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Molecular Mechanisms of Pathogenesis, Host Resistance, and Therapy

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NATURAL AND SYNTHETIC POLYPEPTIDES THAT RECOGNIZE THE CONSERVED LIPID A BINDING SITE OF LIPOPOLYSACCHARIDES

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Bacterial endotoxins (lipopolysaccharides, LPS) induce a variety of biological activities through lipid A (Rietschel E.Th. and Galanos C., 1977), the glycolipid structure which is conserved among LPS originating from different Gram-negative bacteria (Luderitz O. et al, 1978), by interacting with receptor proteins present in the blood and tissues of mammalians. Detoxification of LPS in mice by the peptide antibiotic polymyxin B (PMXB) was a concept first introduced in the mid sixties (Rifkind D., 1967) but the molecular basis of this observation have been provided only a decade later by the demonstration that PMXB binds to lipid A stoichiometrically (Morrison D.C. and Jacobs D.M., 1976). Since then, a huge body of literature has been produced on the efficacy of PMXB in various models of endotoxemic animals, although the human use of this peptide antibiotic has been always limited by its high toxicity. In fact, PMXB contains uncommon amino acids like D-phenylalanine and α,γ-diaminobutyric acid (about 50 % w/w of the structure) which can replace the natural homolog L-lysine in protein synthesis by cultured cells (Christensen H.N and Liang M., 1966).

PMXB diffuses to tissues (Craig W.A. et al., 1974), it is not biodegradable by serine proteases like trypsin and chymotrypsin and as consequence it accumulates in kidneys and in nervous tissues.

We have recently explained on molecular basis the structural features of PMXB that permit binding to lipid A and the consequent detoxification, by using synthetic peptides (SAEP) mimicking the primary and secondary structure of PMXB (Rustici A. et al., 1993). Binding takes place through a preliminary interaction of the lysine or arginine residues of the peptide structure with the anionic phosphate groups of lipid A, but stability of the complex requires hydrophobic interactions, possibly involving the fatty acid residues of lipid A, the hydrophobic amino acids and the alchyl chain of lysine and arginine residues. These conclusions are drawn from the following experimental observations: anionic residues of aspártic and glutamic acid cannot replace the cationic lysine and arginine residues in peptide structures; the complex is stable in a broad range of pH (2-11) and ionic strength (0.01-0.5 M); the primary amino groups of lysine are still reactive to specific reagents after complex formation; the complex is dissociated by treatment with 1% (w/v) SDS. PMXB-related synthetic peptides have shown anti-LPS activity comparable to PMXB in vitro and in endotoxemic mice and rabbits (Rustici A. et al., 1993; Velucchi M. et al., 1994) with the capability to diffuse to tissues and significantly inhibit systemic and local LPS-induced TNF release in target organs like liver, lungs and spleen (Demitri M.T. et al., 1996).

Understanding the physical chemical features required by peptide structures to interact and detoxify the lipid A binding site of LPS has allowed our group to develop an algorithm for predicting similar amino acid sequences in the primary structure of natural polypeptides known to specifically bind LPS (Porro M., 1994 a,b). The algorithm predicts that any cyclic or linear sequence encompassing a minimum of six to seven amino acid residues, respectively, containing a minimum of three aliphatic cationic amino acids with solvent parameter values (Levitt M., 1976) $\geq +1.5$ Kcal/mole (lysine and arginine) and hydrophobic amino acids with solvent parameter values ≥ -1.5 Kcal/mole (tryptophan, phenylalanine, tyrosine, leucine, isoleucine and valine), characterized by the value Rcat/hyd (ratio between the number of cationic versus the number of hydrophobic amino acids) equal to or greater than 1, has high probability to interact by high affinity with the lipid A binding site of LPS. To test the hypothesis, we have therefore searched in the primary amino acid structure of well established LPS-binding proteins, amino acid sequences fulfilling the three requirements of the algorithm. Once found, the candidate anti-LPS sequences have been synthesized and used in a variety of assays for showing binding to lipid A and neutralization of the toxic effects of LPS in vitro and in vivo. The first set of LPS-binding proteins considered, have included phylogenetically different proteins carrying out different biological functions like CD14 (the 55 kD LPS receptor of immunocompetent cells), LBP (the 60 kD LPS binding protein present in plasma), BPI (the 55 kD bactericidal permeability-increasing protein), LALF (the 15 kD factor of Limulus amebocytes) and LEBP-PI (the 12 kD Limulus endotoxin-binding protein-protease inhibitor). In each protein we have found at least one candidate amino acid sequence fulfilling all requirements of the algorithm and the corresponding synthetic peptides have shown the capability to bind and detoxify LPS in various assays (Velucchi M. et al., 1994 ; Porro M., 1994 a,b).

We have therefore applied the algorithm to other natural polypeptides of the animal kingdom with established antibiotic activities, in order to investigate on molecular basis their possible role as anti-LPS molecules. This became of evident interest when direct antibiotic activity as well as synergistic activity with hydrophobic antibiotics, like rifampin and erythromycin, were found to be properties of anti-LPS synthetic peptides (Vaara M. and Porro M., 1996). We have predicted a number of amino acid sequences fulfilling the three criteria of the algorithm in the primary structure of Cecropin A (aa sequence 1-11), Melittin (aa sequence 16-24), Magainin (aa sequence 4-14) and Rabbit Cationic Protein CAP18 (aa sequence 107-125). Another protein which is of relevant interest in the elucidation of the molecular basis of its interaction with the lipid A moiety of LPS is tubulin, the globular protein that forms microtubules in the cytoskeleton and that has been reported to bind LPS (Ding A. et al., 1992). In the primary structure of tubulin, the algorithm predicts seven amino acid sequences fulfilling all the requirements, specifically the amino acid sequence 156-166, 287-299, 335-345, 362-373, 381-390, 393-401 and 408-415. Our laboratory is currently investigating all the above predicted peptides for binding and neutralization of LPS.

Quite recently, we became interested in another category of natural polypeptides which could potentially interact with the lipid A moiety of LPS: the opioid-related peptides. Our interest was based on the observations that lipid A is a pyrogenic molecule with somnogenic activity (Cady A.B. et al., 1989) and that LPS induces, through lipid A, the permeability of the blood-brain barrier (Quagliarello V. et al., 1992), the release of proinflammatory cytokines in the brain tissue (Faggioni R. et al., 1995) and produces effects mediated by opioid peptides (Yirmiya R. et al., 1994). On this regard, it is important to note that the sensation of pain follows the activation of nociceptors, primary sensory neurons located on cells that are targets for opioids and are specialized to detect tissue damage in the peripheric and in the central nervous system (Taddese A. et al., 1995). Nociceptin or Orphanin FQ is a new heptadecapeptide discovered in the brain tissue of mammalians, which resembles the opioid peptide dynorphin A and acts as an endogenous agonist of the Opioid Receptor-Like 1 (ORL_1), a new G-protein coupled receptor (Meunier J.C. et al., 1995; Reinsheid R.K. et al., 1995). ORL_1 is an orphan receptor, resembling opioid receptors, whose cDNA has been identified in brain tissue of humans and mice. Nociceptin increases reactivity to pain by stimulating the ORL_1 receptor, a process that can be therefore defined as nociceptive stimulation of a nociceptor.

We have found that the peptide nociceptin is the one that, within the family of dynorphin-related peptides, fulfils all the criteria required by the algorithm for a peptide sequence efficiently binding lipid A and resulting in its detoxification. We have therefore tested this hypothesis by comparing nociceptin with SAEP in a variety of "in vitro" and "in vivo" assays for neutralization of LPS activity.

Binding competition at the equilibrium (Rustici A. et al., 1993) showed that nociceptin competed efficiently with SAEP for the lipid A binding site of LPS (Figure 1).

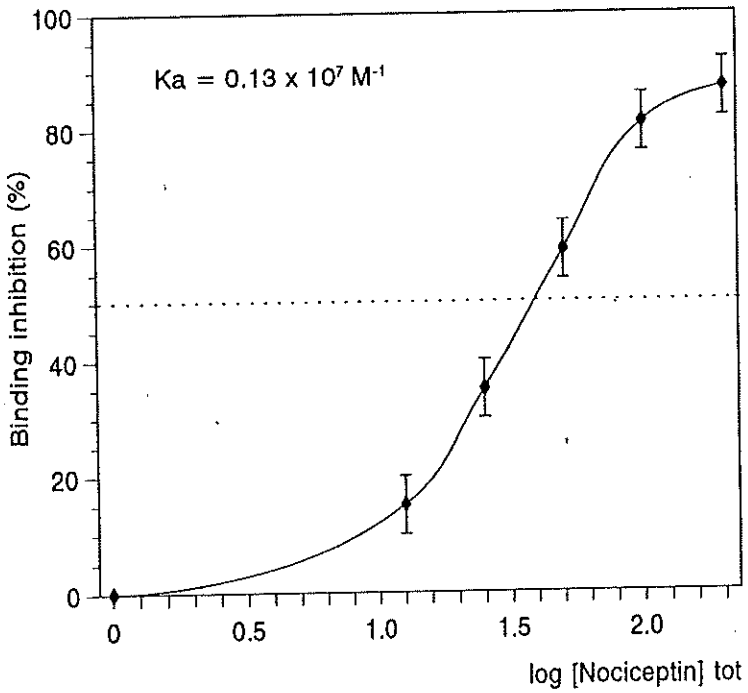
Figure 1. Binding competition of nociceptin with SAEP-6 for E.coli B5 LPS (Sigma). The affinity constant value of nociceptin for B5 LPS was calculated from the concentration (μM) of the peptide displacing 50% of SAEP-6 from LPS complexes (selectivity). Sequences and structural features of the peptides show their relative cationic (+) and hydrophobic (*) amino acids. Peptides were synthesized on solid phase and characterized according to the described methods (Rustici A. et al., 1993).

* * + + + + *
Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-Leu-Ala-Asn-Gln-OH

Nociceptin
(Rcat/hyd = 1.33)

* + + + * * + +
Ile-Lys-Thr-Lys-Lys-Phe-Leu-Lys-Lys-Thr-OH

SAEP-6
(Rcat/hyd = 1.67)



This observation was confirmed by the competitive inhibition of the lipid A-induced clotting of Limulus amebocyte lysate (LAL), the most specific and sensitive enzymatic reaction for LPS detection, where the biological activity of purified Escherichia coli B5 (EC5) LPS at 80 pg/ml was inhibited by preincubation with 80 ng (40 pmoles)/ml of nociceptin (Table 1).

Table 1. Competitive inhibition of LPS-induced LAL clot by nociceptin.

	CLOT
LPS E. coli EC5 80 pg/ml	Positive
LPS E. coli EC5 80 pg/ml+Nociceptin 800 pg/ml	Positive
LPS E. coli EC5 80 pg/ml+Nociceptin 8 ng/ml	Positive
LPS E. coli EC5 80 pg/ml+Nociceptin 80 ng/ml	Negative

When administered in vivo at appropriate stoichiometry with respect to a challenge of high and low dose LPS, nociceptin inhibited the lipid A-induced, cytokine-mediated, local hemorragic necrosis in the skin of rabbits (local Shwartzman reaction) as well as the systemic release of tumor necrosis factor (TNF) in the serum of mice (Figure 2, Table 2). In contrast, the dynorphin-related peptide alpha-endorphin, a pro-opioimelanocortin-derived peptide whose amino acid sequence does not meet the criteria of the algorithm for binding lipid A, did not show inhibitory activity in any of the assays performed .

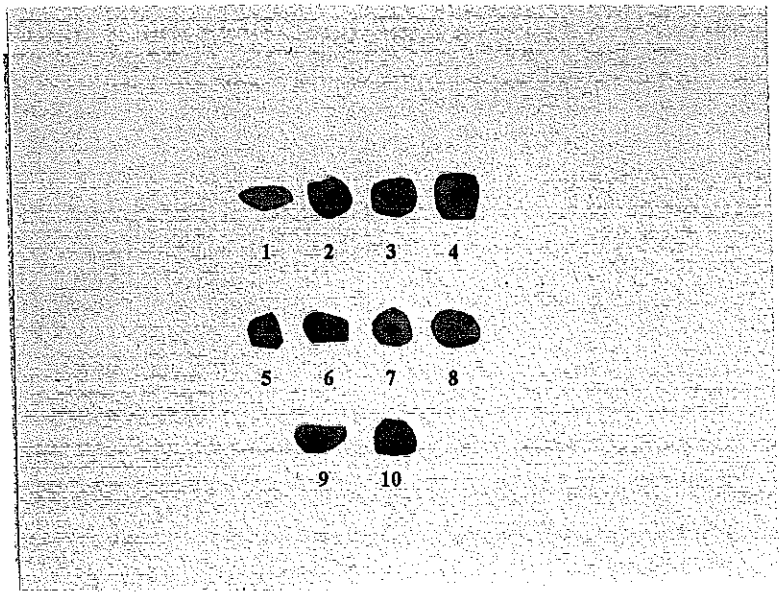


Figure 2. Inhibition of lipid A-induced local hemorragic necrosis in the skin of rabbits by nociceptin, performed in the conditions previously reported (Velucchi M. et al., 1994). Groups of three New Zealand white rabbits were intradermally injected with high-dose Salmonella minnesota Re595 LPS and Neisseria meningitidis A1 LPS alone or in complex with nociceptin at different stoichiometry, along with controls. 72-96 hours after injection, animals were observed for local lesions.
Saline (0.2 ml), 1. Re595 LPS at the doses of 2.5, 5 and 10 μg (0.2 ml), 2, 3 and 4. The selected dose of LPS for inhibition experiments was 5 μg, injected at a ratio 1:250 (w/w) with the various peptides and corresponding to a peptide dose in the range 0.5-1 μmole. LPS injected with SAEP-2 (Rustici A. et al., 1993) or SAEP-6 as positive control, 5; LPS injected with the non-neutralizing peptide 4 (Rustici A. et al, 1993) as negative control, 6; LPS injected with alfa-endorphin (Sigma Chemical Co., St. Louis, MO), 7; LPS injected with nociceptin, 8. Neisseria meningitidis A1 LPS (5 μg/dose) injected with nociceptin respectively at 1:100 and 1:250 w/w, 9 and 10. A1 LPS induced dermal lesions of comparable size to those induced by comparable doses of Re LPS.

Table 2. Inhibition of systemic TNF release in the serum of mice by nociceptin.
Groups of five CD1 mice each, were challenged i.v. by low-dose E.coli B5 (EC5) LPS (100 ng/mouse) 10 min. after an intravenous administration of 500 μg (250 nmoles)/mouse of peptide or saline as control. Animals were bled 90 min. after LPS administration and serum TNF of each mouse was titered by bioassay (Aggarwal B.B. et al., 1985) and reported as mean ± SD. Statistical significance was calculated by "t" test. Data are representative of several independent experiments.

	Serum TNF (pg/ml)	% inhibition (p value)
LPS	4,129 ± 775	
Nociceptin + LPS (-10 min.)	1,874 ± 615	55 ($p<0.01$)

TNF was also significantly inhibited in "in vitro" experiments when peritoneal macrophages were incubated with LPS in the presence of nociceptin, while no inhibitory effect was observed when pre-incubation of nociceptin with macrophages was followed by washing with saline before LPS was added to the system (Table 3).

Table 3. Effect of nociceptin on TNF production in mouse peritoneal macrophages. Nociceptin at the concentration of 100 μg/ml was preincubated with macrophage cells in serum-free D-MEM medium (Galli G and Fratelli M., 1993), 10 min at 37 °C, and then E.coli B5 LPS at 1 μg/ml was added. In order to control the specificity of the results, in a set of experiments macrophage cells preincubated with nociceptin were washed with saline before LPS was added. Then, for all experiments, incubation followed for 4 hours at 37 °C and the produced TNF was titered by bioassay (Aggarwal B.B. et al., 1985) along with controls. The results are expressed as mean ± SD of several independent experiments. Statistical significance was calculated by "t" test.

	TNF (pg/ml)	% inhibition (p value)
LPS	2,306± 373	
Nociceptin + LPS	1,007± 305	56 ($p<0.01$)
Nociceptin + washing + LPS	2,800± 450	n.s.

Altogether these findings suggest that the opioid-related peptide nociceptin might be a potential target of LPS in the central and peripheric nervous system of mammalians. Its capability to interact with LPS, through lipid A, might work as a recognition system alerting the host's defenses on the basis of an imbalance in the equilibrium nociceptin/nociceptor, therefore serving as a physiological defense against the early biological effects which follow an LPS insult. Specific studies are clearly necessary to test this hypothesis.

In conclusion, the results of this study suggest that LPS-binding polypeptides having different biological functions and originating from species phylogenetically different may use a homogeneous recognition system based on amino acid sequences which bind to lipid A, the structurally conserved region of LPS. These amino acid sequences seem reasonably predictable on the basis of an algorithm whose criteria have been postulated from physical chemical considerations of the structures of PMXB-related synthetic peptides. At least in aqueous solvents, the binding thermodynamic and the size of the peptide sequences recognized by lipid A parallel a basic property of the immune system in the Ag-Ab recognition mechanism, that is they are about the size of epitopes recognized by monospecific antibodies (Cygler M. et al., 1991 ; Nnalue N.A. et al.,

1992) and by human major histocompatibility complex class I molecules (Parker K.C. et al., 1992).

Although our results originate from sequences of peptides with and without secondary rearrangement selected from the primary structure of natural polypeptides, the variety of peptide structures studied suggests that their physical chemical features may also represent the general target of lipid A in the conformationally rearranged structure of proteins.

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