the FEBS Journal

Staphylococcal enterotoxin C1-induced pyrogenic cytokine production in human peripheral blood mononuclear cells is mediated by NADPH oxidase and nuclear factor-kappa B

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Keywords
human peripheral blood mononuclear cells; NADPH oxidase; NF-κB; pyrogenic cytokine; staphylococcal enterotoxin C1

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(Received 2 April 2007, revised 17 May 2007, accepted 22 May 2007)

doi:10.1111/j.1742-4658.2007.05896.x

The staphylococcal enterotoxins produced by *Staphylococcus aureus* are associated with pyrogenic response in humans and primates. This study investigates the role of NADPH oxidase and nuclear factor-kappa B (NF-κB) on enterotoxin staphylococcal enterotoxin C1 (SEC1)-induced pyrogenic cytokine production in human peripheral blood mononuclear cells (PBMC). The results indicate that the febrile response to the supernatant fluids of SEC1-stimulated PBMC in rabbits was in parallel with the levels of interleukin-1β and interleukin-6 in the supernatants. The release of interleukin-1β and interleukin-6, nuclear translocation of NF-κB and its DNA binding activity in the SEC1-stimulated PBMC were time-dependent and were completely eliminated by pyrrolidine dithiocarbamate or SN-50 (NF-κB inhibitors). The release of reactive oxygen species in the supernatants and translocation of the NADPH oxidase $p47^{phox}$ subunit to the plasma membrane of SEC1-stimulated PBMC were time-dependent. Administration of apocynin (NADPH oxidase inhibitor) attenuated the febrile response to the supernatants in rabbits and decreased the translocation of NADPH oxidase $p47^{phox}$ subunit and NF-κB activity in the SEC1-stimulated PBMC, and suppressed reactive oxygen species and pyrogenic cytokine production in the supernatants. Taken together, SEC1 may act through an NADPH oxidase mechanism to release reactive oxygen species, which activate NF-κB in PBMC to stimulate the synthesis of pyrogenic cytokines that trigger a fever response in rabbits.

Staphylococcus aureus is a major food-borne pathogen which produces a number of toxins and virulence factors [1]. The staphylococcal enterotoxins produced by *S. aureus* are known to cause staphylococcal food poisoning, fever, and toxic shock syndrome, and also act as immunosuppressors and affect cytokine

Abbreviations
Apo, apocynin; EMSA, electrophoretic mobility shift assay; ETYA, 5,8,11,14-eicosatetraynoic acid; FLAP, 5-LOX-activating protein; HBSS, Hanks' balanced salt solution; HIMO, 1L-6-hydroxymethyl-*chiro*-inositol-2(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate; IL, interleukin; 5-LOX, 5-lipoxygenase; MK 886, 3-[1-(*p*-chlorobenzyl)-5-(isopropyl)-3-*t*-butylthioindol-2-yl]-2,2-dimethylpropanoic acid; NF-κB, nuclear factor-kappa B; PBMC, human peripheral blood mononuclear cells; PDTC, pyrrolidine dithiocarbamate; PI3K, phosphatidylinositol-3-kinase; ROS, reactive oxygen species; SEs, staphylococcal enterotoxins; SEC1, staphylococcal enterotoxin C1; SP, supernatant fluids of SEC1-stimulated PBMC; TSST-1, toxic shock syndrome toxin-1; Wort, wortmannin.

production in humans and primates [2,3]. Staphylococcal enterotoxins are relatively heat stable [2], and ingestion of staphylococcal enterotoxins causes emesis and diarrhea [4]. *Staphylococcus aureus* is also an important microorganism of bovine, ovine and caprine mastitis [5]. Staphylococcal enterotoxins, especially staphylococcal enterotoxin C, in *S. aureus* have been isolated from the dairy products of infected animals, which could cause problems in public health and food safety [6,7]. The staphylococcal enterotoxins are 26–30 kDa proteins, and are classified into different toxin serotypes (SEA, SEB, SEC, SED, SEE, SEG, SEH, SEI, and SEJ, etc.) [8]. More than three SEC subtypes (SEC1, SEC2, and SEC3) may exist [1]. SEA to SEE has been reported to account for approximately 95% of staphylococcal food poisoning outbreaks [8]. Production of SEC1 by *S. aureus* from patients with toxic shock syndrome has been revealed [9]. SEC1 is a member of the pyrogenic toxins family that enhances the susceptibility of host to lethal endotoxin shock [2]. SEC1 has also been suggested to be involved in some cases of sudden infant death syndrome [10].

Nuclear factor-kappa B (NF-κB) is a ubiquitous transcription factor which regulates the expression of genes encoding growth factors, chemokines, cytokines, cell adhesion molecules and some acute phase proteins both in health and in many diseases [11,12]. NF-κB has been identified in various cell types and is regulated by many inducers, such as ultraviolet irradiation, cytokines, and bacterial or viral products [13–16]. NF-κB in its inactive state resides in the cytoplasm bound to an inhibitory protein known as IκB. Activation of NF-κB is triggered by extracellular stimuli. The IκB is then phosphorylated and proteolytically processed by proteasomes and other proteases [17]. This proteolytic process allows translocation of NF-κB from the cytosol to the nucleus, where it binds to the promoter region of target genes [18].

Reactive oxygen species (ROS) have been reported to play a pivotal role in many forms of cell signaling as well as activation of NF-κB [19,20]. ROS, including H_2O_2, superoxide and hydroxyl radicals, are vital for the pathology of inflammatory processes, onset of hypertension and cancer [21–23]. The primary source of ROS, including superoxide radicals and H_2O_2, is through the activation of NADPH oxidase in polymorphonuclear neutrophils [24]. The core enzyme of NADPH oxidase consists of five subunits, $p40^{phox}$, $p47^{phox}$, $p46^{phox}$, $p22^{phox}$ and $gp91^{phox}$. Upon stimulation, the cytosolic $p47^{phox}$ is phosphorylated and moves to the membrane, where it binds to cytochrome b_{558} and becomes an active oxidase [25–27]. Another activation pathway for NF-κB is via 5-lipoxygenase (5-LOX), a 78 kDa protein, which is expressed mainly in leukocytes and mast cells [28]. Stimuli trigger the migration of 5-LOX from the cytoplasm to the plasma membrane, where it associates with 5-LOX-activating protein (FLAP) and metabolizes arachidonic acid to release ROS [28,29]. The phosphatidylinositol-3-kinase (PI3K)/Akt pathway also affects NF-κB activation [30]. Activated PI3K phosphorylates phosphatidyinositol-4,5-bisphosphate to form PIP3, which further activates Akt and affects NF-κB activity [31].

In the present study, SEC1-induced translocation of the NADPH oxidase $p47^{phox}$ subunit, production of superoxide anion, and activation of NF-κB were determined to investigate possible mechanism involved in the release of pyrogenic cytokines from peripheral blood mononuclear cells (PBMC) and the pyrogenic response in rabbits.

Results

Febrile response to the supernatant fluids of the SEC1-stimulated PBMC

To determine whether the supernatant fluids of SEC1-stimulated PBMC (SP) can induce the pyrogenic response, the supernatant fluids obtained from PBMC treated with SEC1 were given intravenously to rabbits. After administration of the SP (1 $mL \cdot kg^{-1}$), colonic temperature began to rise in a SEC1 concentration-dependent manner (Fig. 1A). This febrile response was not affected by polymyxin B (Fig. 1B) but was abolished after heating the SP at 70 °C for 30 min (Fig. 1C). Additionally, intravenous injection of less than 30 $ng \cdot kg^{-1}$ of SEC1 did not induce a febrile response in rabbits (data not shown). Within the range of 10^5–10^8 $cells \cdot mL^{-1}$, the pyrogenic response to the SP was cell number dependent (Fig. 1D). Over the incubation time of 48–96 h, the pyrogenic responses to the SP were incubation time-related (Fig. 1E). Table 1 indicates that the levels of interleukin (IL)-1β and IL-6 in the SP began to rise at 6 h, and reached their peak levels between 48 and 96 h. Over the dose range of 0.2–5.0 $ng \cdot mL^{-1}$ of SEC1, IL-1β and IL-6 in the SP displayed a SEC1 dose-related manner (data not shown). Figure 1F shows that monoclonal antibody to IL-1β or IL-6 had a significant antipyretic effect. The pyrogenic response to the SP was almost completely abrogated by the combination of anti-IL-1β and anti-IL-6 monoclonal IgG but was not affected by the control IgG (Fig. 1F).

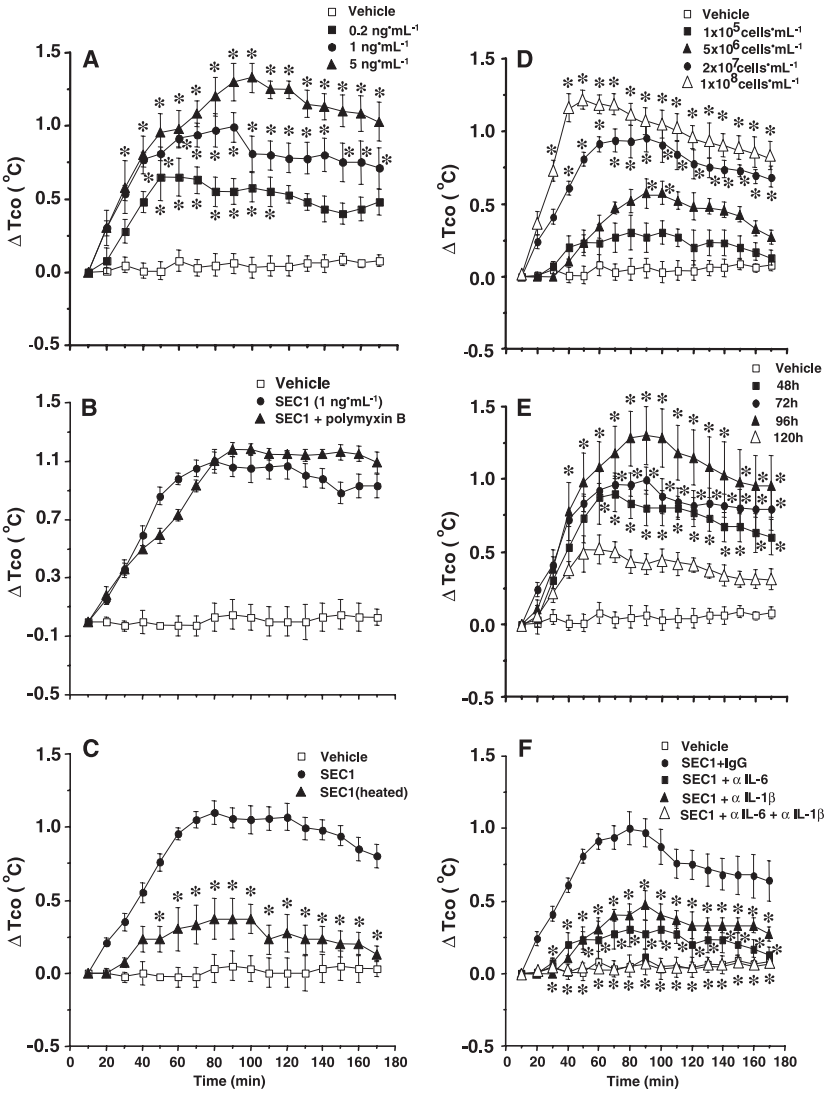


Fig. 1. The pyrogenic response in rabbits induced by the supernatant fluids of SEC1-treated human PBMC. (A, B) Changes in the colonic temperature (Δt_{co}) of rabbits intravenously injected (1 mL·kg^{-1}) with the supernatant fluids obtained from PBMC (1×10^7 cells·mL^{-1}) treated for 72 h with the vehicle, SEC1 or SEC1 plus polymyxin B (50 μg·mL^{-1}). (C) Δt_{co} of rabbits injected with the nonheated supernatant fluids obtained from PBMC treated with the vehicle, or SEC1, or with the heated (70 °C for 30 min) supernatant fluids obtained from PBMC treated with SEC1 (1 ng·mL^{-1}). (D) Δt_{co} of rabbits injected with the supernatant fluids obtained from the indicated concentrations of PBMC with 1 ng·mL^{-1} of SEC1. (E) Δt_{co} of rabbits injected with the supernatant fluids obtained from PBMC with the vehicle for 72 h or with 1 ng·mL^{-1} of SEC1 for the indicated time periods. (F) Δt_{co} of rabbits treated with the supernatant fluids obtained from PBMC with the vehicle, SEC1 (1 ng·mL^{-1}) plus control IgG (100 μg·mL^{-1}), SEC1 plus anti-IL-6 monoclonal IgG (100 μg·mL^{-1}), SEC1 plus anti-IL-1β monoclonal IgG (100 μg·mL^{-1}), or SEC1 plus anti-IL-6 and anti-IL-1β monoclonal IgG. Before injection to rabbits, the indicated antibodies were added to the SEC1-treated supernatants and incubated at 37 °C for 30 min. All experimental groups: $n = 5$, except for those received vehicle ($n = 8$) or antibody ($n = 4$). Normal saline was used as the vehicle. * Significantly different from the corresponding values of the vehicle group except for those received heated supernatant (compared with the nonheated SEC1 group) or antibody (compared with the SEC1-treated PBMC plus IgG group).

SEC1 induces NF-κB activation in PBMC

PBMC were treated in the presence or absence of NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC) or SN-50 prior addition of SEC1. After 24 h of incubation, the supernatant fluids were collected for cytokine analysis and for the fever index of pyrogen test in rabbits. As shown in Table 2, pretreatment of PBMC with PDTC or SN-50 not only attenuated the SEC1-induced production of IL-1β and IL-6 in the SP, but

Table 1. Time course release of the pyrogenic cytokines from SEC1-treated PBMC. The concentrations of pyrogenic cytokines in the supernatant fluids obtained from human PBMC (1×10^7 cells·mL^{-1}) treated with vehicle (normal saline) or SEC1 (1 ng·mL^{-1}) for the indicated time periods were determined according to the manufacturer's instructions. Colorimetric results were read on a multiscan photometer (MRXII, Dynatech, MeLean, VA, USA) 96-well plate reader at a wavelength of 450 nm. Cytokine levels were quantified by comparison with standards. The sensitivity of IL-1β and IL-6 was < 0.1 and < 0.7 pg·mL^{-1}, respectively. Data are expressed as the mean ± SEM of triplicate cultures.* Significantly different from the corresponding values of the vehicle group.

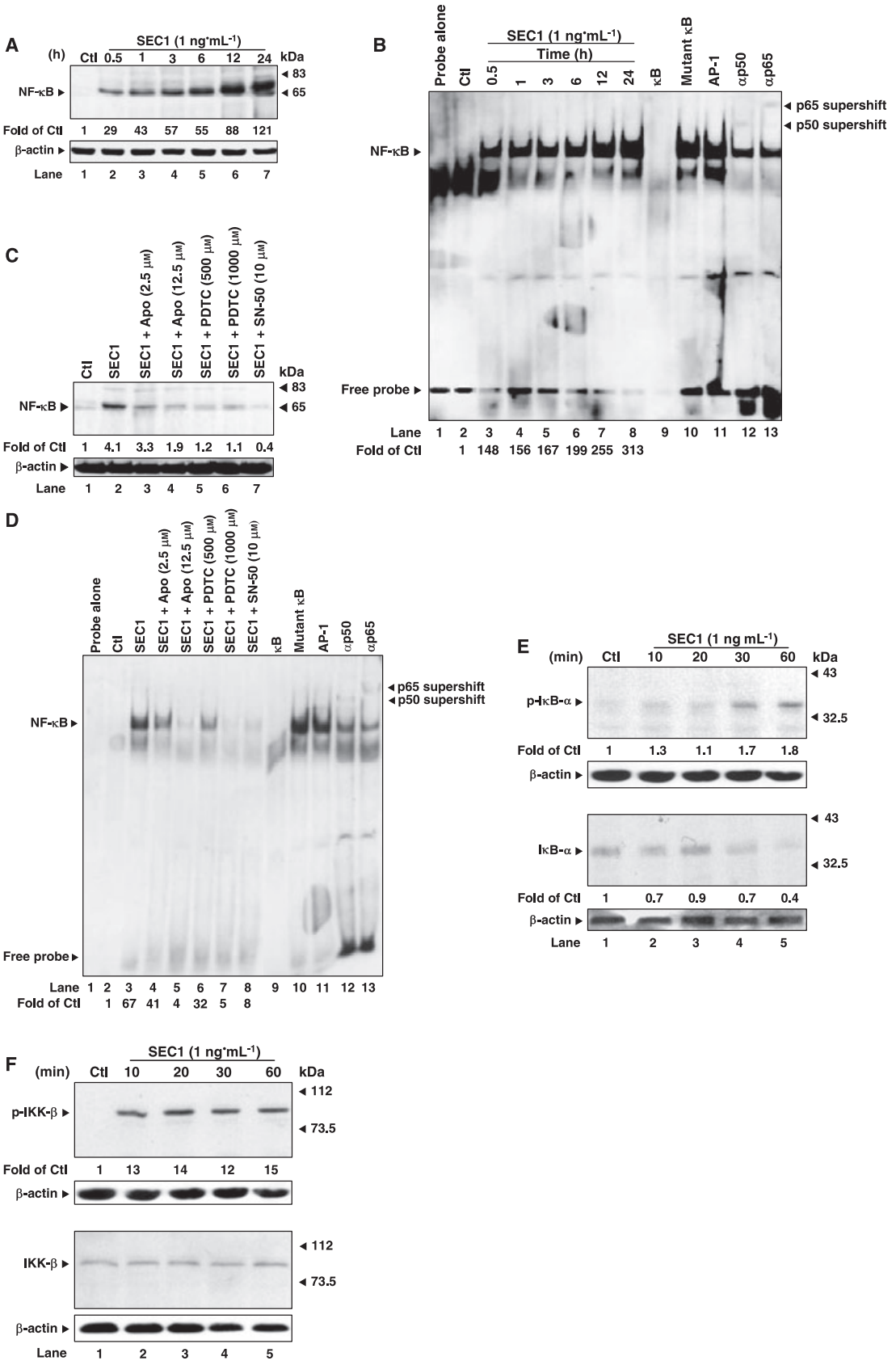
		Cytokine production (pg·mL^{-1})	
Time (h)	Treatment	IL-1β	IL-6
6	Vehicle	12 ± 3	90 ± 9
	SEC1	691 ± 52*	5000 ± 100*
12	Vehicle	7 ± 1	100 ± 12
	SEC1	1725 ± 71*	26200 ± 200*
24	Vehicle	13 ± 2	102 ± 5
	SEC1	2179 ± 49*	33600 ± 210*
48	Vehicle	6 ± 1	82 ± 8
	SEC1	3011 ± 95*	48300 ± 320*
72	Vehicle	5 ± 1	110 ± 12
	SEC1	3187 ± 75*	70000 ± 440*
96	Vehicle	9 ± 5	108 ± 10
	SEC1	3091 ± 60*	64200 ± 360*

Table 2. Effects of NF-κB, PI3K/Akt, 5-LOX/FLAP and NADPH oxidase inhibitors on SEC1 induced pyrogenic cytokine production and fever index in rabbits. Human PBMC (1×10^7 cells·mL^{-1}) were pretreated with or without PDTC (1000 μM), SN-50 (10 μM), Wort (400 nM), HIMO (25 μM), ETYA (60 μM), MK 886 (10 μM) or apocynin (Apo, 12.5 μM) for 1 h prior to addition of SEC1 (1 ng·mL^{-1}). After 24 h of incubation, the supernatant fluids were collected for cytokine analysis and for the fever index of pyrogen test in rabbits. For experiments, 0.01% dimethylsulfoxide (this concentration was tested and revealed to be nontoxic to the cells) was used as the vehicle. Data are expressed as the mean ± SEM of triplicate culture. * Significantly different from the corresponding control values (the vehicle group). † Significantly different from the corresponding control values (the SEC1 group). ‡ Number of rabbits tested.

	Cytokine production (pg·mL^{-1})		
Treatment	IL-1β	IL-6	Fever index (°C)
Vehicle	12 ± 3	160 ± 7	0.18 ± 0.02 (5)‡
SEC1	2141 ± 5*	32500 ± 510*	1.06 ± 0.03 (5)*
PDTC	13 ± 5	100 ± 3	0.21 ± 0.02 (5)
SEC1 + PDTC	176 ± 62†	5500 ± 210†	0.32 ± 0.04 (5)†
SN-50	12 ± 7	271 ± 100	0.11 ± 0.06 (5)
SEC1 + SN-50	92 ± 7†	6800 ± 260†	0.31 ± 0.05 (5)†
Wort	27 ± 2	330 ± 90	0.22 ± 0.04 (5)
SEC1 + Wort	1900 ± 179	37200 ± 960	1.19 ± 0.04 (5)
HIMO	22 ± 1	200 ± 26	0.13 ± 0.05 (5)
SEC1 + HIMO	1931 ± 155	39500 ± 870	1.12 ± 0.04 (5)
ETYA	16 ± 1	403 ± 93	0.11 ± 0.06 (5)
SEC1 + ETYA	1981 ± 252	34400 ± 860	1.07 ± 0.05 (5)
MK 886	25 ± 2	283 ± 100	0.22 ± 0.02 (5)
SEC1 + MK 886	2057 ± 223	38700 ± 870	1.08 ± 0.03 (5)
Apo	2 ± 1	1 ± 1	0.21 ± 0.03 (5)
SEC1 + Apo	32 ± 2†	3 ± 2†	0.25 ± 0.03 (5)†

also inhibited the SEC1-induced febrile response in rabbits. The detection of NF-κB protein in the nucleus became apparent after 30 min and increased dramatically up to 24 h (Fig. 2A). The DNA-binding activity of NF-κB was detected at 30 min of SEC1 treatment and the level kept increasing to 24 h (Fig. 2B). The specificity of the NF-κB band was completely eliminated in the presence of a 100-fold excess of the unlabeled κB oligonucleotide (Fig. 2B, lane 9). Conversely, a 100-fold excess of oligonucleotide probes of the unlabeled mutant κB (Fig. 2B, lane 10) or the unlabeled AP-1 (Fig. 2B, lane 11), a transcription factor containing DNA binding site, had no effect on the ability of the NF-κB to bind to DNA. The NF-κB subunits were characterized by using a specific antibody for the p50 or p65 subunit, and the results indicate that the NF-κB band intensity reduced in the presence of anti-p50 or anti-p65 IgG (Fig. 2B, lanes 12 and 13). Treatment of PBMC with the NF-κB inhibitors, PDTC or SN-50, inhibited the SEC1-induced NF-κB

Fig. 2. NF-κB activation by SEC1. (A) PBMC (1×10^7 cells·mL^{-1}) were treated with the vehicle control (Ctl, normal saline) or SEC1 for western blot analysis using an anti-NF-κB p65 monoclonal IgG. β-actin was similarly assessed to serve as a loading control. The intensity of the individual protein signal was normalized to that of β-actin, with Ctl levels arbitrarily set to 1. (B) PBMC were treated with the vehicle (normal saline) or SEC1 for EMSA. Except for the free probe control, nuclear proteins (10 μg) were used. Specificity was determined by competition of the nuclear protein obtained from the cells treated with 1 ng·mL^{-1} of SEC1 for 24 h. The NF-κB-DNA binding activity (lanes 2–8) was quantified by densitometry. The time-course groups were compared with the Ctl group to obtain the relative binding activity. (C) Western blot analysis shows the inhibition of SEC1-induced NF-κB nuclear translocation activity by apocynin (Apo), PDTC or SN-50 in PBMC. PBMC were pretreated with the vehicle (0.5% ethanol; this concentration was tested and revealed to be nontoxic to the cells) or the indicated inhibitors for 1 h before treatment of SEC1 for 24 h. (D) Analysis of EMSA shows the inhibition of SEC1-induced NF-κB activity by the indicated inhibitors in PBMC. (E, F) PBMC were treated with the vehicle (normal saline) or SEC1. After incubation, whole cell lysates were prepared for western blot analysis using an antiphospho-IκB-α (p-IκB-α), anti-IκB-α (IκB-α), antiphospho-IKK-β (p-IKK-β) or anti-IKK-β (IKK-β) polyclonal IgG.


A
(h)
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3
6
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83
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NF-κB
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Probe alone
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SEC1 (1 ng·mL-1)
Time (h)
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1
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κB
Mutant κB
AP-1
αp50
αp65
p65 supershift
p50 supershift
NF-κB
Free probe
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Fold of Ctl
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148
156
167
199
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313
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Ctl
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SEC1 + Apo (2.5 μM)
SEC1 + Apo (12.5 μM)
SEC1 + PDTC (500 μM)
SEC1 + PDTC (1000 μM)
SEC1 + SN-50 (10 μM)
kDa
83
65
NF-κB
Fold of Ctl
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κB
Mutant κB
AP-1
αp50
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Fold of Ctl
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β-actin
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73.5
p-IKK-β
Fold of Ctl
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β-actin
IKK-β
112
73.5
β-actin
Lane
1
2
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5

nuclear translocation (Fig. 2C, lanes 5–7). The DNA-binding activity of NF-κB induced by SEC1 was completely blocked by 1000 μM of PDTC (Fig. 2D, lane 7) or 10 μM of SN-50 (Fig. 2D, lane 8). PDTC or SN-50 alone did not affect the nuclear translocation or DNA-binding activity of NF-κB (data not shown). Moreover, IκB-α was significantly phosphorylated and degraded at 30 and 60 min, respectively, after treatment of PBMC with SEC1 (Fig. 2E). Phosphorylation of IKK-β in the whole cell lysates of SEC1-stimulated PBMC was rapidly increased within 10 min and sustained for 60 min, whereas the total IKK-β protein expression was not affected (Fig. 2F).

NF-κB activation is mediated by NADPH oxidase

PBMC were pretreated with or without PI3K/Akt specific inhibitor, 1L-6-hydroxymethyl-*chiro*-inositol-2(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate (HIMO) or wortmannin (Wort) prior to the addition of SEC1. After 24 h of incubation, the supernatant fluids were collected for the cytokine analysis and for the fever index of pyrogen test in rabbits. As shown in Table 2, neither Wort nor HIMO affected the production of IL-1β or IL-6 in the SP. The induction of the febrile response in rabbits was not affected in the presence of wortmannin or HIMO. Additionally, these inhibitors did not alter the SEC1-induced expression of nuclear NF-κB protein (Fig. 3A, lanes 5 and 6) and its DNA-binding activity (Fig. 3B, lanes 6 and 7). Similar findings were obtained by using 5-LOX inhibitor, 5,8,11,14-eicosatetraynoic acid (ETYA) or FLAP inhibitor (3-[1-(*p*-chlorobenzyl)-5-(isopropyl)-3-*t*-butylthioindol-2-yl]-2,2-dimethylpropanoic acid; MK 886) (Table 2, Fig. 3A, lanes 3 and 4, and Fig. 3B, lanes 4 and 5). Wort, HIMO, ETYA, or MK 886 alone has no effect on the nuclear translocation or DNA-binding activity of NF-κB (data not shown). Strikingly, treatment of PBMC with NADPH oxidase inhibitor (apocynin) prior to the addition of SEC1 completely blocked the release of these two cytokines in the SP and attenuated the febrile response in rabbits (Table 2). Apocynin also inhibited the SEC1-induced nuclear NF-κB expression (Fig. 2C, lanes 3 and 4) and its DNA-binding activity (Fig. 2D, lanes 4 and 5). Phosphorylation of IKK-β and IκB-α was slightly reduced at 2.5 μM of apocynin and eradicated at 12.5 μM (Fig. 4). The ROS level in the SP increased at 2 min and reached its peak level at 12 min (Table 3) after treatment with SEC1. In the presence of apocynin, but not ETYA or MK 886, the production of ROS was inhibited (Table 4). PI3K/Akt inhibitors also did not change the formation of ROS (data not shown). Figure 5A indicates that the

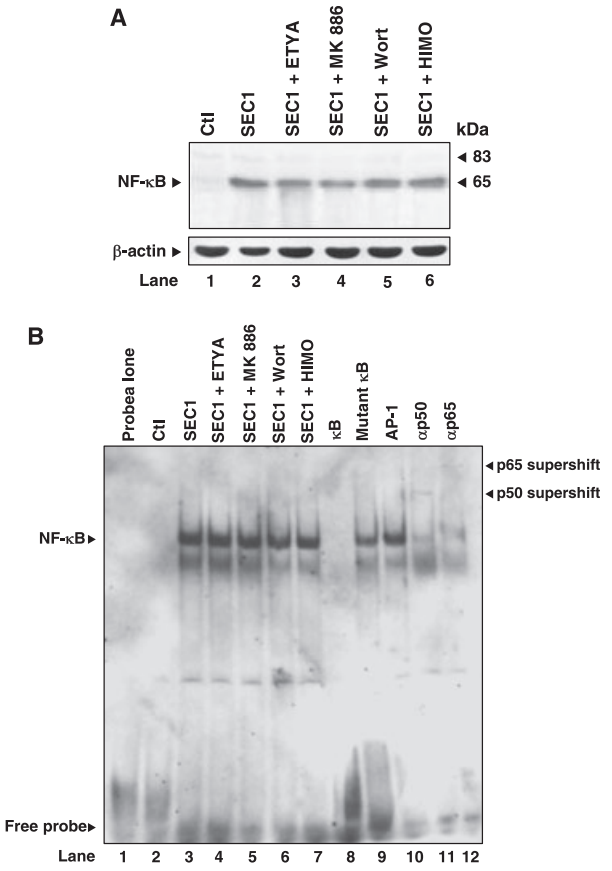

Fig. 3. Effects of 5-LOX/FLAP and PI3K/Akt inhibitors on SEC1-treated NF-κB activity. (A) PBMC were treated with the vehicle (0.01% dimethylsulfoxide; this concentration was tested and revealed not to be toxic to the cells), ETYA (60 μM), MK 886 (10 μM), Wort (400 nM), or HIMO (25 μM) for 1 h before treatment of SEC1 (1 ng·mL^{-1}) for 24 h. Nuclear proteins were subjected to western blot analysis by using an anti-NF-κB p65 monoclonal IgG. (B) PBMC were pretreated with the vehicle control (Ctl, 0.01% dimethylsulfoxide), ETYA, MK 886, Wort, or HIMO for 1 h before treatment of SEC1 for 24 h. Nuclear proteins were subjected to EMSA.

subunit of NADPH oxidase p47^{phox} appeared (four-fold) on the cell membrane at 2 min, reached its peak level (5.8-fold) at 4 min and stayed (3- or 4.4-fold, respectively) at 12 or 24 min following treatment with SEC1. Conversely, the level of p47^{phox} in the cytoplasm of the SEC1-treated PBMC decreased 80% within 2 min and sustained this level for 24 min (Fig. 5A). Figure 5B demonstrates that the treatment of SEC1-stimulated PBMC with apocynin not only profoundly decreased the level of p47^{phox} in the membrane, but also increased the level of p47^{phox} in the cytoplasm. The use of apocynin alone did not affect the translocation of p47^{phox}, production of ROS, nuclear expression of NF-κB or its DNA-binding activity (data not shown).

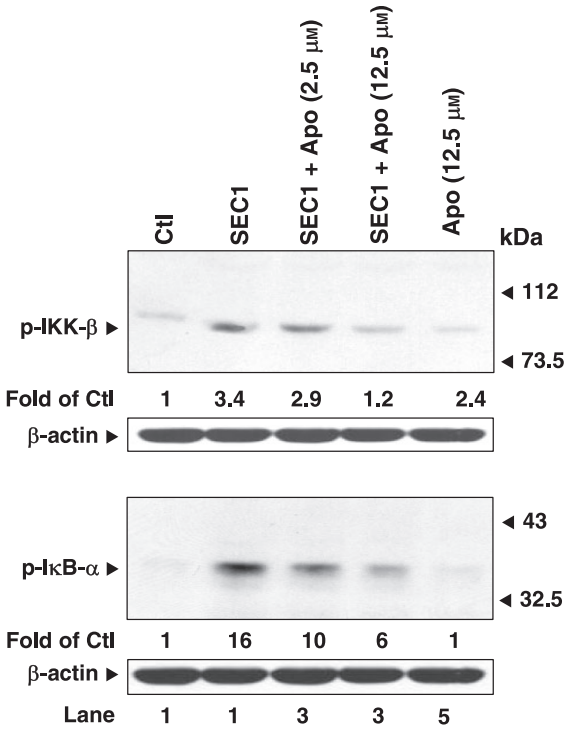

Fig. 4. Effects of NADPH oxidase inhibitor on the phosphorylation of IKK-β and IκB-α. PBMC were pretreated with the vehicle control (Ctl, 0.5% ethanol) or apocynin for 1 h before addition of SEC1 (1 ng·mL^{-1}) for 60 min. Whole cell lysates were prepared for western blot analysis using an antiphospho-IKK-β (p-IKK-β) or antiphospho-IκB-α (p-IκB-α) polyclonal IgG.

Table 3. Time-dependent effects of SEC1 on ROS production in human PBMC. Human PBMC (5×10^5 cells·mL^{-1}) were treated with the vehicle (normal saline) or SEC1 (1 ng·mL^{-1}) for the indicated time periods. After incubation, the supernatant fluids were collected and the contents of ROS were determined. Data are expressed as the mean ± SEM of triplicate culture. * Significantly different from the corresponding values of the 0 min group.

Time (min)	Lucigenin chemiluminescence counts
0	68016 ± 1187
2	223689 ± 18061*
4	312135 ± 27918*
6	457014 ± 52511*
8	464427 ± 44959*
10	496952 ± 36740*
12	584833 ± 30245*

Discussion

The present study demonstrates that the febrile response to the supernatant fluids obtained from SEC1-treated human PBMC in rabbits is associated with the levels of IL-1β, IL-6 and ROS in the supernatant fluids of SEC1-treated human PBMC. Adding

Table 4. Effects of NADPH oxidase and 5-LOX/FLAP inhibitors on ROS production in SEC1-treated human PBMC. Human PBMC (5×10^5 cells·mL^{-1}) were pretreated with or without apocynin (Apo, 12.5 μM), ETYA (60 μM) or MK 886 (400 nM) for 1 h prior to addition of SEC1 (1 ng·mL^{-1}). After 12 min of incubation, the supernatant fluids were collected and the contents of ROS were determined. For experiments, 0.01% dimethylsulfoxide was used as the vehicle. Data are expressed as the mean ± SEM of triplicate culture. * Significantly different from the corresponding values of the SEC1 group.

Treatment	Lucigenin chemiluminescence counts
Vehicle	93070 ± 2801
SEC1	689144 ± 18201
SEC1 + Apo	74963 ± 542*
SEC1 + ETYA	728354 ± 61621
SEC1 + MK 886	758756 ± 15237

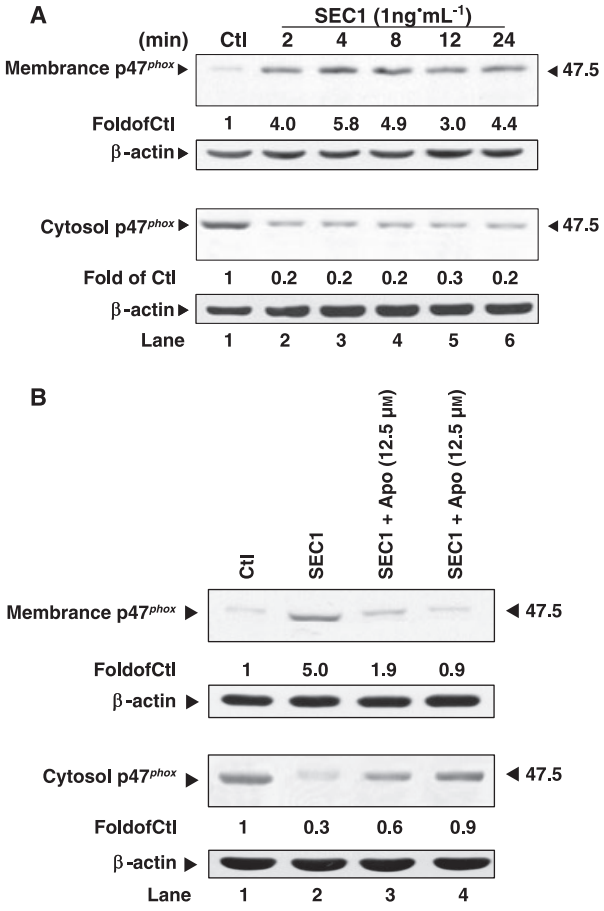

Fig. 5. Membrane translocation of p47^{phox} in SEC1-treated cells. (A) PBMC were treated with the vehicle control (Ctl, normal saline) or SEC1. (B) PBMC were treated with or without apocynin for 1 h prior to addition of SEC1 for 12 min. After incubation, the membrane and cytosol proteins were obtained for western blot analysis using an anti-p47^{phox} polyclonal IgG.

PDTC, SN-50 or apocynin to the SEC1-stimulated PBMC attenuates the febrile response and the levels of IL-1β, IL-6 and ROS in the supernatant fluids. Adding an anti-IL-1β or anti-IL-6 monoclonal IgG to the supernatant fluids significantly decreases the febrile response in rabbits. These data indicate that SEC1 may act through NF-κB and NADPH oxidase mechanisms in the PBMC to stimulate the synthesis or release of IL-1β, IL-6 and ROS.

ROS have recently gained attention as secondary messengers that regulate intracellular signaling cascades and transcription factors. Some investigations have reported that NF-κB can be activated by ROS [32,33] produced by a pathway involving 5-LOX/FLAP [34], NADPH oxidase [35] or PI3K/Akt [30]. During NF-κB activation, phosphorylation of IKK-α/IKK-β heterodimer results in the phosphorylation and degradation of IκB-α, which then leads to the phosphorylation of NF-κB p65 subunit and renders the release of NF-κB p50/p65 heterodimer to translocate from cytosol to the nucleus where it binds and activates various target genes [36]. During NADPH oxidase activation, phosphorylation of $p47^{phox}$ allows the migration of entire cytosolic complex ($p40^{phox}$, $p47^{phox}$ and $p67^{phox}$) to the membrane to associate with cytochrome b_{558} (containing $p22^{phox}$ and $p91^{phox}$) to assemble the active oxidase which catalyzes reduction of oxygen to superoxide and leads to the formation of ROS [25]. ROS-induced activation of NF-κB in T cells has been suggested to proceed in part via SHIP-1-mediated phosphorylation of IKK complex or via Syk-dependent phosphorylation of IκB-α [36]. In stimulated phagocytic cells, ROS produced by NADPH oxidase also activates IKK and NF-κB and induces production of proinflammatory cytokine IL-1β via a Toll-like receptor-mediated pathway [37]. Recently, the primary actions of superantigen staphylococcal enterotoxins have been studied. In T cells, SEB or SEC interacts with specific variable β (Vβ) elements on α/β T cell receptor, such as Vβ 3, 12, 13.2, 14, 15, 17 and 20 [38,39]. Stimulation of a T cell receptor results in ROS production within short period of time (approximately 10 min), which is dependent on the expression of $p47^{phox}$ [40]. In dendritic cells, on the other hand, SEB reacts with Toll-like receptor 2 or 4 [38,41]. Activation of Toll-like receptor increases $p47^{phox}$ expression and elevates ROS formation at a later time point (> 30 min) [42]. Phosphorylation of $p47^{phox}$ has also been suggested to via protein kinase C or via interleukin-1 receptor-associated kinase 4 in a cell-free system [43,44]. In the present study, the cytokine synthesis and febrile response induced by SEC1 is dependent on NADPH oxidase and not on PI3K/Akt or 5-LOX/FLAP because these phenomena are attenuated by apocynin and not by ETYA, MK 886, Wort or HIMO (Table 2). In addition, SEC1 induces the formation of ROS, and the phosphorylation of IKK-β and IκB-α at 2, 10 and 30 min of stimulation, respectively (Table 3 and Fig. 2E,F). An inhibitor for NADPH oxidase attenuates the movement of $p47^{phox}$ (Fig. 5B), translocation of NF-κB (Fig. 2C), DNA-binding activity of NF-κB (Fig. 2D) and phosphorylation of IKK-β, IκB-α (Fig. 4), whereas inhibitors of NF-κB do not affect the ROS generation (data not shown). These phenomena imply that NADPH oxidase resides on the upstream of IKK-β, IκB-α and NF-κB. Therefore, SEC1 may act through the following mechanism to induce pyrogenic cytokine production in PBMC: SEC1 triggers the translocation of $p47^{phox}$ from cytoplasm to plasma membrane to activate NADPH oxidase for ROS production which then causes the phosphorylation of IKK-β and IκB-α, and thus activation of NF-κB.

Consensus DNA-binding motifs for NF-κB proteins exist in the promoters of immunologically relevant genes, such as the genes for IL-1β and IL-6 [45–47]. Cytokine-induced NF-κB complexes containing p50 and p65 subunits have also been demonstrated in many cell types [48,49]. In the present study, SEC1 induces the translocation of NF-κB which may bind to its target genes to trigger the production of IL-1β and IL-6 that is blocked by the NF-κB inhibitor (PDTC or SN-50). Moreover, the NF-κB binding ability is abrogated by binding site competition and antibody supershift analysis. These findings indicate that the stimulatory effect of SEC1 appears to require NF-κB. For the febrile response, two classes of cytokines have been reported: endogenous pyrogenic cytokines (IL-1 and IL-6) and endogenous antipyretic cytokines (IL-10 and tumor necrosis factor-α) [50]. In the present study, SEC1 stimulate the release of pyrogenic cytokines to trigger febrile responses in rabbits. Cytokines stimulated by NF-κB can also directly activate the NF-κB mechanisms and establish a positive autoregulatory loop to amplify the inflammatory reaction [51]. Recently, the production of IL-6 by dendritic cells has been reported [52]. Our parallel study also observed the formation of a large amount of IL-6 when dendritic cells were sorting from the PBMC and stimulated with SEC1 (C.-L. Su & S.-J. Won, unpublished results). The level of IL-6 in the supernatant fluids was increased from 66 $pg \cdot mL^{-1}$ in nontreated to 79372 $pg \cdot mL^{-1}$ in those treated with 1 $ng \cdot mL^{-1}$ of SEC1 for 48 h (C.-L. Su & S.-J. Won, unpublished results). These results suggest that dendritic cells may contribute in part to pyrogenic cytokine production of

PBMC. However, long-term exposure (approximately 10 days) of SEC1 to bovine PMBC has been reported to induce tolerance by increasing IL-10 and transforming growth factor-β, and decreasing IL-2 [53,54]. The major cell type for the IL-10 formation and Th2 shift has been characterized to be T regulatory cells ($CD8^+CD26^+$ or $CD4^+CD25^+$ in bovines; $CD4^+CD25^+$ in humans) [53–55].

NADPH oxidase contains a redox center which catalyzes superoxide formation by transferring electrons from NADPH onto oxygen molecules [56]. A deficiency of one *PHOX* subunit in NADPH oxidase leads to the inhibition of superoxide generation, and results in chronic granulomatous disease [57]. ROS derivatives of superoxide also mediate signaling transduction [22]. In nonphagocytic cells, such as fibroblasts, endothelial cells, vascular smooth muscle cells, cardiac myocytes and thyroid tissue, ROS are produced in one third of neutrophils in response to hormones or local metabolic changes [22]. ROS also amplify the immune response by enhancing the receptor signaling cascades of T cells [58]. In experimental systems, ROS increase IL-2 formation in antigenically or mitogenically stimulated T cells. In the present study, SEC1 induces translocation of $p47^{phox}$ from the cytoplasm to the cell membrane (Fig. 5A), ROS production (Table 3), pyrogenic cytokine formation (Table 1), and the febrile response (Fig. 1A and Table 2). The inhibitor of NADPH oxidase (apocynin) decreases ROS formation (Table 4), pyrogenic cytokines *in vitro* and febrile responses *in vivo* (Table 2). Taken together, the present study demonstrates that the bacterial enterotoxin SEC1 may activate NADPH oxidase in PBMC to produce ROS which may act though NF-κB to trigger the production of pyrogenic cytokine IL-1β and IL-6.

Experimental procedures

PBMC preparation

Human PBMC freshly collected buffy coat fraction of whole blood from healthy donors at the Tainan Blood Bank Center (Tainan City, Taiwan) were isolated by centrifugation over a Ficoll-Paque (Amersham Pharmacia, Uppsala, Sweden) density gradient at 400 ***g*** for 30 min at room temperature in a Sorvall RT6000B (Du Pont, DE, USA) [59]. The cells collected at the interface were washed thrice with serum-free RPMI-1640 (Gibco BRL, Grand Island, NY, USA) and subsequently resuspended in an AIM-V medium (Gibco BRL) containing 50 $\mu g \cdot mL^{-1}$ of gentamicin (Sigma Chemical Co., St Louis, MO, USA). For experiments, the indicated concentration of PBMC was incubated with the different concentrations of the tested agents in a 37 °C incubator. After incubation, the supernatants of PBMC were harvested by centrifugation at 800 ***g*** and stored at −80 °C before use.

Pyrogen assay

As described previously [59], adult male New Zealand White rabbits from the Animal Center of National Cheng Kung University (NCKU, Tainan, Taiwan) with body weight between 2.2 and 3.0 kg were housed individually at an ambient temperature of 22 ± 1 °C under a 12 : 12 h light/dark cycle (lights on 06.00 h). The pyrogen assay was carried out using unanaesthetized animals which were restrained in rabbit stocks. Animal feed and water were provided *ad libitum*. The colonic temperature [60] of each animal was measured every minute with a copper constantan thermocouple connected to a thermometer (HR1300, Yokogawa, Tokyo, Japan) during the experimental period between 09.00 and 20.00 h. Only animals with stable body temperatures in the range 38.6–39.0 °C were used to determine the effect of the tested agents. All animal experiments were approved by the Animal Research Committee of National Cheng Kung University, and were conducted under the guidelines of the National Research Council, Taiwan.

Reagents

All drug solutions were prepared in pyrogen-free glassware that was heated for 5 h before use. All solutions were passed through 0.22 μm filters (Millipore, Bedford, MA, USA). Sterile SEC1 (Toxin Technology, Sarasota, FL, USA) was made up in normal saline solution. The SEC1 used in this study contained $\leq 25\ pg \cdot mL^{-1}$ endotoxin because none of the SEC1 solutions induced gelation in the Limulus amebocyte lysate (Gibco BRL) assay. Chemicals were obtained from Sigma Chemical Co. unless otherwise indicated. SN-50, HIMO and MK 886 were purchased from Calbiochem (San Diego, CA, USA). Polymycin B was obtained from Merck (Darmstadt, Germany). Apocynin was purchased from Fluka (Riedel-de Haen, Germany). SN-50 and PDTC were dissolved in distilled water. Wort, HIMO, MK 886 and ETYA were dissolved in dimethylsulfoxide. Apocynin was dissolved in ethanol. Polymycin B was dissolved in normal saline. Before use, all dissolved chemicals were diluted with AIM-V medium to yield the final desired experimental concentrations.

Primary antibodies including mouse monoclonal NF-κB p65, rabbit polyclonal NF-κB p65, goat and rabbit polyclonal NF-κB p50, and rabbit polyclonal $p47^{phox}$ were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal IκB-α, phospho-IκB-α, IKK-β, and phospho-IKK-α/IKK-β were purchased from Cell Signaling (Beverly, MA, USA). Human monoclonal IL-1β and IL-6 antibodies were obtained from R&D Systems (Minneapolis, MN, USA).

Cytokine secretion assay

Human PBMC (1×10^7 cells·mL^{-1}) were incubated with SEC1 alone or cocultured with the tested inhibitors. After incubation, the collected supernatants were stored at −80 °C and later used for cytokine analysis. The concentrations of IL-1β and IL-6 in the SEC1-stimulated PBMC supernatants were determined by human Colorimetric Sandwich ELISA kits (R&D Systems). The specific activity of IL-1β and IL-6 was 1.3×10^8 U·mg^{-1} and 1×10^6 U·mL^{-1}, respectively.

Preparations of whole cell lysates and nuclear fractions

Human PBMC (1×10^7 cells·mL^{-1}) were treated with or without the tested agents. The protein extraction was performed as previously described [61]. Briefly, the whole cells were lysed with 200 μL lysis buffer containing 1 mM EDTA, 10 mM Tris/HCl, pH 7.4, 0.5% (w/v) SDS, 0.15 M NaCl, 1 mM EGTA, 5 μg·mL^{-1} aprotinin, 2 mM sodium orthovanadate, 5 μg·mL^{-1} leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, and 1% (v/v) Triton X-100 at 4 °C for 35 min. The mixture was centrifuged at 15 000 ***g*** for 10 min, and the resulting supernatant was used as the whole cell lysate for immunoblotting.

Nuclear fractions were prepared as previously described [62]. Agent-treated PBMC (1×10^7 cells·mL^{-1}) were isolated by centrifugation and washed twice with ice-cold NaCl/Pi. The PBMC were then lysed in 400 μL of buffer A (10 mM Hepes, pH 7.9, 3 mM sodium orthovanadate, 5 mM $MgC1_2$, 10 mM KC1, 10 mM NaF, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol and 2 μg·mL^{-1} each of aprotinin, leupeptin, antipain, and pepstatin A), and incubated on ice for 20 min. The nuclear fractions were isolated by centrifugation at 11 000 ***g*** at 4 °C for 10 s. The obtained nuclear pellets were resuspended in 60 μL of buffer B (1.5 mM $MgC1_2$, 420 mM NaCl, 20 mM Hepes, pH 7.9, 0.2 mM EDTA, 10 mM NaF, 25% glycerol, 1 mM sodium orthovanadate, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride and 1 μg·mL^{-1} each of antipain, leupeptin, aprotinin, and pepstatin A) and then incubated for 20 min on ice with occasional mixing. The nuclear debris was removed by centrifugation at 12 000 ***g*** for 16 min at 4 °C. The nuclear protein extracts were used for further assay.

Preparations of cytosolic and membrane fractions

Plasma membranes were enriched by differential centrifugation as described previously [63]. Human PBMC (1×10^7 cells·mL^{-1}) treated with or without the tested agents were washed with NaCl/Pi and scraped with a rubber policeman into an ice-cold lysis buffer (50 mM Tris-HC1, pH 8, 4 mM EDTA, pH 8, 2 mM EGTA 0.05 mM phenylmethylsulfonyl fluoride and 20 μg·mL^{-1} leupeptin). After sitting on ice for 30 min, the mixture was transferred to a Dounce homogenizer. The cells were broken with ten strokes of a pestle. The homogenate was centrifuged at 650 ***g*** for 5 min to remove unbroken cells and nuclei. After centrifugation at 150 000 ***g*** for 45 min, the obtained supernatant was used as the cytosolic fraction. The pellet was resuspended in lysis buffer containing 0.5% Triton X-100 and then sat on ice for 50 min. After centrifugation at 150 000 ***g*** for another 30 min, the resulting supernatant was used as the membrane fraction.

Immunoblotting

The protein contents of the whole cell, cytosolic, plasma membrane and nuclear fractions were determined by a protein assay kit (Bio-Rad, Hercules, CA, USA). All isolated proteins were stored at −80 °C before use. The proteins were resolved using 10–12% SDS/PAGE with a running buffer (25 mM Tris, 192 mM glycine, 3.5 mM SDS, pH 8.3) and subsequently transferred to polyvinylidene fluoride membranes (Millipore) as described previously [64]. The membranes were blocked by incubation in NaCl/TrisT (20 mM Tris, 137 mM NaCl, 0.05% Tween-20, pH 7.4) containing 5% skim milk for 2 h at room temperature. The membrane was then probed with an appropriate first antibody. A secondary probe with horseradish peroxidase-labeled goat antimouse (1 : 5000) or goat antirabbit (1 : 5000) IgG was visualized by exposing to X-ray film after staining with chemiluminescence reagents.

Electrophoretic mobility shift assay (EMSA)

The EMSA of the nuclear extracts was performed as described previously [65]. The probe consisting of a double-stranded oligonucleotide with the consensus binding sequence for NF-κB (5′-AGTTGAGGGGACTTTCCCAG GC-3′) (Promega, Madison, WI, USA) was 3′ end-labeled with digoxigenin-ddUTP using a digoxigenin gel shift kit (Roche Molecular Biochemicals, Mannheim, Germany). The binding reaction was carried out for 30 min at 37 °C according to the manufacturer's protocol for experiments (Roche, Molecular Biochemicals). The specificity of the protein–DNA complexes was proven by immunoreactivity with goat or rabbit polyclonal antibody specific for p65 or p50 (Santa Cruz Biotechnology) of NF-κB. To further demonstrate the specificity, a competition assay was conducted by adding a 100-fold excess of the unlabeled oligonucleotides or unlabeled mutant NF-κB oligonucleotides (5′-AG TTGAGGCGACTTTCCCAGGC-3′; Santa Cruz Biotechnology) to the nuclear extracts. The NF-κB-unrelated oligonucleotide probe control, AP-1 binding site (5′-CGCT TGATGAGTCAGCCGGAA-3′), was purchased from Promega. The gels were transferred to Hybond-N plus

membrane (Amersham Biosciences, Little Chalfont, UK), dried and subjected to autoradiography.

Determination of ROS

ROS, including superoxide radical and H_2O_2, were determined using a lucigenin-enhanced chemiluminescence method as described previously [66,67]. Briefly, human PBMC (5×10^5 cells·mL^{-1}) in Hanks' balanced salt solution (HBSS; pH 7.4, 1.25 mM $CaCl_2$) were treated with or without SEC1, or incubated with or without the tested agents [68]. After the indicated time periods, the sample was prepared by adding 0.2 mL of the cell culture supernatants to 0.1 mL HBSS in the dark and mixed using the Chemiluminescence Analyzing System (TLU-21, Tohoku Electronic Industrial Co., Sendai, Japan). The photon emission from the sample was measured every 10 s at 37 °C. After 200 s, the samples were measured continuously for 12 min after injecting 1 mL of 10 μM lucigenin (Sigma Chemical Co.) in HBSS into the stainless cell of the system. The area under the curve was calculated to obtain total chemiluminescence.

Statistical analysis

The animals were maintained at an ambient temperature of 22 °C for at least 90 min to obtain the thermal balance before any tested agent was injected. The temperature responses were assessed as changes from the preinjection values. The experimental results are presented as mean ± SEM for multiple experiments. The results were compared by one-way analysis of variance using the MINITAB (version 10.2) software package (Minitab Inc., State College, PA, USA). $P < 0.05$ was considered statistically significant. The maximum elevation of the colonic temperature over the preinjection value (Δt_{co}) and the fever index, given by the area under the curve over the 1-h period after the injection of the tested agents, were calculated in terms of °C per 1 h [69].

Acknowledgements

This study was supported by grants from the National Science Council, Taiwan, Republic of China (NSC 89-2320-B-006-113, NSC 90-2314-B-006-123, NSC 93-2320-B-309-002 and NSC 95-2313-B-309-001).

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