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Antimicrobial activity, bactericidal mechanism and LPS-neutralizing activity of the cell-penetrating peptide *p*VEC and its analogs

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p*VEC is a cell-penetrating peptide derived from the murine vascular endothelial-cadherin protein. To evaluate the potential of *p*VEC as antimicrobial peptide (AMP), we synthesized *p*VEC and its analogs with Trp and Arg/Lys substitution, and their antimicrobial and lipopolysaccharide (LPS)-neutralizing activities were investigated. *p*VEC and its analogs displayed a potent antimicrobial activity (minimal inhibitory concentration: 4–16 μM) against Gram-positive and Gram-negative bacteria but no or less hemolytic activity (less than 10% hemolysis) even at a concentration of 200 μM. These peptides induced a near-complete membrane depolarization (more than 80%) at 4 μM against *Staphylococcus aureus* and a significant dye leakage (35–70%) from bacterial membrane-mimicking liposome at a concentration as low as 1 μM. The fluorescence profiles of *p*VEC and its analogs in dye leakage from liposome and membrane depolarization were similar to those of a frog-derived AMP, magainin 2. These results suggest that *p*VEC and its analogs kill bacteria by forming a pore or ion channel in the cytoplasmic membrane. *p*VEC and its analogs significantly inhibited nitric oxide production or tumor necrosis factor-α release in LPS-stimulated mouse macrophage RAW264.7 cells at 10 to 50 μM, in which RAW264.7 were not damaged. Taken together, our results suggest that *p*VEC and its analogs with potent antimicrobial and LPS-neutralizing activities can serve as AMPs for the treatment of microbial infection and sepsis. Copyright © 2011 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: *p*VEC; cell-penetrating peptide; antimicrobial activity; bactericidal mechanism; LPS-neutralizing activity

Introduction

*p*VEC is a recently described cell-penetrating peptide derived from the murine vascular endothelial-cadherin protein [1]. *p*VEC has been shown to translocate efficiently into various mammalian cell lines by a temperature-independent and receptor-independent mechanism without exhibiting cellular toxicity and to carry macromolecular cargoes through plasma membranes [1,2]. It consists of a hydrophobic N-terminus, an arginine-rich middle region and a hydrophilic C-terminus. Cationic and hydrophobic properties of *p*VEC are similar to those of antimicrobial peptides (AMPs) involved in host innate immunity [3]. This fact has prompted us to examine the antimicrobial potential of *p*VEC against microorganisms. In previous studies, *p*VEC was shown to kill bacteria by permeabilizing their membranes [4,5].

In the present study, to evaluate the potential of *p*VEC as AMP, we synthesized *p*VEC and its analogs with Trp and Arg/Lys substitution. Ile^{9} of *p*VEC was substituted by Trp in all analogs because Trp residue preferentially interacts with the interfacial region of bacterial membranes [6]. Two His residues (His^{14} and His^{16}) were replaced by Arg (*p*VEC-a1) or Lys (*p*VEC-a2) residues because their high positive charge facilitates the initial binding to the negatively charged surface of bacterial membranes. Arg-containing peptide (*p*VEC-a3) and its counterpart Lys-containing peptide (*p*VEC-a4) were designed by Arg substitution or Lys substitution, respectively.

In this study, antimicrobial activity of *p*VEC and its analogs against Gram-positive and Gram-negative bacterial strains and hemolytic activity against human red blood cells were examined. Furthermore, to gain insight into the mechanism of bactericidal action of *p*VEC and its analogs, we examined the depolarization of the cytoplasmic membrane potential against intact *Staphylococcus aureus* and the leakage of calcein from bacterial membrane-mimicking liposome.

Bacterial killing by antimicrobial compounds may cause a release of endotoxin [lipopolysaccharide (LPS)] from Gram-negative bacteria, which subsequently might lead to an exaggerated immune response. This can result in early sepsis, in which high levels of cytokines and inflammatory mediators become destructive, causing organ failure and cardiovascular shock and even resulting in death [7]. In recent studies, some AMPs are found to counteract the development of septic shock by neutralizing endotoxin [8]. In this study, therefore, the LPS-neutralizing activity of *p*VEC and its analogs was established by examining the inhibition of nitric oxide (NO) production and tumor necrosis factor-α (TNF-α) release in LPS-stimulated mouse macrophage RAW264.7 cells.

Materials and Methods

Materials

Rink amide 4-methylbenzhydrylamine resin and Fmoc amino acids were obtained from Calbiochem-Novabiochem (La Jolla,

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Journal of PeptideScience

CA, USA). Other reagents used for peptide synthesis included trifluoroacetic acid (Sigma, St. Louis, MO, USA), piperidine (Merck, Darmstadt, Germany), DCC (Fluka, Buchs, Switzerland), HOBt (Aldrich, Milwaukee, WI, USA) and DMF (peptide synthesis grade; Biolab). Phosphatidylethanolamine (PE, from egg yolk), phosphatidylglycerol (PG, from egg yolk) and calcein were purchased from Sigma Chemical Co (St. Louis, MO, USA). $DiSC_3$-5 was obtained from Molecular Probes (Eugene, OR, USA). All other reagents were of analytical grade. The buffers were prepared in double glass-distilled water.

Peptide Synthesis

*p*VEC and its analogs shown in Table 1 were synthesized by the standard Fmoc-based solid-phase method [9] on rink amide 4-methylbenzhydrylamine resin (0.54 mmol/g). DCC and HOBt were used as coupling reagents, and tenfold excess Fmoc amino acids were added during every coupling cycle. After cleavage and deprotection with a mixture of trifluoroacetic acid/water/thioanisole/phenol/ethanedithiol/triisopropylsilane (81.5 : 5 : 5 : 5 : 2.5 : 1, v/v/v/v/v/v) for 2 h at room temperature, the crude peptide was repeatedly extracted with diethyl ether and purified by RP-HPLC on a preparative Vydac C_{18} column (20 × 250 mm, 300 Å, 15-μm particle size) using an appropriate 0–90% water/acetonitrile gradient in the presence of 0.05% trifluoroacetic acid. The final purity of the peptides (>98%) was assessed by RP-HPLC on an analytical Vydac C_{18} column (4.6 × 250 mm, 300 Å, 5-μm particle size). The molecular mass of synthetic peptides was determined by MALDI-TOF MS (Shimadzu, Kyoto, Japan) (Table 1).

Antimicrobial Activity

The antimicrobial activity of the peptides against three Gram-positive bacterial strains and three Gram-negative bacterial strains was examined by using the broth microdilution method [10,11]. Aliquots (100 μl) of a bacterial suspension at 2×10^6 colony-forming units/ml in 1% peptone were added to 100 μl of the peptide solution (serial twofold dilutions in 1% peptone). After incubation for 18–20 h at 37 °C, bacterial growth inhibition was determined by measuring the absorbance at 600 nm with a Microplate autoreader EL 800 (Bio-Tek Instruments, Winooski, VT, USA). The minimal inhibitory concentration (MIC) was defined as the minimum peptide concentration that inhibited bacteria growth. Three types of Gram-positive bacteria (*Bacillus subtilis* [KCTC 3068], *Staphylococcus epidermidis* [KCTC 1917] and *S. aureus* [KCTC 1621]) and 3 types of Gram-negative bacteria (*Escherichia coli* [KCTC 1682], *Pseudomonas aeruginosa* [KCTC 1637] and *Salmonella typhimurium* [KCTC 1926]) were procured from the Korean Collection for Type Cultures (KCTC) at the Korea Research Institute of Bioscience and Biotechnology.

Hemolytic Activity

Fresh human red blood cells (hRBCs) were washed three times with phosphate-buffered saline (PBS) (35 mM phosphate buffer, 0.15 M NaCl, pH 7.4) by centrifugation for 7 min at 1000 *g* and resuspended in PBS. The peptide solutions (serial twofold dilutions in PBS) were added to 100 μl of hRBC suspension [4% (v/v) in final] in PBS to a final volume of 200 μl and incubated for 1 h at 37 °C. Samples were centrifuged at 1000 *g* for 5 min, and hemoglobin release was monitored by measuring supernatant absorbance at 405 nm with a Microplate ELISA reader (Molecular Devices, Sunnyvale, CA, USA). Minimal hemolytic concentration (MHC) is defined as the lowest peptide concentration that causes 5% or more hemolysis. As negative and positive controls, hRBCs in PBS (A_{blank}) and 0.1% Triton X-100 (A_{triton}) were employed, respectively. The hemolysis percentage was calculated according to the following equation:

$$\%\,\text{hemolysis} = 100 \times \left[\left(A_{sample} - A_{blank}\right)/\left(A_{triton} - A_{blank}\right)\right]$$

Membrane Depolarization

The cytoplasmic membrane depolarization activity of peptides was determined as previously described [12], using *S. aureus* strain (KCTC 1621) and the membrane potential-sensitive dye, $DiSC_3$-5. Briefly, *S. aureus* was grown at 37 °C with agitation to mid-log phase ($OD_{600} = 0.4$) and harvested by centrifuge. The cells were washed twice with washing buffer (20 mM glucose, 5 mM HEPES, pH 7.4) and resuspended to an OD_{600} of 0.05 in a similar buffer containing 0.1 M KCl. Thereafter, the cells were incubated with 20 nM $DiSC_3$-5 until a stable reduction of fluorescence was achieved, indicating the incorporation of the dye into the bacterial membrane. Membrane depolarization was then monitored by observing the change in the intensity of fluorescence emission of the membrane potential-sensitive dye $DiSC_3$-5 ($\lambda_{ex.} = 622$ nm, $\lambda_{em.} = 670$ nm) after the addition of peptides. Full dissipation of the membrane potential was obtained by adding melittin (final concentration: 4 μM).

Dye Leakage

Calcein-entrapped large unilamellar vesicles (LUVs) composed of PE/PG (7 : 3, w/w) were prepared by vortexing the dried lipid in

Table 1. Amino acid sequences, calculated and observed molecular masses, net charge and RP-HPLC retention times of *p*VEC and its analogs

Peptides	Amino acid sequences	Molecular MS		Net charge	RP-HPLC retention times (min)[b]
		Calculated	Measured[a]		
*p*VEC	LLIILRRRIRKQAHAHSK-NH_2	2208.7	2208.9	+7	18.5
*p*VEC-a1	LLIILRRRWRKQARARSK-NH_2	2319.9	2320.2	+9	19.4
*p*VEC-a2	LLIILRRRWRKQAKAKSK-NH_2	2263.8	2264.2	+9	19.2
*p*VEC-a3	LLIILRRRWRRQARARSR-NH_2	2375.9	2376.0	+9	19.7
*p*VEC-a4	LLIILKKKWKKQAKAKSK-NH_2	2151.8	2152.2	+9	18.3

Peptides were eluted for 60 min, using a linear gradient of 0% to 90% (v/v) acetonitrile in water containing 0.05% (v/v) trifluoroacetic acid.
[a]Molecular masses were determined by MALDI-TOF MS.
[b]RP-HPLC retention time was measured using a C_{18} reverse-phase analytical Vydac column (4.6 × 250 mm, 300 Å, 5-μm particle size).

dye buffer solution (70 mM calcein, 10 mM Tris, 150 mM NaCl, 0.1 mM EDTA, pH 7.4). The suspension was subjected to ten frozen–thaw cycles in liquid nitrogen and extruded 21 times through polycarbonate filters (two stacked 100-nm pore size filters) with a LiposoFast extruder (Avestin, Inc., Ottawa, Canada). Untrapped calcein was removed by gel filtration on a Sephadex G-50 column. The concentration of calcein-entrapped LUVs was determined in triplicate by phosphorus analysis [13]. Calcein leakage from LUVs was monitored at room temperature by measuring fluorescence intensity at an excitation wavelength of 490 nm and emission wavelength of 520 nm on a model RF-5301PC spectrophotometer. Complete dye release was obtained by using 0.1% Triton X-100.

Cell Culture

RAW 264.7 cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and antibiotic–antimyotic solution (100 units/ml penicillin, 100 μg/ml streptomycin and 25 μg/ml amphotericin B) in 5% CO_2 at 37 °C. Cultures were passed every 3 to 5 days, and cells were detached by brief trypsin treatment and visualized with an inverted microscope.

Cytotoxicity Against RAW 264.7 Cells

Cytotoxicity of peptides against RAW 264.7 cells was determined using the MTT proliferation assay as reported previously with minor modifications [14,15]. RAW 264.7 cells were seeded on 96-well microplates at a density of 2×10^4 cells/well in 150 μl DMEM containing 10% fetal bovine serum. Plates were incubated for 24 h at 37 °C in 5% CO_2. Peptide solutions (20 μl) (serial two-fold dilutions in DMEM) were added, and the plates further incubated for 2 days. Wells containing cells without peptides served as controls. Subsequently, 20 μl MTT solution (5 mg/ml) was added in each well, and the plates were incubated for a further 4 h at 37 °C. Precipitated MTT formazan was dissolved in 40 μl of 20% (w/v) SDS containing 0.01 M HCl for 2 h. Absorbance at 570 nm was measured using a Microplate ELISA reader (Molecular Devices). Cell survival was expressed as a percentage of the ratio of A_{570} of cells treated with peptide to that of cells only.

Measurement of NO Production from LPS-stimulated RAW264.7 Cells

Nitrite accumulation in culture media was used as an indicator of NO production [16]. RAW264.7 macrophages were cultured overnight in a 96-well plate (5×10^5 cells/well). The medium was then removed, followed by the addition to each well of fresh DMEM supplemented with 5% of bovine serum. The cells were stimulated with LPS (20 ng/ml) in the presence (10, 25 and 50 μM) or absence of the peptides. Cells that were stimulated with LPS alone, and untreated cells served as controls. After incubating for 24 h, the amount of NO in the supernatant was estimated from the accumulation of the stable NO metabolite nitrite with Griess reagent according to the manufacturer's instructions (1% sulfanilic acid, 0.1% *N*-1-Naphthylethylenediamine dihydrochloride and 5% phosphoric acid). Absorbance was measured at 540 nm.

Measurement of TNF-α Release from LPS-stimulated RAW264.7 Cells

RAW264.7 cells were cultured overnight in a 96-well plate (5×10^5 cells/well). The cells were stimulated with LPS (20 ng/ml) in the presence (10, 25 and 50 μM) or absence of the peptides. The cells were incubated for 24 h at 37 °C followed by collection of samples of the medium from each well. The TNF-α concentration in each of the samples was evaluated using a mouse TNF-α enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's protocol (R&D Systems, Minneapolis, MN, USA). All experiments were carried out in duplicate.

Results and Discussion

Antimicrobial and Hemolytic Activities

The MICs of *p*VEC and its analogs against Gram-negative and Gram-positive bacteria are shown in Table 2. *p*VEC and its analogs displayed a broad spectrum and potent antimicrobial activity (MIC: 4–16 μM). The hemolytic activity of the peptides against hRBCs was determined by the release of hemoglobin. As shown in Figure 1, *p*VEC and its analogs showed no or little hemolytic activity (less than 10% hemolysis) even at a concentration of 200 μM in some cases.

Table 2. Antimicrobial and hemolytic activities and therapeutic index of *p*VEC and its analogs

Peptides	MIC[a] (μM)						GM[b] (μM)	MHC[c] (μM)	TI[d] (MHC/GM)
	E. coli	*P. aeruginosa*	*S. typhimurium*	*B. subtilis*	*S. epidermidis*	*S. aureus*			
*p*VEC	16	8	4	4	8	4	7.3	>200	54.8
*p*VEC-a1	8	8	4	4	8	4	6.0	200	33.3
*p*VEC-a2	8	8	4	4	8	4	6.0	>200	66.6
*p*VEC-a3	8	8	4	4	8	4	6.0	94	15.7
*p*VEC-a4	8	8	4	4	8	4	6.0	>200	66.6

[a]MIC is defined as the lowest peptide concentration that causes 100% inhibition of microbial growth.
[b]GM of the MIC from six bacterial strains.
[c]MHC is defined as the lowest peptide concentration that produces 5% or more hemolysis.
[d]TI is defined as the ratio MHC/GM. In case of MHC > 200 μM, 400 μM is used for calculation of the TI.

Journal of PeptideScience

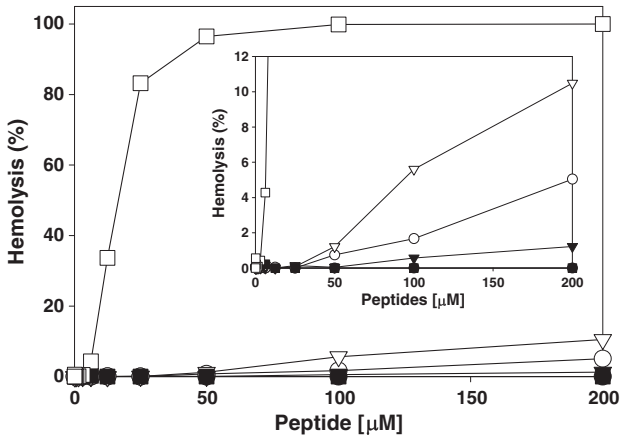

Figure 1. Concentration–response curves of percent hemolysis of *p*VEC and its analogs against human red blood cells. Symbols: *p*VEC (●); *p*VEC-a1 (○); *p*VEC-a2 (▼); *p*VEC-a3 (▽); *p*VEC-a4 (■) and melittin (□).

Cell Selectivity (Therapeutic Index)

To evaluate the peptides for their potential as therapeutic candidates, we evaluated their therapeutic index (TI) values. TI value is useful for relative comparison of cell selectivity or safety between peptides because it represents the balance between antimicrobial and hemolytic activities [17–22]. MHC value was determined as the lowest peptide concentration that produces 5% or more hemolysis. When there was no significant hemolysis at the highest concentration tested (200 μM), 400 μM was used for calculation of the TI because the test was carried out by twofold serial dilutions. TI of *p*VEC and its analogs was calculated as the ratio of the MHC value to the GM (the geometric mean of the MICs of six bacterial strains observed) value (TI = MHC / GM). As shown in Table 2, *p*VEC-a3 showed the lowest TI value because of it having the highest hemolytic activity among peptides. Both *p*VEC-a2 and *p*VEC-a4 exhibited slightly more improved TI value than that of *p*VEC. The order of TI for the peptides follows *p*VEC-a2 = *p*VEC-a4 > *p*VEC > *p*VEC-a1 > *p*VEC-a3. Net charge, α-helicity, hydrophobicity, hydrophobic moment, amphipathicity and polar angle were known as major structural parameters influencing antimicrobial and hemolytic activities of AMPs [23–27]. It was suggested that high hydrophobicity is correlated with increased hemolytic activity and mammalian cell toxicity, whereas antimicrobial activity is less dependent on this parameter [28]. The hydrophobicity of peptides can easily be measured from their RP-HPLC retention time in analytical C_{18} column [28]. A longer retention time is indicative of a higher hydrophobicity. The order of the hydrophobicity of peptides follows *p*VEC-a3 > *p*VEC-a1 > *p*VEC-a2 > *p*VEC > *p*VEC-a4 (Table 1). As expected, *p*VEC-a3 with higher hydrophobicity showed the highest hemolytic activity. It was found that Arg-containing peptide (*p*VEC-a3) is more hydrophobic than its counterpart Lys-containing peptide (*p*VEC-a4). The same result was found in Trp-rich model peptides and tritrpticin analogs [15,29,30]. These results suggest that Lys residues are more effective than Arg residues in the design of novel AMPs with high TI.

Bactericidal Mechanism

Bacterial killing effect of the majority of AMPs is considered to be due to their action on the lipid matrix of bacterial cell membranes, either by forming pore/ion channels, thinning the membrane or disrupting the bilayer (i.e. membrane-targeting AMPs) [31–33]. These cause the lysis of bacterial cells as a result of increased permeability. In contrast, a few AMPs such as buforin 2 and PR-39 were known to penetrate microbial cell membranes without inducing membrane permeabilization and cause bacterial cell death by inhibiting protein, DNA or RNA synthesis (i.e. intracellular-targeting AMPs) [34–36]. *p*VEC was known to enter mammalian and microbial cells and preferentially permeates and kills microbes at a concentration that show no damage to normal human cells [4,5]. This result suggests that the antimicrobial action of *p*VEC may be related to intracellular-targeting mechanism rather than membrane-targeting mechanism.

Therefore, in this study, to investigate the mechanism of antimicrobial activity of *p*VEC and its analogs in more detail, we examined the ability of these peptides to induce depolarization of the cytoplasmic membrane of intact *S. aureus* (Figure 2) and to cause the leakage of a fluorescent dye entrapped within negatively charged PE/PG (7 : 3, w/w) LUVs, which mimics bacterial membranes (Figure 3).

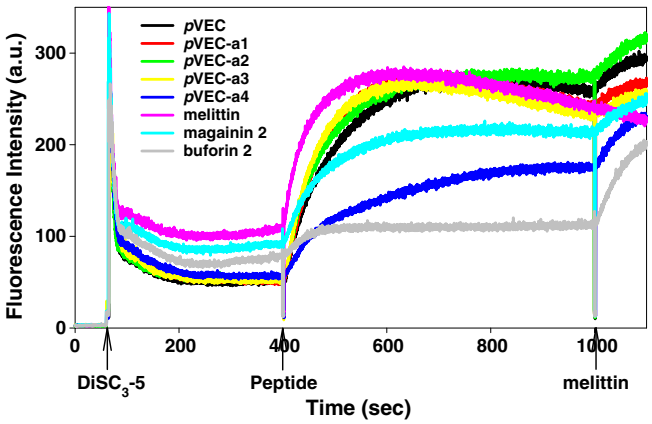

Figure 2. Time-dependent peptide-induced cytoplasmic membrane depolarization against *Staphylococcus aureus* (OD_{600} = 0.05) measured by an increase in fluorescence of the membrane potential-sensitive dye, $DiSC_3$-5. Dye release was monitored at an excitation wavelength of 622 nm and an emission wavelength of 670 nm. In each run, the peptides were added near the 400 s mark (peptide concentration: 4 μM).

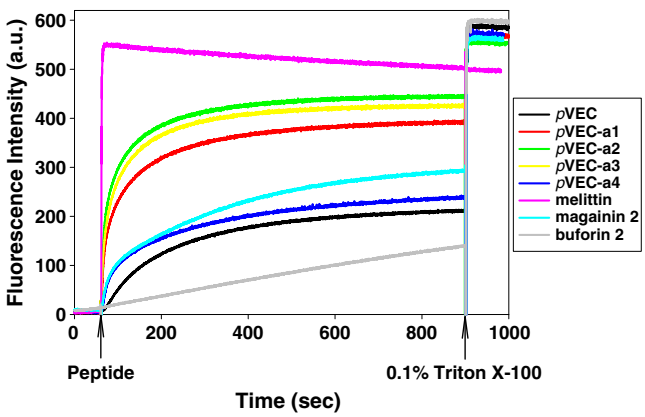

Figure 3. Time-dependent peptide-induced dye leakage from calcein-entrapped negatively charged PE/PG (7 : 3, w/w) LUVs. The concentration of lipid and each peptide was 68 and 1.0 μM, respectively.

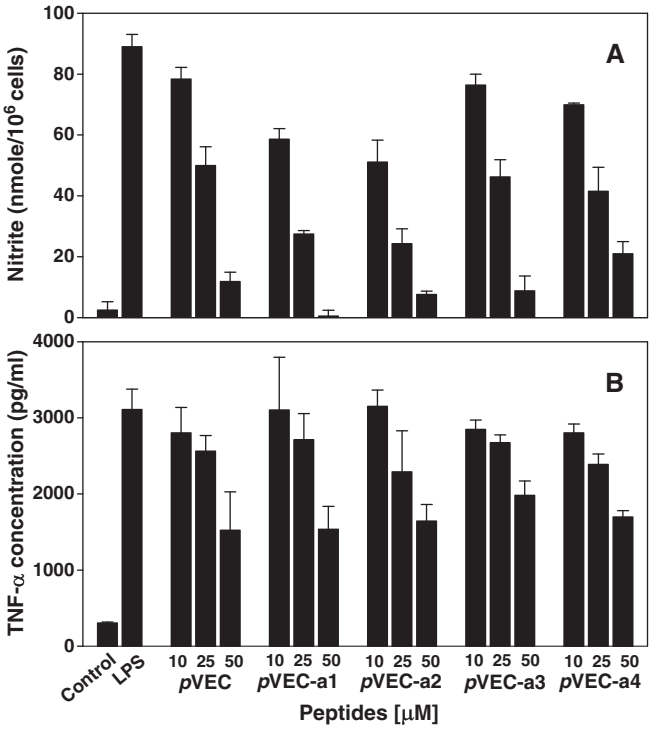

Figure 4. Inhibitory effects of the peptides on LPS-induced nitric oxide production (A) or TNF-α release (B) in RAW264.7 cells. RAW264.7 cells (5×10^5 cells/well) were treated with 20 ng/ml LPS in the absence or presence of various concentrations (10, 25 and 50 μM) of the peptides for 24 h. The error bars represent standard deviations of the mean determined from three independent experiments.

A honey bee-derived AMP, melittin, which has the propensity to disrupt lipid bilayers [37], induced a complete membrane depolarization at MIC (4 μM) against *S. auerus*. As observed in magainin 2, *p*VEC and its analogs led to a near-complete membrane depolarization (>80%). In contrast, buforin 2, the intracellular-targeting AMP caused no or less membrane depolarization. In dye leakage ability from PE/PG (7 : 3, w/w) vesicles, melittin led to a complete dye leakage at a concentration as low as 1 μM (Figure 3). Although much weaker than melittin, *p*VEC and its analogs and magainin 2 showed a significant dye leakage (35–70%). In contrast, buforin 2, induced very little dye leakage. The fluorescence profiles of *p*VEC and its analogs in dye leakage from liposome and membrane depolarization were similar to those of a frog-derived AMP, magainin 2, which has the propensity of a pore/ion channel formation in lipid bilayers [31]. Taken together, these results suggest *p*VEC and its analogs kill microbes via a pore or ion channel formation in the cytoplasmic membrane.

LPS-neutralizing Activity

Sepsis is the major cause of mortality in the intensive care unit, accounting for 200 000 deaths every year in the USA alone [38]. It was demonstrated that release of LPS from antibiotic-treated Gram-negative bacteria can indeed enhance sepsis [39]. Therefore, an effective antimicrobial agent should not only exert antimicrobial activity but also have the ability to neutralize LPS and ameliorate its toxicity. To investigate whether *p*VEC and its analogs might have LPS-neutralizing activity, we assessed their ability to inhibit NO production and TNF-α release in LPS-stimulated mouse macrophage RAW264.7 cells. *p*VEC and its analogs did not show any cytotoxicity against RAW264.7 cells until 100 μM (data not shown). These peptides significantly inhibited NO production or TNF-α release in LPS-stimulated mouse macrophage RAW264.7 cells at 10 to 50 μM, a concentration that RAW264.7 was not damaged (Figure 4).

Collectively, our results suggest that *p*VEC and its analogs kill bacteria by forming a pore or ion channel in the cytoplasmic membrane and can serve as AMPs for the treatment of microbial infection and sepsis.

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