■ Original Article ■

The Effect of Caffeic Acid Phenethyl Ester (CAPE) on Phagocytic activity of septic Neutrophil *in vitro*

Eun-A Jang[1,*], Hui-Jing Han[2,**], Tran Duc Tin[2,**], Eunye Cho[3,**], Seongheon Lee[3,*] and Sang Hyun Kwak[2,3,†,*]

[1]*Department of Anesthesiology and Pain Medicine, School of Dentistry, Chonnam National University
& Chonnam National University Hospital, Gwangju 61469, Korea*
[2]*Brain Korea 21 Project, Center for Creative Biomedical Scientists at
Chonnam National University, Gwangju 61469, Korea*
[3]*Department of Anesthesiology and Pain Medicine, Chonnam National University Medical School &
Hospital, Gwangju 61469, Korea*

Caffeic acid phenethyl ester (CAPE) is an active component of propolis obtained from honeybee hives. CAPE possesses anti-mitogenic, anti-carcinogenic, anti-inflammatory, and immunomodulatory activities in diverse systems, which know as displays antioxidant activity and inhibits lipoxygenase activities, protein tyrosine kinase, and nuclear factor kappa B (NF-κB) activation. This study aimed to investigate the effect of CAPE on lipopolysaccharide (LPS)-induced human neutrophil phagocytosis. Human neutrophils were cultured with various concentrations of CAPE (1, 10, and 100 μM) with or without LPS. The pro-inflammatory proteins (tumor necrosis factor-alpha [TNF-α], interleukin [IL]-6 and IL-8) levels were measured after 4 h incubation. To investigate the intracellular signaling pathway, we measured the levels of mitogen-activated protein kinases (MAPK), including phosphorylation of p38, extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) and c-Jun N-terminal kinase (JNK). Next, to evaluate the potential phagocytosis, neutrophils were labeled with iron particles of superparamagnetic iron oxide nanoparticles (SPIONs, 40 nm) for 1 h in culture medium containing 5 mg/mL of iron. The labeling efficiency was determined by Prussian blue staining for intracellular iron and 3T-wighted magnetic resonance imaging. CAPE decreased the activation of intracellular signaling pathways, including ERK1/2 and c-Jun, and expression of pro-inflammatory cytokines, including TNF-α and IL-6, but had no effect on the signaling pathways of p38 and cytokine IL-8. Furthermore, images obtained after mannan-coated SPION treatment suggested that CAPE induced significantly higher signal intensities than the control or LPS group. Together, these results suggest that CAPE regulates LPS-mediated activation of human neutrophils to reduce phagocytosis.

Key Words: Neutrophil, Caffeic acid phenethyl ester, Phagocytosis, Pro-inflammatory, Mitogen-activated protein kinase

INTRODUCTION

Neutrophils are one of the cell types produced in the bone marrow. They are the most abundant circulating leucocytes in humans, representing about 50~70% of the total leucocyte population in humans, whereas, in mice, they account for only 10~25% (Kolaczkowska and Kubes, 2013). Neutrophils play an important role in the regulation of inflammatory responses not only to diverse pathological conditions but also to invading pathogens, such as bacteria, fungi, and viruses (Hsu et al., 2003). As one of the key cells involved in the immune defense system, they release destructive enzymes to counteract an infection (Navegantes et al., 2017). In the stable condition, neutrophils exist the rest stage, prevent secrete factor or toxic intracellular to harm the host, finally, they undergoing apoptosis within 72 h, are removed by macrophages and dendritic cells (Hsu et al., 2003; Kolaczkowska and Kubes, 2013). However, the rest of neutrophil can become activated by products form invasions of bacteria cytokines or chemokines, such as tumor necrosis factor-alpha (TNF-α), granulocyte-macrophage colony-stimulating factor (GM-CSF), and interleukin (IL)-8 (Wright et al., 2010). During an inflammatory response, neutrophils rapidly infiltrate into damaged tissues, which enhances the secretion of granule components or various pro-inflammatory cytokines and chemokines to maintain the recruitment and activation not only of neutrophils but also other cells of the immune system, such as monocytes, dendritic cells, and T cells (Bordon et al., 2013; Navegantes et al., 2017). The rapid recruitment of neutrophils to tissues is initiated by changes on the surface of endothelium caused by inflammatory mediators released from local leucocytes when they come into contact with a pathogen. At the inflammatory site, neutrophils can remove pathogens by multiple means, both intra- and extracellular (Kolaczkowska and Kubes, 2013). Neutrophils contribute to the killing of a pathogen through three main mechanisms, including phagocytosis, degranulation, and neutrophil extracellular traps (NETs) (Adrover et al., 2020; Sekheri et al., 2020; Rosa et al., 2021).

Neutrophils undergo a progression of proliferation, maturation, and differentiation from the stem cell through myeloblast, promyelocyte, myelocyte, metamyelocyte, band cell, and polymorphonuclear cell. Early in the neutrophil differentiation process, the cells develop phagocytic capacity, followed by the development of oxygen-dependent microbicidal activity, increased adhesiveness, cell motility, chemotactic response, and other cell-type specific traits. At the site of infection, primed neutrophils recognize invading microorganisms, which are taken up and packed into phagosomes. Within the phagosome, the neutrophil kills the pathogens by using NADPH oxygenase-dependent mechanisms (reactive oxygen species [ROS]) and antibacterial proteins (cathepsins, defensins, lactoferrin, and lysozyme) released upon fusion of cytoplasmic granules with microbe-containing phagosomes (degranulation), which together create the environmental conditions non-conducive for survival of bacteria (Rigby and DeLeo, 2012; Bordon et al., 2013; Kolaczkowska and Kubes, 2013).

Caffeic acid phenethyl ester (CAPE), a derivative of caffeic acid, is an active component of honeybee propolis and has been used in traditional medicine for a number of years. The primary active components of propolis are known for their anti-inflammatory, antioxidant, and wound-healing properties. CAPE is a flavonoid-like compound demonstrated to have antiviral, antitumoral, anti-atherosclerotic, and immunomodulatory properties (Przybyłek and Karpiński, 2019; Rojczyk et al., 2020; Forma and Bryś, 2021).

CAPE is unstable in rat plasma; after about 6 h, it is hydrolyzed to caffeic acid; however, this process does not occur in human plasma (Murtaza et al., 2015). Some studies previously mentioned that CAPE can prevent the expression of pro-inflammatory mediator products; inhibit the activation of enzymes, such as lipoxygenase, cyclooxygenase, glutathione-S-transferase, and xanthine oxidase; reduce T cell proliferation; and, moreover, suppress the stimulation of extra- and intracellular signals, such as mitogen-activated protein kinase (MAPK) and nuclear factor kappa B (NF-κB), thus decreasing the inflammatory response (Cho et al., 2014; Armutcu et al., 2015). For these reasons, CAPE is considered a promising therapy for various pathologies, such as inflammation, cancer, sepsis, infection, and neurodegeneration.

In the present experiments, we evaluated whether CAPE attenuates lipopolysaccharide (LPS)-mediated activation of neutrophils through inhibiting intracellular signaling pathways, including MAPK, and expression levels of pro-inflammatory cytokines (IL-6, IL-8, and TNF-α) in neutrophils. With the results, we continued to investigate whether CAPE affects the phagocytic activity of neutrophils.

MATERIALS AND METHODS

Isolation of neutrophils

The peripheral blood of healthy volunteers was used to isolate neutrophils under a protocol approved by Chonnam National University hospital Institutional Review Board (CNUH-2023-154) followed as described previously (Kwak et al., 2005). 6% of Dextran was added, and erythrocytes were sedimented under gravity for 45 min at room temperature, and then leucocyte-enriched pellets were collected by centrifugation at 1,100 rpm for 6 min and resuspended in platelet-poor plasma. Next, leucocyte-enriched plasma was centrifuged with gradient Percoll (3 mL of 42% to 3 mL of 51%) at 1,100 rpm for 20 min. Neutrophils were found at the 42~51% Percoll layer interface. Then, red blood cells (RBC) were removed by RBC lysis buffer, and neutrophils were collected by centrifugation at 3,000 rpm for 5 min. Finally, neutrophils were resuspended in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 1% streptomycin/penicillin (Mediatech, Herndon, VA, USA).

Cytokine ELISA

Neutrophils were cultured with or without LPS from Escherichia coli 055:B5 (LPS 100 ng/mL) and CAPE (1, 10, and 100 μM) in 24-well plates. CAPE and Escherichia coli 055:B5 endotoxins were purchased from Sigma-Aldrich (St. Louis, MO, USA). After 4 h, the levels of inflammatory cytokines (TNF-α, IL-6, and IL-8) were evaluated by ELISA kits (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

Western blot analysis

Neutrophils were cultured with CAPE (100 μM) 1 h before adding LPS (100 ng/mL). Next, neutrophils were incubated for 20~30 min in ice-cold lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM Na_2EDTA, 1% Triton X-100, 1 mM EGTA, 1 mM Na_3VO_4, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM PMSF, and 1 μg/mL leupeptin; pH 7.5). Debris from the lysed cells was pelleted by centrifugation at 14,000 for 20 min, and the supernatant was stored at 80℃ until use. The protein concentration of each sample was analyzed by the BCA kit (Rockford, IL, USA) according to the manufacturer's protocol. The levels of phosphorylated and total p38, extracellular signal-regulated protein kinases 1 and 2 (ERK1/2), and c-Jun N-terminal kinase (JNK) (Cell Signaling Technologies, Beverly, MA, USA) were detected by western blot analysis. For the western blot assay, 50 μg of protein was loaded and run on a 10% Tris-HCl SDS polyacrylamide gel and transferred onto polyvinylidene difluoride membranes, then blocked with 5% nonfat milk powder in 20 mM TBS with 0.1% Tween. After blocking, the membrane was incubated with the specific antibodies overnight at 4℃. Finally, the chemiluminescence system and analysis software (Bio-Rad) were used to analyze the ratio between phosphorylated and total kinase.

Magnetic Labelling of human neutrophils

Histochemical Detection of Mannan-coated SPIONs: Histochemical detection of mannan-coated superparamagnetic iron oxide nanoparticles (M-SPIONs, AMAG Pharmaceuticals, Cambridge, USA) was performed according to previous studies (Metz et al., 2004; Matuszewski et al., 2005; Shanhua et al., 2013). In brief, neutrophils were incubated with M-SPIONs for 1 h and then fixed with 4% formaldehyde. A cytocentrifuged spin preparation of fixed cells (1×10^6 cells) was stained with Prussian blue for iron detection and the presence of M-SPIONs and counterstained with nuclear fast red (NFR) solution (Sigma-Aldrich, St Louis, MO, USA). To this end, neutrophils were fixed in acetone and incubated with a 1:1 (v/v) mixture of 2% potassium ferrous cyanide (Sigma, St Louis, MO, USA) and 2 N HCl (Sigma) for 20 min. Glass slides were rinsed in distilled water and counterstained with NFR solution for 5 min. After that, the slides were washed with distilled water, and the cells were dehydrated with 70, 80, 90, and 95% alcohol
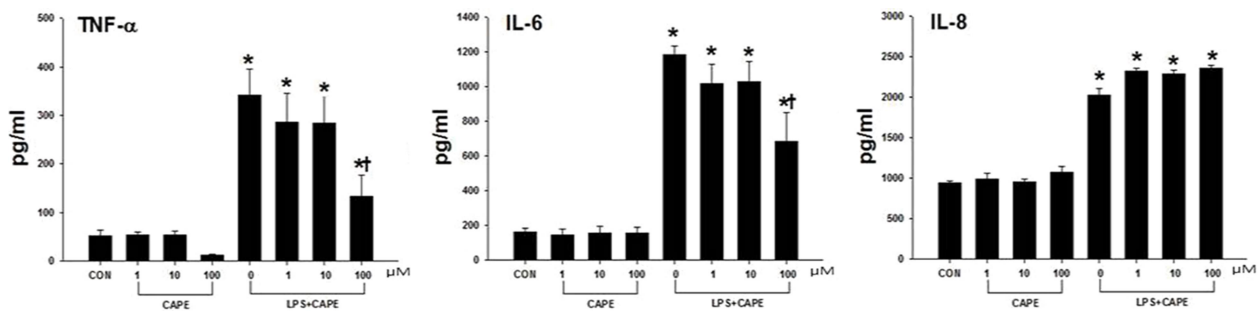
Fig. 1. The effects of CAPE change expressions of TNF-α, IL-6, and IL-8 on septic neutrophils. Protein results were obtained from ELISA. Each bar represents means ± SD. Data are representative of three separate experiments (*: $P < 0.05$ versus control, †: $P < 0.05$ versus LPS alone).

once each and 100% alcohol twice. Subsequently, each slide was immersed for a minimum of 1 min and mounted with Permount solution (Fisher Scientific, NJ, USA).

***In vitro* MR Imaging:** A 0.5 mL phantom tube was used for *in vitro* MR imaging. 200 μL gelation gelatin (10%) put into the button of tube. Cells were counted and take 2.5×10^6 neutrophils into 50 μL 4% HCHO. It was embedded in 50 μL gelatin 10% (1:1), put them in to the in the middle layer and covered the top of the tube with 100 μL gelatin. MR imaging was performed on 3T MR scanner (TimTrio, Simens, Germany). Axial T2-weighted MR imaging was acquired with the following parameters; time of repetition (TE) /echo time (TE): 3,000 ms/178 ms, field of view (FOV): 160 × 160 mm, matrix number: 189 × 384, slice thickness: 2 mm with a 1mm intersection gap. For analysis of relaxation time, regions of interest (circular, 7 mm in diameter) were placed in the center of phantom tube, and excluded the areas with air-susceptibility artifacts. Signal intensity (SI) of T2-weighted MR imaging were obtained by averaging the middle five regions of interest in the phantom volume.

Statistical analysis

Data were expressed as mean ± SEM for each group and analyzed by ANOVA or the Tukey Kramer multiple comparison test or Student's *t*-test. Statistical significance was defined as $P < 0.05$.

RESULTS

The effect of CAPE on LPS-induced cytokine expression by neutrophils

To assess the effect of the possible interaction between CAPE and LPS on neutrophil activation, neutrophils were incubated with various concentrations of CAPE (0, 1, 10, and 100 μM) and LPS (100 ng), and the protein levels of IL-6, IL-8, and TNF-α were determined 4 h later. In these experiments, there were increases in the expression of all cytokines after stimulation with LPS. There was no effect of CAPE alone over the range of concentrations examined. LPS-induced cytokine (TNF-α and IL-6) expression was attenuated in the presence of CAPE (100 μM) (Fig. 1).

The effect of CAPE on LPS-induced activation of p38, ERK1/2, JNK

To examine the effects of CAPE on LPS-induced activation of the p38, ERK1/2, and JNK pathways, LPS-stimulated neutrophils were incubated with or without CAPE (100 μM), and then the levels of active, phosphorylated forms of p38, ERK1/2, and JNK pathways were determined. Exposure of resting neutrophils with CAPE alone had no effect on the activation of the p38, ERK1/2, and JNK pathways. As shown in Fig. 2, the culture of neutrophils with LPS resulted in the activation of all three pathways. The addition of CAPE to LPS-stimulated neutrophils did not affect p38 activation but resulted in attenuated JNK and ERK1/2 phosphorylation (Fig. 2).
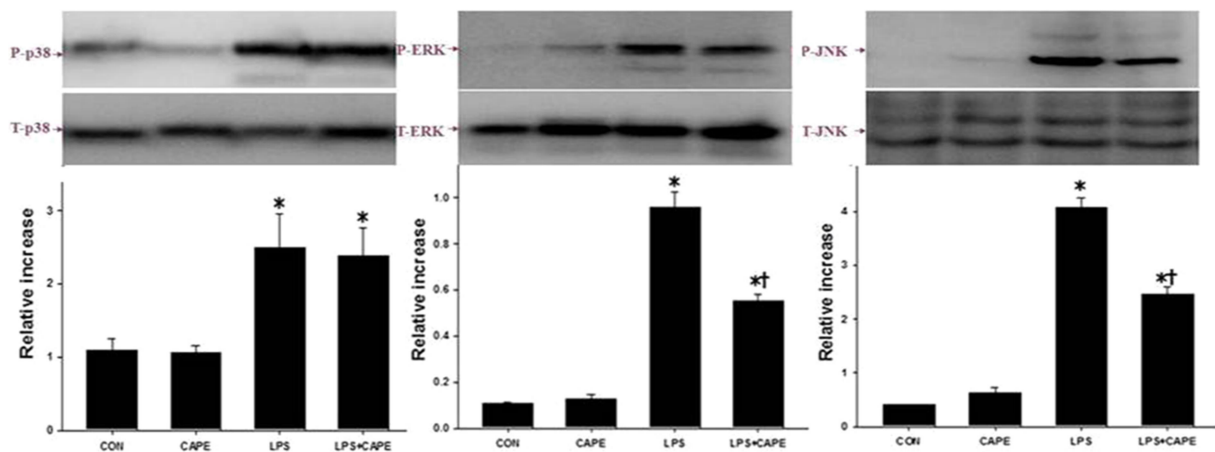
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Fig. 2. The effect of CAPE attenuates MAPKs kinase protein expression induced by LPS on human neutrophil. The ratio of phosphorylated to total ERK1/2, JNK, and p38 pathway was calculated with data from three independent experiments. Densitometry was performed using a chemiluminescence system and analysis software (Bio-Rad, Hercules, CA) to determine the ratio between phosphorylated and total kinase. Each bar represents means $\pm$ SD (*: $P < 0.05$ versus control, †: $P < 0.05$ versus LPS alone).
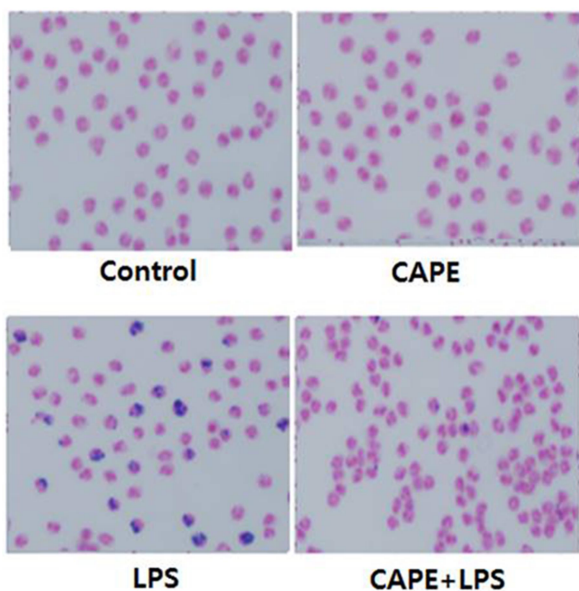

Fig. 3. The uptake iron oxides in human neutrophils. Iron oxide was indicated as blue and cell nuclei as red. Cell were incubated with CAPE (100 μM) and with or without LPS (100 ng/mL) for 2 hours. After then neutrophil incubated with M-SPION displayed uptake of iron oxide for 1 hour.

Fig. 4. Signal intensity (SI) of neutrophils labeled with M-SPIONs in an *in vitro* phantom tube. (A) SI of T2-wighted MR imaging for control neutrophils incubated with M-SPIONs, LPS (100 ng/mL)-induced neutrophils incubated with M-SPIONs, CAPE (100 μM)-pretreated LPS (100 ng/mL)-induced neutrophils incubated with M-SPIONs (B) Graph showing mean SI of neutrophils incubated with M-SPIONs, LPS (100 ng/mL)-induced neutrophils incubated with M-SPIONs, CAPE (100 μM)-pretreated LPS (100 ng/mL)-induced neutrophils incubated with M-SPIONs (*: $P < 0.05$ versus control, †: $P < 0.05$ versus LPS alone).

The effect of CAPE on phagocytosis of LPS-induced neutrophils treated with M-SPIONs

To detect the presence of intracellular iron oxide and the phagocytic activity, neutrophils were stained with Prussian blue staining and detected with 3T magnetic resonance
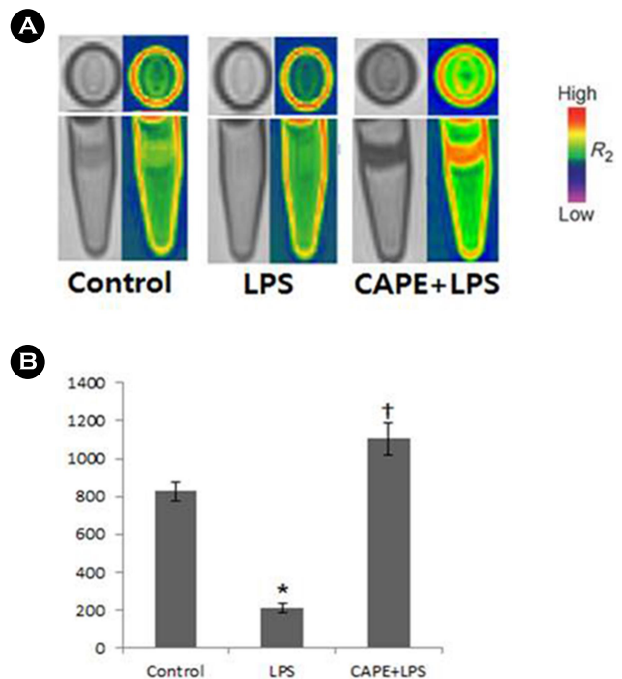
(MR) imaging. The neutrophils were incubated with or without CAPE (100 μM) and LPS (100 ng/mL) for 2 h and then treated with M-SPIONs for 1 h. Neutrophils in the control group showed no positive staining for iron, and 80~90% of the LPS-induced neutrophils incubated with M-SPIONs showed intracellular iron was present in the cytosol of neutrophils. The addition of CAPE to LPS-stimulated neutrophils decreased the Prussian blue staining (Fig. 3). The MR T2 signal intensities were 828 ± 50.6 for control neutrophils incubated with M-SPIONs, 210 ± 26.6 for LPS-induced neutrophils, and 1106 ± 86.1 for CAPE-pretreated LPS-induced neutrophils (Fig. 4). The mannose-receptor blocking CAPE pretreated LPS group demonstrated an intracellular iron uptake that was much less than that of the non-blocking LPS group, as determined by Prussian blue staining.

DISCUSSION

Previous studies have demonstrated that CAPE has a role in the modulatory activation of neutrophils (such as anti-inflammatory and antioxidant) and enhances the host defense system (Przybyłek and Karpiński, 2019; Rojczyk et al., 2020; Forma and Bryś, 2021). However, how CAPE downregulates the expression of various pro-inflammatory cytokines and activators of signal transduction pathways, specifically the MAPK signaling pathway, to affect neutrophil-mediated phagocytosis remains unclear. Thus, in our study, we sought to understand how CAPE regulates the phagocytic activity of LPS-stimulated neutrophils. Neutrophil-mediated phagocytosis was detected by M-SPIONs. Cellular uptake is decided by particle size, coatings, and surface charge. Neutrophils use their pattern recognition receptors (PRRs), such as mannose receptors on the surface, which can bind to bacteria components (as mannan is one of the components on the cell wall of microorganisms) to detect the infection, but simultaneously it also is used to make markers for M-SPION sense in the diagnosis of the septic model. LPS-induced neutrophils can stimulate the activation of mannose receptors, leading to the accumulation of M-SPIONs in neutrophils. Finally, T2-weighted MR imaging signal intensity can detect changes in the uptake of iron oxide (Shanhua et

al., 2013). Based on that, we evaluated whether CAPE affected neutrophil-mediated phagocytosis by analyzing the T2-weighted MR imaging signal intensity and cellular uptake of iron oxide with Prussian blue staining.

Inflammation is the process that release of inflammatory mediators include biogenic amines, metabolites of arachidonic acid, platelet aggregation factors, pro-inflammatory cytokines and free oxygen radicals from injured tissues and migratory cells (Armutcu et al., 2015). During inflammation, neutrophils are one of the first cells to migrate toward the site of infection, where they ingest invading microorganisms by phagocytosis (Hayashi et al., 2003).

The process was realized by pattern recognition receptors (PRRs) expressed on the surface of the neutrophils, which bind with secreted bacterial products such as PGN, lipoproteins, lipopolysaccharide, CpG-containing DNA, and flagellin to recognize and cause adaptive immune (Rigby and DeLeo, 2012). Toll-like receptors (TLRs) are one of the PRRs that have an important role in the innate immune recognition of pathogens, immune cellular activation, and cytokines release. Human neutrophils express various TLRs, such as TLR1, 2, and 4~10. However, phagocytosis is based mainly on the expression of TLR4 and TLR9 (Hayashi et al., 2003).

Neutrophils respond to LPS through the upregulation of various pro-inflammatory cytokines, and the release of inflammatory mediators and antibacterial products are hallmarks of the involvement of these cells in the pathophysiology of sepsis (Arndt et al., 2004). In addition, LPS is presented in TLR 4 in cell membranes, bring out the activation of MAPK kinase and enhancing expression of cytokine (TNF-α, IL-6, IL-8 and, IL-1β) (Zhang and Dong, 2005).

TLR stimulation leads to increased phagocytosis and the number of cytokine products, priming of superoxide generation, and a reduction in chemotaxis (Hayashi et al., 2003). Hellberg et al. reported that TNF-α strongly stimulates phagocytosis by neutrophils, whereas several cytokines, including IL-1β, IL-2, IL-6, IL-8, IL-10, IL-12, and IL-17, had no significant effect (Hellberg et al., 2011). The binding of TNF-α to cell surface receptors increases the expression of signal transduction pathways, specifically MAPK, which

induces the secondary inflammatory response to contribute to the biological activity of TNF-α (Armutcu et al., 2015). The MAPK kinase pathway such as p38 and JNK pathways involve regulating the stress response, whereas ERK1/2 pathway responses to growth factors and chemoattractants (Avdi et al., 2001).

The studies reported that p38 is phosphorylated, which has an association with the bacteria ingestion and support the multiple signaling cascades of cells during phagocytosis (Hsu et al., 2003; Rossi and Lord, 2013). However, another study revealed that the p38 pathway did not regulate phagocytosis (Yeh et al., 2010). Thus, the role of the p38 pathway in phagocytosis by neutrophils is controversial. Mei-Chun Yeh et al suggest that the phagocytosis by neutrophil depend on JNK pathway, which plays a key role in increasing the adhesion of bacteria to neutrophils and up-regulating the phagocytosis for the killing of bacteria by cells. When JNK pathway is inhibited by the protein transduction domain of HIV-TAT (TAT-JIP), bring out reducing neutrophils phagocytosis and preventing the recognition of neutrophils to bacteria (Yeh et al., 2010), result in JNK pathway modulates up-stream events in the microbial killing during the phagocytosis. The others study of Alessandra Rossi et al showed that ERK1/2 pathway was necessary to uptake bacteria and maintain phagocytosis by neutrophil. The ERK1/2 pathway was blocked by PD98059, which induced to suppress neutrophil phagocytosis (Rossi and Lord, 2013). The evidence presented demonstrates that the ERK1/2 pathway plays a major role in phagocytosis by neutrophils. Our study confirmed that LPS activation of neutrophils leads to the upregulation of not only members of MAPK (p38, ERK1/2, and JNK) but also the levels of inflammatory mediators (IL-6, IL-8, and TNF-α). Moreover, we showed that SPION-labeled LPS-induced neutrophils appeared as lower signal intensity on T2-weighted MR imaging and neutrophil uptake higher iron oxide than group control. Taken together, we suggest that the phagocytic activity of neutrophils is related to the expression of intracellular signaling pathways (ERK1/2 and JNK pathways) and pro-inflammatory cytokines (TNF-α). Thus, by modulating these pathways, the phagocytic activity and release of toxic cytokine products of neutrophils can be controlled, and the suppression of these activities is considered a potential strategy for the treatment of inflammatory diseases.

CAPE possesses anti-mitogenic, anti-carcinogenic, anti-inflammatory, and immunomodulatory activities, which have been attributed to its antioxidant activity and inhibition of lipoxygenase activities, protein tyrosine kinase, and NF-κB activation (Mirzaei et al., 2021; Elumalai et al., 2022; Oršolić and Jazvinšćak Jembrek, 2022).

CAPE suppresses the synthesis of pro-inflammatory cytokines (IL-6, IL-8, and TNF-α) and attenuates the activity of the ERK1/2 and JNK pathways during neutrophil contact with LPS (Murtaza et al., 2015). Our study asserts again the key role of CAPE on neutrophils during sepsis; CAPE significantly inhibited signaling events mediated by the ERK1/2 and JNK pathways and attenuated the expression of IL-6 and TNF-α compared with the group treated with LPS alone, leading to the activation of adaptive immune systems in the host. As mentioned above, the ERK1/2 and JNK pathways have a crucial role in the phagocytosis by neutrophils, so suppressing these signaling pathways leads to the attenuation of the phagocytic activity of neutrophils. Moreover, although there were no dramatically differences signaling pathway p38 and cytokine IL-8 between group treat LPS alone and group CAPE combine LPS, we suggest that CAFE has the effective inhibitor of neutrophils phagocytosis, which evidence was the decline of uptake iron oxide on neutrophils and the signal intensity on T2-weighted MR imaging higher than group treated with LPS alone. The p38 pathway and IL-8 play an important role in the inflammatory response; however, they do not contribute to the phagocytic activity of neutrophils. Based on the results, we demonstrated that the inhibition neutrophils secrete inflammatory mediators (IL-6 and TNF-α) by CAPE through signaling the ERK1/2 and JNK pathways, not the p38 pathway; is the close association with the phagocytic activity of neutrophils. The degree of phagocytosis activation by neutrophils creates reactive oxygen species (ROS) products (Bordon et al., 2013), cause harmful tissue during inflammatory. Therefore, CAPE controlled the process, which is considered a new strategy for treating sepsis and acute inflammatory diseases applied.

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