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Research article

The role of polar and facial amphipathic character in determining lipopolysaccharide-binding properties in synthetic cationic peptides

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Two series of peptides, designated K and NK were synthesized and tested for lipid A binding and neutralizing properties. K_2, which has an 11-residue amphiphilic core, and a branched N-terminus bearing two branched lysinyl residues does not bind lipid A, while NK_2, also with an 11-residue amphiphilic core comprised entirely of non-ionizable residues, and a similarly branched, cationic N-terminus, binds lipid A very weakly. Both peptides do not inhibit lipopolysaccharide (LPS) activity in the *Limulus* assay, nor do they inhibit LPS-induced TNF-α and NO production in J774 cells. These results are entirely unlike a homologous peptide with an exclusively hydrophobic core whose LPS-binding and neutralizing properties are very similar to that of polymyxin B [David SA, Awasthi SK, Wiese A *et al*. Characterization of the interactions of a polycationic, amphiphilic, terminally branched oligopeptide with lipid A and lipopolysaccharide from the deep rough mutant of *Salmonella minnesota*. *J Endotoxin Res* 1996; **3**: 369–379]. These data suggest that a clear segregation of charged and apolar domains is crucial in molecules designed for purposes of LPS sequestration and that head-tail (polar) orientation of the cationic/hydrophobic regions is preferable to molecules with mixed or facial cationic/amphipathic character.

Introduction

Endotoxins, or lipopolysaccharides (LPS), structural components of the outer membranes of Gram-negative bacteria,[1] play a pivotal role in the pathogenesis of sepsis syndrome.[2,3] Upon recognition of circulating LPS by a variety of cell types in the body, important among which is the monocyte/macrophage,[4] numerous inflammatory mediators are produced in response. These include TNF-α,[5] IL-1β, and IL-6.[6] Other cells, such as the endothelial cell, produce NO.[7,8] It is the unregulated overproduction of such pro-inflammatory mediators rather than LPS itself[9] that leads to the systemic inflammatory response culminating in the septic shock syndrome.[10] More than 300,000 cases of septic shock occur each year in the US, at least half of which are caused by Gram-negative organisms.[11]

The absence of specific therapeutic modalities for the treatment of septic shock has engendered a variety of experimental approaches, one of which being to target LPS itself, by the use of an agent that would bind to, and sequester, this potent microbial product, thereby preventing its recognition by effector cells. The strategy of sequestering LPS, historically, has been addressed by the use of either polyclonal or monoclonal antibodies raised against the structurally conserved regions of the molecule.[12,13] However, several clinical studies[13–16] have, to date, failed to establish unequivocal clinical value. Macromolecules of non-immunological origin such as

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Abbreviations: CD, circular dichroism; DOSPER, 1,3-di-oleoyloxy-2-(6-carboxyspermyl)-propylamide; IL-1β, interleukin-1β; LAL, *Limulus* amebocyte lysate; LPS, lipopolysaccharide; NO, nitric oxide; PBS, phosphate-buffered saline; PMB, polymyxin B; SDS, sodium dodecyl sulfate; TNF-α, tumor necrosis factor-α

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the neutrophil granule-associated bactericidal/permeability increasing protein (BPI)[17–23] and a *Limulus*-derived protein (endotoxin neutralizing protein)[24–27] are also being evaluated for possible therapeutic effects.

During the last few years, we,[28–32] as well as others,[33–35] have explored the possibility of sequestering LPS using small molecules. The interaction of polymyxin B (PMB), a peptide antibiotic, with LPS and lipid A[36–41] has served as a central paradigm and our strategy has been to first evaluate peptides designed to be functional analogs of PMB, and then utilize the observations in developing non-peptidic small molecules. The relative ease of solid-phase synthesis and the possibility of selectively modulating the physicochemical properties by the introduction of specific functionalities render peptides as valuable test-cases, parallels of which can then be attempted with non-peptide molecules. Several iterations of this approach has allowed us to incrementally refine the heuristics – the rules of thumb – of developing LPS-sequestering agents. Recently, we had reported a peptide (designated G-2) with a short hydrophobic core bearing terminally-branched cationic residues is an effective LPS-sequestrant of low toxicity.[42] Structurally analogous to this peptide are certain members of lipopolyamines, a class of commercially available compounds originally developed for purposes of transfecting DNA into mammalian cells,[43,44] and we have recently shown that the lipopolyamines bind and neutralize LPS potently *in vitro* and *in vivo* and are essentially non-toxic.[45]

The lipopolyamines, while effective and non-toxic, bind LPS with an affinity of approximately one-tenth that of polymyxin B,[45] and in our continuing efforts to further refine the structural correlates in optimal LPS-binding ligands, we now report the characterization of some analogs of G-2 in which the hydrophobic core has been replaced by slightly longer amphipathic segments containing either charged residues interspersed with hydrophobic residues, or containing exclusively residues of varying polarity with non-ionizable side-chains. These studies address the relationship of the mode of amphipathicity – the spatial organization of hydrophobic and cationic/hydrophilic domains – and the LPS-binding properties of these peptides. We show that, for optimal LPS binding, a clear segregation of cationic and hydrophobic domains is mandatory and that peptides with mixed cationic-amphipathic character are unsuitable, and discuss the implications for small-molecule design.

Materials and Methods

Smooth LPS, Re-chemotype LPS and diphosphoryl hexacyl-type lipid A from *Escherichia coli* K12 D31m4 were obtained from List Biologicals (Campbell, CA, USA). Monodansylcadaverine and polymyxin B were from Sigma Chemical Co. (St Louis, MO, USA).

Peptide design, synthesis and characterization

Four peptides, designated K_0, K_1, K_2 and NK_2 were synthesized, whose sequences are shown in Figure 1. All four peptides contain an amphipathic core of 11 amino acids, and the numerical suffix refers to the number of branched lysine (K) residues at the N-terminus (Fig. 1). These peptides are longer than G-2,[42] and yet not long enough to be membrane-active.[46,47] The hydrophobicity profiles of the K and NK peptide cores are shown in Figure 2. The sequences of both peptide cores were designed to maximize the hydrophobic moment,[48–50] a quantitative measure of amphipathicity,[51] by an algorithm which sequentially chooses residues 100° apart in a hydrophobicity polar plot[52] (David SA, manuscript in preparation). In the K-series, two pairs of hydrophilic

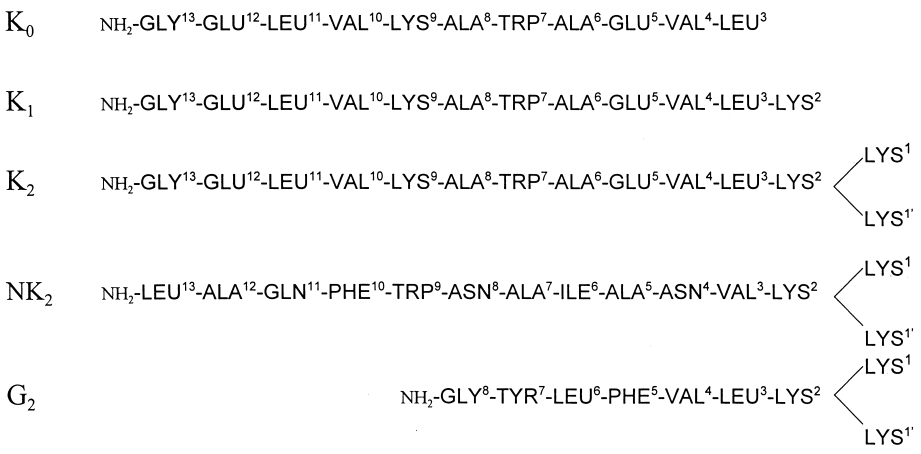

Fig. 1. Sequences of the peptides. Residues are numbered from the N-termini. C-termini are amidated. For comparison, the sequence of G-2[42] is also shown.

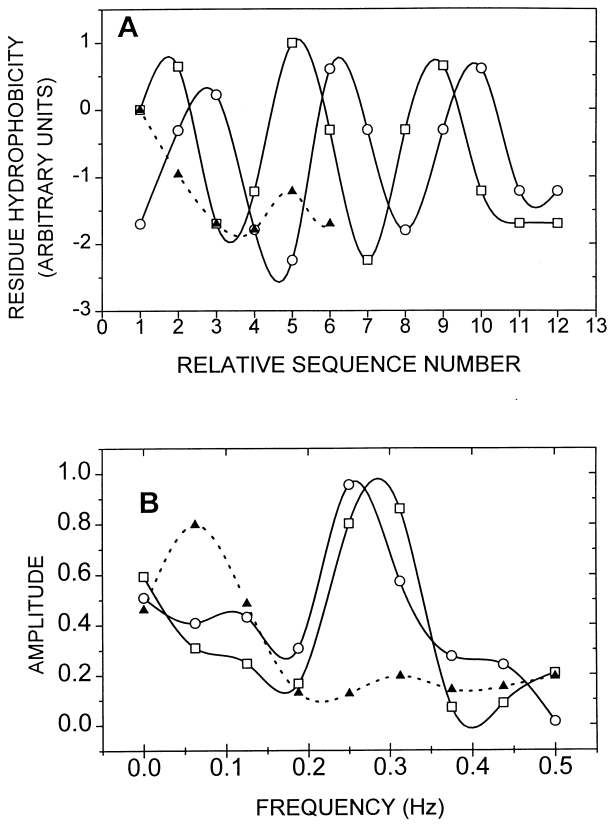

Fig. 2. (**A**) Hydrophobicity profiles of the amphipathic cores of K and NK peptides. The Cornette Scale[50] for residue hydrophobicity is used. The mean hydrophobicity for the K (open squares), NK (open circles) and G-2 (filled triangles) cores are, respectively, –0.678, –0.791, and –1.23 units. (**B**) Discrete Fourier transform of the data points in (A), showing periodicity for K and NK at 0.27 Hz (3.6 residues). The G-2 core[42] lacks this periodicity.

residues at positions *i* and *i* +3 (or 4) are oppositely charged so as to facilitate helical stabilization via ion-pair formation.[53–56] In NK_2, there are no charged residues, and the amphipathicity is achieved only by using residues with non-ionizable side-chains. Both series contain a single tryptophan residue in the middle of the core, designed to serve as an intrinsic probe, in order to facilitate the monitoring of peptide binding via fluorescence spectroscopy. The peptides were synthesized on an LKB Biolynx semi-automated solid-phase synthesizer using conventional Fmoc chemistry as reported earlier.[42] Reverse-phase HPLC on a C_{18} column using a acetonitrile/water/0.1% trifluoroacetic acid gradient indicated a purity of at least 95%. The retention times for K_0, K_1, K_2 and NK_2 were, 35.2, 34.5, 29.2, and 30.2 min, respectively. The peptides were characterized by amino acid analyses, [^{1}H]-NMR, absorption and fluorescence spectroscopy, and verified by mass spectrometry to be of the expected masses (1213.44, 1341.62, 1598.98 and 1646.92 Da, respectively).

Fluorescence spectroscopy

All fluorescence experiments were carried out in 2 mM Tris-HCl, pH 7.2 at 25°C using an Hitachi fluorescence spectrophotometer. Effective bandpasses were 5 nm for both excitation and emission monochromators in all experiments. Monodansylcadaverine was used as a fluorescent displacement probe for quantitating the relative affinities of ligand binding to lipid A or Re-LPS exactly as described earlier.[29–31,37] The effect of lipid A addition on the intrinsic tryptophanyl fluorescence of the peptide was performed at an excitation wavelength of 276 nm and scanning the emission from 320–400 nm. Changes in intensity are reported as $F - F_0/F_0 \times 100$, where F and F_0 are the intensities at maximal emission (362 nm) at a given lipid A concentration, and in the absence of peptide, respectively.

Circular dichroism

CD experiments were performed on a Jasco J2000 spectropolarimeter. Small aliquots of concentrated stock solutions/suspensions of lipid A, smooth LPS, or SDS were added incrementally to the peptide solution (~10 μM in 10 mM PBS, pH 7.2) in a 1 mm path length cuvette, and 10 scans were accumulated and averaged from 190–300 nm at a scan rate of 10 nm/min.

Limulus *amebocyte lysate (LAL) assay*

A quantitative kinetic chromogenic version of the *Limulus* amebocyte lysate assay (QCL-1000) from BioWhittaker (Walkersville, MD, USA) was used. A constant concentration of smooth LPS from *E. coli* K12 D31m4 (50 ng/ml; 50 μl) was mixed with an equal volume of varying concentrations of the peptides (or polymyxin B as control) in endotoxin-free water in a 96-well endotoxin-free microtiter plate. A 0.1 ml aliquot of reconstituted LAL reagent (coagulogen + chromogenic substrate) was then added, and the absorption at 410 nm was monitored continuously for 60 min with a Dynatech MR5000 plate reader equipped with kinetic software. Standard curves were constructed by plotting log LPS concentration against the logarithm of time (min) required to reach a target optical density (arbitrarily taken as 1.0 absorbance unit) and were linear from 0.2–500 ng/ml. The kinetic method thus allowed a much wider dynamic range of quantitation than the more conventional endpoint method (linearity range, 0.5–20 ng/ml). All standards and samples were assayed in quadruplicate, and experiments were carried out at 25°C in order to retard the chromogenic reaction, thereby allowing an acceptable data sampling rate (1 plate read/min).

Cytokine and nitric oxide assays

LPS-responsive murine macrophage-like J774 cells (American Tissue Type Collection, Washington, DC, USA) were seeded in a 96-well tissue culture plate at 5 x 10^5 cells/well. Following overnight culture in RPMI-1640 supplemented with L-glutamine, 10% fetal bovine serum, penicillin and streptomycin, the cells were stimulated for 8 h with LPS alone (smooth LPS from *E. coli* K12 D31m4; 50 ng/ml), or LPS pre-incubated with graded concentrations of the peptides K_2 and NK_2, or polymyxin B (control). Supernatants were harvested and assayed for TNF-α by ELISA (Genzyme, Cambridge, MA, USA). Nitric oxide was measured as nitrite using the Griess reagent.[57]

RESULTS AND DISCUSSION

In the dansylcadaverine fluorescent probe displacement experiments which were employed as an initial biophysical screen, we employed purified diphosphoryl lipid A rather than native smooth LPS since the pronounced heterogeneity of the latter hinders precise quantitation. None of the K-series peptides bind lipid A with appreciable affinity (ED_{50} = 40 μM; Fig. 3) while NK_2 binds with low affinity (ED_{50} = 7.5 μM). The relative binding affinity of polymyxin B, used as control, is about 0.3 μM, consistent with results we had obtained previously.[29,30,37,42] It should be noted that the dansylcadaverine displacement method that we have used is heavily biased toward electrostatic interactions, and is not an adequate descriptor of hydrophobic interactions since the displacement profiles of polymyxin B and its nonapeptide derivative are virtually indistinguishable,[58] while the two compounds behave quite differently in terms of neutralizing LPS activity.[59] The intrinsic tryptophan residue serves as a useful 'reporter' in monitoring ligand interactions and we had previously employed this method in characterizing the binding of lipid A to melittin[28] and human serum albumin.[60] In fluorimetric titrations of lipid A with the peptides, K_0, K_1 and K_2 quench tryptophan emission intensity in a concentration-dependent manner, the degree of quenching being in the order $K_2 > K_1 > K_0$ (Fig. 4). For K_1 and K_2, but not for K_0, inflections are observed at a peptide:lipid A molar ratio of 1:1. However, no shifts in emission wavelength were observed (data not shown). This indicates that the fluorophore does not sense a micro-environment of low polarity in the presence of lipid A,[28] suggesting that the amphiphilic cores of the peptides do not penetrate the lipid A superstructures. While the inflections in the fluorescence intensities suggest a 1:1 peptide:lipid A stoichiometry, the feeble intensity changes and the absence of accompanying blue-shifts suggest that the apparent

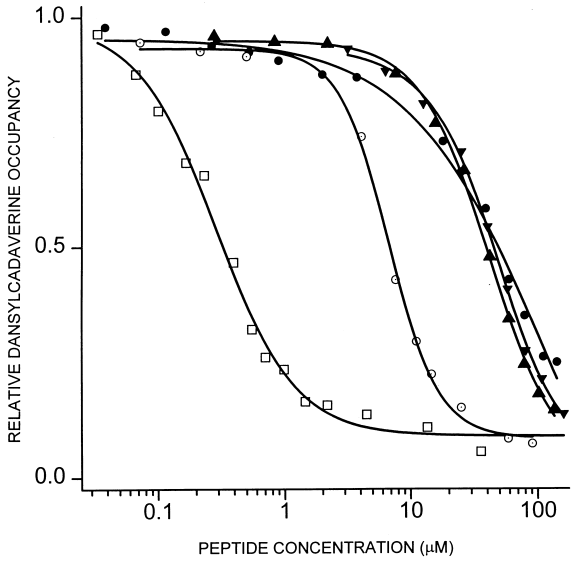

Fig. 3. Relative binding affinities of the peptides. Dansylcadaverine displacement activity. K_0 (inverted filled triangles), K_1 (filled triangles), K_2 (filled circles), NK_2 (open circles), polymyxin B (control; open squares). Lipid A, 25 mM; probe, 10 μM; 2 mM Tris-HCl, pH 7.2.

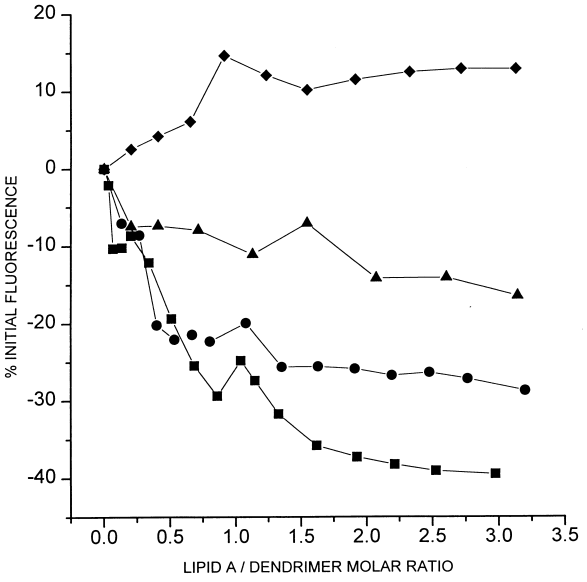

Fig. 4. Tryptophan quenching by lipid A. K_0 (filled triangles), K_1 (filled circles), K_2 (filled squares), NK_2 (filled diamonds). Excitation, 276 nm (5 nm bandpass). Intensity is reported as % change of initial peptide fluorescence (12.5 μM).

'binding' is, in fact, merely an adsorption process, driven by electrostatic attractive forces between the charged double-layer on the lipid A assemblies,[61] and those on N-termini of the peptides. This would be consistent with the observed correlation of quenching potency with charge density ($K_2 > K_1 > K_0$), and the fact

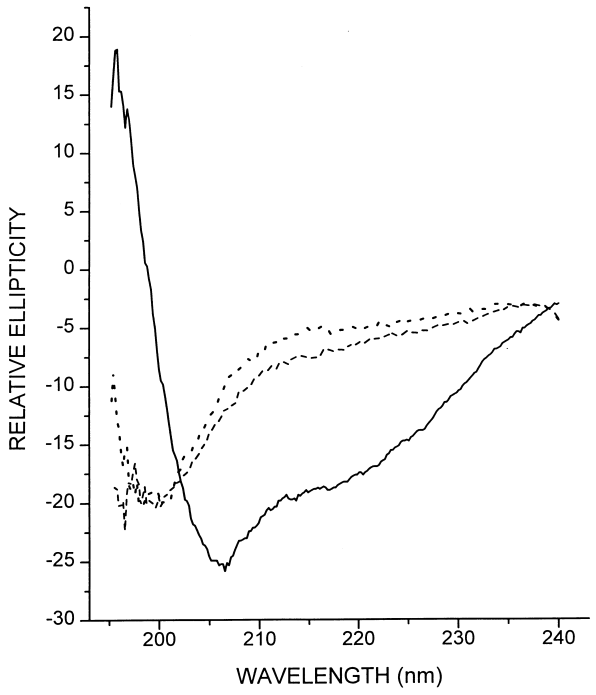

Fig. 5. Representative circular dichroic spectra of 50 μM K_2. In 50 mM PBS, pH 7.2 (dotted line); 50 mM peptide + 125 μM lipid A (dashed line); K_2 (50 μM) in 0.5% SDS (full line), showing a strong induction of ellipticity at 208 nm, indicative of helical structure in the presence of SDS.

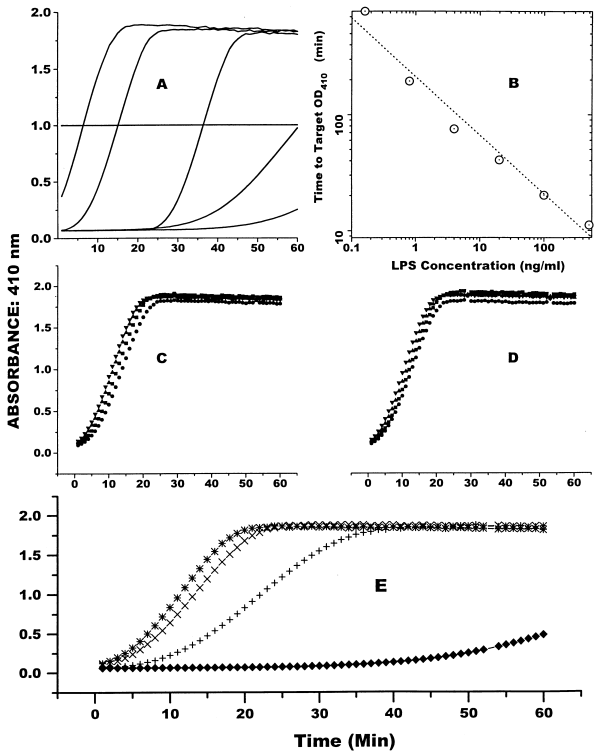

Fig. 6. Chromogenic kinetic *Limulus* assay. (**A**) Rise in OD profiles for endotoxin standards. Curves (left to right) represent chromogenic responses to 500, 100, 20, 2.5, and 0.5 ng/ml of standard LPS. Target OD was arbitrarily set to 1.0 absorbance units. (**B**) Standard curve constructed from the rise in OD profiles shown in (A). *Limulus* activation profiles of LPS (200 ng/ml) incubated with graded concentrations of K_2 (**C**) and NK_2 (**D**). Peptide concentrations in μM: filled circles, 50; filled squares, 12.5; filled triangles, 3.125; inverted filled triangles, 0.78125. (E) Polymyxin B control for experiments in (C) and (D). Polymyxin B concentrations in μM: stars, 0.195; crosses, 0.391; +, 3.125; filled diamonds, 50.

that the quenching is greatly attenuated when titrations are performed at high ionic strength (50 mM NaCl; data not shown). Furthermore, the lack of correspondence between the observed apparent 'stoichiometry' and the number of positive charges in the peptides suggest that the fluorescence data are indicative of a non-specific polyelectrolyte-type adsorption process. The addition of lipid A to NK_2 results in minimal intensity enhancements (Fig. 4), also with an inflection at 1:1 peptide:lipid A molar ratio, and accompanied with small (~5 nm) blue-shifts in emission wavelength, indicative of very weak interactions.

These peptides were designed to be amphiphilic; many such peptides (including melittin[62–64]) although disordered in dilute solutions of low ionic strength, become distinctly helical when bound to ligands of opposite charge. We, therefore, employed circular dichroism to evaluate the effect of lipid A, as well as rough (Re) and smooth LPS on the secondary structure of K_2 and NK_2. Neither peptide undergoes any appreciable secondary structural changes when titrated with lipid A or LPS, but assumes distinct helical conformations in the presence of SDS, indicating that the peptides interact with neither lipid A nor LPS, and that the peptides are, indeed protohelical under appropriate conditions. Figure 5 shows representative CD spectra of K_2. Both K_2 and NK_2 fail to inhibit either LPS-induced LAL activity (Fig. 6), or TNF-α and NO production by J774 cells (Fig. 7).

These results, although 'uninteresting' in that they are negative and do not immediately suggest leads, are instructive. Both K_2 and NK_2 differ from G-2[42] only in that the former peptides possess amphipathic cores while that of G-2 is comprised entirely of hydrophobic residues. While G-2 is as active as polymyxin B in terms of apparent binding constants as well as LPS-inhibitory activities, the new peptides are inactive. The K-series peptides, bearing two anionic residues in the core region do not bind lipid A at all, presumably due to the strongly unfavorable Born energy[65,66] of internalizing the charge in the lipid interior of very low dielectric constant, while NK_2, with no ionizable residues in its core, binds lipid A only feebly, and does not inhibit LPS activity. Similar results have been observed previously with peptides containing repeating Lys–Phe–Phe motifs[35] and random co-polymers of Lys–Leu (unpublished data). These results, therefore, suggest that, for amphipathic molecules designed to bind LPS, a clear segregation of the charged and apolar domains is crucial, and that head-tail (polar) orientation of

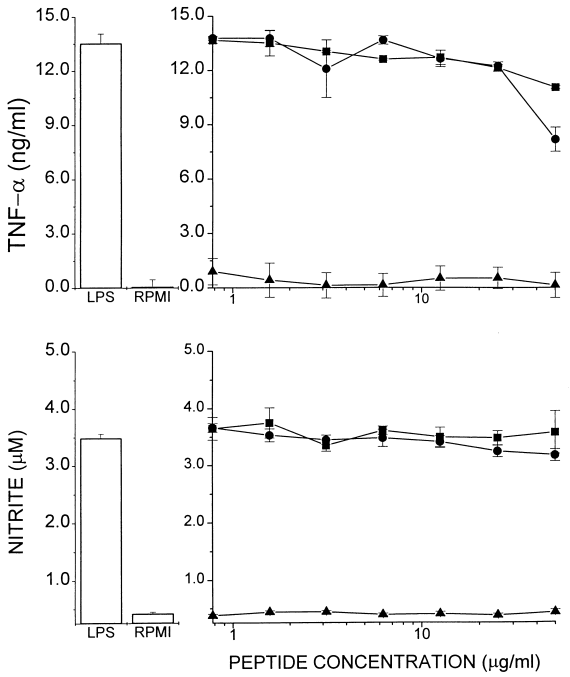

Fig. 7. Effect of K_2 (filled squares) and NK_2 (filled circles) on TNF-α (top panel) and nitric oxide (bottom panel) production in J774 cells stimulated with 50 ng/ml LPS for 20 h in the presence of graded doses of the peptides. Polymyxin B (control; filled triangles). Bar graphs on left indicate LPS-alone and medium-alone internal controls. Error bars correspond to SD calculated on quadruplicates.

the cationic/hydrophobic regions is preferable to molecules with mixed cationic/amphipathic character. An example of the former ('polar amphipaths') would be certain members of the lipopolyamine class which have been shown to sequester LPS and neutralize its activities both *in vitro* and *in vivo*.[45] An example of a cationic amphipath with non-segregated hydrophobic/cationic character would be squalamine[67,68] and some of its analogs.[69,70] Squalamine was first isolated as a broad-spectrum antimicrobial principle from the stomach of the dogfish shark,[67] and has subsequently spawned a great deal of interest in steroidal-polyamine conjugates. We have been interested in evaluating these molecules as candidate LPS-sequestering agents because of their cationic/amphipathic character. The results obtained from the studies reported in this paper, however, suggest that squalamine may not be a potent LPS-binding agent because of the sulfonate moiety which renders it zwitterionic. Several analogs of squalamine and related steroidal amines have been synthesized in which both the polyamine appendage and the steroid backbone have been systematically modified.[69,71,72] Of particular interest are the latter analogs some of which, lacking the sulfonate moiety, have very distinct 'facial' amphipathic backbones arising as a consequence of the stereochemistry of hydrophilic substituents.[69] Based on these results, it would appear that such molecules may not be attractive candidates as LPS-sequestrants. Some of these molecules are becoming available and offer a direct means of testing this hypothesis. Such studies would be useful in extending and refining our efforts aimed at rationally developing non-toxic, clinically useful anti-LPS molecules.

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