Lipid-mediated regulation of pro-inflammatory responses induced by lipopolysaccharide

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Abbreviations: AP-1 – activator protein-1; BMP – lysobisphosphatidic acid; DGKε – diacylglycerol kinase-ε; IL – interleukin; LBP – LPS-binding protein; LPS – lipopolysaccharide; NFκB – nuclear factor κB; NRLP3 – NLR family, pyrin domain-containing 3; 17ODYA – 17-octadecynoic acid; PI(4)P – phosphatidylinositol 4-monophsophate; PI(4,5)P_2 – phosphatidylinositol 4,5-bisphosphate; PIP5KI – type I phosphatidylinositol 4-phosphate 5-kinase; PI4KII – type II phosphatidylinositol 4-kinase; TNF-α – as tumor necrosis factor-α; TLR – Toll-like receptor

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ABSTRACT

Lipopolysaccharide (LPS, endotoxin) is the component of the outer membrane of Gram-negative bacteria which upon infection induces the body's inflammatory reaction facilitating eradication of pathogens. However, exaggerated reactions to LPS can lead to potentially deadly sepsis while chronic, low-grade inflammation is linked with the development of several metabolic diseases, like type 2 diabetes. These processes are initiated by the binding of LPS to CD14 protein and the TLR4/MD2 receptor complex located in the plasma membrane of immune cells and also by the activation of a cytoplasmic multi-protein complex called the inflammasome. Recent studies have shown that lipids of the plasma membrane and endomembranes are important regulators of LPS-triggered signaling pathways. In this review we summarize those data emphasizing the role of phosphatidylinositols and modification of proteins by palmitoylation. Dysregulation of the lipid-dependent steps of the LPS-induced signaling can lead to excessive production of cytokines during sepsis and metabolic diseases linked with endotoxemia.

INTRODUCTION

Lipopolysaccharide (LPS) is a component of the outer membrane of Gram-negative bacteria which upon infection strongly induces pro-inflammatory reactions of immune cells, such as macrophages. LPS stimulates macrophages owing to a chain of reactions including binding of LPS monomers to CD14 protein located in the plasma membrane and its transfer to Toll-like receptor 4 (TLR4) in the TLR4/MD2 complex [1]. The pro-inflammatory responses induced by LPS are beneficial to the organism, helping it to combat the infection, however, exaggerated responses to LPS can lead to a potentially fatal sepsis. According to the guidelines of the Surviving Sepsis Campaign of 2016, sepsis is recognized when an infection leads to life-threatening organ failure, while persistent circulatory and metabolic abnormalities are the basis for diagnosing septic shock. The mortality of severe sepsis and septic shock reaches 30-50% [2]. Paradoxically, sepsis is a growing problem in highly developed countries. Thus, in the UK sepsis affects about 250 000 people each year (http://sepsistrust.org/facts-sources) and it is also an increasing problem in Poland (http://pokonacsepse.org). The growing incidence of sepsis is related to the increased frequency of use of modern medical techniques, such as surgical procedures and immunosuppressive therapies, all of which increase the risk of bacterial infections. The elderly and children are at a higher risk of sepsis also outside the hospital. The potentially deadly outcome of sepsis drives a strong interest in elucidating the molecular mechanisms of the pro-inflammatory reactions triggered by LPS. In this review we summarize data pointing to regulator role of lipids in those processes and discuss our team's contribution to advancing this field.

TLR4 AND CD14, TWO MAJOR PLAYERS IN LPS-INDUCED PRO-INFLAMMATORY REACTIONS

TLR4, the LPS receptor, belongs to a family of Toll-like receptors which trigger innate immune responses after recognition of a variety of microbial "signatures", so-called pathogen-associated molecular patterns. These include bacterial wall and membrane components, and also microbial nucleic acids. The discoveries concerning the role of TLRs have had a major impact on innate immunology and were honored with the Nobel Prize to Jules Hoffmann and Bruce Beutler in 2011. In a typical scenario, activation of TLR4 requires a cascade of events starting from an interaction of LPS aggregates released from bacteria with LPS-binding protein (LBP) in the serum. LBP changes organization of the LPS aggregates which facilitates interaction of LPS monomers with CD14 protein found in the plasma membrane of macrophages and other cells of myeloid origin (Fig. 1). Subsequently, CD14 transfers the LPS to MD2 in the TLR4/MD2 complex. By simultaneous binding of MD2 and the adjacent TLR4 receptor,

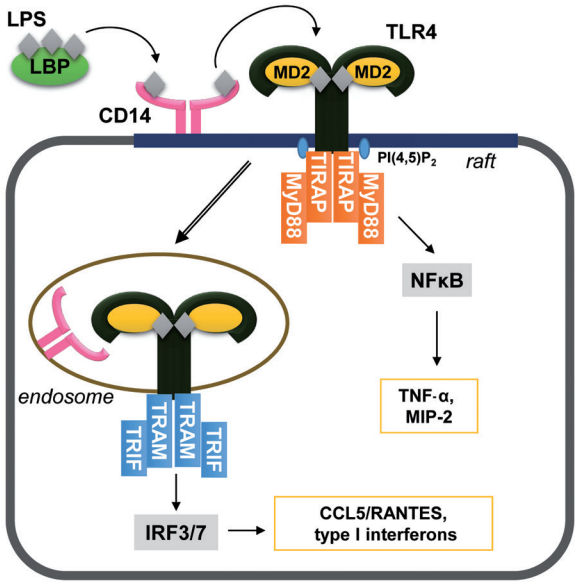

Figure 1. Stimulation of macrophages with LPS. LPS aggregates are bound in blood serum by LBP which facilities the interaction of LPS monomers with CD14 protein in the plasma membrane. Subsequently, CD14 transfers the LPS to TLR4/MD2 complex. The receptor complex dimerizes, recruits TIRAP and MyD88 adaptor proteins triggering downstream signaling leading to activation of transcription factor NFκB and cytokine production. After internalization, TLR4 binds TRAM and TRIF adaptor proteins and initiates endosomal signaling pathway aiming at activation of transcription factors IRF3/7 and synthesis of another set of cytokines.

LPS mediates the formation of "M" shaped dimers of two TLR4/MD2 complexes [3]. Dimerized TLR4 recruits a pair of TIRAP and MyD88 adaptor proteins which guide the assembly of a signaling complex called the myddosome comprised of IRAK4 and IRAK1/2 kinases [4]. The assembly of the myddosome triggers a signaling cascade that eventually leads to the activation of transcription factors, including nuclear factor κB (NFκB) and activator protein-1 (AP-1), and production of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α [5]. Subsequently, the TLR4/MD2 complexes undergo CD14-controlled internalization [6–8]. In the endosome, after TIRAP/MyD88 dissociation, TLR4 binds TRAM and TRIF adaptor proteins. The signaling pathway triggered thereby leads to activation of IRF3/7 transcription factors and expression of type I interferons and other cytokines exemplified by chemokine CCL5/RANTES. Also, late phase activation of NFκB occurs as a result of the TRIF engagement [9,10]. Beside this classical mode of action, LPS also participates in the induction of pro-inflammatory responses *via* activation of a multi-protein complex called the NLRP3 inflammasome (NLRP3 stands for NLR family, pyrin domain-containing 3), as described in the next chapter.

CD14 is a GPI-anchored protein, and such proteins concentrate in specific regions of the plasma membrane rich in sphingolipids and cholesterol called rafts. The concept that sphingolipids and cholesterol self-assemble into dynamic domains which separate laterally from the glycerophospholipid-rich bulk of the plasma membrane and some internal membranes was proposed by Kai Simons in 1997 [11]. From that time on, as a result of intensive biochemical and microscopic studies, it is concluded that the rafts are nanoscale dynamic assemblies of lipids and selected proteins contributing to the raft assembly. Upon cell stimulation, the rafts can merge into larger platforms facilitating interactions of "raftophilic" receptors with their effector proteins. This mode of action is common to some immuoreceptors, as exemplified by T cell receptor [12], and our earlier studies have indicated that among those receptors is also Fcγ receptor IIA. This receptor recognizes Fc fragment of IgG and thereby induces phagocytosis and destruction of pathogens covered (opsonized) by IgG antibodies. We found that upon ligand binding Fcγ receptor IIA associates which rafts which at the same time coalesce into micrometric structures encompassing several GPI-anchored proteins and myristoylated/palmitoylated tyrosine kinases of the Src family. As a result, the receptor undergoes tyrosine phosphorylation which initiates downstream signaling leading to local synthesis of phosphatidylinositol 4,5-bisphosphate [$PI(4,5)P_2$] in rafts and the actin cytoskeleton rearrangement required for phagocytosis [13-17]. Our experience with raft-based signaling turned our attention to the potential contribution of raft lipids to TLR4 activation by LPS. We critically discussed the data pointing to the raft engagement in LPS-induced signaling in a review by Płóciennikowska *et al*. [12] and suggested that rafts can be of special importance for CD14-dependent events in those processes. Our later studies confirmed those suggestions revealing a signaling activity of CD14 that is related to phosphatidylinositol turnover [18,19], as we discuss below.

LIPIDS AS MODULATORS OF LPS-INDUCED SIGNALING

Recent years have brought about ample data showing that the pro-inflammatory activity of LPS goes beyond the TLR4-dependent activation of cells during infection and revealed a cross-talk of LPS-induced signaling with other pro-inflammatory processes [20,23]. If LPS enters the cytoplasm of macrophages, it binds to caspase 4/5/11 and leads to direct non-canonical activation of the NLRP3 inflammasome. Its activity executes death of cells by pyroptosis and leads to the release of pro-inflammatory interleukin (IL)-1 [21,22]. LPS and CD14/TLR4 also act in synergy with lipids, palmitic acid and oxidized LDL-cholesterol, in inducing pro-inflammatory responses. Notably, these lipids are major components of the high-fat westernized diet. Palmitic acid is commonly found in natural fats, which is why it is the main fatty acid consumed. In the USA it accounts for 60% of the total consumption of saturated fatty acids [24]. The diet-induced low-grade inflammation induced by LPS is called metabolic endotoxemia and is linked with the development of several metabolic diseases. It is caused by the entry of low concentrations of LPS derived from intestinal bacteria into the blood stream that is facilitated by the increased permeability of the gut epithelium developing as a result of the diet [25,26]. The consequences of metabolic endotoxemia are linked with TLR4 and CD14 activity since TLR4 or CD14 knock-out mice kept on high-fat diet were free of pathological features observed in their wild-type counterparts, e.g., there was no increased expression of the pro-inflammatory cytokines (e.g., TNFα) in their tissues and no insulin resistance appeared. Of interest, CD14-depleted mice did not gain weight despite being fed the high-fat diet [25–27].

There is also a link between high-fat diet-induced obesity and sepsis, therefore, obesity is considered a factor contributing to morbidity and mortality from sepsis. The mortality of morbidly obese surgical patients is 7.4-fold higher than the average and the duration of stay of obese patients in intensive care units (which increases their susceptibility to sepsis) is longer than of the lean ones [28–30]. These data show the influence of dietary lipids on the inflammatory reactions of the organism providing a rationale for pro-health changes in dietary habits. They also justify the interest in the molecular mechanisms of the pro- and anti-inflammatory properties of dietary components, as we discussed in [30,31].

As indicated above, palmitic acid and oxidized LDL may act synergistically with LPS in inducing the release of pro-inflammatory cytokines. At the molecular level this synergy is a result of the canonical activation of NLRP3 inflammasome, that is associated with the development of type 2 diabetes and atherosclerosis [32–34]. Palmitic acid also leads to the activation of TLR4 signaling pathways by direct binding to a hydrophobic pocket of MD2 protein that associates with TLR4 [35]. Another possibility, especially intriguing to us, is the involvement of palmitic acid in protein palmitoylation which changes the functioning of proteins in pro-inflammatory signaling cascades, as found recently for CD36 protein [36]. Yet another mechanism for the induction of inflammation by palmitic acid has been proposed by Huang et al. [37]. They postulate that palmitic acid, which is a component of lipids located in the rafts of the plasma membrane, affects formation of these membrane nanodomains. After incorporation of palmitic acid to the membrane, size and stability of those domains could increase, thus contributing to the formation of signaling platforms that promote TLR4 activation [37]. A similar mechanism is proposed to be initiated by excess cholesterol accumulating in the plasma membrane. [38, 39].

Taking into account the influence of cholesterol on TLR4 activity we studied the function of lysobisphosphatidic acid (BMP), a unique endosomal lysophospholipid which affects the cellular trafficking of cholesterol [40, 41]. It was found that incorporation of exogenous BMP into the plasma membrane and intracellular membranes of macrophages inhibited the endosomal TLR4 receptor signaling pathway. These anti-inflammatory properties of BMP arise from reduced lipid packing in membranes, as revealed by measurements of fluorescence of the lipophilic Laurdan dye. The membrane disordering by BMP was a consequence of abnormal cholesterol level in the BMP-enriched membranes. These studies have revealed anti-inflammatory properties of BMP, and from a molecular point of view indicated the important role played by the BMP – cholesterol relationship in the organization of the cell membranes, and thus in the activation of TLR4 in macrophages [42].

The data discussed above, ranging from epidemiological to those obtained at the cellular and molecular level, clearly indicate that lipids modulate the pro-inflammatory signaling triggered by LPS and that some of them act in synergy with LPS while others counteract its pro-inflammatory activity. These data change the view of the role of lipids in LPS-induced responses which was underappreciated for a long time and most studies in this field used to focus on the protein components of TLR4 signaling cascades. Our interest in the engagement of lipids in LPS-induced signaling was reinforced by the knowledge we gained when studying the role of raft lipids in the activation of Fcγ receptor IIA. Therefore, in this new field we aimed at deciphering the role of plasma membrane rafts in the pro-inflammatory responses induced by LPS in macrophages.

CD14-DEPENDENT SYNTHESIS OF PI(4,5)P_2 IN LPS-STIMULATED CELLS

Taking into account the role played by plasma membrane rafts in LPS-induced signaling we focused our attention on the role of raft-residing CD14 protein in the stimulation of cells with LPS. Our early studies indicated that CD14 together with scavenger receptors allows binding of high doses of LPS to the surface of J774 macrophage-like cells and controls the balance between the following uptake of LPS mediated by scavenger receptors leading to LPS detoxification and the pro-inflammatory reaction involving TLR4 [43,44]. In the latter case, CD14 is required for the endosomal (TRIF-dependent) signaling of TLR4 [43]. Those data were in agreement with other studies showing that CD14 controls the endocytosis of LPS-activated TLR4 required for the TRIF-dependent signaling to occur [7]. It has been recently proposed that CD14 undergoes constitutive endocytosis while TLR4 joins it upon binding of LPS which also accelerates the process [7,8]. Our further studies have added an important piece to these findings by unraveling the origin of the biological activity of CD14. We discovered that upon binding of LPS, CD14 triggers the turnover of PI(4,5)P_2, a plasma membrane lipid of signaling significance [18,19]. This finding changed our view of the CD14 functioning in LPS-stimulated cells by clearly showing that it goes beyond passive LPS binding. We also provided evidence that the signaling abilities of CD14 leading to PI(4,5)P_2 synthesis are related to CD14 localization in plasma membrane rafts [18,19].

PI(4,5)P_2 is generated primarily by phosphorylation of PI(4)P catalyzed by type I phosphatidylinositol 4-phosphate 5-kinase (PIP5KI) isoforms Iα, Iβ and Iγ. PI(4,5)P_2 is a plasma membrane anchor for TIRAP adaptor protein which is involved in the MyD88-dependent signaling cascade of TLR4 [10]. Next, in the course of LPS action PI(4,5)P_2 undergoes hydrolysis catalyzed by phospholipase Cγ2 to diacylglycerol and inositol 1,4,5-trisphosphate (affecting cytoplasmic Ca^{2+} concentration) or, alternatively, PI(4,5)P_2 is phosphorylated to PI(3,4,5)P_3 by the 110δ isoform of PI3-kinase. All these events are required for the internalization of LPS-activated TLR4/MD2 and initiation of the TRIF-dependent signaling in endosomes [45,46]. It is worth mentioning that PI(4,5)P_2 is also a plasma membrane anchor for gasdermin D, a protein forming pores in the plasma membrane. Upon inflammasome activation the gasdermin D pores are formed allowing the release of pro-inflammatory IL-1 [47,48]. Thus, PI(4,5)P_2 controls many aspects of LPS-induced processes and the fact that PI(4,5)P_2 level in LPS-stimulated cells is controlled by CD14 puts this protein at the apex of several of them.

We found that upon binding to CD14, LPS triggers a biphasic accumulation of PI(4,5)P_2 in cells with the first peak

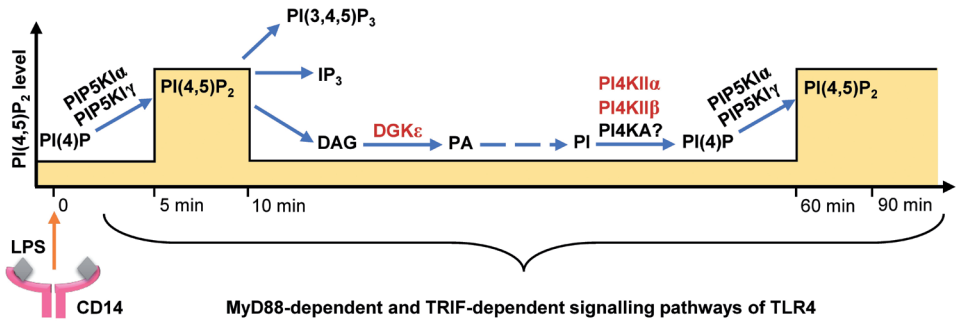

Figure 2. LPS-induced biphasic changes of $PI(4,5)P_2$ level in macrophages.

Upon binding of LPS, CD14 undergoes clustering in the plasma membrane and triggers biphasic accumulation of $PI(4,5)P_2$ required for production of pro-inflammatory cytokines in MyD88- and TRIF-dependent signaling pathways of TLR4. Palmitoylated enzymes catalyzing indicated steps of $PI(4,5)P_2$ turnover are shown in red. DAG, diacylglycerol; PA, phosphatidic acid; PI, phosphatidylinositol; PI4KA, type III phosphatidylinositol 4-kinase α.

at 5-10 min, followed by $PI(4,5)P_2$ depletion resulting from its hydrolysis and phosphorylation, and the second peak of accumulation at 60-90 min (Fig. 2). The biphasic accumulation of $PI(4,5)P_2$ in LPS-stimulated macrophages allows their maximal pro-inflammatory response in both the MyD88- and TRIF-dependent manner [18, 19]. The $PI(4,5)P_2$ fluctuations are likely to control a proper timing of the engagement of $PI(4,5)P_2$-binding proteins and also the onset of Ca^{+2}- and $PI(3,4,5)P_3$-dependent events of LPS-induced signaling. Accordingly, the interference with $PI(4,5)P_2$ synthesis by silencing of PIP5KIα and PIP5KIγ [18] as well as two kinases which catalyze PI(4)P formation (see below) inhibited the LPS-induced production of pro-inflammatory cytokines.

The explanation of how CD14 protein lacking an intercellular domain can trigger signaling events was brought about by ultrastructural studies. We used an unconventional electron microscopy analysis with sheets of the plasma membrane obtained by cell cleavage and double gold labeling of the membrane to display CD14 localization on the cell surface and $PI(4,5)P_2$ in the inner leaflet of the plasma membrane. The studies showed that binding of LPS induces rapid and transient clustering of CD14 in the plasma membrane and the time of the clustering coincided with the first peak of $PI(4,5)P_2$ accumulation. The lipid and its precursor PI(4)P and also PIP5KIα and PIP5KIγ were observed in the vicinity of the aggregates of CD14 protein indicating that the LPS-induced CD14 aggregation in the plasma membrane is a signal for the production of $PI(4,5)P_2$. The increased production of $PI(4,5)P_2$ is a specific response of cells to the clustering of CD14 in the plasma membrane and does not require TLR4 participation [18]. Further studies performed in HEK293 cells devoid of endogenous CD14 and TLR4 but sensitized to LPS by ectopic expression of these proteins at various ratios revealed that TLR4 can fine-tune the CD14-induced $PI(4,5)P_2$ fluctuations, e.g., by affecting CD14 trafficking in cells [19].

A pool of $PI(4,5)P_2$-generating kinases, the $PI(4,5)P_2$ newly formed in LPS-stimulated cells and CD14, all accumulated in the cellular fraction insoluble in nonionic detergents, enriched in the components of membrane rafts [18,49]. A few other studies, including those on Fcγ receptor IIa signaling, indicated that $PI(4,5)P_2$ can be enriched in rafts of the plasma membrane [17,50] and that clustering of GPI-anchored CD59 protein can induce $PI(4,5)P_2$ hydrolysis and the release of Ca^{2+} from intracellular stores [51]. Furthermore, in LPS-stimulated dendritic cells CD14 of rafts triggers activation of transcription factor NFAT without TLR4 involvement [52]. Those reports support our findings that in LPS-stimulated macrophages the plasma membrane rafts in which aggregation of CD14 occurs are sites of $PI(4,5)P_2$ generation and turnover which controls the both signaling pathways of TLR4.

PALMITOYLATION OF ENZYMES CONTROLLING PHOSPHATIDYLINOSITOL TURNOVER IN LPS-STIMULATED CELLS

The data on $PI(4,5)P_2$ turnover in LPS-stimulated cells pointed to CD14-bearing rafts as the sites of these processes. One of the factors controlling the accumulation of proteins in the rafts is their palmitoylation. Our interest in the modification of proteins with palmitic acid is also related to the synergy found between the pro-inflammatory signaling triggered by LPS and this dietary fatty acid (see above). Therefore, we performed a global analysis of palmitoylated proteins in RAW264 macrophage-like cells and found that LPS induced up-regulation of some palmitoylated enzymes that control $PI(4,5)P_2$ synthesis, which indicated that this modification of proteins is crucial for the LPS-induced signaling to occur.

Palmitoylation, frequently referred to as *S*-palmitoylation, is defined as the posttranslational attachment of a palmitic acid (C16:0) residue to a cysteine residue of the protein through a thioester bond [24]. Palmitate is transferred onto the thiol group of cysteine from cytosolic palmitoyl-CoA by palmitoyl acyltransferases zDHHC, enzymes containing the zinc finger DHHC domain named after the highly conserved Asp-His-His-Cys motif. In mammals, the zDHHC enzyme family consists of 23 members [53]. The zDHHC enzymes have at least four transmembrane helices and are located in the plasma membrane, endoplasmic reticulum and the Golgi apparatus. In the process opposite to palmitoylation, the thioester bond is cleaved by several depalmitoylating enzymes [54]. Beside *S*-palmitoylation, palmitate can also be attached (by enzymes other than zDHHC) to the hydroxyl group of serine or threonine via an oxyester linkage in a process called *O*-palmitoylation. It can be also attached to the amine group of N-terminal amino acids or to the amine group of the lysine side chain giving *N*- or *εN*-palmitoylation. Other fatty acid residues can be used for *S*-, *O*-, *N*- and *εN*-acylation of proteins as well. *S*-palmitoylation affects a diversity of protein functions, including membrane binding, cellular trafficking and localization, stability, and protein–protein interactions and similarly profound are the consequences of protein *O*- and *N*-acylation [24]. The most dramatic changes of localization concern cytosolic proteins which upon *S*-palmitoylation

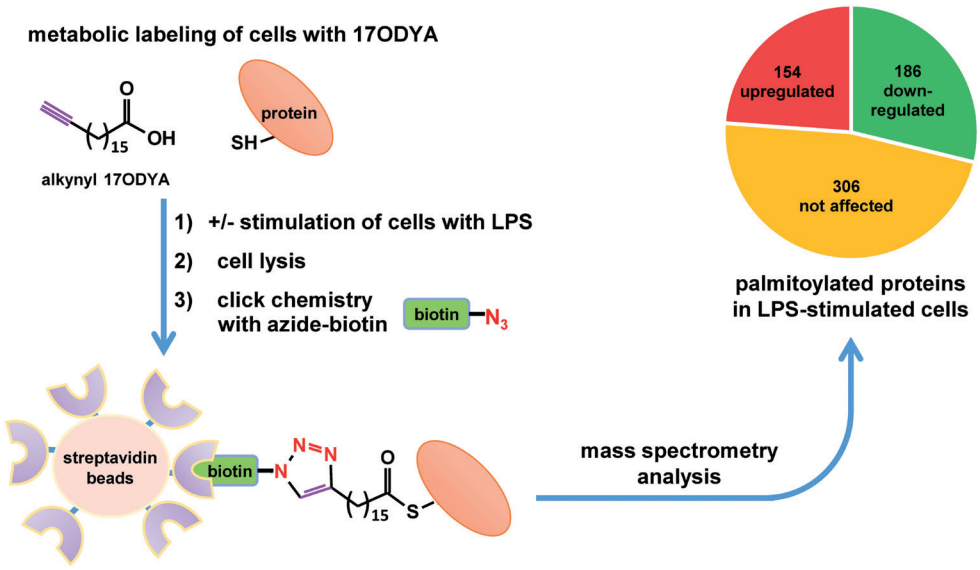

Figure 3. Click-chemistry based approach to studying changes of the level of palmitoylated proteins in LPS-stimulated RAW264 cells.

The procedure begins with incubation of alive cells with 17ODYA, a palmitic acid analogue, followed by stimulation of cells with LPS (control cells are left unstimulated), cell lysis and tagging of 17ODYA-labeld proteins with biotin in so-called "click" reaction. Biotinylated proteins are enriched on streptavidin-bearing beads and analyzed by mass spectrometry.

acquire a hydrophobic anchor facilitating their docking into membranes [55]. The saturated nature of the palmitic acid residue facilitates anchoring of palmitoylated proteins within ordered lipid rafts. Tyrosine kinases are well known examples of such proteins. However, several integral membrane proteins also undergo *S*-palmitoylation, often on cysteine residue(s) located in the proximity of the junction of the transmembrane and cytoplasmic domains of the protein. Such modification can determine in which cellular membrane and in which part of the membrane the modified protein will accommodate without a hydrophobic mismatch potentially caused by the thickness of the bilayer [56,57]. The number of palmitoylated integral membrane proteins is vast, including several adaptor proteins involved in immunoreceptor signaling (LAT, Cbp/PAG) and hemagglutinin of influenza A virus [58,59]. It should be borne in mind that not all palmitoylated proteins are located in rafts and a prominent example of the opposite is transferrin receptor. The final destination of a protein is governed not only by its palmitoylation but also by the length and hydrophobicity of its transmembrane domain [60]. The influence of palmitoylation on cellular localization of proteins suggested that this modification can affect LPS-induced signaling pathways which are linked with redistribution of CD14 and TLR4/MD2.

The last decade has brought about a methodological breakthrough in the identification of palmitoylated proteins owing to the development of two non-radioactive methods based, respectively, on so-called "click" chemistry and acyl-biotin exchange [61-63]. These techniques have paved the way for high through-put mass spectrometry-based proteomic analysis of protein palmitoylation in various cells and tissues [64-67] and facilitated identification of novel palmitoylated proteins of both pathogens and host cells involved in the innate immune responses, such as TLR2 [68].

Our proteomic approach aiming at identification of palmitoylated proteins affected by LPS used the click chemistry developed by Hang, Martin and Cravatt [62,63]. RAW264 cells were metabolically labeled with an alkynyl palmitic acid analogue, 17-octadecynoic acid (17ODYA), stimulated or not with LPS, and lysed. Next, the 17ODYA-labeled proteins were tagged with azide-biotin (the "click" reaction), captured on streptavidin beads and analyzed by mass spectrometry (Fig. 3). Using this proteomic approach we identified a total of 646 palmitoylated proteins in RAW264 cell, in agreement with other studies on protein palmitoylation in these cells [64,69,70]. Among those proteins, 154 were up-regulated and 186 down-regulated in cells stimulated with 100 ng/ml LPS for 60 min. This is a time window where the signaling events related to both the MyD88- and TRIF-dependent pathways of TLR4 occur [49]. The obtained results indicated a global character of changes in the palmitoylation of proteins associated with the stimulation of cells by LPS. The palmitoylated proteins affected were involved in several processes, including transcription and translation, amino acid and lipid metabolism, cell reorganization and intercellular interactions [49]. Among them was also Lyn tyrosine kinase of the Src family. A further detailed analysis indicated that palmitoylation of Lyn is required to promote its redistribution and local accumulation in rafts of LPS-stimulated macrophages. The accumulation of Lyn kinase in membrane rafts determines the inhibitory influence of the kinase on TLR4 signaling pathways [71].

Most importantly, using the proteomic approach we found eight palmitoylated enzymes/proteins engaged in the phosphatidylinositol cycle and revealed that LPS affected the level of some of them. The up-regulated enzymes included two kinases, type II phosphatidylinositol 4-kinase, isoforms IIα and IIβ (PI4KIIα and PI4KIIβ), which phosphorylate phosphatidylinositol to PI(4)P which, in turn, is a substrate for the production of PI(4,5)P_2 but also affects several cellular events by itself. Both kinases contain the evolutionarily conserved catalytic domain with the CCPCC motif, which is the site of their palmitoylation. Attachment of a palmitic acid residue to the PI4KIIα and PI4KIIβ kinases causes these enzymes to behave as integral membrane proteins in terms of their susceptibility to extraction. Also, palmitoylation is required for their enzymatic activity [72-74]. The palmitoylation and hence the activity of PI4KIIα kinase was found to be constitutive while palmitoylation/activity of PI4KIIβ kinases was induced by LPS. The factor identified so far inducing the recruitment of PI4KIIβ kinase to the plasma membrane and its activation is platelet derived growth factor and its effector protein – GTPase Rac1 [75,76].

To verify the data on the PI4KIIα and PI4KIIβ involvement in LPS-induced signaling we either silenced or increased their expression. Overproduction of palmitoylatable forms of both kinases up-regulated the endosomal TRIF-dependent signaling of TLR4, and conversely, depletion of the kinases inhibited especially strongly this signaling pathway. These data indicate that LPS induces palmitoylation and activation of PI4KIIβ kinase, which together with PI4KIIα produces PI(4)P participating in signaling pathways that control the synthesis of pro-inflammatory cytokines [19,49].

In addition to the PI4KIIα and PI4KIIβ kinases, the other palmitoylated proteins identified by us and involved in the phosphatidylinositol metabolism include Sac1 phosphatase, which dephosphorylates PI(4)P to phosphatidylinositol [78]. The amount of palmitoylated Sac1, like that of PI4KIIβ kinases, increases in LPS-stimulated cells. These data suggest that the level of PI(4)P is strictly regulated under these conditions. Up-regulation of diacylglycerol kinase-ε (DGKε) is also noteworthy. It is an enzyme that specifically phosphorylates one type of diacylglycerol, 1-stearoyl-2-arachidonyl-glycerol, which is released by the hydrolysis of $PI(4,5)P_2$. In turn, the phosphatidic acid produced by DGKε is an intermediate in the phosphatidylinositol resynthesis. DGKε exhibits the properties of a surface protein as well as those of an integral membrane protein [79]. Our results suggest that palmitoylation of DGKε kinase may contribute to its binding to the plasma membrane or the endoplasmic reticulum, as is the case with membrane binding of PI4KIIβ kinase. In contrast to PI4KIIβ and DGKε, the level of palmitoylated type 1 and 2 phosphatidylinositol 4,5-bisphosphate 4-phosphatases (Tmem55b and Tmem55a, respectively), which dephosphorylate $PI(4,5)P_2$ to PI(5)P, and also of inositol-3-phosphate synthase 1, which is the rate-limiting enzyme in *de novo* synthesis of *myo*-inositol, were not changed in LPS-stimulated cells. Furthermore, palmitoylated sodium/*myo*-inositol transporter 1, mediating the uptake of extracellular *myo*-inositol was down-regulated under the influence of LPS. Taken together, these findings directly link palmitoylation of proteins with the LPS-induced $PI(4,5)P_2$ turnover and *de novo* phosphatidylinositol synthesis.

CONCLUSIONS

Recent studies have revealed lipid-dependent mechanism controlling the pro-inflammatory response of macrophages to LPS. Among others, CD14-dependent biphasic increase of $PI(4,5)P_2$ level was uncovered in LPS-stimulated macrophages which determines the production of cytokines by the cells. On the other hand, it was found that the phosphatidylinositol turnover is controlled by the palmitoylation of enzymes catalyzing various steps of this cycle. These include phosphatidylinositol kinase PI4KIIβ, whose palmitoylation (hence activity) determines the maximum pro-inflammatory response in LPS-stimulated cells. Several other proteins involved in anti-bacterial defense are palmitoylated, including TLR2 and the major pro-inflammatory cytokine TNFα [68,80]. Further studies on protein palmitoylation are likely to unravel new important players governing efficient transduction of pro-inflammatory signaling in LPS-stimulated cells. From this point of view, the data indicating that high-fat diet can trigger pro-inflammatory reactions of body tissues owing to increased palmitoylation of proteins is of special interest [36]. These results have expanded the spectrum of the pro-inflammatory activity of palmitic acid. The data obtained thus far suggest that dysregulation of the lipid-dependent steps of LPS-induced signaling cascades can lead to excessive production of cytokines which underlies sepsis and metabolic endotoxemia.

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Udział lipidów w regulacji prozapalnych szlaków sygnałowych indukowanych przez lipopolisacharyd

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Słowa kluczowe: lipopolisacharyd, receptory Toll-podobne, błona komórkowa, fosfatydyloinozytol, palmitoilacja białek

STRESZCZENIE

Lipopolisacharyd (LPS, endotoksyna) jest składnikiem błony zewnętrznej bakterii Gram-ujemnych, który w czasie infekcji wywołuje reakcję zapalną ułatwiającą zwalczenie bakterii. Nadmierna odpowiedź organizmu na LPS może prowadzić do potencjalnie śmiertelnej sepsy, a chroniczny stan zapalny o niskim natężeniu jest łączony z rozwojem szeregu schorzeń metabolicznych, np. cukrzycy typu 2. Odpowiedź zapalna jest uruchamiana przez wiązanie LPS z białkiem CD14 i kompleksem receptora TLR4/MD2 zlokalizowanymi na błonie komórkowej komórek układu odpornościowego, a także przez aktywację cytoplazmatycznego kompleksu białek nazywanego inflamasomem. Badania ostatnich lat wykazały, że lipidy błony komórkowej i błon wewnątrzkomórkowych modulują szlaki sygnałowe uruchamiane przez LPS. W niniejszej pracy omawiamy te dane poświęcając szczególną uwagę przemianom fosfatydyloinozytoli i modyfikacji białek przez palmitoilację. Rozregulowanie zależnych od lipidów etapów kaskad sygnałowych indukowanych przez LPS może leżeć u podstaw sepsy i schorzeń metabolicznych powiązanych z endotoksemią.