

STAPHYLOCOCCAL ENTEROTOXIN A MODULATES INTRACELLULAR Ca^{2+} SIGNAL PATHWAY IN HUMAN INTESTINAL EPITHELIAL CELLS

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Abstract To clarify whether SEA can affect any changes of cellular signalling pathways in intestinal epithelial cells, we performed experiments to see if SEA can modulate the intracellular signaling pathway in intestinal epithelial cells. We demonstrate here that SEA induces an increase in intracellular calcium ($[\text{Ca}^{2+}]_i$) in human intestinal epithelial cells and the $[\text{Ca}^{2+}]_i$ is released from intracellular stores. SEA-induced increase of $[\text{Ca}^{2+}]_i$ was clearly inhibited by treatment with a nitric oxide synthase (NOS) inhibitor, N^G -monomethyl-L-arginine. Intestinal epithelial cells express endothelial NOS (eNOS) in resting cell condition, and express inducible NOS (iNOS) after stimulating with tumor necrosis factor (TNF)- α . TNF- α -pretreated cells showed a significant increase in $[\text{Ca}^{2+}]_i$ that was also inhibited by the NOS inhibitor. These results demonstrate that SEA induces an increase in intracellular Ca^{2+} concentration in human intestinal epithelial cells, and the expressions of both eNOS and iNOS could be responsible for the increase in intracellular Ca^{2+} concentration in the cells induced by SEA. It is important to study the relationship of NOS expression, Ca^{2+} signal pathway and emesis for understanding the mechanism of SEA-induced food poisoning.

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Staphylococcal enterotoxins (SEs) cause staphylococcal food poisoning and toxic shock syndrome in humans and other species^{1,2)}. Over the past few decades, several studies have been conducted on the nature of SEs and the molecular basis of the superantigen activities of SEs has been extensively studied^{3,4)}. However, the pathogenesis of SEs-mediated food poisoning, which causes diarrhea and vomiting within about 4-6 h of ingesting contaminated food, is not clearly understood⁵⁾. Little is known about how the toxins enter the body via the intestine and cause food poisoning, and relationship between the superantigenic and emetic activities. Previous studies have shown that SEA did not behave as a bacterial cytotoxin to intestinal

epithelial cells in morphologic feature⁶⁾. However, the enterotoxin could cross the intestinal epithelium in immunologically intact form⁵⁾ and participate in the initiation, exaggeration or reactivation of enteric inflammatory disease^{7,8)}. Superantigen-mediated activation of the immune system affects the form and function of the gut, and suggested that these bacterial products can initiate or exacerbate a secretory or inflammatory enteropathy^{4,9)}. The question remains whether exposure to SEs has any significance for human gut dysfunction or disease. In the present study, we performed experiments to see if SEA can modulate the intracellular signaling pathway in intestinal epithelial cells. Our results demonstrated for the first time that SEA could

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induce an increase in intracellular calcium ($[Ca^{2+}]_i$) in intestinal epithelial cells, and the increase is originated from intracellular calcium stores. We further investigated the involvement of nitric oxide synthase (NOS) in modifying calcium transients, and suggested that the increase in $[Ca^{2+}]_i$ evoked by SEA is dependent on NOS expression.

Recombinant SEA expressed by the *Escherichia coli*¹⁾ was used in this study. The purity of recombinant SEA was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The concentration of purified recombinant SEA was determined by the method of Bradford assay (Bio-Rad Laboratories) at 595 nm, and the purified SEA was stored at -20 °C until to use.

Henle-407, a human intestinal epithelial cell line, was maintained in RPMI 1640 medium (Nissui Pharmaceutical) supplemented with 10% heat-inactivated fetal calf serum, 1 μ M L-glutamine, 100 U/ml penicillin G and 100 μ g/ml streptomycin. Cells were cultured in disposable tissue culture flasks or 24-well tissue culture plates in a humidified 5% CO₂ atmosphere at 37°C. For measurement of $[Ca^{2+}]_i$ concentration, the cells were cultured on poly-L-lysine coated glass cover slips (Matsunami Glass) in 35-mm diameter cell culture dishes.

Fura-2 acetoxymethylester (Fura-2/AM) (Dojindo, Kunamoto) and fluorometric microscopy system were used to measure $[Ca^{2+}]_i$. Henle 407 cells, grown on poly-L-lysine-coated glass cover slips, were washed once with RPMI 1640 medium and then incubated for 45 min at 37 °C in RPMI 1640 medium containing 0.5 mg/ml bovine serum albumin (BSA, fraction V, Sigma Chemical Co.) and 1.5 μ M Fura-2/AM as described previously¹⁰⁾. Fura-2/AM loaded cells were washed twice with PBS and then placed in a perfusion chamber connected to an inverted epifluorescence microscope (Zeiss, Axiovert 135). Upon assay, the cells were exposed to a perfusion of purified SEA. SEA was diluted 1:10 (final concentration

of 4 μ M) in PBS with or without 1.5 mM Ca^{2+} . One single cell, isolated optically by the microscope, was analyzed by using a 100 \times /1.3 NA oil-immersion objective. Each experiment was repeated at three to five independent occasions. The microscope was connected to an F-4500 fluorescence spectrophotometer (Hitachi, Ltd) for dual wavelength excitation fluorimetry. Upon binding to Ca^{2+} , fura-2 shifts excitation maximum from 380 nm to 340 nm. The ratio between the fluorescence intensity at 340 and 380 nm (F340/380) gives a value of free Ca^{2+} in the cytosol. The background fluorescence was measured and subtracted before calculation of $[Ca^{2+}]_i$.

Human intestinal cells cultured on glass cover slips were loaded with Fura-2/AM. After stabilization of the baseline signal with PBS containing 1.5 mM Ca^{2+} , 8, 4, 1 and 0.4 μ M SEA in PBS containing 1.5 mM Ca^{2+} was introduced to the cell monolayer with a flow rate of 0.5 ml/min and fluorescence intensity of Fura-2/AM in a single cell was determined on fluorescence spectrophotometer. SEA-induced significant increase in $[Ca^{2+}]_i$ in the Henle 407 cells was dose-dependent, and application of SEA resulted in calcium peak and then returned to the baseline (Figure 1A, B). Eighty-five percent of analyzed cells (17/20 cells) at the concentration of 4 μ M SEA and 79.2% (19/24) of the cells at 0.4 μ M SEA showed responses. However, 4 μ M GST in 1.5 mM Ca^{2+} -containing PBS did not induce an increase in $[Ca^{2+}]_i$ in the cells (0/21 cells) (Figure 1C). Furthermore, the increase in $[Ca^{2+}]_i$ in the cells was inhibited by anti-SEA antibody (Figure 1D). These results indicated that SEA could trigger transient increase of intracellular calcium concentration in Henle 407 cells.

To confirm that the $[Ca^{2+}]_i$ transient in the cells was mediated by open channels in the plasma membrane or by release of Ca^{2+} from intracellular stores, Fura-2/AM loaded cells were

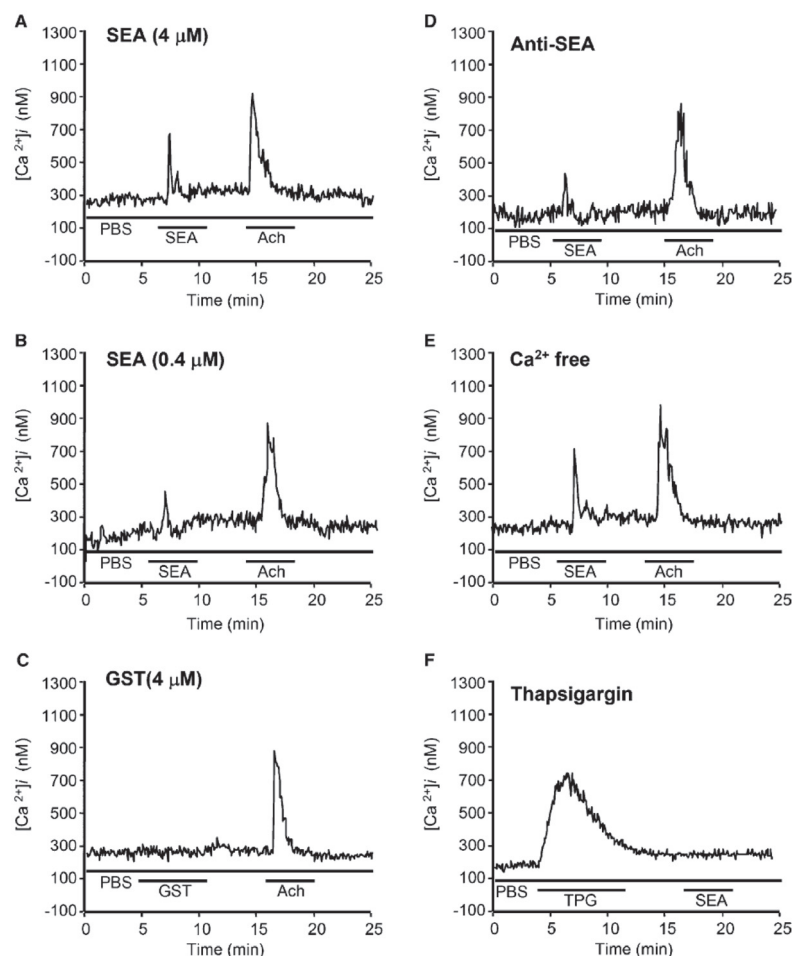


Figure 1 SEA induces an increase in cytosolic calcium in human intestinal epithelial cells. (A, B) Henle 407 cells were loaded with Fura-2/AM, washed, and placed in PBS. SEA and Ach were added to the monolayer at the indicated time (for 5 min) for evaluate the Ca^{2+} capacity of the cells. Data are representative of 3 identical experiments of total 20 (A) and 24 (B) cells. For each identical experiment, 5-10 cells were analyzed. (C) Fura-2/AM-loaded cells were added 4 μM GST at the indicated time as controls. Data are representative of 3 identical experiments of total 21 cells. For each identical experiment, 6-8 cells were analyzed. (D) Fura-2/AM-loaded cells were added 4 μM SEA that has been incubated with anti-SEA IgG (1 mg/ml) for 1 h in 37 $^{\circ}\text{C}$. Data are representative of 3 identical experiments of total 24 cells. (E) Fura-2/AM loaded cells were washed, and placed in Ca^{2+} -free PBS and added 4 μM SEA. Data are representative of 3 identical experiments of total 25 cells. (F) Fura-2/AM-loaded cells were added 2 μM thapsigargin for 10 min and then added 4 μM SEA at the indicated time. Ach was used for confirming the cell conditions and as a control to raise intracellular Ca^{2+} . Data are representative of 3 identical experiments of total 22 cells.

washed and placed in the Ca^{2+} -free PBS, and then exposed to SEA in Ca^{2+} -free condition. The fluorescence intensity of Fura-2/AM in a single cell was determined in the condition without extracellular calcium. Application of SEA also resulted in $[\text{Ca}^{2+}]_i$ peak and then returned to the baseline (Figure 1E). Furthermore, emptying the ER intracellular stores with thapsigargin (2

μM) showed the absence of the signal (Figure 1F). These results indicated that SEA-induced increase $[\text{Ca}^{2+}]_i$ is originated from intracellular calcium stores.

Acetylcholine chloride (Ach, Wako Pure Chemicals), adenosine-3',5'-cyclic monophosphorothioate, triethyl ammonium salt (Rp-cAMP; BIOLOG), N^G -monomethyl-L-arginine

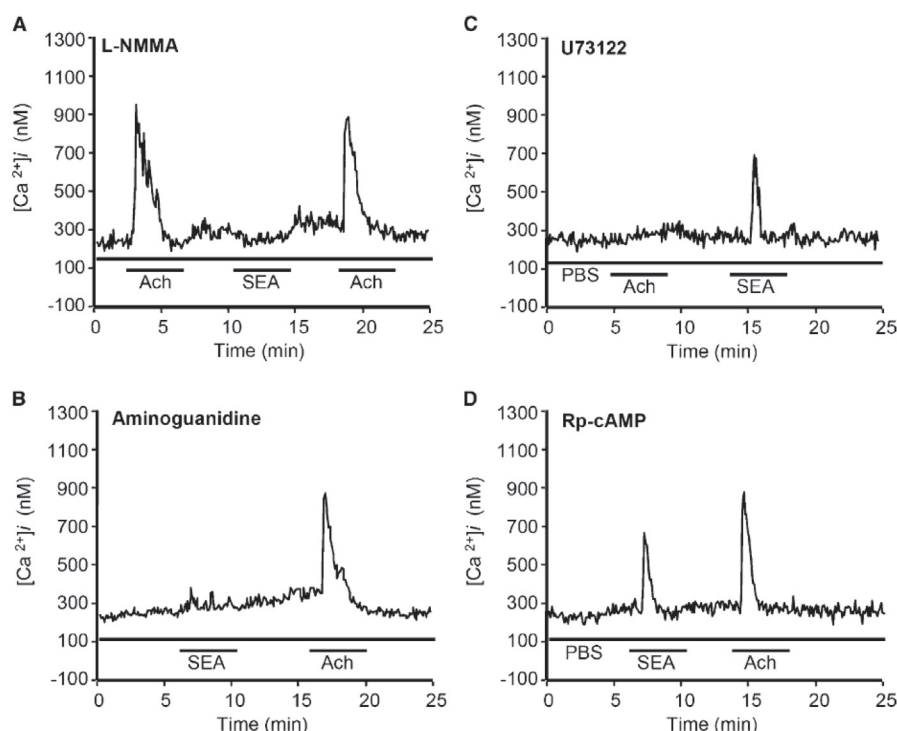


Figure 2 L-NMMA, but not U73122 or Rp-cAMP, inhibited SEA-induced $[Ca^{2+}]_i$ elevation in intestinal epithelial cells. (A) Henle 407 cells were loaded with $1.5 \mu M$ Fura-2/AM washed, and placed in Ca^{2+} -free PBS. After stabilization of the baseline signal for 300 s with PBS containing 10 mM L-NMMA, 10 μM Ach or 4 μM SEA in the presence 10 mM L-NMMA was added to the monolayer to evaluate the Ca^{2+} capacity of the cells at the indicated time (for 5 min). Data are representative of 5 identical experiments of total 32 cells. (B) Henle 407 cells were loaded with $1.5 \mu M$ Fura-2/AM washed and placed in Ca^{2+} -free PBS. After stabilization of the baseline signal with PBS containing 0.1 mM aminoguanidine, 4 μM SEA or 10 μM Ach in the presence 0.1 mM aminoguanidine was added to the monolayer to evaluate the Ca^{2+} capacity of the cells at the indicated time (for 5 min). Data are representative of 2 identical experiments of total 14 cells. (C) 4 μM SEA or 10 μM Ach in the presence of 10 mM U73122 was added to the monolayer to evaluate the Ca^{2+} capacity of Henle 407 cells at the indicated time. Data are representative of 3 identical experiments of total 25 cells. (D) Henle 407 Cells were loaded with $1.5 \mu M$ Fura-2/AM and incubated with 10 μM Rp-cAMP for 30 min, washed, and placed in Ca^{2+} -free PBS. 4 μM SEA or 10 μM Ach was added to the monolayer at the indicated time. Data are representative of 3 identical experiments of total 24 cells.

(L-NMMA) (Wako), and U73122 (Wako) were used to treat or stimulate the cells in inhibition experiments. Human intestinal cells were loaded with Fura-2/AM and stabilized the base level signal with PBS containing 10 mM L-NMMA. 4 μM SEA and 10 μM Ach were added to the monolayers to evaluate the Ca^{2+} capacity of the cells, respectively. L-NMMA clearly inhibited SEA-induced in an increase $[Ca^{2+}]_i$ (Figure 2A). In contrast, L-NMMA did not affect the increase in $[Ca^{2+}]_i$ induced by Ach which raises $[Ca^{2+}]_i$ through phospholipase C signaling pathway. Next, we examined other inhibitors for an increase in

$[Ca^{2+}]_i$, Rp-cAMP (a protein kinase A inhibitor) and U73122 (a phospholipase C inhibitor). None of them inhibited the increase in $[Ca^{2+}]_i$ during SEA administration (Figure 2C, D). Furthermore, to confirm the effect of NOS, another NOS inhibitor, aminoguanidine (0.1 mM), was used for the blocking experiments. Aminoguanidine also significantly inhibited SEA-induced in an increase $[Ca^{2+}]_i$ (Figure 2B). These results suggested that SEA-induced calcium increase may be the result of NO and NOS expression.

To investigate whether the NOS expression is involved in the increase in $[Ca^{2+}]_i$ induced

with SEA in human intestinal epithelial cells, mRNA expressions of eNOS and iNOS in resting cells and the cells activated with human tumor necrosis factor- α (TNF- α) and interferon- γ (IFN- γ) were determined by RT-PCR. Henle-407 cells were cultured in 24-well culture plates at a density of 1×10^5 cells per well for 24 h and then stimulated with or without SEA, TNF- α (Dainippon Pharmaceutical) or IFN- γ (Peppo Tech.). The cells were harvested after indicated periods of incubation and total RNA was extracted with a nucleic acid purification kit, MagExtractor RNA (Toyobo). First-strand cDNA was generated in 20 μ l reverse transcription mixture containing 1 μ g total RNA using random primers (Takara) and reverse transcriptase M-MLV (Invitrogen). Quantitative real-time PCR was performed using following primer sets: eNOS primers (sense, 5'-CAGTGTCCAACATGCTGCTGGAAATTG-3'; anti-sense, 5'-TAAAGGTCTTCTTCCTGGTGATGCC-3'), iNOS primers (sense, 5'-GCCTCGCTCTGGAAAGA-3'; anti-sense, 5'-TCCATGCAGACAACCTT-3') and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers (sense, 5'-TGAAGGTCGGTGTGAACGGATTTGG-3'; anti-sense, 5'-ACGACATACTCAGCACCAGCATCAC-3'). Two microliters of the RT product, 0.2 μ M of each primer and iQ SYBER Green Supermix (Bio-Rad) which includes AmpliTaq gold DNA polymerase, SYBER Green I Dye, dNTPs with dUTP were included in the 20 μ l reaction mixture for real-time RT-PCR. Thirty-five PCR cycles of amplification were performed. Each cycle consisted of a 30 s (for eNOS and GAPDH) or 60-s (for iNOS) at 94 $^{\circ}$ C for denaturing, 30 s at 55 $^{\circ}$ C (eNOS and GAPDH) or 57 $^{\circ}$ C (iNOS) for annealing, and 120 s at 72 $^{\circ}$ C for extension. Henle 407 cells in resting cell condition expressed eNOS, but did not express iNOS. After being stimulated with TNF- α or IFN- γ , the cells expressed iNOS (Figure 3). These results indicated that Henle 407 cell could express eNOS in resting cells and

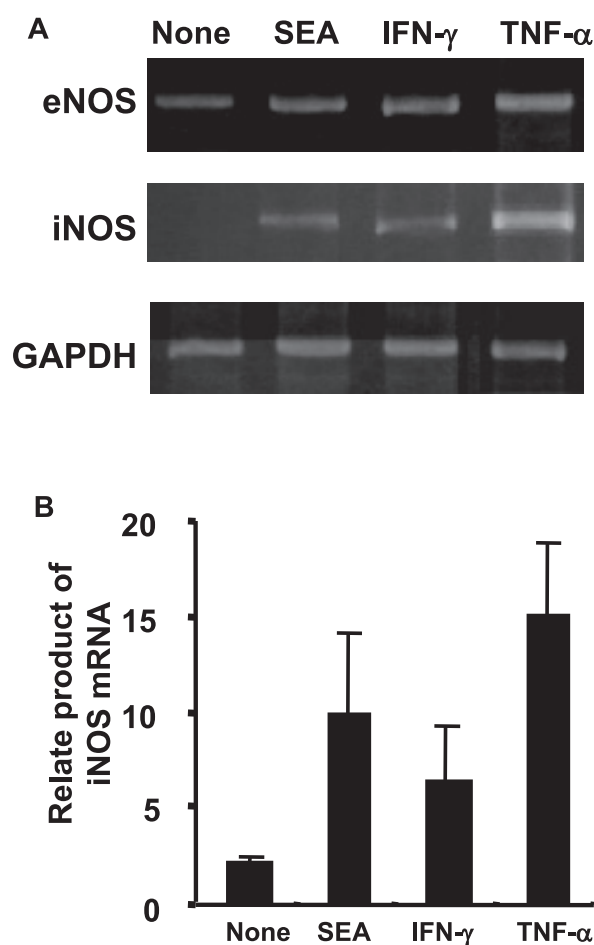


Figure 3 Expression of eNOS and iNOS mRNA in resting cells and cells pre-stimulated with IFN- γ and TNF- α was determined by RT-PCR (A) and quantitative real-time RT-PCR (B).

express both eNOS and iNOS in the cells pre-stimulated with TNF- α and IFN- γ .

Furthermore, Henle 407 cells were incubated with TNF- α for 6 h before the determination of the $[Ca^{2+}]_i$ response. The cells pretreated with TNF- α showed the significant higher increase in $[Ca^{2+}]_i$ in comparison with the cells that were not pretreated (Figure 4A and B). In addition, the NOS inhibitor, L-NMMA, also clearly inhibited SEA-induced increase in $[Ca^{2+}]_i$ in the cells pretreated with TNF- α (Figure 4C). These results indicated that SEA-induced $[Ca^{2+}]_i$ increase in pretreated cells might be the result of the expression of both eNOS and iNOS.

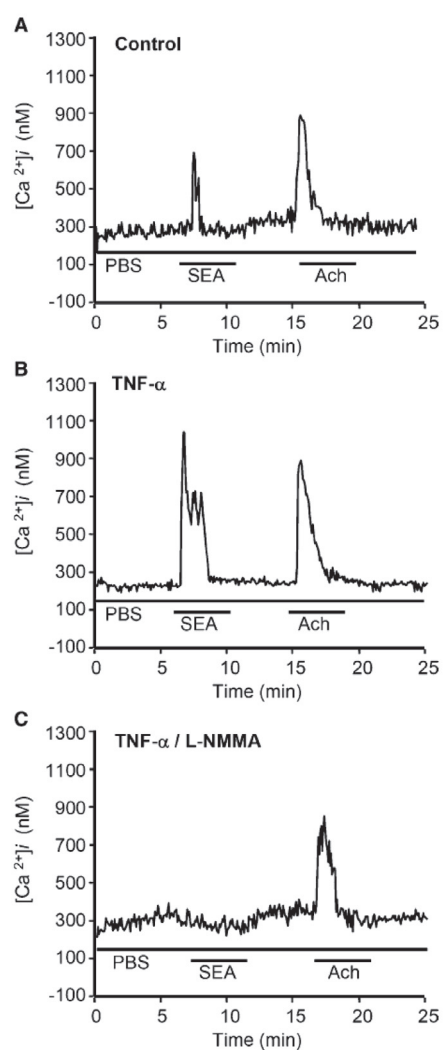


Figure 4 Enhanced response of $[Ca^{2+}]_i$ increase in intestinal epithelial cells after treatment with $TNF-\alpha$. Cells were cultured without (A) or with $TNF-\alpha$ (B, C) for 6 h, and then were loaded with Fura-2/AM. Four μM SEA was added to the monolayer in the absence (A, B) or the presence (C) of 10 mM L-NMMA to evaluate the Ca^{2+} capacity at the indicated time. Fluorescence intensity of Fura-2/AM in a single cell was determined as Figure 1 legend. Data are representative of 3 identical experiments of total 21 (A), 24 (B) and 18 (C) cells.

SEA is a major cause of symptoms of food poisoning in humans^{11,12}. Despite their molecular characteristics and superantigenic activities have been elucidated, relatively little is known about the mechanism how the toxins can induce the symptoms of food poisoning, which in turn hampers the design of protective measures and

antitoxic drugs. Oral administration of SEA has not been shown to cause gastrointestinal mucous membrane damage such as edema, cytolysis, tissue sloughing, luminal necrotic tissue or alternations in epithelial mitotic morphology¹³. These made it difficult to study mechanism of SEA-induced food poisoning that results in vomiting and diarrhea. Recently, use of an in vitro coculture model comprising monolayers of the T84 epithelial cell line and peripheral blood mononuclear cells showed that the SE-induced activation of immune cells resulted in increased epithelial permeability, reduced responses to pro-secretory stimuli and increased production of the T cell and monocyte chemoattractants^{7,9,14}. Hamad *et al.*⁵ have shown that SEA and SEB can cross the epithelium in an immunologically intact form, in some cases by a facilitated mechanism. These studies suggest that SEA may directly affect the function of intestinal epithelial cells. In the present study, our data showed that SEA increases $[Ca^{2+}]_i$ concentration in the human intestinal epithelial cells. These results indicated that SEA has a direct effect on the cellular signaling pathway of intestinal epithelial cells.

$[Ca^{2+}]_i$ is an important regulator of many physiological processes and pathological responses¹⁵⁻¹⁸. It has been reported that high storages of Ca^{2+} are kept in the endoplasmic reticulum and are released upon signals and/or receptor activation in resting epithelial cell¹⁹. Previous studies have shown that alterations of Ca^{2+} store site-related Ca^{2+} mobilizations are involved in the cellular mechanisms of endothelial migration²⁰, and that Ca^{2+} homeostasis plays a significant role in regulating endothelial functions including angiogenesis²¹. Inhibition of Ca^{2+} pathways suppressed endothelial growth, adhesion, migration and in vitro tube formation²¹. In the present study, the results showed that SEA, a bacterial exotoxin protein, induces an increase in $[Ca^{2+}]_i$ concentration in human intestinal epithelial cells, suggesting that this event may be important

for studying the mechanism how SEA induces food poisoning.

Our results showed that NOS inhibitors significantly reduced the SEA-induced $[Ca^{2+}]_i$ increase. Release of Ca^{2+} from intracellular stores by NO is consistent with similar findings reported for other cell types including endothelial cells²²⁾, rat pancreatic β -cells²³⁾ and interstitial cells²⁴⁾, and with the ability of ryanodine to block NO-induced Ca^{2+} release^{23,25)}. Watson et al.²⁶⁾ have shown that arachidonic acid induced Ca^{2+} release was inhibited by the NOS inhibitor, 7-nitroindazole. NO is an intracellular and intercellular messenger with important functions in a number of physiologic and pathobiologic process within gastroenterology including gastrointestinal tract motility, mucosal function, inflammatory responses, gastrointestinal malignancy and blood flow regulation²⁷⁾.

In conclusion, this study demonstrated for the first time that SEA could increase Ca^{2+} signal in human intestinal epithelial cells. The increase in cytosolic Ca^{2+} occurred after the cells were exposed to SEA, and the increase in Ca^{2+} is originated from intracellular stores. The SEA-induced $[Ca^{2+}]_i$ increase was significantly reduced by a NOS inhibitor, L-NMMA. In addition, the intestinal epithelial cells could express eNOS in resting cells and express iNOS after being stimulated with TNF- α or IFN- γ as well as SEA. The cells stimulated with TNF- α showed the stronger increase in a Ca^{2+} signal. These results indicated that NOS expression is involved in the $[Ca^{2+}]_i$ increase evoked by SEA. It is important to study the relationship of NOS expression, $[Ca^{2+}]_i$ signal pathway and emesis for understanding the mechanism of SEA-induced food poisoning.

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