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Effects of Two Magainin Peptides on Eicosanoid Release from Rat Peritoneal Macrophages

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Magainins are novel polycationic peptides with broad-spectrum antimicrobial activity, including activity against gram-negative bacteria. Gram-negative bacteremia can elicit endotoxic shock that is associated with the increased formation of eicosanoids. Inhibition of eicosanoid synthesis has been shown to improve the outcome of experimental endotoxic shock. The aim of the present study was to test the in vitro effects of two magainin peptides, MSI-97 (M1) and MSI-98 (M2), on eicosanoid synthesis by rat peritoneal macrophages (Mϕ) stimulated by *Salmonella enteritidis* lipopolysaccharide (LPS; 50 μg/ml) and *Salmonella minnesota* lipid A (5 μg/ml) and to compare their effects on LPS reactivity with a metachromatic dye. M1 (100 μg/ml) significantly ($P < 0.05$) reduced LPS-stimulated synthesis of thromboxane B_2 (TXB_2), without changing 6-keto-prostaglandin $F_{1\alpha}$ in Mϕ. Similarly, M2 (10 μg/ml) significantly attenuated Mϕ synthesis of TXB_2 stimulated by either LPS or lipid A. However, at a higher concentration (100 μg/ml), M2 but not M1 significantly augmented LPS-induced increases in TXB_2 and 6-keto-prostaglandin $F_{1\alpha}$. Polymyxin B (40 μg/ml) inhibited LPS production and lipid A-stimulated TXB_2 production. M1 (100 μg/ml) and polymyxin B (10 and 40 μg/ml) also inhibited calcium ionophore A23187 (10 μM)-induced synthesis of TXB_2. The lipid A moiety of LPS reacts with dimethylmethylene blue dye, providing a metachromatic assay of LPS. This metachromatic reaction with lipid A was significantly reduced by polymyxin B and M2 at all concentrations. M1 was effective only at the highest M1:lipid A concentration ratio (2:1). Thus, M1 and M2 share some similarities with polymyxin B in inhibiting lipid A reactivity with the dye, which suggests that these magainins may also bind to lipid A. However, M1 was devoid of any inhibitory effects on dye reactivity with *S. enteritidis* LPS and M2 was inhibitory at only one concentration ratio (1:5). In conclusion, the varied effects of the magainin peptides on LPS, lipid A, and Mϕ eicosanoid synthesis appear to depend on the type of peptide used and on its concentration.

Despite aggressive therapeutic interventions including the use of specific antibiotics, the mortality rate from septic shock is approximately 50% (6). Treatment of gram-negative bacterial sepsis is complicated by the increasing occurrence of antibiotic-resistant bacterial strains and the systemic release of free endotoxin following antibiotic-induced lysis of gram-negative bacteria (21). The use of antibiotics with both bactericidal and antiendotoxin activities may provide a more effective strategy for the treatment of severe gram-negative sepsis.

Magainins are a family of novel peptides with a broad range of antimicrobial activity against gram-negative and gram-positive bacteria (2, 22, 29). These peptides were originally isolated from the skin of the African clawed frog (*Xenopus laevis*). The most important natural peptides have been designated magainins 1 and 2, each consisting of a 23-amino-acid sequence, with molecular weights of 2,407 and 2,464, respectively (22). Several peptide analogs have now been synthesized (3, 29); these peptide analogs mimic the activities of magainins 1 and 2 but display greater selective toxicities and potencies to bacteria than do the natural compounds. Selective toxicities and potential therapeutic applications are supported by evidence that the concentrations of magainins that produce hemolysis of human erythrocytes are one to two orders of magnitude greater than the doses that produce optimum antimicrobial effects (5). The mechanisms of the antimicrobial activities of magainins remain to be fully characterized, but studies suggest that magainins are membrane active and interact readily with acidic phospholipids (14).

Polymyxins are another class of peptide polycationic antibiotics that may share some characteristics with magainins. These antibiotics affect gram-negative bacteria by disrupting their membranes and increasing cell wall permeability. Polymyxin B has been shown to be protective in experimental gram-negative sepsis (8). An additional beneficial action of polymyxin B is its ability to bind stoichiometrically to the lipid A moiety of bacterial lipopolysaccharide (LPS) (16) and inhibit its biological actions, including stimulation of arachidonic acid metabolism (12).

Macrophages (Mϕ) are major producers of eicosanoids, such as thromboxane and prostaglandins, which play an important role as mediators of endotoxic and septic shock (6, 13). The aim of the present study was to determine the effects of two magainin compounds on endotoxin-induced in vitro synthesis of thromboxane B_2 (TXB_2) and 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) by Mϕ. The calcium ionophore A23187, a potent stimulator of TXB_2 and 6-keto-$PGF_{1\alpha}$ synthesis by Mϕ, was used as a nonspecific stimulus. The results obtained with the magainins were compared with those obtained with polymyxin B, which was used as a positive control. Since polymyxin B also has been shown to inhibit LPS reactivity with the dye dimethylmethylene blue (DMB) by binding to lipid A (11), we also studied the effects

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of the magainins as well as those of polymyxin B on the LPS and lipid A reactivities with this metachromatic dye.

MATERIALS AND METHODS

In vitro experiments. Male Long-Evans rats (weight, 250 to 300 g) were used as a source of Mϕ, which were removed by peritoneal lavage with RPMI 1640 medium (GIBCO, Grand Island, N.Y.) with L-glutamine containing penicillin (50 U/ml), streptomycin (50 μg/ml), and sodium heparin (10 U/ml). The cell suspension was kept on ice and the Mϕ were counted, adjusted to a concentration of 10^6 cells per ml, and plated onto six-well plates (flat-bottom, 35-mm-width well) at 2 ml of cell suspension per well. After 2 h of incubation at 37°C in 95% room air–5% CO_2, nonadherent cells were removed by rinsing (three times) the plate wells with sterile 5% glucose.

The magainin peptides MSI-97 (Gly-Ile-Gly-Lys-Phe-Leu-His-Ser-Ala-Gly-Lys-Phe-Gly-Lys-Ala-Phe-Val-Lys-Ile-Met-Lys-Ser-NH_2), designated M1, and MSI-98 (Gly–Ile–Gly–D-Lys–D-Phe–Leu–His–Ser–Ala–D-Lys–D-Lys–D-Phe–Gly–D-Lys–Ala–D-Phe–Val–Gly–Glu–Ile–Met–Asn–Ser–NH_2), designated M2, were supplied by Magainin Sciences Inc., Plymouth Meeting, Pa. The magainins were dissolved in RPMI 1640 (with antibiotics) at a concentration of 1 mg/ml immediately before their use and were then diluted in RPMI 1640 to a final concentration of 100 or 10 μg/ml for in vitro incubation. Concentrations of the magainins much greater than 100 μg/ml were not used because of reduced cellular viability as determined by trypan blue exclusion. The calcium ionophore A23187 (Calbiochem, La Jolla, Calif.) was freshly prepared at a concentration of 10 μM. *Salmonella enteritidis* LPS (Boivin extraction; lot 764190; Difco Laboratories, Detroit, Mich.) was dissolved in sterile 5% glucose at a concentration of 500 μg/ml. Lipid A from *Salmonella minnesota* Re 595 (lot 703882; Calbiochem, La Jolla, Calif.) was dissolved in sterile 5% glucose containing 0.5% triethylamine at a concentration of 50 μg/ml. Polymyxin B sulfate (Sigma Chemical Co., St. Louis, Mo.) was dissolved in dimethyl sulfoxide and further diluted in RPMI 1640 (100 or 400 μg/ml) immediately before use.

Ten minutes before the addition to the cells, each magainin solution was mixed with an equal volume of A23187, LPS, or lipid A solutions; an appropriate amount of the mixture was added to each well in order to have a final 1:10 dilution of these solutions. The final concentrations of polymyxin B (10 and 40 μg/ml) were selected on the basis of the previously reported inhibitory doses in vitro (12).

After 3 h of incubation, the medium from each well was collected separately, the samples were centrifuged, and the supernatants were saved and stored at −20°C for assay. TXB_2 and 6-keto-$PGF_{1\alpha}$ were measured by radioimmunoassay as described previously (4, 27). Cellular viability was determined by trypan blue exclusion and was ascertained to be >95%.

Metachromatic reaction. Polymyxin B has been reported to bind to the lipid A region of LPS and to produce a reduction in the metachromatic reaction between LPS and DMB dye, measured as the decrease in the optical density (OD) (at 535 nm) of the reaction mixture (11). DMB was obtained from Serva, Heidelberg, Germany, and the DMB reagent was prepared as reported previously (11). *S. enteritidis* LPS was dissolved and diluted in sterile water for injection, USP (Travenol Laboratories, Deerfield, Ill.). *S. minnesota* lipid A was dissolved and diluted in 0.5% triethylamine. Polymyxin B sulfate (lot 9N1374; Burroughs Wellcome Co., Research Triangle Park, N.C.) was dissolved and diluted in sterile water. Magainin peptides were dissolved and diluted in sterile water.

Serial dilutions of magainins or polymyxin B were incubated for 180 min at 37°C in a sterile Falcon 24-well culture plate (Becton & Dickinson, Oxnard, Calif.) with *S. enteritidis* LPS or *S. minnesota* lipid A at concentration ratios of 1:50, 1:5, and 2:1 (compound:LPS or compound:lipid A). Control incubations were combinations of magainin or polymyxin B solvent (sterile water) and *S. enteritidis* LPS or *S. minnesota* lipid A or combinations of magainin or polymyxin B plus LPS solvent (sterile water) or lipid A solvent (0.5% triethylamine). After 180 min, 0.1-ml samples were taken from each well and tested for DMB-reactive material (11).

Experiments were carried out in triplicate. The value of the OD (at 535 nm) of magainin plus sterile water or magainin plus 0.5% triethylamine was subtracted from the corresponding OD value of magainin plus LPS or magainin plus lipid A. The resulting values were averaged and compared with the OD values of magainins without LPS or lipid A. The same schedule was followed for the experiments with polymyxin B and LPS or lipid A.

Data were expressed as means ± standard errors of the mean and were subjected to analysis of variance. Significant differences between experimental groups were determined by using the Fisher protected least-squares difference test, unless specified otherwise. A P value of less than 0.05 was considered significant.

RESULTS

The lower concentration of M1 (10 μg/ml) induced a modest but significant increase in the *S. minnesota* lipid A-induced TXB_2 synthesis, but it had no effect on basal level-, ionophore-, or LPS-induced TXB_2 synthesis (Fig. 1A). M1 (100 μg/ml) significantly ($P < 0.05$ versus control) stimulated basal level-induced TXB_2 synthesis but reduced LPS-induced TXB_2 synthesis by rat Mϕ. A23187-induced TXB_2 production was also reduced by M1 (Fig. 1B). M1 (100 μg/ml) did not change the *S. enteritidis* LPS-induced increase in 6-keto-$PGF_{1\alpha}$. M1 (100 μg/ml) significantly ($P < 0.05$) increased basal levels but decreased A23187-induced synthesis of the 6-keto-$PGF_{1\alpha}$ (Fig. 1C).

M2 (10 μg/ml) (Fig. 2A) slightly reduced the basal level-induced but not the A23187-induced TXB_2 synthesis. However, this concentration of M2 reduced ($P < 0.05$) both the *S. enteritidis* LPS-stimulated and the *S. minnesota* lipid A-induced increase in TXB_2 levels. M2 (100 μg/ml) significantly ($P < 0.05$) increased *S. enteritidis* LPS-induced TXB_2 and 6-keto-$PGF_{1\alpha}$ synthesis; however, it did not affect the basal level-induced or the A23187-induced synthesis of these eicosanoids (Fig. 2B and C).

Polymyxin B (40 μg/ml) significantly ($P < 0.05$) decreased the basal level-, A23187-, and *S. enteritidis* LPS- or *S. minnesota* lipid A (Fig. 3A)-induced TXB_2 synthesis. The lower concentration of polymyxin B (10 μg/ml) significantly ($P < 0.05$) reduced the A23187-induced TXB_2 increase, but it had no effect on either basal level- or *S. enteritidis* LPS-induced TXB_2 or 6-keto-$PGF_{1\alpha}$ values (Fig. 3B and C).

Since magainins and polymyxin B share several chemical and biological characteristics, magainins might also form a complex with LPS and reduce the metachromatic reaction between LPS and DMB. Both M1 (1:5 and 2:1 concentration ratios) and M2 (all concentration ratios) inhibited the metachromatic reactivity of lipid A as determined by OD changes (Table 1). M1 had no effect on LPS reactivity with DMB, and

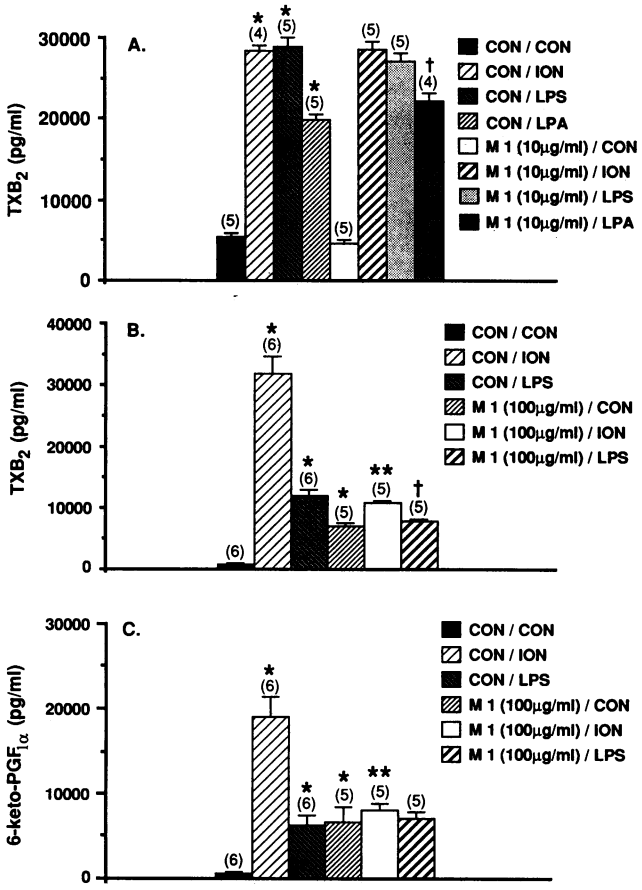

FIG. 1. (A) Effect of M1 (10 μg/ml) on calcium ionophore A23187 (ION; 1 μM)-, *S. enteritidis* LPS (50 μg/ml)-, and *S. minnesota* lipid A (LPA; 5 μg/ml)-stimulated rat peritoneal Mϕ synthesis of TXB_2. Mϕ were incubated for 3 h in RPMI 1640 medium (CON/CON) or in RPMI 1640 containing A23187 (CON/ION), LPS (CON/LPS), LPA (CON/LPA), M1 (M1/CON), M1 plus A23187 (M1/ION), M1 plus LPS (M1/LPS), or M1 plus LPA (M1/LPA). Values in parentheses are number of experiments. Data are expressed as means ± standard errors of the means. *, $P < 0.05$ versus CON/CON; †, $P < 0.05$ versus CON/LPA. (B) Effect of M1 (100 μg/ml) on A23187 (ION; 1 μM)- and LPS (50 μg/ml)-stimulated in vitro rat peritoneal Mϕ synthesis of TXB_2. For details, see legend for panel A. *, $P < 0.05$ versus CON/CON; **, $P < 0.05$ versus CON/ION; †, $P < 0.05$ versus CON/LPS. (C) Effect of M1 (100 μg/ml) on A23187 (ION; 1 μM)- and LPS (50 μg/ml)- stimulated in vitro rat peritoneal Mϕ synthesis of 6-keto-$PGF_{1\alpha}$. For details see legend for panel A. *, $P < 0.05$ versus CON/CON; **, $P < 0.05$ versus CON/ION.

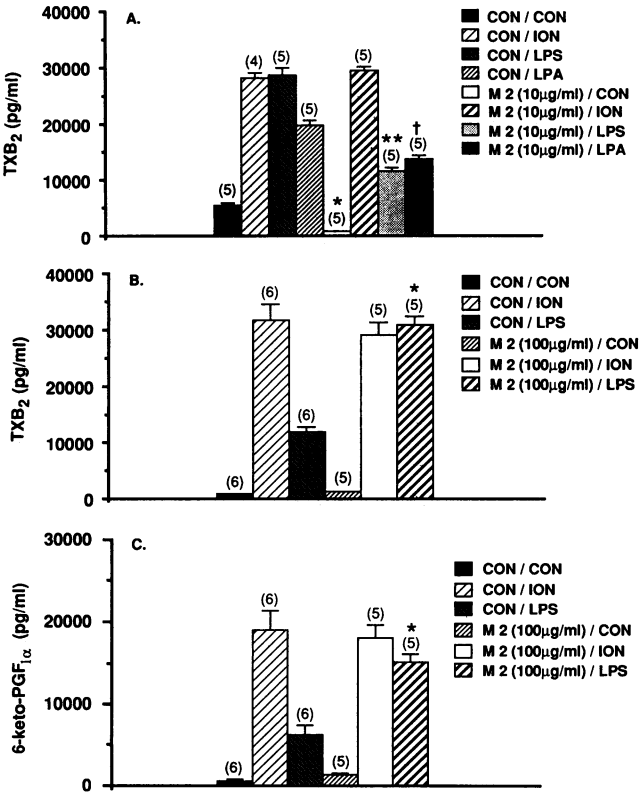

FIG. 2. (A) Effect of M2 (10 μg/ml) on calcium ionophore A23187 (ION; 1 μM)-, *S. enteritidis* LPS (50 μg/ml)-, and *S. minnesota* lipid A (LPA; 5 μg/ml)-stimulated in vitro rat peritoneal Mϕ synthesis of TXB_2. *, $P < 0.05$ versus CON/CON. Values in parentheses are number of experiments. **, $P < 0.05$ versus CON/LPS; †, $P < 0.05$ versus CON/LPA. (B) Effect of M2 (100 μg/ml) on calcium ionophore A23187 (ION; 1 μM)- and *S. enteritidis* LPS (50 μg/ml)-stimulated in vitro rat peritoneal Mϕ synthesis of TXB_2. Values in parentheses are number of experiments. *, $P < 0.05$ versus CON/LPS. (C) Effect of M2 (100 μg/ml) on calcium ionophore A23187 (ION; 1 μM)- and *S. enteritidis* LPS (50 μg/ml)-stimulated in vitro rat peritoneal Mϕ synthesis of 6-keto-$PGF_{1\alpha}$. Values in parentheses are number of experiments. *, $P < 0.05$ versus CON/LPS. See legend to Fig. 1A for abbreviation definitions.

M2 had an effect on LPS metachromatic reactivity only at the 1:5 concentration ratio. Polymyxin B at all concentration ratios showed a significant ($P < 0.05$) decrease in lipid A metachromatic reactivity, and at 1:5 and 2:1 concentration ratios, it reduced LPS metachromatic reactivity.

DISCUSSION

The present study demonstrated that the magainin peptides can significantly influence the metabolism of arachidonic acid to TXB_2 and 6-keto-$PGF_{1\alpha}$ by Mϕ. The mechanism(s) of these effects is uncertain. Previous studies have shown that polymyxin B binds stoichiometrically to lipid A (16) and inhibits lipid A stimulation of arachidonic acid metabolism (12). Synthetic magainin peptides also bind to *Salmonella typhimurium* (18, 19). Thus, one notion investigated in the present study was that the inhibitory effects of polymyxin B and magainins on LPS- and lipid A-induced eicosanoid synthesis may be due to LPS and/or lipid A binding to the magainins. The reactivity of the DMB metachromatic dye with LPS or lipid A was variably inhibited by M1, M2, and polymyxin B. M1 had no effect on the metachromatic reactivity of LPS, and M2 had an effect on the metachromatic reactivity of LPS only at a 1:5 concentration ratio. Polymyxin B blocked the metachromatic activity of LPS at the two highest concentrations. In contrast to LPS, the three compounds with the exception of the lowest M1 concentration ratio blocked the metachromatic reactivity of lipid A. The less effective inhibition of the metachromatic reactivity of LPS by magainins might reflect greater steric hindrance with intact LPS as opposed to that with purified lipid A. These composite observations also suggest that the effects of M1 and M2 on LPS-induced TXB_2 synthesis cannot be explained solely by the binding of magainins to

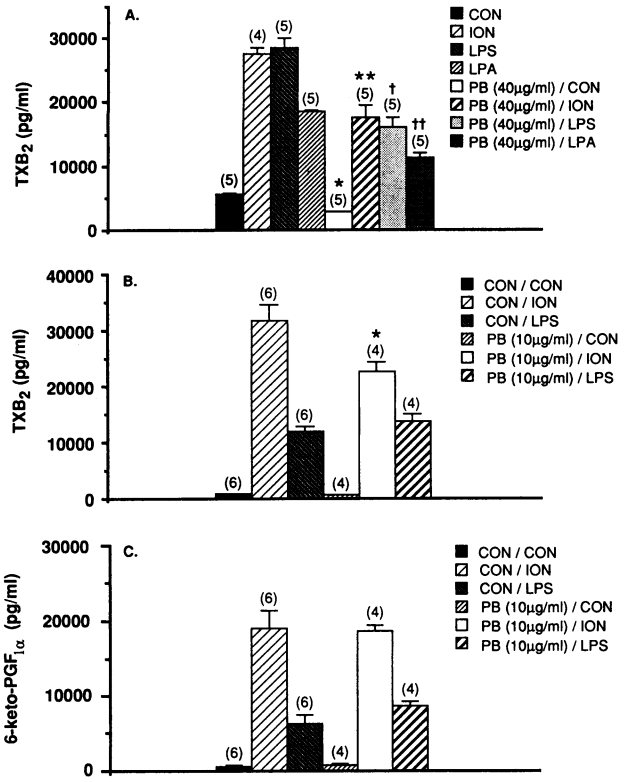

FIG. 3. (A) Effect of polymyxin B (PB; 40 μg/ml) on calcium ionophore A23187 (ION; 1 μM)-, *S. enteritidis* LPS (50 μg/ml)-, and *S. minnesota* lipid A (LPA; 5 μg/ml)-stimulated in vitro rat peritoneal Mϕ synthesis of TXB_2. *, $P < 0.05$ versus Mϕ incubated in RPMI 1640 medium (CON/CON). Values in parentheses are number of experiments. **, $P < 0.05$ versus CON/ION; †, $P < 0.05$ versus CON/LPS; ††, $P < 0.05$ versus CON/LPA. (B) Effect of polymyxin B (PB; 10 μg/ml) on calcium ionophore A23187 (ION; 1 μM)-, *S. enteritidis* LPS (50 μg/ml)-, and *S. minnesota* lipid A (LPA; 5 μg/ml)-stimulated in vitro rat peritoneal Mϕ synthesis of TXB_2. Values in parentheses are number of experiments. *, $P < 0.05$ versus CON/ION. (C) Effect of polymyxin B (PB; 10 μg/ml) on calcium ionophore A23187 (ION; 1 μM)-, *S. enteritidis* LPS (50 μg/ml)-, and *S. minnesota* lipid A (LPA; 5 μg/ml)-stimulated in vitro rat peritoneal Mϕ synthesis of 6-keto-$PGF_{1\alpha}$. Values in parentheses are number of experiments. See legend to Fig. 1A for abbreviation definitions.

LPS. M2 (10 μg/ml) decreased LPS- and lipid A-induced but not A23187-stimulated TXB_2 synthesis. M1 (100 μg/ml) also inhibited LPS-induced TXB_2 synthesis, but this effect was not specific for LPS since A23187-induced TXB_2 synthesis was also inhibited. Polymyxin B (40 μg/ml), like M1, also inhibited LPS- and A23187-induced TXB_2.

It appears that the effects of the magainins on eicosanoid synthesis are due to something other than direct complexing with LPS. The magainins may inhibit the enzymes necessary for metabolism of arachidonic acid to eicosanoids. Protein kinase C has been shown to be involved in the activation of arachidonic acid metabolism by LPS (20). LPS-induced synthesis of TXB_2 in Mϕ cultures is decreased by the protein kinase C inhibitors H7, staurosporine (9), and polymyxin B (15, 28). Therefore, since magainins share some of the same physical-chemical properties as polymyxin B, they may reduce Mϕ eicosanoid synthesis by inhibiting protein kinase C activity. Indeed, certain synthetic analogs of the magainins have recently been shown to be potent inhibitors of protein kinase C in the rat brain (17).

TABLE 1. Effects of M1, M2, or polymyxin B on the metachromatic reactivity of *S. enteritidis* LPS and *S. minnesota* lipid A with DMB

Compound	Concn ratio[a]	OD at 535 nm[b]	
		S. enteritidis LPS	*S. minnesota* lipid A
Sterile water		0.168 ± 0.003	0.210 ± 0.025
M1	1:50	0.155 ± 0.018	0.202 ± 0.012
M1	1:5	0.165 ± 0.008	0.143 ± 0.002[c]
M1	2:1	0.140 ± 0.010	0.033 ± 0.025[c]
M2	1:50	0.155 ± 0.007	0.153 ± 0.012[c]
M2	1:5	0.082 ± 0.008[d]	0.050 ± 0.020[c]
M2	2:1	0.148 ± 0.015	0.003 ± 0.006[c]
PB[e]	1:50	0.168 ± 0.010	0.158 ± 0.007[c]
PB	1:5	0.108 ± 0.009[d]	0.028 ± 0.002[c]
PB	2:1	0.067 ± 0.006[d]	0.003 ± 0.021[c]

[a] Concentration ratio of compound:LPS or compound:lipid A.
[b] Data are means ± standard errors of the means.
[c] $P < 0.05$ versus lipid A plus sterile water by the Fisher protected least-squares difference test.
[d] $P < 0.05$ versus LPS plus sterile water by the Fisher protected least-squares difference test.
[e] PB, polymyxin B.

Magainins also affect membrane ion fluxes (30), which could directly or indirectly influence eicosanoid production. Magainin antimicrobial activity has been suggested to occur as a result of membrane anion channel formation, which alters membrane potential and function (7, 10). Thus, in addition to protein kinase C inhibition, the altered calcium ionophore stimulation of TXB_2 synthesis by M1 could be a result of altered membrane ion conductances. Other metabolic effects of the magainins include disruption of respiratory control in isolated liver mitochondria (25). The potential impact of these metabolic changes on arachidonic acid metabolism is complex and likely multifactorial.

In contrast to the inhibitory effects of M2 (10 μg/ml) and M1 (100 μg/ml), M2 (100 μg/ml) augmented LPS-induced eicosanoid synthesis. The mechanisms for the latter effect are uncertain. The effects of nonspecific priming on LPS cell activation and augmented eicosanoid metabolism have been demonstrated with other agents, e.g., phorbol esters and ionophores (1, 20).

We have no direct evidence regarding the antimicrobial activity of M1 used in the present study. The primary structure of this peptide is very similar to that of the originally discovered magainin 1 (29). However, the 18th and 19th amino acids (Gly and Glu) have been deleted and replaced with one lysine residue. A reduction from 23 to 22 amino acids on the peptide carboxyl terminus did not reduce substantially the antibacterial activity of magainin (30), while the insertion of extra lysine residues has been reported to increase the antibacterial activity of magainin. Therefore, M1 antibacterial activity should be no lower than that reported for the originally discovered magainin. MICs range between 100 and 500 μg/ml for gram-negative organisms (5). The antimicrobial activity of M2, which is the [D-Lys4,10,11, 14, D-Phe5,12,16]-magainin 2 carboxy-terminal amide, has been reported to be substantially reduced compared with that of the originally discovered magainin 2, with MICs of about 1,000 μg/ml (22). However, all-D magainins, when compared with their all-L enantiomers, showed much longer

half-lives because of their resistance to enzymatic degradation (24).

In conclusion, our data demonstrate that magainin peptides can affect endotoxin-induced Mϕ eicosanoid metabolism in vitro, depending on the type and concentration of the peptide. The results of the present study suggest that magainins not only directly affect lipid A but may also alter Mϕ responses to LPS. Because of the potential use of magainins as antimicrobial agents, further studies on their cellular mechanisms of action are warranted.

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