

CEL-I, an *N*-Acetylgalactosamine (GalNAc)-Specific C-Type Lectin, Induces Nitric Oxide (NO) Production in RAW264.7 Mouse Macrophage Cell Line

Tomohiro Yamanishi¹, Tomomitsu Hatakeyama², Kenichi Yamaguchi¹, and Tatsuya Oda^{1*}

¹Division of Biochemistry, Faculty of Fisheries, and ²Department of Applied Chemistry, Faculty of Engineering, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852-8521

Running title: Induction of NO Production by C-type Lectin CEL-I

Field: Biochemistry

Topic: Biochemistry General

*To whom correspondence should be addressed.

Fax: +81-95-819-2831, E-mail: t-oda@nagasaki-u.ac.jp

SUMMARY

We found that CEL-I, a GalNAc-specific C-type lectin isolated from the marine invertebrate Holothuroidea (*Cucumaria echinata*), induces inducible nitric oxide synthase (iNOS) expression and NO production in RAW264.7 cells. The NO production was inhibited by an iNOS inhibitor, L-NAME, but was not by a lipopolysaccharide (LPS) inhibitor, polymyxin B. In the presence of 0.1 M GalNAc, increased NO production by CEL-I-treated RAW264.7 cells was observed rather than the inhibition. Bovine serum albumin (BSA) significantly inhibited the CEL-I-induced NO production as well as the binding of FITC-labeled CEL-I on RAW264.7 cells. Three MAP kinase inhibitors (specific to extracellular regulated kinase, c-jun NH₂-terminal kinase, and p38 MAP kinase) inhibited CEL-I-induced NO production with different extents. Heat-treatment of CEL-I resulted in a decreased activity of CEL-I depending on the temperature. These results suggest that CEL-I induces NO production in RAW264.7 cells through the protein-cell interaction rather than the binding to the specific carbohydrate chains on the cell surface.

Key words: CEL-I, C-type lectin, *Cucumaria echinata*, inducible nitric oxide synthase (iNOS), nitric oxide (NO).

Abbreviations: C-type lectin, Ca²⁺-dependent lectin; DMEM, Dulbecco's modified Eagle's minimal essential medium; ERK, extracellular regulated kinase; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; F-CEL-I, FITC-labeled CEL-I; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; G-CSF, granulocyte colony-stimulating factor; IFN- γ , interferon- γ ; iNOS, inducible nitric oxide synthase; JNK, c-jun NH₂-terminal kinase; L-NAME, NG-nitro-L-arginine methyl ester; LPS,

lipopolysaccharide; MAP kinase, mitogen-activated protein kinase; NO, nitric oxide; PBS, phosphate-buffered saline; PHA-L, phytohemagglutinin-L (*Phaseolus vulgaris* agglutinin); TNF, tumor necrosis factor; WGA, wheat germ agglutinin.

INTRODUCTION

Lectins are a group of proteins or glycoproteins that are capable of specific recognition and reversible binding to carbohydrate moiety of glycoproteins or glycolipids on cell surface. Thus, lectins are not only interesting bioactive molecules but also useful tools in studies of cell surface, oligosaccharide, and glycoconjugate structures (1).

In addition to huge numbers of plant lectins, a few lectins are also found in animals and microorganisms, and especially animal lectins have been studied along with their physiological functions (2, 3). Ca^{2+} -dependent (C-type) and independent (galectin) lectins are known as major classes of animal lectins (4-6). The C-type animal lectins have characteristic carbohydrate-recognition domain (CRD) consisting of 120-130 amino acids residues, which exhibits some degree of sequence homology between species. Several C-type lectins have been found in marine invertebrates (7-10). Hatakeyama *et al.* have isolated four Ca^{2+} -dependent galactose/GalNAc-specific lectins (CEL-I, II, III, and IV) from the marine invertebrate *Cucumaria echinata* (Holothuroidea) (11). Interestingly, it has been demonstrated that one of these lectins, CEL-III, is a novel Ca^{2+} -dependent lectin that exhibits potent hemolytic activity and cytotoxicity, and membrane damage through the formation of ion-permeable pores in the plasma membrane is proposed to be the underlying cytotoxic and hemolytic mechanisms of CEL-III (12-14). On the other hand, CEL-I is the smallest Ca^{2+} -dependent lectin in *C. echinata* which is composed of two identical subunits of 16 kDa linked by a single disulfide bond. CEL-I shows very high specificity for GalNAc, and the binding affinity of CEL-I for GalNAc is estimated to be approximately 1000-fold higher than that for galactose (11, 15). Furthermore, previous study has demonstrated that CEL-I is highly cytotoxic to MDCK, HeLa, and XC cells, whereas CHO, L929, and RAW264.7 cells were relatively resistant (16, 17). Since neither

hemolytic activity nor a pore-forming property of CEL-I have been found so far, the cytotoxic mechanism of CEL-I seems to be different from that of CEL-III. Based on the dramatic cellular morphological change accompanied with an increase in membrane permeability in CEL-I-treated MDCK cells, it is considered that CEL-I may cause the profound change in the plasma membrane structure which may partly responsible for the cytotoxicity (17).

In addition to the potent cytotoxicity, recent studies have demonstrated that CEL-I induces the secretion of TNF- α and G-CSF from mouse macrophage cell line RAW264.7 in a dose-dependent manner (18). For the cytokine-inducing activity of CEL-I, the specific cell surface binding or plasma membrane attack seems to be important rather than endocytotic internalization and subsequent vesicle trafficking through the Golgi complex. The involvement of MAP kinase system in the process leading to cytokine secretion was also suggested (18).

It has been reported that some lectins such as Con A, PHA (19), and mistletoe lectin (20) induce nitric oxide (NO) production in macrophages as well as cytokine secretion. NO, a gaseous free radical, is known to be one of the most important effector molecules of innate immune cells. NO is synthesized by three different classes of NO synthase: inducible NOS (iNOS), endothelial NOS (eNOS), and neuronal NOS (nNOS) which are involved in vasodilatation, inflammation, and immune responses (21-23). In macrophages, NO production is induced after inflammatory stimulation and subsequent expression of iNOS (24, 25). It is well documented that a stimulatory pathway in macrophages is initiated by binding of a bacterial cell wall component (lipopolysaccharide, LPS) to the CD14-toll-like receptor that triggers a complex kinase cascade and eventually leads to gene activation and subsequent expression of iNOS (26).

In this study, we investigated whether or not CEL-I can induce NO production in RAW264.7 cells. The results indicated that CEL-I was able to induce NO production through the activation of the iNOS gene expression.

MATERIALS AND METHODS

Materials – CEL-I was purified from an aqueous extract of *C. echinata* by means of column chromatography on Lactosyl-Cellulofine, GalNAc-Cellulofine, and Sephadex G-75, essentially as reported previously (11, 12), and stored at -80°C until use. Phytohemagglutinin-L (PHA-L) was obtained from Seikagaku Co., Tokyo, Japan and Wheat germ agglutinin (WGA) and MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) were obtained from Sigma Chem. Co., St. Louis, MO, USA. Anti-mouse TNF- α monoclonal antibody was purchased from Endogen, Inc., Boston, MA. Murine-recombinant interferon- γ (IFN- γ) was purchased from PharMingen Co., San Diego, CA, USA. Sulfanilic acid and N-1-naphtyl-ethylenediamine dihydrochloride were purchased from Wako Pure Chemical Industry, Co., Ltd., Osaka, Japan. Fluorescein isothiocyanate isomer I (FITC), and NG-nitro-L-arginine methyl ester (L-NAME), an inhibitor for NO synthase, were purchased from Dojin Chemical Laboratories, Kumamoto, Japan. PD98059, SB202190, and SP600125, which are specific inhibitors for extracellular regulated kinase (ERK), p38 mitogen-activated protein (MAP) kinase, and c-jun NH₂-terminal kinase (JNK), respectively, were obtained from Calbiochem Co., La Jolla, CA, USA.

Cell Culture – RAW264.7 (mouse macrophage) cells were obtained from the American Type Culture Collection, Rockville, MD, USA, and cultured in Dulbecco's modified Eagle's minimum essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin G (100 IU/ml), and streptomycin (100 μ g/ml) as described previously (18).

Measurement of Cytotoxicity of Lectins – Cytotoxicity of lectins on RAW264.7 cells was measured by MTT tetrazolium cytotoxicity assay. In brief, 3×10^4 cells/well in a 96-well plate were cultured with varying concentrations of each lectin in the serum-free DMEM for 24 h, and then incubated with MTT for 20 min. After aspiration of the medium, dimethylsulfoxide was added to dissolve the MTT formazan reaction product and the optical density was measured at 535 nm using a multiwell scanning spectrophotometer (Multiskan Spectrum, Thermo Electron Co., Vantaa, Finland).

Nitrite assay for estimation of NO production – Adherent cells in 96-well plates (3×10^4 cells/well) in serum-free DMEM were treated with CEL-I at 37°C. After the incubation for the indicated periods of time, NO production was determined by assaying the culture medium for NO_2^- , the stable reaction product of nitric oxide (NO) with molecular oxygen using Griess reagent (3 mM sulfanilic acid, 30 μM N-1-naphthyl-ethylenediamine dihydrochloride, 25% glacial acetic acid). The supernatants of cultured cells were mixed with twice volume of Griess reagent. After incubation at room temperature for 20 min, the absorbance at 550 nm was then measured using a spectrophotometer (Thermo Electron). The NO_2^- concentrations were estimated from a reference to a standard curve for serial twofold dilution of NaNO_2 .

Western blot analysis for detection of iNOS expression – Adherent cells in 96-well plates (3×10^4 cells/well) in serum-free DMEM were treated with CEL-I at 37°C. After the incubation for the indicated periods of time, the cells were washed three times with ice cold PBS, and were lysed with 100 μl extraction buffer (10 mM HEPES, 150 mM NaCl, 1 mM EGTA, 1% CHAPS, and 1% Triton X-100) containing protease inhibitor cocktail (Nakarai tesque Co., Kyoto, Japan). After shaking for 30 min at 4°C, the cytosolic fraction was obtained from the supernatant after centrifugation at 15,000 rpm for 10 min, and the protein concentrations were determined with the BCA assay kit

(Sigma) by using BSA as the standard. The extract was mixed with the equal volume of SDS-sample buffer (1% SDS, 1% 2-mercaptoethanol, 10 mM Tris-HCl, pH 6.8, 5% glycerin) and incubated at 60°C for 30 min. Samples containing 10 µg of protein were subjected to SDS-PAGE in a 10% polyacrylamide gel. The proteins were then electrotransferred to a polyvinylidene difluoride (PVDF) membrane, and the membrane was blocked with 1% skim milk in TBS-0.1% Tween 20 (TBST). Immunostaining of the blot was performed with anti-iNOS (Upstate Biotechnology, Lake Placid, NY, USA) and Goat anti-rabbit IgG-horseradish peroxidase conjugate (Upstate). The blot was developed by using ECL Plus Western Blotting Detection Reagents (Amersham Biosciences, Piscataway, NJ, USA).

RNA isolation and reverse transcription polymerase chain reaction (RT-PCR) for detection of iNOS mRNA expression – Adherent cells in 12 wells plates (1 x 10⁶ cells/well) in serum-free DMEM were treated with CEL-I at 37°C. After the incubation for the indicated periods of time, total RNA was isolated from the cells with Trizol Reagent (Invitrogen Co., Carlsbad, CA, USA). Total RNA (1 µg) was reverse transcribed with an oligo dT primer in a 10-µl reaction volume using PrimeScript 1st strand cDNA Synthesis Kit (TaKaRa, Co., Ltd., Kyoto, Japan) according to the manufacturer's instructions. PCR was performed with 1 cycle of 70 sec at 95°C, 20 cycles of 55 sec at 93°C, 45 sec at 61°C, 40 sec at 72°C, and 1 cycle of 100 sec at 72°C, in a 25 µl reaction mixture containing 12.5 µl of GoTaq Green Master Mix (Promega, Co., Madison, WI, USA), 0.5 µl of forward and reverse primers (1 µM each), 0.5 µl of 1st strand cDNA, and 11 µl of nuclease-free water. The primer sets for iNOS, respectively, were synthesized as described by Imanishi et al (27). Each PCR reaction (25 µl) was run on 2% agarose gels containing 0.1 µg/ml ethidium bromide, and the amplified products (231 bp for iNOS) were observed by Light capture (ATTO Co.,

Tokyo, Japan).

FITC Labeling of CEL-I – FITC-labeled CEL-I (F-CEL-I) was prepared by essentially the same method as described previously (14). In brief, 2 mg of FITC was added to 1 ml of 0.5 M sodium bicarbonate buffer, pH 8.3, containing 10 mg of CEL-I and 0.1 M GalNAc. After stirring for 4 h at 4°C, the reaction mixture was applied to a column (1.5 x 10 cm) of Sephadex G-25 previously equilibrated with phosphate-buffered saline, pH7.4 (PBS), followed by dialysis against PBS. F-CEL-I retained the original hemagglutinating activity toward rabbit erythrocytes and NO inducibility.

Binding of FITC-Labeled CEL-I – Cell monolayers (2×10^5 cells/well of 48-well plates) were incubated with 25 µg/ml of F-CEL-I at 37°C for 2 h in serum-free DMEM. After removal of the medium by aspiration, the cells were washed three times with ice-cold PBS. The washed cells were solubilized in 0.5 ml of 20 mM Tris-HCl buffer, pH 8.5, containing 0.1% SDS. The fluorescence intensity of the solubilized cell lysate was measured with a Hitachi F-2500 fluorescence spectrophotometer (Hitachi Instruments, Tokyo, Japan) at an excitation wavelength of 490 nm and an emission wavelength of 520 nm.

RESULTS

Cytotoxicities of CEL-I, PHA-L, and WGA on RAW264.7 cells – In our previous study, we found that CEL-I showed cytotoxic effects on several cell lines depending on the cell lines. Among the cell lines tested, RAW264.7 cells were relatively resistant to CEL-I cytotoxicity (17). Consistent with this, the viability of RAW264.7 cells was more than 60% even after 24 h exposure to 100 µg/ml of CEL-I. PHA-L and WGA also showed dose-dependent cytotoxicity on RAW264.7 cells, and the order of the cytotoxic potency of these lectins was WGA>PHA-L>CEL-I (Fig. 1).

Induction of NO Production by CEL-I in RAW264.7 Cells – As shown in Fig. 2, CEL-I induced NO production in RAW264.7 cells in a concentration-dependent manner. Under the same conditions, PHA-L also induced the production of NO, but the level of NO was much lower than the levels induced by CEL-I. WGA had no such activity up to 100 µg/ml. Consistent with time-dependent increase in NO production in the CEL-I-treated RAW264.7 cells, increased expressions of iNOS mRNA and NOS protein were observed in a time-dependent manner as examined by RT-PCR and Western blotting, respectively (Fig. 3).

Effects of Polymyxin B, L-NAME, and anti-TNF- α antibody on CEL-I-Induced NO Production in RAW264.7 Cells – To confirm that CEL-I-induced NO production is not due to the contamination of trace amount of endotoxin in CEL-I sample, the effect of polymyxin B, an inhibitor for LPS, was examined. As shown in Fig. 4A, in the presence of polymyxin B, only a slight reduction of NO production in CEL-I-treated RAW264.7 cells was observed, while LPS-induced NO production was strongly inhibited. Furthermore, to avoid the effects of polymyxin B itself, CEL-I solution was applied to a polymyxin B-column, and then examined the NO-inducing activity. More than 90% of the activity remained after having passed through the column (data not shown). On the other hand, both CEL-I- and LPS-induced NO productions were diminished in the presence of L-NAME, a NO synthase (NOS) inhibitor, in a concentration-dependent manner (Fig. 4B). Since our previous study demonstrated that CEL-I is capable of inducing TNF- α secretion from RAW264.7 cells (18), and TNF- α is known to provoke NO production from macrophages (28), there is a possibility that CEL-I-induced NO production is a secondary phenomenon by the TNF- α induced by CEL-I first. To verify this possibility, the effects of anti-TNF- α antibody on the CEL-I-induced NO production was examined. In the presence of 10 µg/ml of anti-TNF- α antibody, no significant reduction in the NO level induced by CEL-I was observed, even though this concentration of anti-TNF- α antibody completely prevented the detection of 100 ng/ml of TNF- α (data not shown), which is far more higher than the maximum

level of TNF- α induced by 100 μ g/ml of CEL-I from RAW264.7 cells (18).

Effect of IFN- γ on CEL-I- and LPS-induced NO production – It has been reported that synergistic enhancement of NO production is observed when RAW264.7 cells were stimulated by LPS in combination with IFN- γ (29). To ascertain whether or not CEL-I-induced NO production by RAW264.7 cells is also influenced by IFN- γ , CEL-I-induced NO levels in the presence of varying concentrations of IFN- γ were examined. As shown in Fig. 5, IFN- γ enhanced CEL-I-induced NO levels in a concentration-dependent manner as seen in LPS-stimulated RAW264.7 cells. In both cases, the maximum synergistic effect was attained at 1,000 U/ml of IFN- γ .

Effects of various saccharides on CEL-I-Induced NO Production in RAW264.7 Cells – Previous studies have demonstrated that the biological activities of CEL-I were inhibited by GalNAc with different extents depending on the activities. To investigate if the specific binding of CEL-I on the macrophage cell surface saccharide chains is involved in the NO production, the effects of GalNAc and other saccharides were examined at the molar ratio of nearly 100,000:1 between saccharide and CEL-I. Unexpectedly, in the presence of GalNAc (0.1 M), increased NO production in CEL-I-treated RAW264.7 cells was observed rather than the inhibition. The effect of GalNAc was dose-dependent, and even 0.01 M GalNAc showed a slight enhancement effect (data not shown). Other monosaccharides such as glucose, GlcNAc, mannose, and galactose also showed similar enhancement effects. The enhancement of NO production by these saccharides was also observed in LPS or PHA-L induced NO production (Fig. 6).

Effects of BSA on CEL-I-Induced NO Production in RAW264.7 Cells and on the Binding of FITC-labeled CEL-I to RAW264.7 Cells – To gain insight into the involvement of protein-cell interaction in the CEL-I-induced NO production in RAW264.7 cells, the effect of BSA as a typical inert protein was examined. As shown in Fig. 7A, BSA inhibited the CEL-I-induced NO production. Furthermore, the binding of FITC-labeled CEL-I to RAW264.7 cells was also inhibited by BSA (Fig. 7B).

Effects of MAP Kinase inhibitors on the CEL-I-Induced NO production –

To investigate the involvement of MAP kinase system in the CEL-I-induced NO production in RAW264.7 cells, the effects of the specific inhibitors for ERK (PD98059), p38 (SB202190), and JNK (SP600125) MAP kinase were examined. As shown in Table I, these inhibitors showed the inhibitory effects with different extents, and, and ERK inhibitor was the most effective.

Effects of Heat-Treatment of CEL-I on the NO-inducing Activity in RAW264.7 Cells – After the treatment of CEL-I at various temperatures (37-100°C) for 15 min, the NO-inducing activity of CEL-I was examined (Table II). The activity decreased depending on the temperature. After 15 min treatment at 100°C, the activity decreased to less than 50% of the original activity.

DISCUSSION

Our previous studies have demonstrated that CEL-I stimulates RAW264.7 cells to produce high level of cytokines such as TNF- α and G-CSF. In the present study, we found that CEL-I also induces the production of NO in RAW264.7 cells at no significant cytotoxic concentration range. The increased NO production by RAW264.7 cells in response to CEL-I was concentration- and time-dependent. Since only a slight inhibitory effect of polymyxin B, an inhibitor of LPS, on the CEL-I-induced NO production was observed, CEL-I itself is responsible for the activity. Furthermore, anti-TNF- α antibody had almost no effect on the NO level induced by CEL-I. Thus it seems likely that the direct effect of CEL-I on RAW264.7 cells is mainly responsible for the induction of NO production. Since both CEL-I- and LPS- induced NO production were synergistically enhanced by IFN- γ , IFN- γ -mediated signaling may be involved in the action of CEL-I and LPS leading to eventual NO production as a common pathway.

The presence of an inducible pathway for NO production from L-arginine catalyzed by inducible NO synthase (iNOS) has been well documented in monocytes and macrophages in response to cytokines and LPS (30, 31). In fact, CEL-I-induced NO production in RAW264.7 cells was inhibited by L-arginine analog NOS inhibitor L-NAME in a concentration-dependent manner as seen in LPS-induced NO production. Furthermore, increases in the expression of iNOS mRNA and iNOS protein were observed in CEL-I-treated cells along with the incubation time.

Thus far, few investigations have shown the effects of lectins on NO production. It has been reported that Con A and PHA but not WGA induced increased NO production in mouse peritoneal macrophages in dose- and time-dependent manners (19). Similar to CEL-I observed in this study, it has been shown that Con A and PHA treatment of macrophages induced transcription of iNOS gene and the enhanced expression of iNOS protein. A glucose/mannose-specific lectin isolated from emperor banana has been reported to induce NO production by mouse peritoneal macrophages (32). On the other hand, it has been reported that galectin-1 elicited a dose- and time-dependent inhibition of LPS-induced NO production, accompanied by a decrease in iNOS expression (33). Although the exact mechanisms of lectin-induced NO production including the case of CEL-I are still unclear, these findings suggest that the specific biochemical characteristics of lectin molecules are important for the effects of lectin on macrophages in terms of the induction of NO production rather than the simple carbohydrate recognition specificity.

Unexpectedly, in the presence of GalNAc, an increase in NO level in CEL-I-treated cells was observed rather than the inhibitory effect, even though 0.1 M GalNAc inhibited the other biological activities of CEL-I such as hemagglutination (16), cytotoxicity (17), and induction of cytokine secretion (18). The exact reason for the

inability of GalNAc to inhibit the CEL-I-induced NO production is unknown now, but one possible speculation is that still unknown functional sites on CEL-I molecule apart from the carbohydrate-recognition sites are involved in the induction of NO production. In general, lectins exert certain biological activities through the recognition of the specific carbohydrate moieties on the target cell surface. However, there are several lines of evidence as well as speculation in favor of non-carbohydrate lectin interaction with cell surface molecules (34, 35). For instance, it has been reported that heat denatured *Abrus* agglutinin, which lost any sugar binding activity, acted as an adjuvant to induce antigen specific humoral immune response in rat (36) and also stimulated murine peritoneal macrophages to release NO, superoxide anion, IL-1, and TNF (37). Further studies have demonstrated that *Abrus* agglutinin on heat denaturation lost its carbohydrate binding activity, but it could still exert the immunomodulating activities (38). In addition, it has been reported that both the A and the B chains, the constituent subunits of Korean mistletoe lectin, induced macrophage-mediated NO production much greater than hololectin, even though the A chain has no carbohydrate binding activity (20). In the case of CEL-I-induced NO production, CEL-I may be recognized by RAW264.7 cells through the specific protein moieties of CEL-I molecule apart from the binding of CEL-I on the carbohydrate chains on the cell surface. The inhibitory effect of BSA on CEL-I-induced NO production as well as on the binding of FITC-labeled CEL-I on RAW264.7 cells may support this notion. Although the effective concentration is quite higher than that of CEL-I, BSA has been reported to induce NO production from macrophages (39). Thus macrophages may have specific recognition sites for certain structures of BSA, which can be linked with the intracellular signaling pathway leading to NO production. Probably, BSA and CEL-I may compete such recognition sites on RAW264.7 cells each other. Since the heat-treatment of CEL-I

resulted in the significant decrease in the activity to induce NO production in RAW264.7 cells depending on the temperature, the intact conformation of CEL-I may be necessary for such specific interaction of CEL-I with the cell surface. As revealed by X-ray crystallographic analysis (40), CEL-I is composed of homodimer of the C-type carbohydrate-recognition domains (5), each of which contains a carbohydrate-binding site at its outward end. Because the specific sugars, such as GalNAc, did not inhibit the NO production, the interaction with RAW264.7 cell surface might be mediated by the lateral face of CEL-I. In fact, CEL-I has a dumbbell-like shape with some shallow pockets around its middle portion, which might be involved in interaction with the target molecules on the cell surface. Further studies are required to clarify this point especially from the viewpoint of structure-activity relationship of CEL-I.

In addition to GalNAc, Glucose, GlcNAc, Galactose, and Mannose also showed enhancement effects on CEL-I-induced NO production. Furthermore, similar enhancement effects of these monosaccharides were observed in LPS- and PHA-L-induced NO productions. It has been reported that LPS-induced iNOS gene expression in rat glial cells was enhanced by extracellular glucose concentration in a dose-dependent manner, and had suggested that glucose metabolism is linked to the regulation of iNOS gene expression (41). Therefore, GalNAc and other monosaccharides may influence the glucose metabolism which in turn leads to the increase in NO production *via* the enhancement of iNOS gene expression.

It has been demonstrated that protein phosphorylation mediated by tyrosine kinase and the serine/threonine are correlated with the production of NO in macrophages activated by LPS and interleukins (31). Based on the analysis using various specific inhibitors, it has been suggested that in addition to the common pathways, Con A and PHA may stimulate their own specific signaling pathway leading

to NO production in macrophages. Namely, p38 MAP kinase is mainly involved in Con A, whereas JNK MAPK is involved in PHA induced NO production (19). Our previous study using Bio-Plex beads assay has suggested that temporal increase in phosphorylation of extracellular regulated kinase (ERK), c-jun NH₂-terminal kinase (JNK), and p38 MAP kinase occurred at relatively early time following CEL-I treatment in RAW264.7 cells, and the CEL-I-induced cytokine secretion was inhibited by specific inhibitors for these MAP kinases (18). Among the three MAP kinase inhibitors, ERK inhibitor showed the most potent inhibitory effect on CEL-I-induced NO production. Thus it seems likely that CEL-I and PHA may use similar MAP kinase pathway leading to NO production in which ERK kinase may play a key role in common.

In conclusion, we found that CEL-I induced NO production in RAW264.7 cells through the increase in iNOS gene expression. The exact mechanism of CEL-I is still unknown, but non-carbohydrate CEL-I interaction with macrophage cell surface may be responsible for the activity rather than the simple binding of CEL-I on carbohydrate chains on the cell surface.

REFERENCES

1. Sharon, N. and Lis, H. (1972) Lectins: cell-agglutinating and sugar-specific proteins. *Science* **177**, 949-959
2. Komano, H., Mizuno, D., and Natori, S. (1980) Purification of lectin induced in the hemolymph of *Sarcophaga peregrina* larvae on injury. *J. Biol. Chem.* **255**, 2919-2924
3. Yamazaki, M., Ikenami, M., Komano, H., Tsunawaki, S., Kamiya, H., Natori, S., and Mizuno, D. (1983) Polymorphonuclear leukocyte-mediated cytolysis induced by animal lectin. *Gann* **74**, 576-583
4. Dodd, R.B. and Drickamer, K. (2001) Lectin-like proteins in model organisms: implications for evolution of carbohydrate-binding activity. *Glycobiol.* **11**, 71R-79R
5. Drickamer, K. (1988) Two distinct classes of carbohydrate-recognition domains in animal lectins. *J. Biol. Chem.* **263**, 9557-9560
6. Barondes, S.H., Castronovo, V., Cooper, D.N.W., Cummings, R.D., Drickamer, K., Feizi, T., Gitt, M.A., Hirabayashi, J., Hughes, C., Kasai, K., Leffler, H., Liu, F.T., Lotan, R., Mercurio, A.M., Monsigny, M., Pillai, S., Poirer, F., Raz, A., Rigby, P.W. J., Rini, J.M., and Wang, J.L. (1994) Galectins: A family of animal β -galactoside-binding lectins. *Cell* **76**, 597-598
7. Giga, Y., Ikai, A., and Takahashi, K. (1987) The complete amino acid sequence of echinoidin, a lectin from the coelomic fluid of the sea urchin *Anthocidaris crassispina*. *J. Biol. Chem.* **262**, 6197-6203
8. Muramoto, K. and Kamiya, H. (1990) The amino-acid sequences of multiple lectins of the acorn barnacle *Megabalanus rosa* and its homology with animal lectins. *Biochim. Biophys. Acta* **1039**, 42-51
9. Suzuki, T., Takagi, T., Furukohri, T., Kawamura, K., and Nakauchi, M. (1990) A

- calcium-dependent galactose-binding lectin from the tunicate *Polyandrocarpa misakiensis*. *J. Biol. Chem.* **265**, 1274-1281
10. Himeshima, T., Hatakeyama, T., and Yamasaki, N. (1994) Amino acid sequence of a lectin from the sea cucumber, *Stichopus japonicus*, and its structural relationship to the C-type animal lectin family. *J. Biochem.* **115**, 689-692
 11. Hatakeyama, T., Kohzaki, H., Nagatomo, H., and Yamasaki, N. (1994) Purification and characterization of four Ca²⁺-dependent lectins from the marine invertebrate, *Cucumaria echinata*. *J. Biochem.* **116**, 209-214
 12. Hatakeyama, T., Nagatomo, H., and Yamasaki, N. (1995) Interaction of the hemolytic lectin CEL-III from the marine invertebrate *Cucumaria echinata* with the erythrocyte membrane. *J. Biol. Chem.* **270**, 3560-3564
 13. Hatakeyama, T., Furukawa, M., Nagatomo, H., Yamasaki, N., and Mori, T. (1996) Oligomerization of the hemolytic lectin CEL-III from the marine invertebrate *Cucumaria echinata* induced by the binding of carbohydrate ligands. *J. Biol. Chem.* **271**, 16915-16920
 14. Oda, T., Tsuru, M., Hatakeyama, T., Nagatomo, H., Muramatsu, T., and Yamasaki, N. (1997) Temperature- and pH-dependent cytotoxic effect of the hemolytic lectin CEL-III from the marine invertebrate *Cucumaria echinata* on various cell lines. *J. Biochem.* **121**, 560-567
 15. Hatakeyama, T., Matsuo, N., Shiba, K., Nishinohara, S., Yamasaki, N., Sugawara, H., and Aoyagi, H. (2002) Amino acid sequence and carbohydrate-binding analysis of the *N*-acetyl-D-galactosamine-specific C-type lectin, CEL-I, from the Holothuroidea, *Cucumaria echinata*. *Biosci. Biotechnol. Biochem.* **66**, 157-163
 16. Hatakeyama, T., Shiba, K., Matsuo, N., Fujimoto, T., Oda, T., Sugawara, H., and Aoyagi, H. (2004) Characterization of recombinant CEL-I, a GalNAc-specific

- C-type lectin, expressed in *Escherichia coli* using an artificial synthetic gene. *J. Biochem.* **135**, 101-107
17. Kuramoto, T., Uzuyama, H., Hatakeyama, T., Tamura, T., Nakashima, T., Yamaguchi, K., and Oda, T. (2005) Cytotoxicity of a GalNAc-specific C-type lectin CEL-I toward various cell lines. *J. Biochem.* **137**, 41-50
 18. Yamanish, T., Yamamoto, Y., Hatakeyama, T., Yamaguchi, K., and Oda, T. (2007) CEL-I, an invertebrate *N*-acetylgalactosamine-specific C-type lectin, induces TNF- α and G-CSF production by mouse macrophage cell line RAW264.7 cells. *J. Biochem.* **142**, 587-595
 19. Keshewani, V. and Sodhi, A. (2007) Differential activation of macrophages *in vitro* by lectin concanavalin A, phytohemagglutinin and wheat germ agglutinin: production and regulation of nitric oxide. *Nitric Oxide* **16**, 294-305
 20. Kang, T.B., Yoo, Y.C., Lee, K.H., Yoon, H.S., Her, E., Kim, J.B., and Song, S.K. (2007) Korean mistletoe lectin (KML-IIU) and its subchains induce nitric oxide (NO) production in murine macrophage cells. *J. Biomed. Sci.* **15**, 197-204
 21. Moncada, S., Palmer, R.M., and Higgs, E.A. (1991) Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.* **43**, 109-142
 22. Nathan, C. Nitric oxide as a secretory product of mammalian cells. *FASEB J.* **6**, 3051-3064
 23. Lowenstein, C.J. and Snyder, S.H. (1992) Nitric oxide, a novel biologic messenger. *Cell* **70**, 705-707
 24. Xie, Q. and Nathan, C. (1994) The high-output nitric oxide pathway: role and regulation. *J. Leukoc. Biol.* **56**, 576-582
 25. Deng, W., Thiel, B., Tannenbaum, C.S., Hamilton, T.A., and Stuehr, D.J. (1993) Synergistic cooperation between T cells lymphokines for induction of the nitric

- oxide synthase gene in murine peritoneal macrophages. *J. Immunol.* **151**, 322-329
26. Paul, A., Pendereigh, R.H., and Plevin, R. (1995) Protein kinase C and tyrosine kinase pathways regulate lipopolysaccharide-induced nitric oxide synthase activity in RAW 264.7 murine macrophages. *Br. J. Pharmacol.* **114**, 482-488
27. Imanishi, N., Andoh, T., Sakai, S., Satoh, M., Katada, Y., Ueda, K., Terasawa, K., and Ochiai, H. (2005) Induction of inducible nitric oxide (NO) synthase mRNA and NO production in macrophages infected with influenza A/PR/8 virus and stimulated with its ether-split product. *Microbiol. Immunol.* **49**, 41-48
28. Silva, J.S., Vespa, G.N., Cardoso, M.A., Aliberti, J.C., and Cunha, F.Q. (1995) Tumor necrosis factor alpha mediates resistance to *Trypanosoma cruzi* infection in mice by inducing nitric oxide production in infected gamma interferon-activated macrophages. *Infect. Immun.* **63**, 4862-4867
29. Inoue, T., Takeshita, K., and Fukushima, K. (2001) Effects of KE-758; an active metabolite of the new anti-rheumatic drug KE-298, D-penicillamine, bucillamine and auranofin on the proliferation of murine lymphocytes, and the production of nitric oxide by murine macrophages. *Int. Immunopharmacol.* **1**, 833-842
30. Sethi, G. and Sodhi, A. (2004) In vitro activation of murine peritoneal macrophages by ultraviolet B radiation: up regulation of CD18, production of NO, proinflammatory cytokines and signal transduction pathway. *Mol. Immunol.* **40**, 1315-1323
31. Eason, S. and Martin, W. (1995) Involvement of tyrosine kinase and protein kinase C in the induction of nitric oxide synthase by lipopolysaccharide and interferon-gamma in J774 macrophages. *Arch. Int. Pharmacodyn.* **330**, 225-236
32. Wong, J.H. and Ng, T.B. (2006) Isolation and characterization of a glucose/mannose-specific lectin with stimulatory effect on nitric oxide production

- by macrophages from the emperor banana. *Int. J. Biochem. Cell Biol.* **38**, 234-243
33. Correa, S.G., Sotomayor, C.E., Aoki, M.P., Maldonado, C.A., and Rabinovich, G.A. (2003) Opposite effects of galectin-1 on alternative metabolic pathways of L-arginine in resident, inflammatory, and activated macrophages. *Glycobiol.* **13**, 119-128
34. Gabius, H.J. (1994) Mini review. Non-carbohydrate binding partners/domains of animal lectins. *Int. J. Biochem.* **26**, 469-477
35. Oldenburg, K.R., Loganathan, D., Goldstein, I.J., Shultz, P.G., and Gallop, M.A. (1992) Peptide ligands for a sugar-binding protein isolated from a random peptide library. *Proc. Nat. Aca. Sci.* **89**, 5393-5397
36. Suryakala, S., Maiti, T.K., Sujatha, N., and Sashidhar, R.B. (2000) Identification of a novel protein adjuvant isolated from *Abrus precatorius*. *Food Agri. Immunol.* **12**, 87-96
37. Tripathi, T. and Maiti, T.K. (2003) Stimulation of murine macrophages by native and heat denatured lectin from *Abrus precatorius*. *Inter. Immunopharmacol.* **3**, 233-244
38. Tripathi, T. and Maiti, T.K. (2005) Immunomodulatory role of native and heat denatured agglutinin from *Abrus precatorius*. *Inter. J. Biochem. Cell Biol.* **37**, 451-462
39. Poteser, M. and Wakabayashi, I. (2004) Serum albumin induces iNOS expression and NO production in RAW 264.7 macrophages. *Br. J. Pharmacol.* **143**, 143-151
40. Sugawara, H., Kusunoki, M., Kurisu, G., Fujimoto, T., Aoyagi, H. and Hatakeyama, T. (2004) Characteristic recognition of N-acetylgalactosamine by an invertebrate C-type Lectin, CEL-I, revealed by X-ray crystallographic analysis. *J. Biol. Chem.* **279**, 45219-45225

41. Won, J.S., Im, Y.B., Key, L., Singh, I., and Singh, A. (2003) The involvement of glucose metabolism in the regulation of inducible nitric oxide synthase gene expression in glial cells: possible role of glucose-1-phosphate dehydrogenase and CCAAT/enhancing binding protein. *J. Neurosci.* **23**, 7470-7478

FIGURE LEGENDS

Fig. 1. Cytotoxicities of CEL-I (●), PHA-L (○), and WGA (■) on RAW264.7 cells.

Adherent cells (3×10^4 cells/well in 96-well plates) were treated with various concentrations of each lectin in serum-free DMEM at 37°C. After 24 h, the cell viabilities were measured by MTT assay as described under "MATERIALS AND METHODS". Each point represents the average of triplicate measurements.

Fig. 2. NO production by RAW264.7 cells treated with various concentrations of

CEL-I (●), PHA-L (○), and WGA (■). Adherent cells (3×10^4 cells/well in 96-well plates) were treated with the indicated concentration of each lectin in serum-free DMEM at 37°C. After 24 h, the NO level in the supernatant of the cells treated under each condition was measured as described under "MATERIALS AND METHODS". Each point represents the average of triplicate measurements.

Fig. 3. Time course analysis of NO production (A), expression of iNOS protein (B), and expression of iNOS mRNA (C) in CEL-I-treated RAW264.7 cells.

(A) Adherent cells (3×10^4 cells/well in 96-well plates) were incubated with 25 μ g/ml of CEL-I for the indicated periods of time, and then the supernatant was withdrawn from each well and subjected to the measurement of NO level, and the cells were subjected to the analysis of iNOS protein (B) as described under "MATERIALS AND METHODS". Each point represents the average of triplicate measurements. Adherent cells (1×10^6 cells/well in 12-well plates) were incubated with 25 μ g/ml of CEL-I for the indicated periods of time, and then the cells were subjected to the analysis of iNOS mRNA levels (C) as described under "MATERIALS AND METHODS".

Fig. 4. Effects of polymyxin B (A) and L-NAME (B) on CEL-I- and LPS-induced NO production in RAW264.7 cells. Adherent cells (3×10^4 cells/well in 96-well plates) were pre-incubated in the presence of indicated concentrations of polymyxin B (A) or L-NAME (B) in serum-free DMEM at 37°C for 10 min, followed by the addition of CEL-I (●) (final 25 $\mu\text{g/ml}$) or LPS (Δ) (final 1 ng/ml). After 24 h incubation at 37°C, the NO level in the supernatant of the cells treated under each condition was measured as described under "MATERIALS AND METHODS". Each point represents the average of triplicate measurements.

Fig. 5. Effects of $\text{INF-}\gamma$ on CEL-I- and LPS-induced NO production in RAW264.7 cells. Adherent cells (3×10^4 cells/well in 96-well plates) were treated with CEL-I (■) (final 25 $\mu\text{g/ml}$), LPS (\square) (final 1 ng/ml) or only DMEM () in the presence of the indicated concentrations of $\text{INF-}\gamma$ in serum-free DMEM at 37°C for 24 h, and then the NO level in the supernatant of the cells treated under each condition was measured as described under "MATERIALS AND METHODS". Each point represents the average of triplicate measurements.

Fig. 6. Effects of GalNAc, Glucose, GlcNAc, Galactose, and Mannose on CEL-I- (A), PHA-L- (B) and LPS- (C) induced NO production in RAW264.7 cells. Adherent cells (3×10^4 cells/well in 96-well plates) were treated with CEL-I (final 25 $\mu\text{g/ml}$), PHA-L (final 100 $\mu\text{g/ml}$) or LPS (final 1 ng/ml) in the presence (\square) or absence (■) of each saccharide (final 0.1 M) in serum-free DMEM at 37°C for 24 h, and then the NO level in the supernatant of the cells treated under each condition was measured as described under "MATERIALS AND METHODS". Each point represents the average of triplicate measurements.

Fig. 7. Effects of BSA on the CEL-I- and LPS-induced NO production in

RAW264.7 cells and on the binding of FITC-labeled CEL-I on RAW264.7 cells. (A) Adherent cells (3×10^4 cells/well in 96-well plates) were treated with CEL-I (■) (final 25 $\mu\text{g/ml}$) or LPS (□) (final 1 ng/ml) in the presence of the indicated concentrations of BSA in serum-free DMEM at 37°C for 24 h, and then the NO level in the supernatant of the cells treated under each condition was measured as described under "MATERIALS AND METHODS". Each point represents the average of triplicate measurements. (B) Adherent cells (2×10^5 cells/well in 48-well plates) were incubated with FITC-labeled CEL-I (final 25 $\mu\text{g/ml}$) in the presence of the indicated concentrations of BSA for 2 h at 37°C in serum-free DMEM. After incubation, the cells were washed three times with PBS and then the amount of cell-associated FITC-labeled CEL-I was measured as described under " MATERIALS AND METHODS ". Each point represents the average of triplicate measurements

Table I . Effects of MAP kinase inhibitors on CEL-I-induced NO secretion by RAW264.7 cells.

Inhibitor	Level of NO ₂ ⁻ (% of control) ^a
PD 98059	34 ± 1.4
SB 202195	60 ± 3.2
SP 600125	45 ± 5.9

Adherent cells (3×10^4 cells/well in 96-well plates) were pre-incubated in the presence or absence of each inhibitor (final 20 mM) in serum-free DMEM at 37°C for 1 h, followed by the addition of CEL-I (final 25 µg/ml). After 24 h incubation at 37°C, NO level in the supernatant of the cells treated under each condition was measured as described under "MATERIALS AND METHODS". Each value represents the average of triplicate measurements.

^a 100 % level of NO detected in the culture supernatant of CEL-I-treated RAW264.7 cells was $42.1 \pm 0.1 \mu\text{M}$.

Table II. Effects of heat-treatment on the NO-inducing activity of CEL-I in RAW264.7 cells.

Temperature (°C)	Level of NO ₂ ⁻ (% of control) ^a
70	62 ± 1.7
80	50 ± 2.7
90	50 ± 0.1
100	31 ± 1.2

CEL-I in PBS (final 1mg/ml) was treated for 15 min at the indicated temperature, and then the NO-inducing activity of each treated CEL-I (final 25 µg/ml) was measured as described in the legend of Fig. 2. Each value represents the average of triplicate measurements.

^a 100 % level of NO detected in the culture supernatant of CEL-I-treated RAW264.7 cells was 37.8 ± 1.3 µM.

Fig. 1

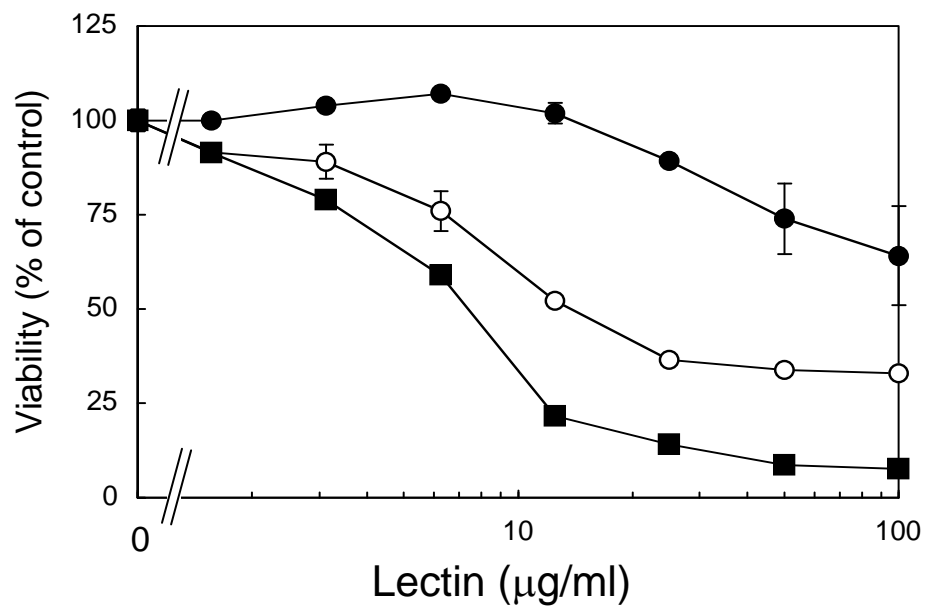


Fig. 2

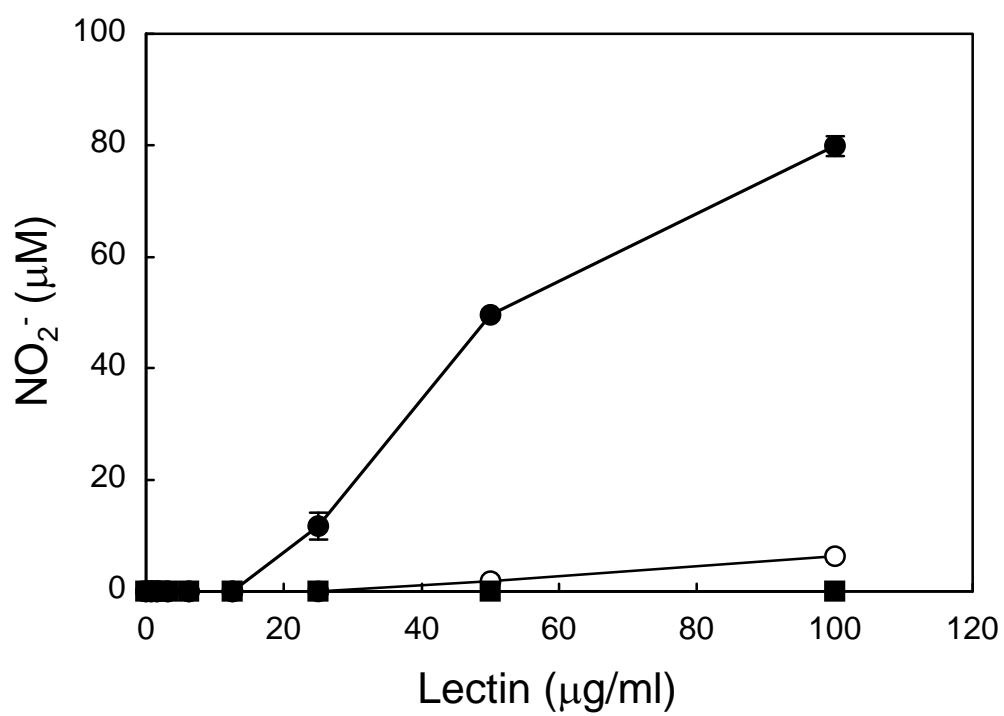


Fig. 3

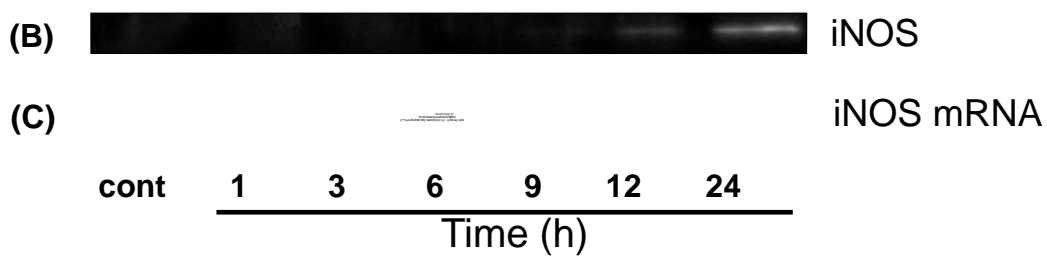
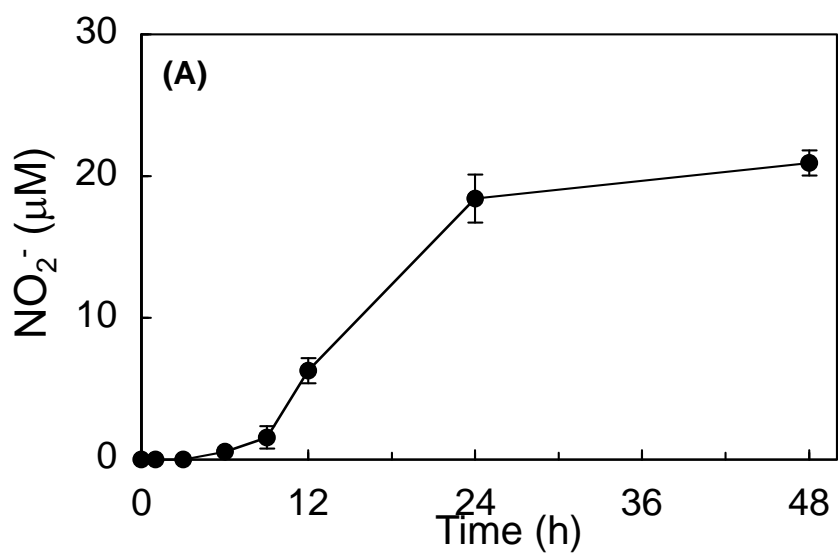


Fig. 4

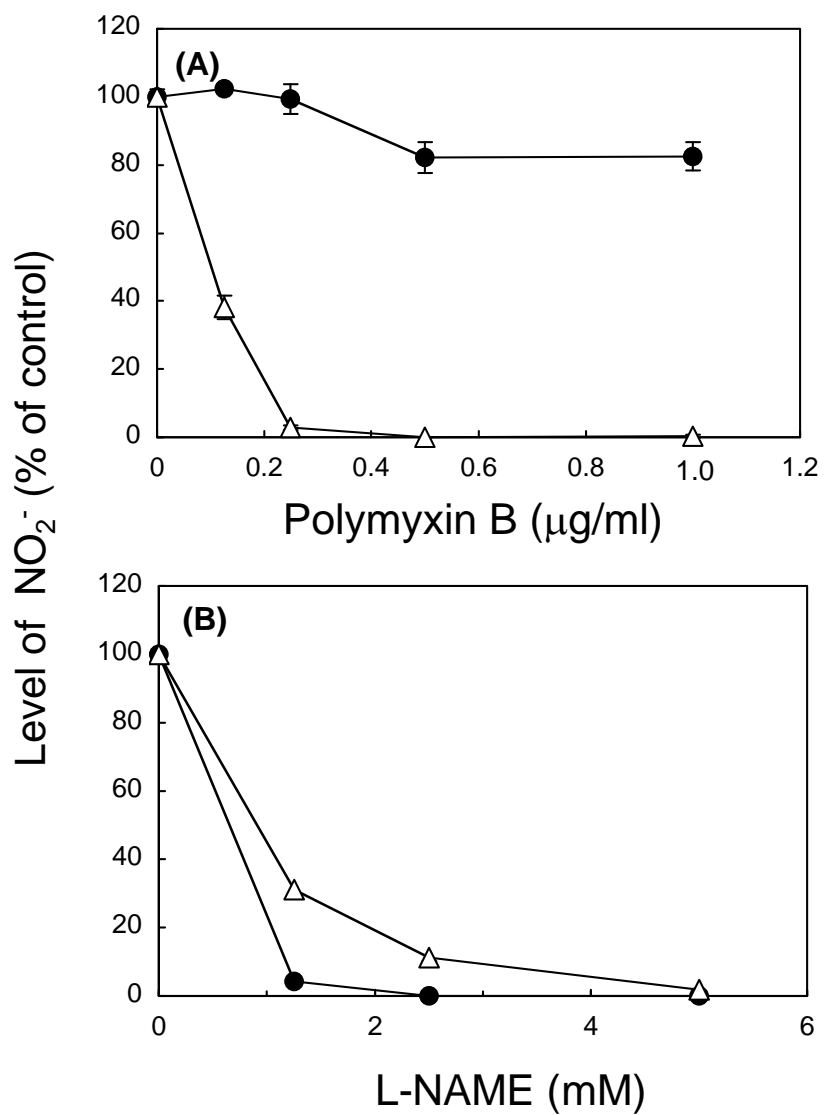


Fig. 5

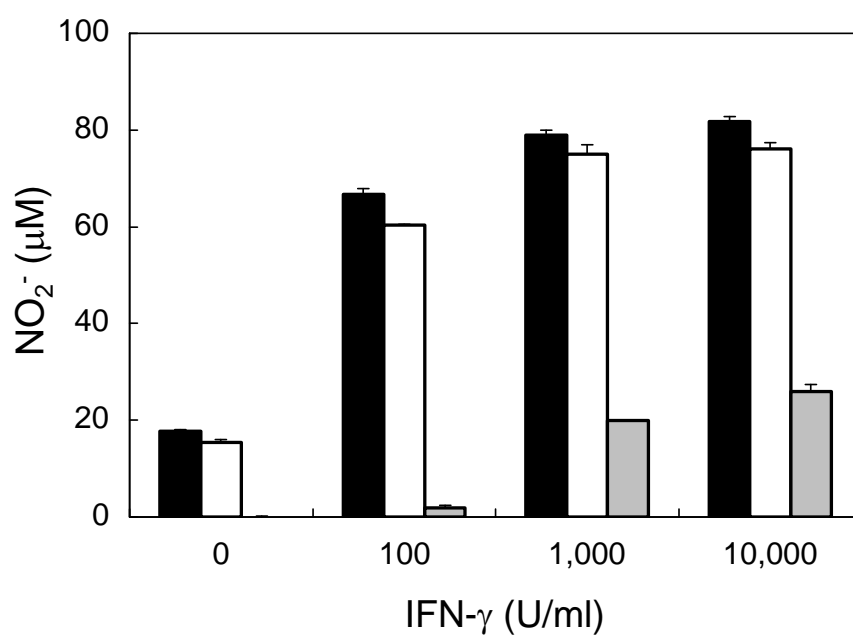


Fig. 6

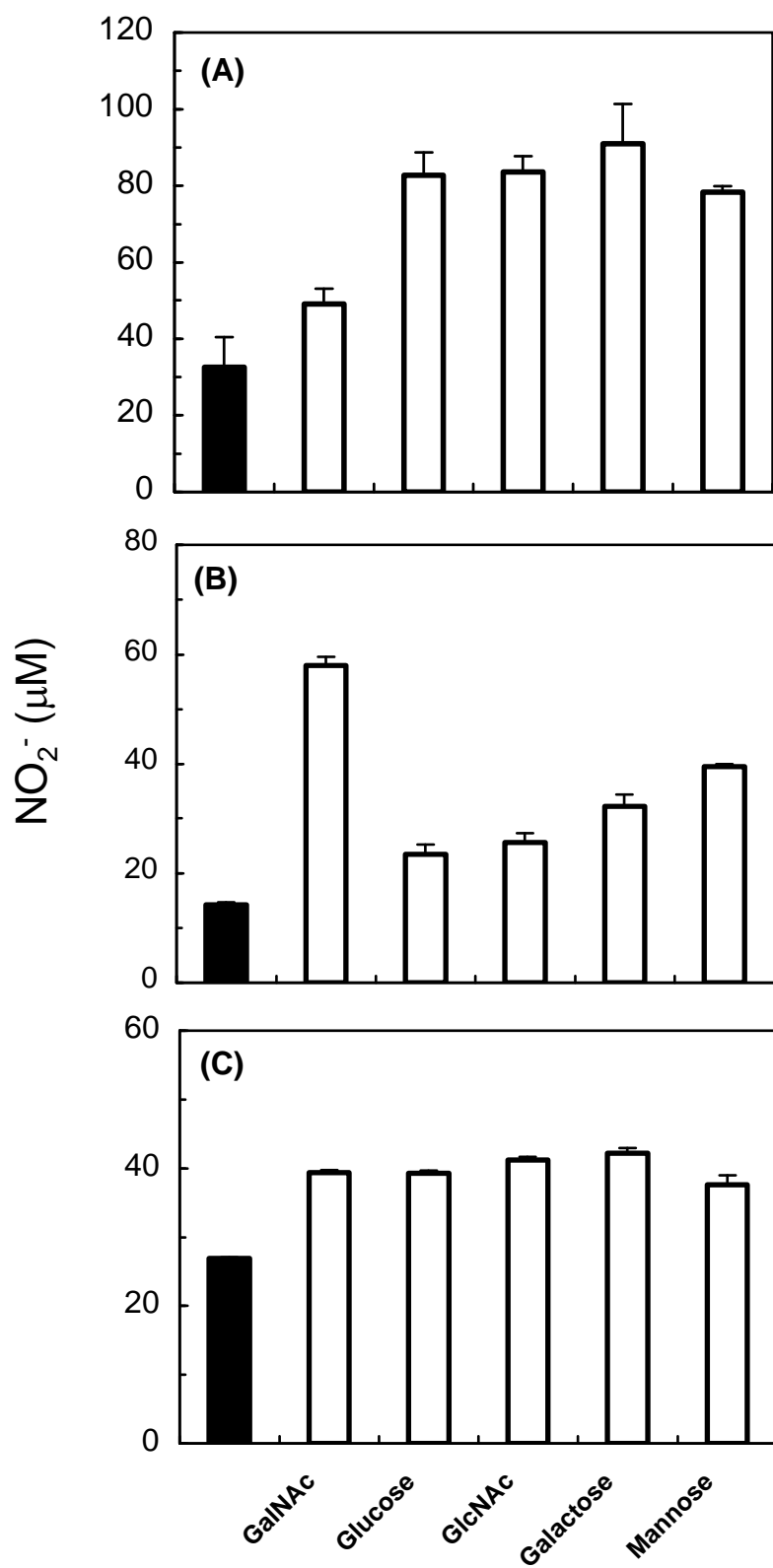


Fig. 7

