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MINI-REVIEW

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Molecular mechanisms of endotoxin activity

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Abstract Endotoxin (lipopolysaccharide, LPS), a constituent of the outer membrane of the cell wall of gram-negative bacteria, exerts a wide variety of biological effects in humans. This review focuses on the molecular mechanisms underlying these activities and discusses structure-function relationships of the endotoxin molecule, its interaction with humoral and cellular receptors involved in cell activation, and transmembrane and intracellular signal transduction pathways.

Key words Endotoxin · Lipopolysaccharide · CD14, LPS-binding protein

Abbreviations *GPI* Glycosyl phosphatidylinositol · *HDL* High-density lipoproteins · *IL* Interleukin · *KDO* 2-Keto-3-deoxyoctonic acid · *LPS* Lipopolysaccharide · *dLPS* Deacylated LPS · *LBP* LPS-binding protein · *MAPK* Mitogen-activated protein kinase · *PTK* Protein tyrosine kinase · *TNF* Tumor necrosis factor

Introduction

About a century ago Richard Pfeiffer used heat-inactivated lysates of *Vibrio cholerae* bacteria to induce a range of pathophysiological reactions in guinea pigs (Pfeiffer 1892). He named the toxic principle “endotoxin” to distinguish it from the then already well-known heat-sensitive proteinaceous exotoxins.

Since then endotoxin has attracted the attention of many scientists and has been studied intensively. Chemically, endotoxins constitute lipopolysaccharides, which are present in the outer cell wall of all gram-negative bacteria. Today the terms endotoxin and lipopolysaccharide (LPS) are used synonymously.

Mammals, including humans, are in permanent contact with gram-negative bacteria and their LPS. Low doses of LPS are thought to be beneficial for the host, e.g., in causing immunostimulation and enhanced resistance to infections and malignancies (Vogel and Hogan 1990). On the other hand, the presence of a large amount of LPS in the bloodstream, as observed during severe gram-negative bacterial infections (notably after application of antibiotics) or as caused by translocation of enterobacteria from the gut, leads to dramatic pathophysiological reactions such as fever, leukopenia, tachycardia, tachypnea, hypotension, disseminated intravascular coagulation, and multi-organ failure. The resulting septic shock syndrome has a mortality rate of about 20–50% and causes 100,000 deaths annually in the USA (Nogare 1991). The obvious biomedical significance of endotoxin explains the still expanding scientific interest in this molecule, particularly in its chemical structure and in the mechanisms of its biological action.

Endotoxin exerts its profound biological effects in an indirect manner. It stimulates host cells (mainly monocytes/macrophages, but also endothelial cells, smooth muscle cells, and neutrophils) to produce and release endogenous mediators. These include bioactive lipids (e.g., platelet-activating factor and thromboxane A_2), reduced oxygen species (e.g., NO), and, in particular, proteins such as interleukin-1(IL-1), IL-6, and tumor necrosis factor alpha (TNFα; Fig. 1). The presence of high amounts of LPS leads to the release of these mediators in large quantities, resulting in the described pathophysiological reactions.

This review concentrates on the problem of how LPS stimulates cells. The structural requirements for the expression of LPS toxicity are discussed, as well as the interaction of LPS with humoral binding proteins and cellular receptors and the intracellular events leading to cell activation.

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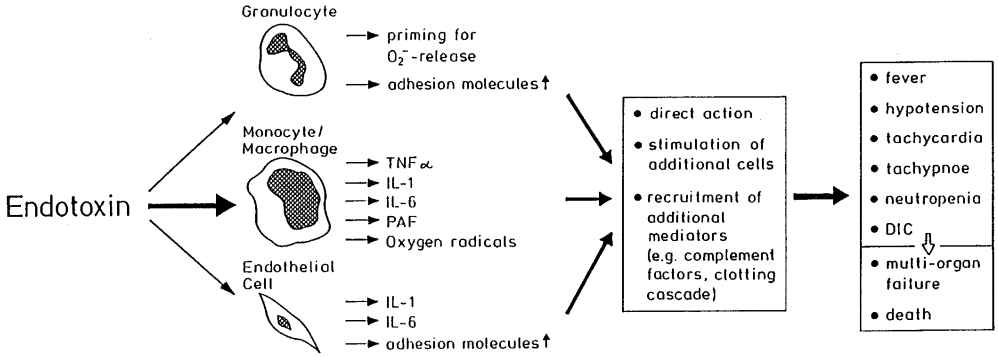


Fig. 1 Scheme of the mode of action of LPS in the pathogenesis of septic shock. *DIC* disseminated intravasal coagulation

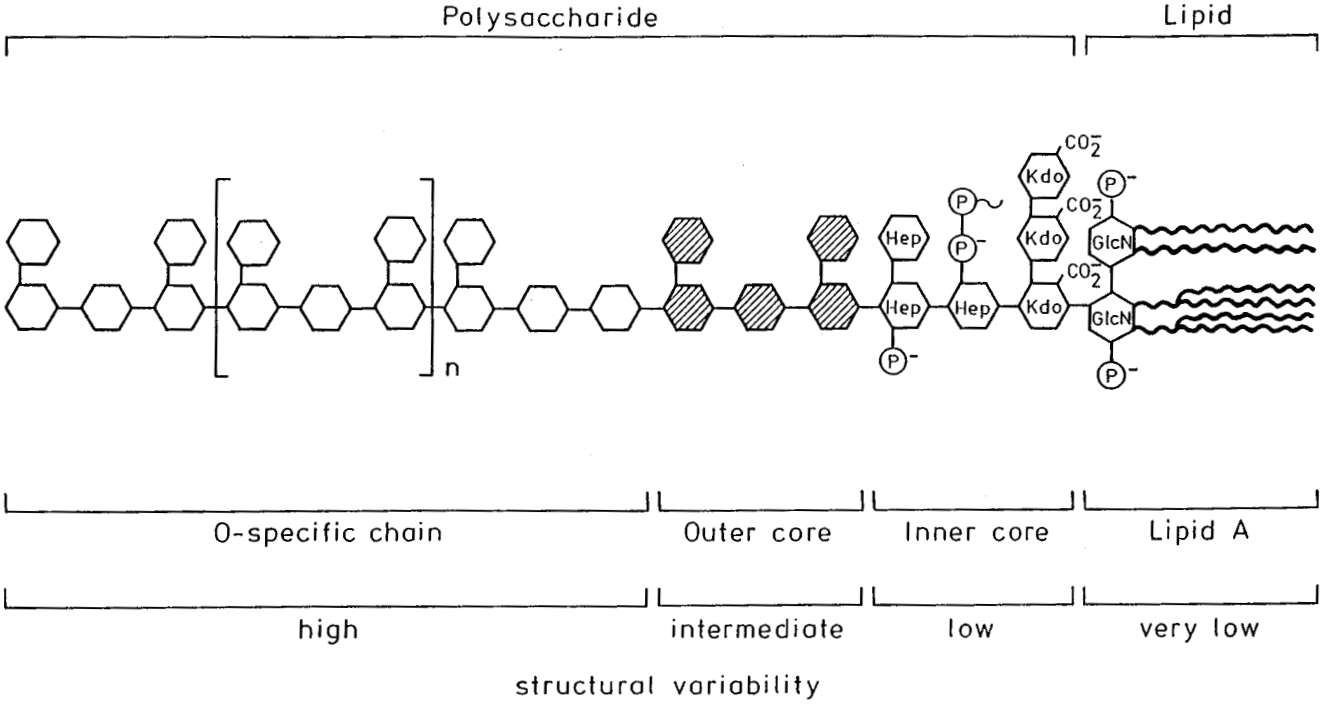
The LPS molecule – structural requirements for bioactivity

A scheme of the principal structure of an enterobacterial LPS is shown in Fig. 2. As the term lipopolysaccharide implies, endotoxin consists of a lipid and a polysaccharide portion.

The polysaccharide part comprises the O-specific chain and the core. The O-specific chain, consisting of up to 50 repeating oligosaccharide units, is characteristic and unique for each bacterial strain and highly antigenic. It determines the serological specificity, i.e., the bacterial serotype. The core oligosaccharide displays far less diversity. Notably, the inner core is structurally rather conserved as the majority of gram-negative bacteria contain a heptose residue and at least one Kdo (2-keto-3-deoxyoctonic acid) group in this region. The lipid part, termed lipid A, constitutes the least variable portion. Not all bacterial strains express LPS having the complete polysaccharide region shown in Fig. 2, but they all contain lipid A. The minimal LPS structure required for the viability of gram-negative bacteria consists of lipid A carrying one Kdo residue (Helander et al. 1988).

The lipid A component has been identified as the LPS component responsible for endotoxic activity (Galanos et al. 1985), i.e., it constitutes the minimal structure that expresses the same biological properties as LPS. This has been proven with LPS-derived "natural" polysaccharide-free lipid A and also with a chemically synthesized counterpart (Galanos et al. 1985). Figure 3a shows the structure of *Escherichia coli*-type lipid A (Zähringer et al. 1994), which is able to stimulate human monocytes to release cytokine in doses comparable to that of *E. coli* LPS. In order to explore the structure-function relationships, synthetic lipid A partial structures differing, for example,

Fig. 2 Schematic chemical structure of an enterobacterial lipopolysaccharide. *Hep* heptose, *Kdo* 2-keto-3-deoxyoctonic acid, *GlcN* N-acetyl glucosamine, *P* phosphate. Modified after Rietschel et al. (1994)



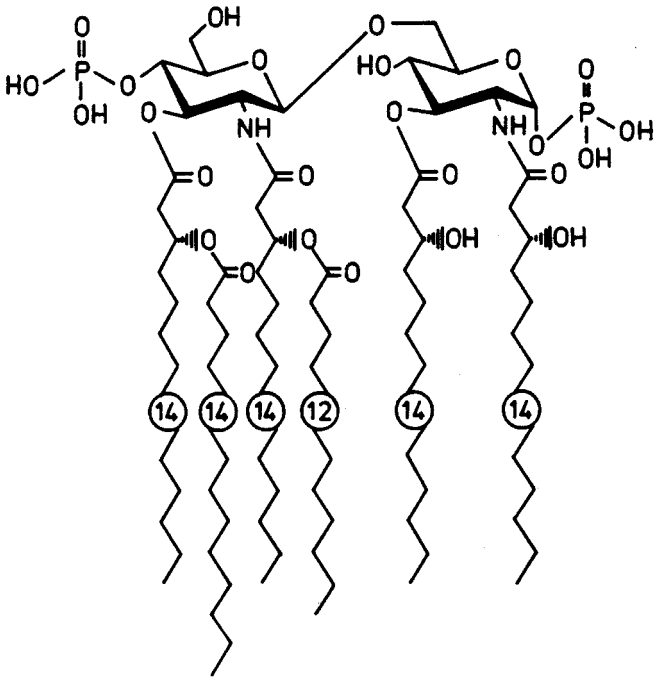


Fig. 3 Primary chemical structure of *Escherichia coli*-type lipid A. Modified after Zähringer et al. (1994)

in their acylation or phosphorylation patterns have been biologically analyzed. Changes in the chemical composition lead to changes in biological activity, which in some cases are dramatic. Any alteration of the hydrophilic backbone (monophosphoryl/monosaccharide structures) or in the hydrophobic part (deletion, addition, or dislocation of fatty acids) results in partial or total loss of activity (Rietschel et al. 1994).

For example, the tetraacylated compound 406, which differs from lipid A by the absence of the two secondary fatty acids, completely lacks cytokine-inducing capacity in human cells (Loppnow et al. 1989; Ulmer et al. 1992). Nevertheless, this compound is able to inhibit the binding of labelled LPS to cells specifically (Ulmer et al. 1992; Heine et al. 1994a). This inhibition is dose-dependent and can be overcome by excess LPS. The data of LPS binding in the presence and absence of the inhibitor plotted in a Lineweaver-Burk plot yield two regression lines, both of which cross at the *Y*-axis (Heine et al. 1994a). These data led to the conclusion that the inhibition is based on competetive binding of compound 406. However, Kitchens and Munford (1995) came to somewhat different results when they used enzymatically deacylated LPS (dLPS) bearing tetraacyl lipid A to antagonize LPS binding to the THP-1 human monocyte cell line. The binding of LPS to the receptor CD14 (see below) was only blocked at high concentrations of dLPS (100 ng/ml). At low dLPS concentrations (1 ng/ml), LPS-induced IL-8 release was inhibited without blocking of LPS-CD14 binding, suggesting that dLPS antagonizes LPS at a different site.

Obviously, for the induction of cytokine release of cells two different preconditions have to be fulfilled: (1) specific binding to a receptor and (2) capacity for subsequent cell activation. Distinct changes in the lipid A structure result in the creation of lipid A/LPS antagonists such as compound 406, which lacks cell activation capacity despite unchanged binding capacity. This is further supported by the fact that the naturally occurring and endotoxically inactive lipid A species of *Rhodobacter sphaeroides* and *R. capsulatus*, which differ from *E. coli* lipid A in the acylation pattern, have also been shown to work antagonistically in LPS-induced cell activation (Loppnow et al. 1990; Qureshi et al. 1991). Based on the structure of *R. capsulatus* lipid A, a compound named E5531 has been synthesized, which acts as a potent LPS antagonist in vitro and in vivo (Christ et al. 1995).

In conclusion, for the expression of maximal biological activity a particular molecular structure of the lipid A is required. The resulting three-dimensional structure of the lipid A molecule has been elucidated by X-ray diffraction (Seydel et al. 1994). This peculiar conformation of lipid A was termed the "endotoxic conformation." Notably, the endotoxically completely inactive lipid A of *R. capsulatus* possesses a different conformation (Brandenburg et al. 1993), which is likely to resemble that of compound 406.

These stringent structural requirements point toward a highly specific interaction of LPS/lipid A with humoral and/or cellular binding structures. Recently, it has been shown that not the aggregated form, but monomeric LPS is the biologically active unit (Takayama et al. 1994). Thus, LPS binding to and activating cells can be described in terms of LPS monomer-receptor interactions.

Interaction of LPS with soluble and membrane proteins

Because of the amphipathic character of LPS, it was earlier postulated that it interacts nonspecifically with responsive host cells by hydrophobic insertion into their cell membrane. However, in the last decade considerable progress has been made in defining molecules that specifically bind to and recognize LPS. Today the concept of specific receptor-mediated interaction of LPS with host factors for generation of biological effects is widely accepted.

In the search for LPS-recognizing molecules, the interest of researchers concentrated on areas of possible localization of LPS during the process of cell stimulation, i.e., serum and target cell membranes. Since monocytes/macrophages play a key role in the host response to LPS (Freudenberg et al. 1986), these cell types have been the subject of particularly intensive research.

Several membrane proteins bind LPS. Among these are members of the CD18/CD11 β-integrin family that recognize surface-bound LPS, resulting in phagocytosis, and the scavenger receptor that mediates the uptake of LPS. Engagement of these receptors leads to clearance and degradation of LPS but not necessarily to activation of cells (Wright 1991).

So far, only the 55-kDa glycoprotein CD14 has been definitely shown to be involved in cell activation, leading

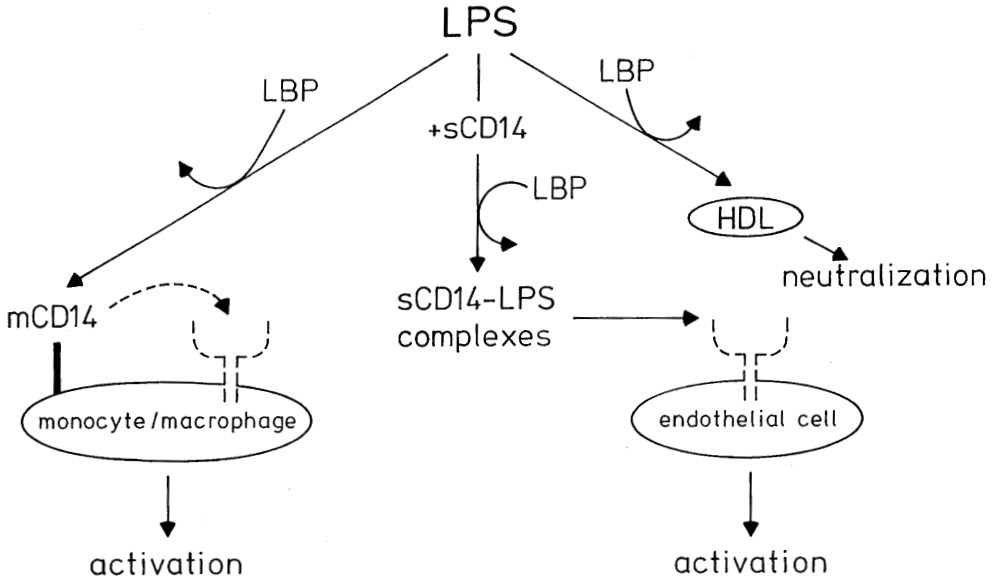


Fig. 4 Initial events in cell activation by endotoxin. *HDL* high-density lipoprotein, *LBP* lipopolysaccharide-binding protein, *mCD14* membrane-bound CD14, *sCD14* soluble CD14

to a secretory response after interaction with LPS (Wright et al. 1990). CD14 exists as a membrane-bound form (mCD14) present on myeloid cells and as a soluble form (sCD14) present in serum.

The binding of LPS to mCD14 on monocytic cells leads to the production and release of cytokines such as IL-1, IL-6, and TNFα. LPS binding is facilitated by a serum factor termed lipopolysaccharide-binding protein (LBP; Schumann et al. 1990).

LBP, a 60-kDa glycoprotein, is present in normal human serum at a concentration of 5–10 μg/ml; this level increases up to 200 μg/ml during the acute-phase response (Tobias et al. 1992). It forms high-affinity complexes with LPS, which are recognized by CD14. The importance of LBP in the mediation of LPS effects is evident from the finding that anti-LBP antibodies protect mice from low LPS dose endotoxemic shock (Gallay et al. 1994).

The binding of LPS to mCD14 has been thoroughly investigated. It takes place via the lipid A domain and is of high affinity (K_D about 3×10^{-8} M; Kirkland et al. 1993); the LPS-binding domain of CD14 has been defined very recently (Viriyakosol and Kirkland 1995; Juan et al. 1995). This domain (amino acids 57–64) overlaps with an amphipathic region analogous to the LPS-binding motif of the *Limulus* anti-LPS factor (Hoess et al. 1993). LPS binding and cytokine induction can be blocked by anti-CD14-antibodies (Schumann et al. 1990).

Despite the unequivocally essential role of CD14 in LPS binding and cell activation, several aspects of LPS-host cell interactions remain to be elucidated. At high concentrations of LPS, binding and cell stimulation can no longer be blocked by anti-CD14 antibodies (Beaty et al. 1994), suggesting the existence of additional mCD14-independent receptor(s). Thus, LPS may directly interact with a membrane molecule other then CD14, or LPS may be internalized and reacts with an intracellular receptor.

Furthermore, mCD14 is not an intrinsic membrane protein with a transmembrane domain; it is linked to the plasma membrane via a glycosyl phosphatidylinositol (GPI) anchor. The GPI tail is not required for cellular activation (Lee et al. 1993). For transmembrane signalling at least one additional molecule must be assumed to exist, which constitutes, most probably together with CD14, the functional LPS receptor (Ulevitch and Tobias 1994).

The soluble form of CD14 (sCD14) lacks the GPI anchor and is present in serum at a concentration of 3–6 μg/ml (Bazil et al. 1986). It also binds LPS, and this binding is enhanced by LBP (Hailman et al. 1994). The resulting sCD14-LPS complexes are able to stimulate cells not bearing mCD14 (such as endothelial cells and smooth muscle cells) to release mediators (Frey et al. 1992; Loppnow 1994). Therefore, it has been postulated that a receptor that recognizes the sCD14-LPS complexes and mediates cell activation exists on these CD14-negative cells. However, this putative receptor has not yet been characterized.

A general model (Fig. 4) for the initial events in the stimulation of cells by LPS could be described as follows: LBP catalyzes the binding of monomeric LPS to the soluble and to the membrane-bound form of CD14. CD14 alone is not capable of mediating further signalling; rather, it collects LPS, perhaps to present it adequately to a second receptor. The interaction of the CD14-LPS complex with this additional molecule leads to signal transduction and cell activation.

So far, neither the hypothetical signal-transducing molecule (on monocytes) nor the receptor for LPS-sCD14 complexes (on endothelial cells) has been identified. The identification and characterization of these molecules are very important for the understanding of the complex mechanism of LPS-induced cell activation and are, therefore, currently the subjects of intensive research. Recently an 80-kDa protein present in membranes of human monocytes and endothelial cells was identified; in a ligand-blotting assay it binds lipid A and LPS only in the presence of sCD14 and LBP (Schletter et al. 1995). Therefore, this

protein represents a possible candidate for the hypothetical signal-transducing element.

A new aspect of the role of LBP has been suggested by the recent finding that it also catalyzes the transfer of LPS to high-density lipoproteins (HDL; Wurfel et al. 1994). HDL is known to bind and neutralize LPS (Flegel et al. 1993), and in this case, LBP may act as a cofactor in the inhibition of LPS bioactivity. Which of the two opposing effects of LBP – potentiating or dampening of endotoxin activity – predominates under various physiological conditions remains to be explored.

Intracellular signalling after LPS-stimulation

Comparatively little is known about intracellular mechanisms of signal transduction initiated by LPS. The knowledge accumulated so far does not allow the construction of a continuous order of events from the event of LPS binding to known cell responses, such as cytokine release. Therefore, the discussion below represents a listing of results dealing with single steps of various signalling cascades. How these steps are linked to each other remains to be established.

The first group comprises experimental results about mechanisms known to directly follow receptor-ligand binding:

1. The involvement of G proteins has been shown in the human promonocytic cell line U937; LPS responsiveness is induced by de novo synthesis of Gi_2. However, the only LPS response parameter examined is enhanced IL-1 production (Daniel-Issakani et al. 1989).

2. Several investigators describe LPS-triggered activation of phospholipase C (PLC) in murine macrophages (Chen et al. 1992). Whether PLC is also activated in human cells, and whether it is functionally important, remains to be established.

3. Furthermore, a possible role of LPS as a second messenger has been proposed (Joseph et al. 1994). LPS is able to bind and stimulate the ceramide-activated protein kinase, thus imitating the second messenger molecule ceramide.

Phosphorylation of proteins appears to play a significant role in LPS signalling pathways. In this process, various protein kinases seem to be involved:

1. Proteinkinase A (PKA) and proteinkinase C (PKC) have been shown to act selectively in LPS induction of cytokines in monocytes (Geng et al. 1993). Whereas inhibition of PKA results in diminished IL-6 production, inhibition of PKC leads to reduced TNFα formation.

2. Protein tyrosine kinase (PTK) activity appears to be the essential step in the induction of IL-1, IL-6, and TNFα and in the activation of the transcription factor NF_KB (Geng et al. 1993). Two members of the *src* tyrosine kinases (hck, lyn) may be involved (Beaty et al. 1994) and, interestingly, the p53/56 lyn tyrosine kinase co-precipitates with CD14 (Stefanova et al. 1993).

3. LPS induces rapid phosphorylation of the tyrosine residues of the 42- and 44-kDa isoforms of the mitogen-activated protein kinase (MAPK1 and 2; Weinstein et al. 1992) and of the MAPK isoform p38 (Han et al. 1994). Phosphorylation activates these serine/threonine protein kinases, which have been shown to play a part in, for example, regulation of transcription factors (Blenis 1993).

4. Recently the phosphorylation of two cytosolic proteins of 36 and 38 kDa, which are distinct from MAPK, was shown to occur after the stimulation of human monocytes by LPS. This phosphorylation corresponds to cytokine release, depends (at low LPS concentrations) on CD14, and is influenced by ADP-ribosylation (Heine et al. 1994b).

LPS causes the activation of the transcription factors NF_KB and cRel/p50 and the translocation to the nucleus of active components (Cordle et al. 1993). These factors bind DNA elements in the enhancer region of various genes, e.g., those for cytokines (Müller et al. 1993), thereby influencing their transcription.

Obviously, so far our understanding of how cells generate and transmit LPS-initiated signals is only fragmentary. The known facts suggest that this process is very complex and that LPS induces more than one kind of intracellular signalling. The definition of the sequence of and interactions between the intracellular events that enable cellular response to LPS activation certainly represents one of the most important goals of endotoxin research.

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