammatory disease and have important effects on the activation of other inflammatory cells, dysregulation of immune response (Proft and Fraser, 2003; Guzik et al., 2005). Thus, the suppression of intracellular responses mediated by SEB can resolve the inflammation. In this study, I investigated the influence of functional or structural change of SEB on the inflammatory responses of immune cells.

Carbamylation is the post translation modification of protein *in vivo* and results from the covalent binding of cyanic acid to proteins. The post-translational modifications of proteins are called posttranslational modification-derived products (PTMDPs). PTMDPs are responsible for alterations of protein structural and functional properties (Jaisson and

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Gillery, 2010). Carbamylation can change the structure of proteins, and modify the activity of enzymes, cofactors, hormones and antibodies (Kuckel et al., 1993; Inoue et al., 2001). Recently, the carbamylated proteins induce the inflammation in various diseases, including chronic kidney disease, atherosclerosis and coronary artery disease (Apostolov et al., 2011). However, some researches, including our previous studies, were determined that the carbamylated proteins have potential anti-inhibitory effect against the inflammations (Bianchi et al, 2006; Chang, 2013; Yang and Chang, 2014).

In the present study, I investigated the effect of carbamylated SEB (CSEB) on the inflammatory response of HL-60 cells. To determine the anti-inflammatory effect of CSEB, I firstly proceeded the carbamylation of SEB. And then, I examined whether CSEB involved in cytokine releases and apoptosis of LPS-stimulated HL-60 cells. For identification of CSEB-mediated mechanism, intracellular signaling pathway mediated by CSEB was demonstrated in these cells. HL-60 cells are the human promyleocytic leukemia cell line and are also considered as a granulocytic inflammatory cell in this study.

MATERIALS AND METHODS

Reagents

Potassium cyanate (KOCN) (Sigma Aldrich, St. Louis, MO) was dissolved in sterile distilled water to prepare the stock solutions (10 mg/mL). Staphylococcal enterotoxin B was purchased from Sigma Aldrich (St. Louis, MO). RPMI 1640 medium and FBS were purchased from Life Technologies, Inc. (Gaithersburg, MD). Annexin V-fluoresceint isothiocyanate (FITC) apoptosis detection kit was purchased from BD biosciences (San Diego, USA). OptEIA Set human IL-6 and IL-8, TNF-α and IL-1β were purchased from BD biosciences. AKT 1/2/3 SimpleStep ELISA™ kit was purchased from Abcam Inc. (Cambridge, USA). EZ-Detect™ transcription factor kits for NF-κB was purchased from PIERCE (Rockford, IL).

Carbamylated SEB synthesis

Carbamylated SEB (CSEB) was synthesized by the reaction with potassium cyanate (KCN) (Leist et al., 2004). 1 μM KCN was added to a mixture of 500 μL of 1 mg/mL SEB and 500 μL of 1 M sodium borate. The mixture was incubated at 37℃ for 24 h to form the reaction solution. The reaction solution was concentrated by ultrafiltration (membrane cut-off, 10 kDa).

Cell culture

HL-60 cells were the human promyelocytic leukemia cell line and were purchased from American Type Culture Collection (Rockville, MD, USA). These cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL), and were incubated at 37℃ in 5% CO_2 incubator.

MTT assay

MTT assay was performed to determine cell proliferation using the cell proliferation kit (Roche, Penzberg, Germany). After treatment, HL-60 cells in 100 μL of the culture medium were plated into a 96-well culture plate. 10 μL of MTT solution was added in each well. After incubation of the plate at 37℃ for 4 h, 100 μL of solubilization solution was added to each well. After 24 h incubation, the absorbance was measured using an ELISA reader (Bio-Tek Instruments, Winooski, VT) at 550 nm.

Enzyme linked immunosorbent assay

IL-6, IL-8, TNF-α and IL-1β in the cultured supernatant of HL-60 cells were measured with a sandwich ELISA using OptEIA™ set human IL-6, IL-8, TNF-α and IL-1β according to the manufacturer's instructions. All assays were performed in triplicate. The concentration of each protein was calculated from the standard curve.

HL-60 cells were washed with ice-cold PBS and lysed with lysis buffer (10 mM HEPES, 10 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 1% NP-40, 0.5 mM PMSF, 0.1 mM DTT, 0.1 mM Na_3VO_4, and protease inhibitors). To determine the activation of AKT in the HL-60 cells, phosphorylation of AKT 1/2/3 was measured by AKT 1/2/3 SimpleStep ELISA™ kit, following the manufacturer's instructions. All assays were performed in triplicate. The concentration of each protein was calculated from the

standard curve.

Cell apoptosis

For measurement of the apoptosis, the cells were incubated with the FITC-labeled annexin V and propidium iodide (PI) for 15 min at room temperature. Annexin V is a marker for phosphatidylserine (PS), which exposed on plasma membrane at the initial stage of apoptosis. PI is a marker for membrane permeability in the late-apoptosis and necrotic cells. Apoptotic cells were analyzed by flow cytometry using CellQuest software and were defined as the cells in the right quadrant that stained positive for annexin V with/without PI. To analyze, 10,000 events were collected for each sample.

NF-κB p65 transcription factor assay

HL-60 cells were washed with ice-cold PBS and were lysed with lysis buffer. To determine the activity of NF-κB in the cell lysates, I was performed a luminescent assay using EZ-Detect™ transcription factor kits for NF-κB p65, following the manufacturer's instructions. Chemiluminescent detection was performed using a luminometer.

Statistical analysis

All data were expressed as mean ± SD. Data were analyzed by student's t-test using SPSS statistical software package (Version 10.0, Chicago, IL). A *P* values less than 0.05 was considered statistically significant.

RESULTS

SEB induces the inflammatory response of HL-60 cells

In the present study, I firstly examined the effect of SEB on the inflammatory-related reaction of HL-60 cells. To determine the optimal concentration of SEB for cell treatment, cytotoxicity of SEB was tested in dose-dependent manner. After addition of SEB in HL-60 cells, cytotoxicity was detected using the MTT assay. I found that SEB at concentration up to 1,000 ng/mL did not affect the viability of HL-60 cells (Fig. 1A). So based on Fig 1A, 1,000 ng/mL of SEB was applied to this study.

And then, I determined SEB-mediated the inflammatory

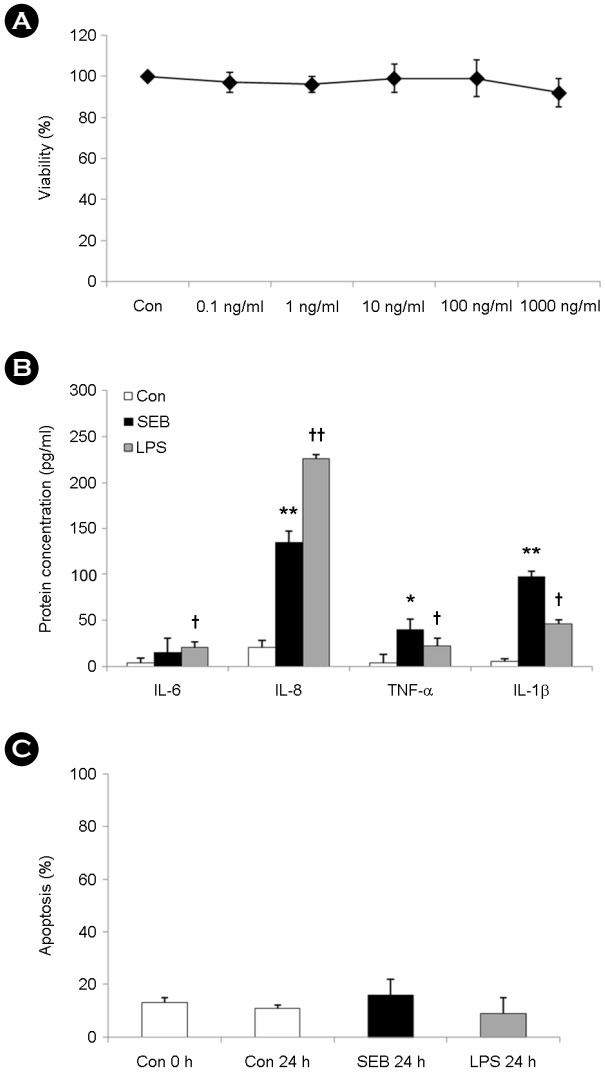

Fig. 1. The inflammatory responses mediated by SEB on HL-60 cells. (A) HL-60 cells were incubated for 24 h in the absence and presence of SEB (0.1, 1, 10, 100 and 1,000 ng/mL). The cytotoxicity of SEB on these cells was measured by performing MTT assay. The data are expressed as the relative ration to the absorbance of the untreated cell (Con), which was set at 100%. (B) HL-60 cells were treated with SEB (1 μg/mL) and LPS (10 μg/mL) for 24 h, respectively. After treatment, the supernatants of HL-60 cells were collected and protein levels were analyzed by ELISA. **P* < 0.05 and ***P* < 0.01 indicate a significant difference between the control and SEB-treated groups. †*P* < 0.05 and ††*P* < 0.01 indicate a significant difference between the control (Con) and LPS-treated groups. (C) HL-60 cells were incubated for 24 h in the presence and absence of SEB (1 μg/mL) and LPS (10 μg/mL), respectively. Apoptosis of these cells was analyzed by measuring the binding of annexin V-FITC and PI. All data are expressed as the means ± SD in three individual experiments.

alterations in HL-60 cells. After the cells were treated with SEB (1 μg/mL) for 24 h, the supernatant of cells were

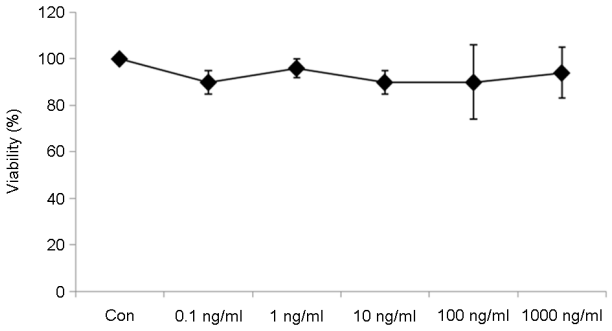

Fig. 2. The cytotoxic effect of carbamylated SEB on HL-60 cells. HL-60 cells were incubated for 24 h in the absence and presence of carbamylated SEB (CSEB) (0.1, 1, 10, 100 and 1,000 ng/mL). The cytotoxicity of CSEB on these cells was measured by performing MTT assay. The data are expressed as the relative ration to the absorbance of the untreated cell (Con), which was set at 100%. All data are expressed as the means ± SD in three individual experiments.

collected and analyzed by ELISA for the determination of cytokine levels. To investigate the apoptotic cells, the cells were collected and analyzed by flow cytometry. In HL-60 cells, the protein expressions of IL-8, TNF-α and IL-1β were significantly increased by stimulation with SEB (Fig. 1B). LPS was treated as a positive control for the inflammatory molecules in HL-60 cells (Chandrasekaran et al., 2011). However, HL-60 cell apoptosis was not changed by SEB and LPS stimulation (Fig. 1C). Apoptosis in inflammatory situations is an important mechanism for the resolution of inflammation and prevention of cancer development (Webb et al., 2004). These results indicate that SEB induces the releases of inflammatory cytokines, including IL-8, TNF-α and IL-1β in HL-60 cells and also considers as an inflammatory molecule in HL-60 cells.

Carbamylated SEB inhibits LPS-mediated cytokine secretion, unlike the inflammatory effect of SEB, in HL-60 cells

To determine the other effect of carbamylated SEB (CSEB) on LPS-mediated inflammation of HL-60 cells, I examined the effect of CSEB on various cytokine productions, including IL-6, IL-8, TNF-α and IL-1β. Before the effects of CSEB were examined, cytotoxicity of CSEB in HL-60 cells was detected using the MTT assay. After CSEB treatment in the dose-dependent manner, cell viability

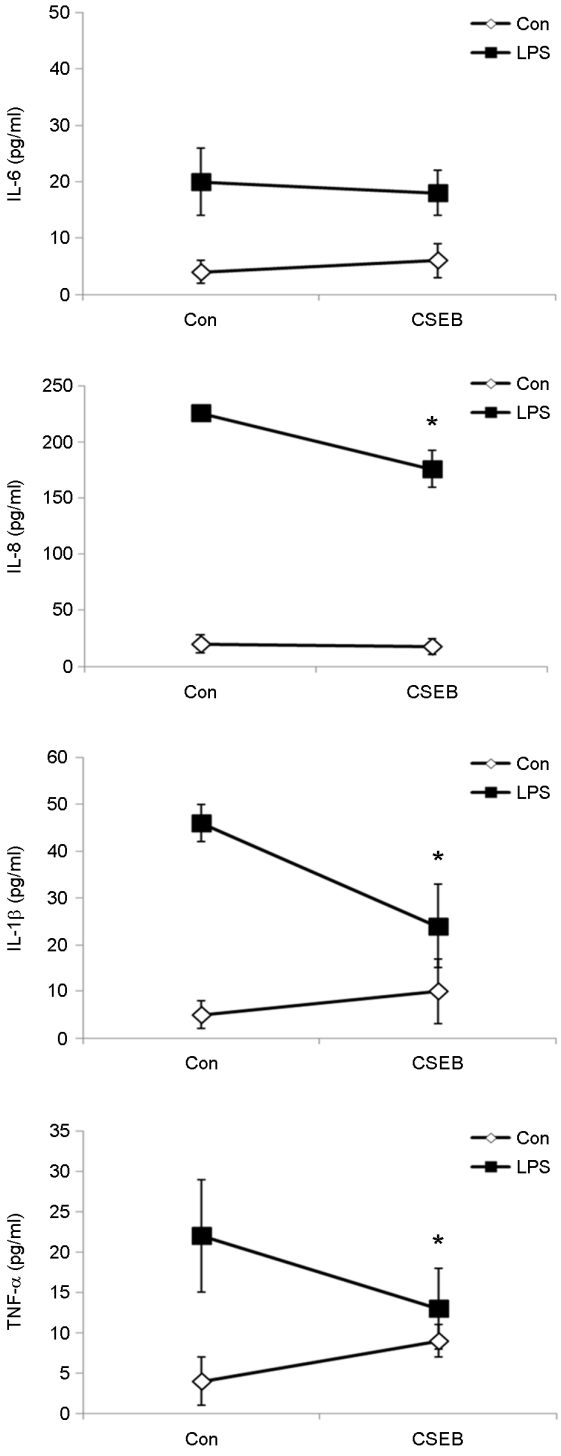

Fig. 3. The inhibitory effect of CSEB on the cytokine release induced by LPS in HL-60 cells. HL-60 cells were pretreated with or without LPS (10 μg/mL) for 1 h. After pretreatment, HL-60 cells were incubated with 1 μg/mL of CSEB for 24 h. The supernatant was collected and protein levels (IL-6, IL-8, TNF-α and IL-1β) were analyzed by ELISA. Data are expressed as the means ± SD in three individual experiments. *$P < 0.05$ indicates a significant difference between the CSEB-untreated cells and the CSEB-treated cells in LPS-treated group.

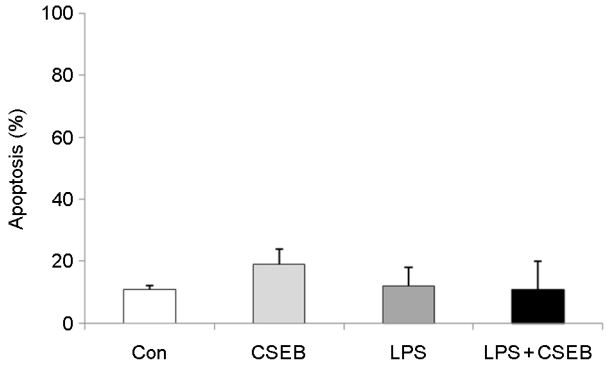

Fig. 4. The effect of CSEB on the survival and the apoptosis of HL-60 cells. HL-60 cells were pretreated with or without LPS (10 μg/mL) for 1 h. After pretreatment, HL-60 cells were incubated with 1 μg/mL of CSEB for 24 h. Apoptosis of these cells was analyzed by measuring the binding of annexin V-FITC and PI. Data are expressed as the means ± SD in three individual experiments.

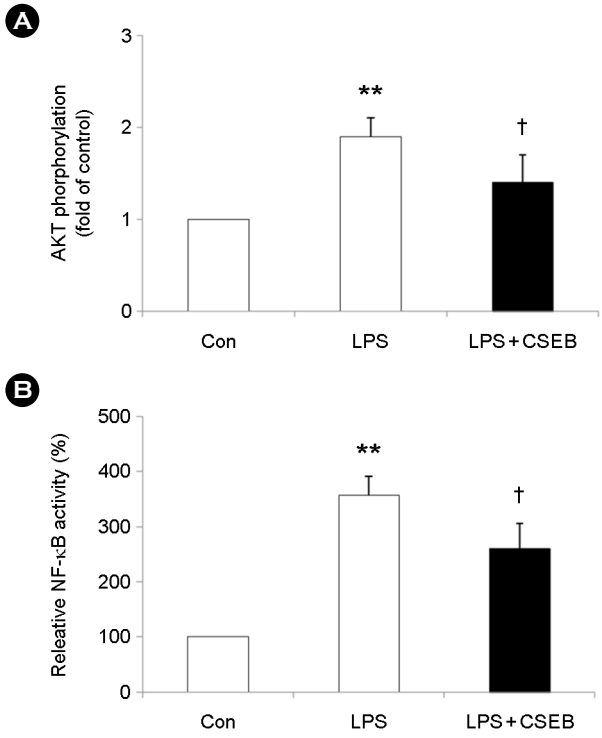

Fig. 5. The suppressive effect of CSEB on the cytokine release in response to LPS occurs through the inhibition of activities of AKT and NF-κB. (A) HL-60 cells were treated with or without LPS (10 μg/mL) and CSEB (for 1 μg/mL) for 1 h. After treatment, Cell lysates were analyzed for Akt phosphorylation by using AKT 1/2/3 SimpleStep ELISATM kit. Data were expressed as the fold change over untreated control group (Con). (B) HL-60 cells were treated with or without LPS (10 μg/mL) and CSEB (for 1 μg/mL) for 2 h. After treatment, the nuclear fraction was extracted from the cells. The NF-κB activation was assessed using an EZ-Detect™ kit. Data were presented in relation to the control (Con), which was set at 100%. Data are expressed as the means ± SD in three individual experiments. $^{**}P < 0.01$ indicates a significant difference between the control (Con) and LPS-treated groups. $\dagger P < 0.05$ indicates a significant difference between the CSEB-untreated cells and the CSEB-treated cells in LPS-treated group.

did not altered by the treatment of CSEB at concentration up to 1,000 ng/mL (Fig. 2). So based on this result, 1,000 ng/mL of CSEB was applied to this study.

To examine the effects of CSEB on cytokine releases and apoptosis, HL-60 cells were pretreated with LPS (10 μg/mL) for 1 h and then were incubated with 1 μg/mL of CSEB for 24 h. The supernatant of cells were collected and analyzed by ELISA for the determination of cytokine levels and the cells were collected and analyzed by flow cytometry for the investigation of the apoptosis. Although the expression level of IL-6 was not changed by the CSEB, CSEB considerably decreases the elevated levels of IL-8, TNF-α and IL-1β in LPS treated HL-60 cells (Fig. 3). However, CSEB had not any effect on the apoptosis of HL-60 cells (Fig. 4). These results suggest that SEB carbamylated by KCN acts as an anti-inflammatory molecule on the cytokine releases of LPS-treated HL-60 cells.

Carbamylated SEB blocks the Akt and NF-κB activation in LPS-treated HL-60 cells

LPS induces the activation of various intracellular signaling molecules and involves in many inflammatory responses. In intracellular signaling molecules activated by LPS, NF-κB the transcription factor is essential for inflammation and involves in LPS-mediated cellular responses (Choi et al., 2006). Akt plays the important role in LPS-mediated inflammation and Akt phosphorylation stimulates NF-κB activation (Shao and Lin, 2008; Lee et al., 2011). To evaluate the alteration of intracellular signaling molecules on CSEB-treated HL-60 cells, I investigated the Akt and NF-κB activation in LPS-stimulated HL-60 cells treated with CSEB. As shown in Fig. 5, CSEB considerably blocked the activations of Akt and NF-κB in LPS-treated HL-60 cells. These results indicate that the inhibitory effect of CSEB in LPS-mediated cytokine releases is occurred by blocking the activition of Akt and NF-κB.

DISCUSSION

In this study, I identified the effect of CSEB as a potent anti-inflammatory molecule on inflammatory phage of HL-60 cells. CSEB reduces the releases of cytokines of HL-60 cells in response to LPS stimulation. CSEB was changed the structure of SEB, a bacterial toxin and an inducer of inflammatory responses, by KCN-mediated carbamylation. Cyanic acid of KCN is derived from urea and normally present in human blood plasma and is not harmful to normal cells (OK et al., 2005). Cyanic acid is reacts irreversibly with the N-terminal groups of amino acid, peptides within proteins in the process of carbamylation. Among intracellular or extracellular processes for the regulation of biological activity of cells, carbamylation can change the structure of proteins, and modify the functional activity of proteins (Kuckel et al., 1993; Inoue et al., 2001). The carbamylation potential of protein depends on various factors, including structure of protein, accessibility of amino groups and the protein lifespan. Recently, the carbamylated proteins induce cell death in various diseases, including chronic kidney disease, atherosclerosis and coronary artery disease (OK et al., 2005; Apostolove et al., 2011). Whereas, carbamylated proteins react as the inhibitory molecules in various inflammatory diseases. In the human fibrosarcoma cells, collagen carbamylation inhibits invasive migration of tumor cell without affecting their proliferation (Said G et al., 2012). Carbamylated erythropoietin is nonerythropoietic, but it has neuroprotective action in chemotherapy-induced peripheral neurotoxicity (CIPN) (Bianchi et al., 2006). In our previous studies, KCN induces apoptosis of HCT 116 cells, the human colorectal cancer cell line (Yang and Chang, 2014). These previous studies indicate that carbamylated proteins have different effect from original proteins.

In the LPS-stimulated HL-60 cells, CSEB decreased the protein levels of IL-8, TNF-α and IL-1β (Fig. 3). Like the inflammatory effect of LPS, SEB is well-known pro-inflammatory bacterial toxin and induces the release of Th1/Th2 cytokines, including IL-6, IL-8, TNF-α and IL-1β in various immune cells. These cytokines have tissue damaging effects and enhance other inflammatory responses (Krakauer, 2013). Cytokine releases induced by SEB and LPS are associated with various intracellular signaling pathways. In pro-inflammatory molecules-associated intracellular signaling pathways, the predominant signaling proteins are phosphoinositide 3 kinase (PI3K), Akt (also known as protein kinase B, PKB) and NF-κB (Meng et al., 2002). PI3K activates Akt and mammalian target of rapamycin (mTOR), and regulates various biological processes, including cell survival, proliferation, differentiation and metabolism (Krakauer, 2013). And then, LPS-mediated NF-κB activation often requires Akt activation. A key regulatory pathway in NF-κB activation is the activation of IκB kinase (IKK) complex. The activation of IKK complex is induced by IKK phosphorylation involved by Akt (Meng et al., 2002). In our study, LPS induces the Akt activation and then NF-κB activation (Fig. 5). However, CSEB blocked Akt/NF-κB activated by LPS stimulation (Fig. 5).

In conclusion, CSEB regulates the LPS-mediated inflammatory response by inhibition of the cytokine release. The process of carbamylation can change the pro-inflammatory effect of SEB and can improve the regulatory system for the resolution of inflammation. The regulatory system of CSEB is occurred via suppression of intracellular signaling pathway, such as Akt and NF-κB. These properties of CSEB can also enhance the immune system.

REFERENCES

Apostolov EO, Ray Debarti, Alovuia WM, Mikhailova MV, Wang X, Basnakian AG, Shah SV. Endonuclease G mediates endothelial cell death induced by carbamylated LDL. Am J Physiol Heart Circ Physiol. 2011. 300: H1997-H2004.

Baker BS. The role of microorganisms in atopic dermatitis. Clin Exp Immunol. 2006. 144: 1-9.

Bianchi R, Brines M, Lauria G, Savino C, Gilardini A, Nicolini G, Rodriguez-Menendez V, Oggioni N, Canta A, Penza P, Lombardi R, Minoia C, Ronchi A, Cerami A, Ghezzi P, Cavaletti G. Protective effect of erythropoietin and its carbamylated derivative in experimental Cisplatin peripheral neurotoxicity. Clin Cancer Res. 2006. 12: 2607-2612.

Chandrasekaran CV, Thiyagarajan P, Deepak HB, Agarwal A. *In vitro* modulation of LPS/calcimycin induced inflammatory and allergic mediators by pure compounds of *Andrographis*

paniculata (King of bitters) extract. Int Immunopharmacol. 2011. 11: 79-84.

Chang JH. The effect of potassium cyanate (KCN) on radiation treatment of the colorectal cancer cell line, HCT 116. J Exp Biomed Sci. 2013. 19: 98-104.

Choi EK, Jang HC, Kim JH, Kim HJ, Kang HCm Paek YW, Lee HC, Lee SH, Oh WM, Kang IC. Enhancement of cytokine-mediated NF-κB activation by phosphatidylinositol 3-kinase inhibitors in monocytic cells. Int immunopharmacol. 2006. 6: 908-915.

Guzik TJ, Bzowska M, Kasprowicz A, Czerniawska-Mysik G, Wojcik K, Szmyd D, Adamek-Guzik T, Pryjma J. Persistent skin colonization with *Staphylococcus aureus* in atopic dermatitis: relationship to clinical and immunological parameters. Clin Exp Allergy. 2005. 35: 448-455.

Inoue EN, Nagano I, Ichinohasama R, Asato N, Kondo Y, Iinuma K. Bimodal effects of platelet-derived growth factor on rat mesangial cell proliferation and death, and the role of lysophosphatidic acid in cell survival. Clin Sci (Lond). 2001. 101: 11-19.

Jaisson S, Gillery P. Evalution of nonenzymatic posttranslational modification-derived products as biomarkers of molecular againg of proteins. Clin Chem. 2010. 56: 1401-1412.

Kotzin BL, Leung EYM, Kappler J, Marrack P. Superantigens and their potential role in human disease. Adv Immunol. 1993. 54: 99-166.

Kuckel CL, Lubit BW, Lambooy PK, Farnsworth PN. Methylisocyanate and actin polymerization: The in vitro effects of carbamylation. Biochim Biophys Acta. 1993. 1162: 143-148.

Krakauer T. Update on Staphylococcal superantigen-induced signaling pathways and therapeutic interventions. Toxins. 2013. 5: 1629-1654.

Leist M, Ghezzi P, Grasso G, Bianchi R, Villa P, Fratelli M, Savino C, Bianchi M, Nielsen J, Gerwien J, Kallunki P, Larsen AK, Helboe L, Christensen S, Pedersen LO, Nielsen M, Torup L, Sager T, Sfacteria A, Erbayraktar S, Erbayraktar Z, Gokmen N, Yilmaz O, Cerami-Hand C, Xie QW, Coleman T, Cerami A, Brines M. Derivatives of erythropoietin that are tissue protective but not erythropoietic. Science. 2004. 305: 239-242.

Lee YG, Lee J, Byeon SE, Yoo DS, Kim MH, Lee SY, Cho JY. Functional role of Akt in macrophage-mediated innate immunity. Front Biosci. 2011. 1: 517-530.

Meng F, Liu L, Chin P, D'Mello SR. Akt is a downstream target of NF-kappaB. J Biol Chem. 2002. doi: 10.1074/jbc.M112464200.

OK E, Basnakian AG, Apostolov EO, Barri YM, Shah SV. Carbamylated low-density lipoprotein induces death of endothelial cells: A link to atherosclerosis in patients with kidney disease. Kidney Int. 2005. 68: 173-178.

Pinchuk IV, Beswick EJ, Reyes VE. Staphylococcal enterotoxins. Toxins. 2010. 2: 2177-2197.

Proft T, Fraser JD. Bacterial superantigens. Clin Exp Immunol. 2003. 133. 299-306.

Said G, Guilbert M, Millerot-Serrurot E, Bulick LV, Terryn C, Garnotel R, Jeannesson P. Impact of carbamylation and glycation of collagen type I on migration of HT1080 human fibrosarcoma cells. Int J Oncol. 2012. 40: 1797-1804.

Shao DZ, Lin M. Platonin inhibits LPS-induced NF-κB by preventing activation of Akt and IKKβ in human PBMC. Inflmm research. 2008. 57: 601-606.

Skov L, Olsen JV, Giorno R, Schlievert PM, Baadsgaard O, Leung DY. Application of Staphylococccal enterotoxin B on normal and atopic skin induces up-regulation of T cells by a superantigen-mediated mechanism. J Allergy Clin Immunol. 2000. 105: 820-826.

Webb PR, Wang KQ, Scheel-Toellner D, Pongracz J, Salmon M, Lord JM. Regulation of neutrophil apoptosis: a role for protein kinase C and phosphatigylinositol-3-kinase. Apoptosis. 2000. 5: 451-458.

Yang EJ, Chang JH. TNF-α Regulates Potassium Cyanate-induced Apoptosis via NF-κB Activation in HCT 116 Cells. Biomed Sci Letters. 2014. 20: 32-38.