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Forum Review

Oxidative Stress, Lipid Rafts, and Macrophage Reprogramming

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ABSTRACT

Oxidant stress, induced under a variety of conditions, is known to lead to the molecular reprogramming of the tissue-fixed macrophage. This reprogramming is associated with an altered response to subsequent inflammatory stimuli, such as lipopolysaccharide (LPS), leading to enhanced liberation of proinflammatory chemokines and cytokines. Due to this altered response, dysregulated immunity ensues, leading to the development of clinical syndromes such as multiple organ dysfunction syndrome (MODS). Although the mechanisms responsible for this altered macrophage activity by oxidant stress remains complex and poorly elucidated, it appears, based on recent research, that early and direct alterations within lipid rafts are responsible. This early and direct interaction with lipid rafts by oxidants leads to the mobilization of annexin VI from lipid raft constructs, leading to the release of calcium. This increased cytosolic concentration of this secondary messenger, in turn, results in the activation of calcium-dependent kinases, leading to further alterations in lipid raft lipids and eventually lipid raft proteins. Due to these lipid raft compositional changes, preassembly of receptor complexes occur, leading to enhanced proinflammatory activation. Within this review, the complexity of oxidant-induced reprogramming within the tissue fixed macrophage as currently understood is explained. *Antioxid. Redox Signal.* 9, 1485–1497.

INTRODUCTION

Ischemia and reperfusion results in the activation of the innate immune system characterized by the systemic inflammatory response syndrome (SIRS) (3, 37, 124). Although this state may persist, resulting in early development of multiple organ dysfunction syndrome (MODS), the majority of patients suffering from ischemia and reperfusion develop a compensatory response that is characterized by a state of dysregulated immune responsiveness (7, 74, 91, 98). During this state of dysregulated responsiveness, patients are at increased risk for the development of opportunistic or nosocomial infections (74, 98). If invasive infection occurs, an exaggerated inflammatory response ensues, leading to the development of MODS (2, 15, 58, 87, 123).

The mechanism responsible for this dysregulated response remains poorly understood. This state has been modeled and characterized by the "two-hit" hypothesis (79, 101). According to this hypothesis, ischemia and reperfusion result in reprogramming of immune cells, so that during subsequent infection an exaggerated host response occurs, resulting in MODS. Although several factors are critical to the development of this dysregulated response, oxidant stress appears to play a pivotal role.

The tissue-fixed macrophages, in response to oxidant stress, demonstrate altered activation of the Toll-like receptors (TLRs) (6, 53, 115). Activation of these receptors by inflammatory factors, such as lipopolysaccharide (LPS) from the cell wall of Gram-negative bacteria, leads to the liberation of various inflammatory mediators that are partly responsible for eradication of invading organisms. However, when exaggerated, as the case following oxidant stress, liberation of the factors leads to subsequent tissue injury and the development of MODS.

The mechanism in which the TLRs are activated and affected

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by oxidant stress remains an area of intense investigation. Recently, it has been demonstrated that activation of the TLRs, in particular TLR4, requires the formation of a receptor complex with CD14 and other constituents on lipid rafts (16). In particular, attenuation and augmentation of this receptor complex formation following oxidant stress results in dysregulated inflammatory mediator production (118).

TOLL-MEDIATED SIGNALING

The tissue-fixed macrophage is activated by pathogen-associated molecular patterns (86). These are structures that are characteristic of large groups of microorganisms, such as bacterial cell wall components and nucleic acid motifs. Unlike the adaptive immune response which requires antigen-specific antibodies, innate immune cells are able to respond rapidly to invading organisms without the need for prior exposure.

In mammalian cells, the key components to this response are the TLRs. These receptors are responsible for the recognition of pathogen-associated molecular patterns and lead to the subsequent activation of the macrophage. The founding member of the TLR family is the *Drosophila* protein, Toll, which was initially identified through its ability to control dorsoventral patterning in the fruit fly (112). Subsequent analysis demonstrated that fruit flies with Toll mutations are unable to produce drosomycin, a key antifungal peptide (31, 69). This mutation is associated with reduced survival during fungal infections. Recognition of the importance of Toll in the *Drosophila* innate immune response has prompted exploration for a possible mammalian counterpart.

Currently, a total of 10 human TLRs have been identified that share structural homology and signaling components (68). Each of the described TLRs, except for TLR9, are transmembrane molecules. The extracellular amino termini have variable leucine-rich repeat domains, which are involved in the recognition of pathogen-associate molecular patterns. The intracellular domains contain a conserved Toll/interleukin-1 (IL-1) receptor (TIR) domain (127). The TIR domain, a defining characteristic of the Toll/IL-1 receptor superfamily, is involved in the association with downstream signaling molecules that mediate the response to TLR stimulation.

Toll-like receptor 4 is part of a complex that recognizes LPS. During Gram-negative infections, the highly conserved lipid A component of LPS activates the immune system, leading to generalized inflammation, manifested clinically as sepsis and septic shock (25, 66). Activation of TLR4 requires the binding of LPS to the acute phase protein, LPS binding protein (LBP) (62). Binding to LBP allows LPS to interact with the glycosylphosphatidyl-inositol (GPI)-anchored recognition receptor, CD14, which is found on lipid rafts (32). Once CD14 bound, LPS interacts with TLR4 and MD2, resulting in activation of several different signaling cascades, leading to inflammatory mediator liberation (Fig. 1) (43). Several kinases are thought to be essential for optimal activation, such as interleukin-1 receptor-associated kinase (IRAK) and mitogen-activated protein kinases (MAPK), consisting of p38, ERK 1/2 and JNK/SAPK (23, 51). Kinase activation is followed by activation of various nuclear factors that are responsible for transcription of mediators required for immune regulation. Although this is critical, the mechanisms underlying receptor complex formation following initial infectious remains poorly defined but appears to require binding of LPS to CD14 on lipid rafts (17, 118).

Membrane-bound CD14 is a 53-kDa glycoprotein present on the surface of myelomonocytic cells, and embedded in the plasma membrane via a GPI-anchor (107). CD14 is essential as both a functional receptor and scavenger for LPS. This dichotomy is based on the observation that certain anti-CD14 monoclonal antibodies (mAbs) suppress LPS-induced cell activation but do not interfere with LPS internalization, whereas other mAbs suppress LPS internalization without affecting LPS-induced cell activation (36, 125). Moreover, these observations indicate that while LPS signal transduction and LPS clearance utilize both LBP and CD14, these two pathways bifurcate after LPS binding to CD14. LPS is internalized within minutes in monocytic cells, and the initial rate and extent of internalization increase with the size of LPS aggregates (34, 61). Although the functional role of CD14 was first established using mAbs, recent work has verified this role through the transfection of CD14-negative cells with CD14 demonstrating enhanced sensitivity to LPS (67). Similarly, mice with a disrupted CD14 gene do not respond to low doses of LPS (46). Under physiological conditions, LPS-induced cell activation involves the formation of a ternary complex with LBP and CD14 within lipid rafts (36).

LIPID RAFTS

The classical fluid mosaic model proposed by Singer and Nicolson in 1972 has been modified in recent years to accommodate a role for distinct microdomains in the cell membrane, which appear to serve as signaling platforms (109). The cell membrane is composed of glycerophospholipids, sphingolipids, and cholesterol. The headgroups of sphingolipids trigger a lateral association of lipids of this class with one another, which is further enhanced by hydrophobic interactions between saturated side chains. Cholesterol seems to fill voids between the large glycerosphingolipids, and tightly interacts with sphingolipids, in particular sphingomyelin, by hydrogen bonding. The tight interaction of sphingolipids with one another and cholesterol results in the segregation of these lipids into discrete membrane structures characterized by a gel-like phase, while glycerophospholipids in the bulk of the cell membrane reside in a more fluid liquid-disordered phase (8).

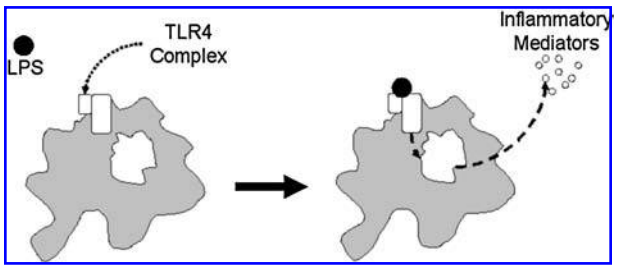

FIG. 1. LPS mediated signaling. Diagram representing LPS-mediated intracellular signaling through the TLR4 receptor.

These distinct membrane microdomains are considered to be floating in an "ocean" of phospholipids, and hence have been termed lipid rafts (109). In addition to the selective lipid composition, selected proteins are preferentially targeted or constitutively found within the lipid raft. Within mononuclear cells, these proteins modified are composed of saturated acyl-chain proteins, including GPI-anchored proteins, such as CD14, and double acylated proteins (5, 77, 90). Other receptor proteins, such as the TLRs, are not constitutively found on rafts, but during activation they are recruited into rafts through an unclear mechanism, resulting in the formation of receptor complexes and the presentation of the inciting stimulus (16, 119). This proposed assembly is consistent with previous data that demonstrated that LPS activation occurs in the plasma membrane by lateral diffusion of the intercalated LPS molecules to transmembrane proteins that then initiate signaling by steric stress (106).

Lipid rafts were originally identified by their resistance to nonionic detergent lysis (109). The existence of rafts was initially controversial, but following an array of analysis their existence in both intact cells and model membrane systems has been verified (14). Rafts appear more prominent and more central to the function during activation of various cells, including the monocyte and macrophage (16, 35, 45, 64, 85, 119). In resting cells, rafts appear small and unstable, and are smaller than the optical diffraction limit (250 nm) (65). Upon stimulation, the raft-preferring receptors are clustered through a poorly defined mechanism leading to the generation of lipid raft macrodomains, allowing LPS to be briefly released into the lipid bilayer where it finally interacts with the complex of receptors, including TLR4 (Fig. 2). Although the mechanism responsible for this clustering remains incomplete, it appears that modulation of lipid raft fluidity through the incorporation of ceramide is critical to this event.

CERAMIDE

Ceramide generation within the macrophage requires the activation of sphingomyelinase that occurs through the rapid hydrolysis of lipid raft sphingomyelin (70). Mammalian cells utilize three distinct forms that are discriminated by their pH optimum, the acid, neutral and alkaline sphingomyelinases, to effect ceramide generation (88, 116). Only the acid and neutral sphingomyelinases are known to be involved in signal transduction in mammalian cells. However, only acid sphingomyelinases appear critical to mononuclear cell activation (40, 63, 71, 92, 113, 122).

Ceramide and acid sphingomyelinase play critical roles in a number of different biological systems. Although many of these systems are involved in the institution of apoptosis, some involve internalization of bacteria, differentiation, and cellular activation (41). Common to these various biological systems is the reorganization of lipid rafts. Recent work has demonstrated the consumption of sphingomyelin within rafts to generate ceramide results in a dramatic alteration of these small rafts (44). Ceramide, which has the unique property of fusing membranes, appears to drive the coalescence of raft microdomains to form large, ceramide-enriched membrane platforms, which exclude cholesterol (8). A mechanism for the formation of ceramide-enriched membrane platforms was indicated from *in vitro* studies of Nurminen and colleagues (84). They demonstrated, using a phosphatidylcholine/shingomyelin-composed unilamellar vesicle, that ceramide generation was followed by the formation of ceramide patches that coalesced rapidly into a macrodomain. Their study suggested that this fusagenic function resulted from hydrogen bonding and van der Waal forces between ceramide molecules themselves.

The formation of these ceramide-enriched membrane platforms serves to cluster specific receptor molecules, and potentially exclude other receptor complexes (44). The best studied within mononuclear cells involves the crosslinking of the Fcγ receptor IIA and IIC (FcγRII) (1). In a series of studies, FcγRII crosslinking resulted in the membrane localization and activation of acid sphingomyelinases. Activation of acid sphingomyelinase resulted in ceramide generation, which fused within the lipid raft forming macrodomains and the recruitment of FcγRII receptors into the raft. Exogenous exposure of C_{16}-ceramide resulted in augmentation of the macrodomain formation and recruitment initiated by FcγRII crosslinking. Similarly

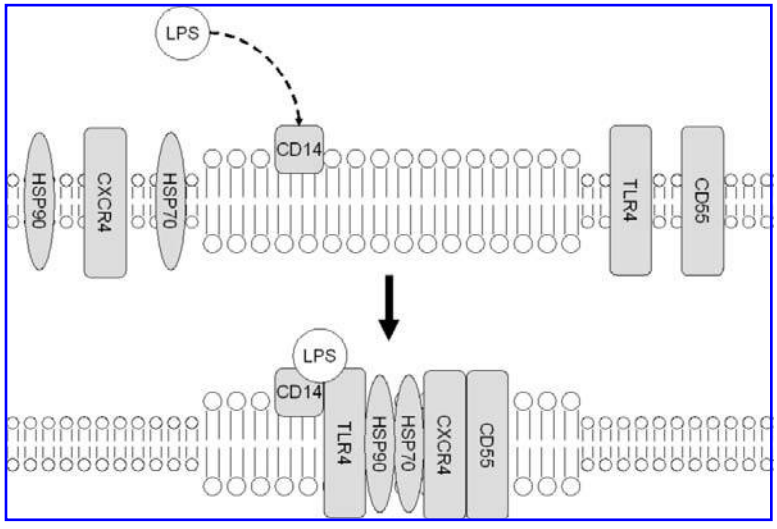

FIG. 2. Lipid raft receptor clustering. Diagram representing LPS-mediated TLR4 receptor clustering consisting of CD14, TLR4, HSP70, HSP90, CD55, and CXCR4.

to these events, we have demonstrated that initial binding of LPS to CD14 results in the activation of acid sphingomyelinase, resulting in the liberation of ceramide, and the formation of the TLR4 raft complex (20). The mechanism, however, responsible for acid sphingomyelinase activity remains unresolved but appears to occur through the activation of phosphatidylcholine (PC)-specific phospholipase C (PC-PLC).

Activation by LPS is associated with the activation of several lipid modulating enzymes in addition to acid sphingomyelinase, most notably PC-PLC (13, 78, 102, 120, 130). Activation of PC-PLC was first described in peritoneal macrophages stimulated with LPS (42). At the time, the potential effect of PC-PLC activation was uncertain. It was, however, associated with the early induction of diacylglycerol (DAG). It was not until recently that an association with PC-PLC and ceramide liberation was proposed. In a recent series of experiments, it has been demonstrated that PC-PLC activation occurs within alveolar macrophages stimulated with LPS (78). This activation was essential to the early production of ceramide by acid sphingomyelinase. Although these studies focused on the subsequent role that PC-PLC played in ERK 1/2 activation, we have demonstrated that PC-PLC activation plays a critical role in TLR4 complex assembly on lipid rafts (18). In these series of experiments, we were able to demonstrate that early activation of PC-PLC results in the sequential generation of DAG, the activation of acid sphingomyelinase and the generation of ceramide (Fig. 3). Interestingly, the activation of PC-PLC by LPS was only dependent on binding to CD14 and not TLR4.

Although the CD14-dependent mechanism for the activation of PC-PLC by LPS remains incomplete, it appears based on recent work by us that membrane activation of the phospholipid flippase, p-glycoprotein (P-gp), is required. This specific flippase found on the plasma membrane, within nonclassic lipid raft components, is responsible for the externalization of phosphatidylserine (PS) and the internalization of PC (47, 72, 96). As a result of this internalization of PC, the substrate for PC-PLC is provided, leading to its activation and generation of DAG. In fact, the activation of PC-PLC and eventual generation of ceramide is prevented by the P-gp inhibitor LY335979 (Fig. 3). Thus, initial binding of LPS/LBP to CD14 results in the sequential activation of several kinases eventually leading to the formation of lipid raft macrodomains through the generation of ceramide (Fig. 4).

As a result of the generation of ceramide and alterations in sphingolipid content within lipid rafts, marked fluidity changes occur within the raft leading to alterations in lipid raft protein content. The full characterization of the protein content within lipid rafts remains incomplete, and has lead to a number of investigations attempting to determine the changes associated with LPS exposure and other inflammatory conditions.

RAFT-ASSOCIATED PROTEINS

The high degree of organization observed within lipid raft structures, coupled with their dynamic nature, appears to be important in modulating and integrating signals by providing a signaling microenvironment that is tailored to produce specific biological responses (118). Changes in protein or lipid composition, size, structure, or membrane localization could potentially affect the functional capabilities of these domains in signaling with important consequences. Thus, clustering of lipid rafts and receptor proteins appears to be an efficient means in signal regulation. This alteration, induced in part by ceramide, may be involved in not only augmenting signaling but could also negatively regulate signaling by sequestering or excluding signaling components in an inactive state. Among the proteins that are targeted to form clusters within rafts are those anchored in part on the outer leaflet of the membrane and can covalently attach to the GPI-protein, CD14 (119). Examples of such proteins include TLR4, HSP70, HSP90, CXCR4, and CD55 (119).

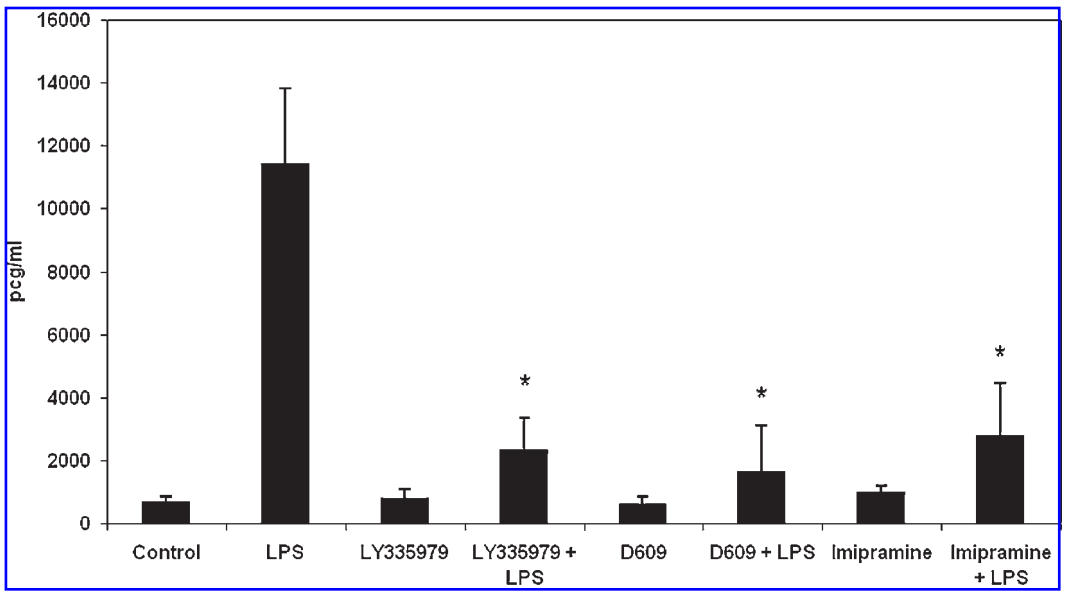

FIG. 3. LPS-mediated ceramide generation. PMA differentiated THP-1 ceramide production following 100 ng/ml LPS was determined by HPLC from cellular lipids extracted following 5 min of exposure. Selected cells were pretreated for 30 min with 0.5 μM LY335979 (a P-gp inhibitor), 100 μM D609 (a PC-PLC inhibitor), or 5 μM imipramine (an acid sphingomyelinase inhibitor). Values represent the mean + SEM for four separately performed experiments (*$p < 0.05$ compared to LPS treated).

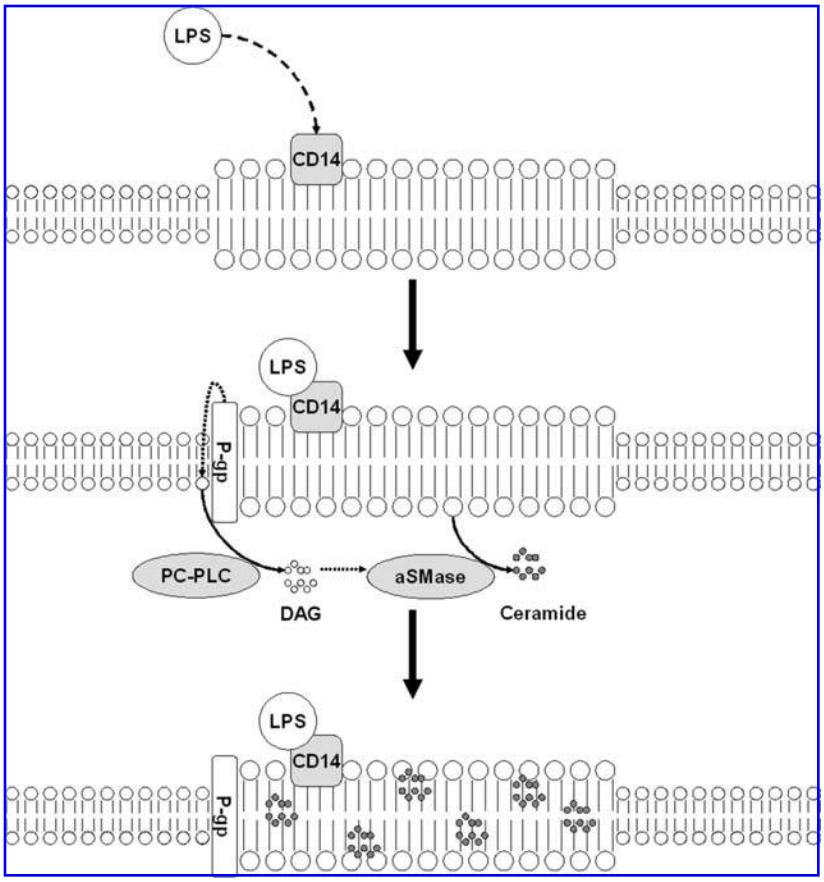

FIG. 4. Mechanism of ceramide generation. Diagram representing mechanism responsible for ceramide generation following initial binding of LPS/LPB to CD14.

Other proteins that are linked to saturated acyl chains, such as the SRC family of kinases, in particular Lyn and HCK, and various integrins, such as Cdc42, CD11b, and CD18, are also targeted to rafts and may additionally affect raft morphology and function (56, 57, 128). In fact, substantial alterations in the lipid raft protein content following LPS exposure of a variety of different TLR receptor complex proteins occurs, including CD14 (Fig. 5). Although formation of this complex is critical to activation, the effect of ischemia and reperfusion on the formation of these complexes is not well characterized.

In addition to these essential receptor components, we have recently demonstrated and characterized alterations in lipid raft CD16 content. The importance of CD16 lipid raft expression and its role in inflammation has only been recently discovered. The composition of CD16 within mononuclear cells has led to the categorization into two subpopulations. These populations consist of cells with CD16 surface expression but diminished CD14 expression (CD14+CD16+) and one without CD16 expression (CD14++CD16−). The population of CD14+CD16+ mononuclear cells normally represents about 10% of the population in healthy adults (131). These CD14+CD16+ cells demonstrate proinflammatory features characterized by enhanced and altered liberation of proinflammatory factors, increased HLA-DR expression and little to no anti-inflammatory factors (132). Although not well investigated following ischemia and reperfusion, the percentages and absolute number of CD14+CD16+ mononuclear cells have been shown to be significantly increased in patients with monocytosis associated with cancer, septicemia, and chronic renal failure undergoing dialysis (33, 52, 94, 97, 105, 108, 110, 114). These findings, in addition to those following trauma, suggest that CD14+CD16+ cells may play a key regulatory role during oxidative stress (54).

OXIDATIVE STRESS REPROGRAMMING

Oxidative stress is known to reprogram macrophages for increased responsiveness to subsequent stimuli, such as LPS. This reprogrammed state leads to increased susceptibility to infection and sepsis, leading to MODS development (79, 80, 104). Patients suffering from ischemia and reperfusion appear to have dysregulated immunity which is central to the development of these clinical syndromes. The effect of ischemia and reperfusion on mononuclear cell phagocytosis, killing of microorganisms, antigen presentation, cytokine production, and induction of cytotoxic effector cells has been characterized (89, 103, 126). However, the mechanisms responsible remain unknown due to both exaggerated pro- and anti-inflammatory responses. Insights into the mechanisms involved have partially been determined through both *in vitro* and *in vivo* modeling of factors induced during oxidant stress.

The effect of oxidative stress on the regulation of TLR4 signaling, although not fully elucidated, appears to result in enhanced activation of macrophage to subsequent stimulation by LPS (Fig. 6). This enhanced activation has been demonstrated to result in excessive generation of various proinflammatory molecules, including TNF-α and IL-8 (26, 28,

29). The mechanism responsible appears to result in enhanced TLR4 mediated signaling through augmented nuclear translocation of NF-κB, leading to altered transcription and translation of proinflammatory genes. This altered signaling has been demonstrated to be associated with a preceding activation of Src family of kinase members (59). Activation of the phosphoinositide (PI) 3-kinase due to SRC activation appears to be involved in the altered downstream signaling events leading to augmented NF-κB activation (60). Due to these findings, several different hypotheses have been suggested and studied, attempting to elucidate the mechanism responsible for this altered signaling. These have included alterations in membrane components, including both lipid raft lipids and proteins.

OXIDANT-INDUCED LIPID RAFT SIGNALING

Although the signaling effects induced by oxidants remains incomplete, mobilization of the secondary messenger calcium appears critical to the initial signaling induced by oxidant exposure. Although several studies have demonstrated this transient change in intracellular calcium following oxidant exposure, the source of calcium remained controversial (48, 49, 60, 81). Several potential sources included extracellular transport through selective plasma membrane calcium channels, or intracellular sources from the endoplasmic reticulum and/or mitochondria through IP3-sensitive gating. However, a less commonly discussed source can occur through the membrane dissociation and cytoplasmic mobilization of the phospholipid and calcium-bound protein annexin VI. This potential source of oxidant-induced calcium flux has been characterized previously in a series of studies that demonstrated significant membrane dissociation of annexin VI following exposure to *t*-butyl hydroperoxide within macrophages (50). As a result of this dissociation, it is believed that calcium bound to annexin VI is released transiently into the cystol inducing a cytosolic calcium flux.

In a series of experiments, we were able to verify that increases in intracellular calcium occurs following oxidant exposure (19, 22). The source of this calcium flux did not appear to be dependent on calcium stores from either the endoplasmic reticulum or the extracellular environment. Rather, this increase in calcium is associated with a loss in lipid raft-associated annexin VI. These data are consistent with a previous observation by Hoyal and colleagues (50). Novel to these observations is that annexin VI is found within lipid rafts, and it is this fraction that is mobilized into the cytosolic component following oxidant exposure (Fig. 7). Furthermore, the integrity of the lipid raft is essential for the responsiveness of the macrophage to oxidant exposure. This was demonstrated by cholesterol depletion by methyl-β-cyclodextrin (MβCD), attenuating the mobilization of annexin VI and bound calcium into the cytosol, but was associated with redistribution from the lipid raft to other cellular locations. This redistribution of annexin VI in response to cholesterol modulation is consistent with a previous observation in CHO cells treated with the cholesterol sequestering agent, digitonin (24).

Although these data clearly demonstrate an increase in intracellular calcium from the lipid raft component of the plasma membrane, it provides little insight into the potential effects that calcium may have as a secondary messenger. Previously, we demonstrated that following either adherence or exposure to PAF, mononuclear cells are reprogrammed through CaMK II activation (21). In a similar fashion, we have recently demonstrated that oxidant exposure is associated with the phosphorylation and activation of CaMK II, and that this molecular event requires a stabile lipid raft and an increase in cytosolic calcium (22). As a result of these events, significant alterations in both lipid raft lipids and proteins occur that appears to be partially responsible for the reprogramming induced following oxidant exposure.

OXIDATIVE EFFECT ON LIPID RAFT LIPIDS

Oxidant stress is associated with significant alterations in membrane lipids. Oxidant exposure is known to result in oxidation of lipids, and is responsible for several clinical conditions such as atherosclerosis (4, 27, 76). The role of this lipid alteration in inflammatory mediated events, however, has not been as thoroughly investigated. Recent data has demonstrated that oxidant exposure results in the oxidation and externalization of PS (4, 27, 76). This event is critical to apoptosis, however, it may also be responsible for alterations in P-gp and generation of ceramide (12, 55, 82, 100, 111, 121).

Ceramide generation is important to changes in lipid raft fluidity that may be responsible for changes in lipid raft protein kinetics. Generation of ceramide following oxidant exposure is well established in a number of different cell types, and appears critical to programmed cell death. In fact, generation of ceramide within these cell types are dependent on acid sphingomyelinase activation (39, 129). However, generation of ceramide following oxidant exposure within the macrophage has not been as well documented. As a result, we have set out to determine if oxidant exposure in addition to the effects on PS results in the generation of ceramide through acid sphingomyelinase activation.

Our current unpublished data demonstrates that oxidant exposure results in the activation of acid sphingomyelinase.-Following the activation of acid sphingomyelinase, ceramide is generated, resulting in the generation of lipid raft macrodomains. The generation of ceramid and lipid raft macrodomains appears dependent on acid sphingomyelinase activation. However, the generation of ceramide and lipid raft macrodomain occurs to a lesser extent than that demonstrated by LPS. But this generation of ceramide is associated with significant alterations in lipid raft proteins.

OXIDATIVE EFFECT ON LIPID RAFT PROTEINS

As a result of the alterations in lipid composition and fluidity within lipid rafts following oxidant exposure, protein composition appears markedly altered. This alteration is believed to be responsible for the subsequent altered activation induced by subsequent stimuli, such as LPS. Recent work has focused on the molecules clustered in the TLR4 receptor that include CD14, HSP70, HSP90, CXCR4, and CD55.

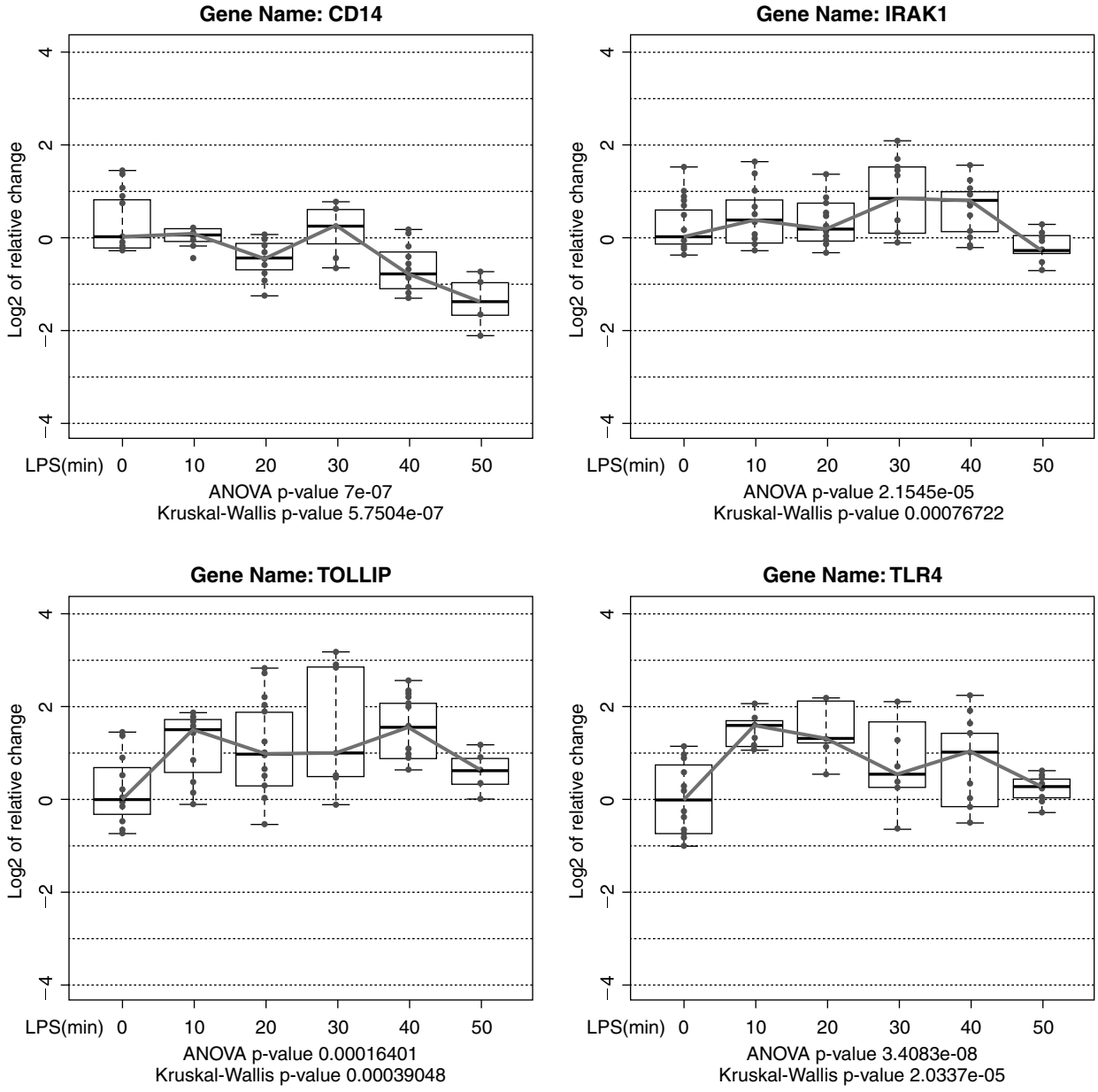

FIG. 5. LPS-mediated lipid raft protein alterations. PMA differentiated THP-1 lipid raft protein alterations were determined following 100 ng/ml LPS exposure for 60 min by reverse phase liquid chromatography by capillary RPLC coupled to an 11-tesla Fourier Transform Ion Cyclotron Resonance mass spectrometer. Representative TLR4 receptor complex components are demonstrated.

Recent data by Powers and colleagues have demonstrated that mobilization of TLR4 into cell surface lipid rafts occurs during oxidant stress (93). This mobilization within their model was critical to the augmented cellular responsiveness seen to subsequent LPS. Mobilization of TLR4 to lipid rafts by oxidative stress, similar to that seen following LPS, was prevented by the cholesterol-depleting agent MβCD (16), thus demonstrating that maintenance of lipid raft microdomain integrity is critical for TLR4 recruitment to the plasma membrane by oxidant stress. It appears that the oxidative-induced recruitment occurs through clustering of SNARE proteins within the raft domain resulting in TLR4-containing vesicles to translocate to the plasmalemmal rafts directly (38, 95). Interestingly, the full effects of oxidant induced signaling were not fully attenuated by MβCD treatment, thus suggesting other potential effects that are directly induced by oxidant exposure.

Although this work provides significant insight into the mechanism responsible for oxidative-induced reprogramming of macrophages, this enhanced surface expression of TLR4 remains inconsistent and has not been validated in human cells. We have therefore focused on the other proteins involved in TLR4 signaling, most notably HPS70, HSP90, and CD55. To study this, we have used an *in vitro* model that consists of exposing PMA-differentiated THP-1 cells to hydrogen peroxide. With this model, we have consistently demonstrated mobilization of each of these components to lipid rafts (Fig. 8). Inter-

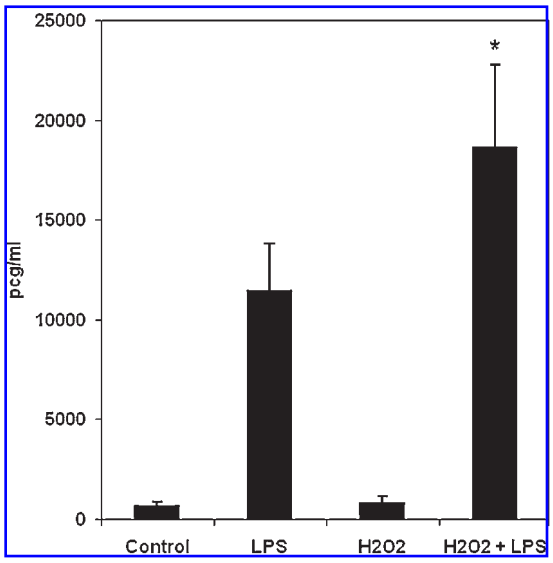

FIG. 6. Oxidant effect on LPS-mediated cytokine production. PMA differentiated THP-1 TNF-α production following 100 ng/ml LPS for 8 h was determined by ELISA (Assay Design, Inc., Ann Arbor, MI). Selected cells were pretreated for 60 min with 100 m*M* hydrogen peroxide. Values represent the mean ± SEM for four separately performed experiments ($*p <$ 0.05 compared to LPS treated).

estingly, we have not been able to demonstrate the mobilization of TLR4 to these raft components. However, similar to the previous report, exposure to MβCD results in attenuation in the mobilization of these proteins.

It is therefore our hypothesis that oxidant exposure results in the assembly of TLR4 receptor components, prior to subsequent LPS exposure, such that the receptor complex is poised to respond rapidly when subsequently exposed to LPS (Fig. 9). This preassembly, in addition to altered lipid raft fluidity, results in enhanced activation leading to altered signaling and enhanced LPS-mediated inflammatory mediator production. This hypothesis is supported by recent evidence demonstrating the responsiveness of macrophage to LPS and its analogues is dependent on the protein composition of the LPS receptor complex that varies with stimulus and may influence downstream signaling (118).

In addition to these findings, we have also been able to clearly demonstrate marked alterations in the lipid raft content of the proinflammatory marker CD16. As previously described, surface expression of CD16 is only present on ~10% of mononuclear cells. Exposure to oxidant stress, either *in vitro* or *ex vivo*, results in a marked alteration with ~25–30% of cells demonstrating surface expression of CD16 (unpublished observations). Additionally, these specific subsets of cells appear to liberate preferential proinflammatory factors in response to inflammatory stimuli.

OXIDANT-INDUCED CYTOSKELETAL AND MEMBRANE ALTERATIONS

Although the effects of oxidant stress may be merely attributed to alterations in lipid raft fluidity, these alterations may actually be due to alterations in protein synthesis. In various other cell types, oxidant stress contributes to cell surface receptor density through alteration in the stability of newly synthesized protein in the endoplasmic reticulum, by inducing translocation of new proteins from the endoplasmic reticulum

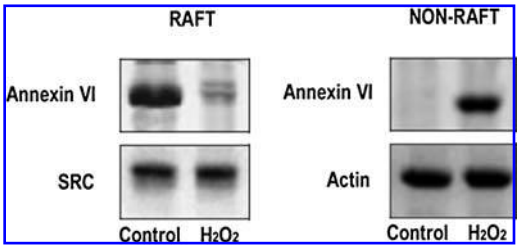

FIG. 7. Oxidant effect on lipid raft annexin VI. PMA differentiated THP-1 cells exposed to 100 m*M* hydrogen peroxide for 5 min underwent lipid raft protein extraction by sucrose gradient centrifugation. Annexin VI was determined by immunoblot analysis. Selected representative gel demonstrated.

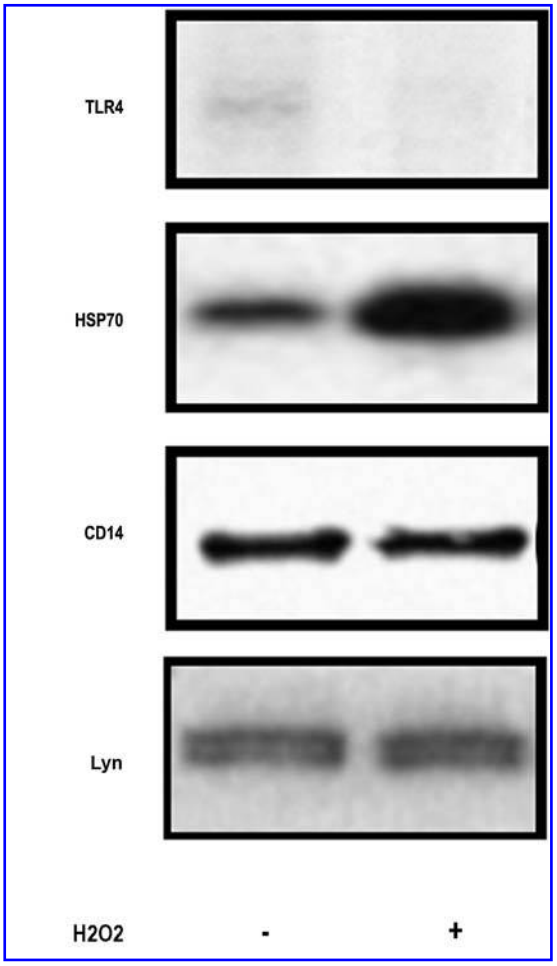

FIG. 8. Oxidant induced TLR4 receptor component mobilization to lipid rafts. PMA differentiated THP-1 cells exposed to 100 m*M* hydrogen peroxide underwent immunoblot analysis of lipid raft proteins isolated by sucrose gradient centrifugation. Representative gel demonstrated.

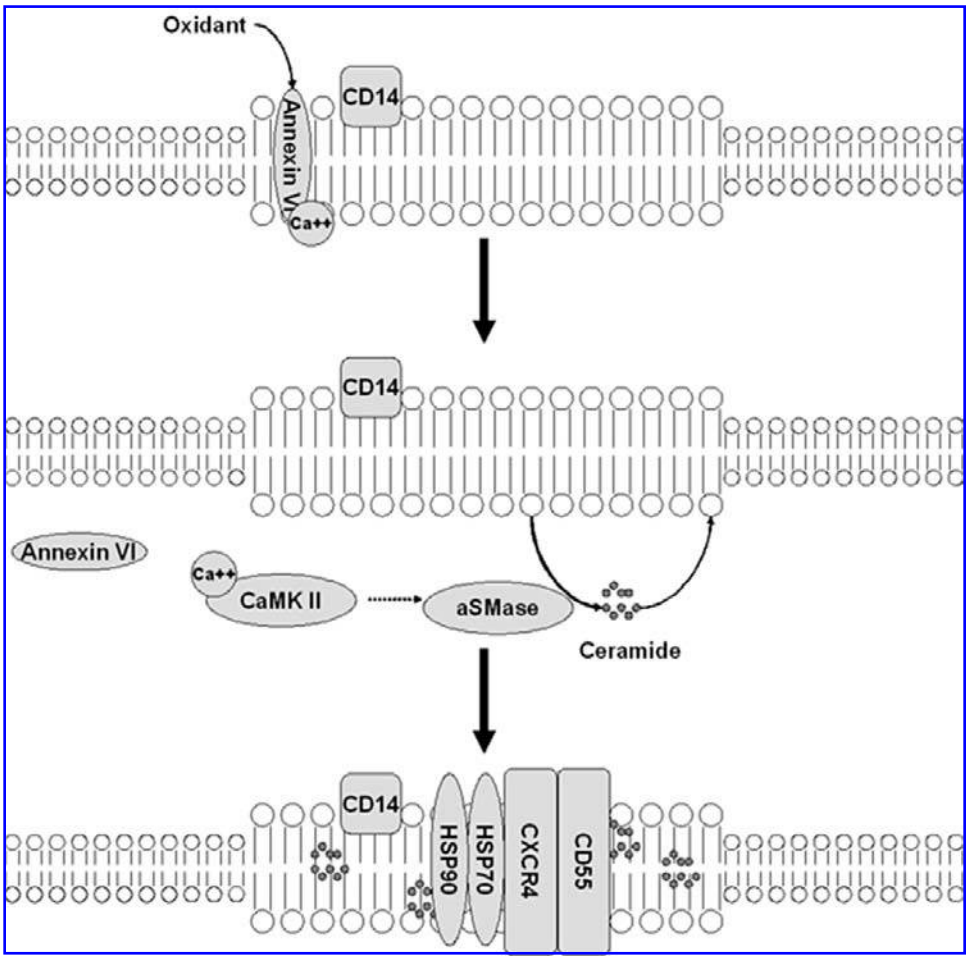

FIG. 9. Mechanism of oxidant induced preassembly of lipid raft receptor components. Diagram representing effects of oxidant exposure on receptor clustering.

to the Golgi compartment and vesicular transport to the plasmalemma (75). Consistent with this mechanism, two distinct strategies aimed at preventing exocytosis of CD11b-containing intracellular vesicular compartments in macrophages, also precluded oxidant-induced upregulation of TLR4 components (73). Specifically, polymerization of actin cytoskeleton with cytochalasin D and calcium depletion inhibit the preassembly of TLR4 components on lipid rafts by oxidant stress.

In addition to these cytoskeletal changes, recent evidence suggests that the flippase P-gp membrane expression is increased following oxidant exposure (unpublished observation). This flippase appears critical to receptor complex formation; however, it remains uncertain if increased expression is associated with any change in substantial change in phospholipid movement. As a result, further investigation is obviously required.

ANTIOXIDANT-INDUCED LIPID RAFT ALTERATIONS

Demonstrating the overall effect induced by oxidant exposure itself, this raises the question if antioxidant exposure can result in attenuation in the potential responses, thus limiting subsequent tissue injury resulting in improved outcome. As demonstrated previously, production of inflammatory cytokines is tightly regulated because excessive production would lead to an amplified inflammatory response and devastating inflam-

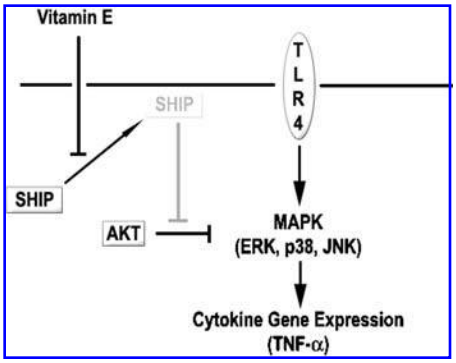

FIG. 10. Mechanism of antioxidant effect on macrophage activation. Diagram of mechanism responsible for anti-inflammatory effect of α-tocopherol succinate.

matory states. Previously, we demonstrated that autocrine release of oxidants is critical to inflammatory-mediated regulation of the macrophage (9, 10). It has been demonstrated that pretreatment with the antioxidant vitamin E results in attenuated endotoxin-mediated proinflammatory mediator production. This seems to result from the specific membrane colocalization of vitamin E within the lipid bilayer (11).

Vitamin E in the form of α-tocopherol succinate includes an aromatic chromanol head and a 16-carbon hydrocarbon tail. The antioxidant function is localized to a phenolic hydroxyl group on the chromanol head, whereas the hydrocarbon tail is important for rapid uptake and localization with the cell membrane (117). This localization within the membrane is critical to the biological effects attributed to α-tocopherol succinate. In addition to the antioxidant effects, α-tocopherol succinate also results in inhibition of various kinases, including protein kinase C (PKC) (99). Together, the current data suggest that the effect of α-tocopherol succinate is caused by membrane localization of this lipid.

Although the regulation of TLR4-mediated signaling is complex, recent reports have suggested that the PI3K/AKT pathway plays a negative regulatory role. Previous observations demonstrated that AKT activation is enhanced by antioxidant exposure, and reversal of this activation attenuates the antioxidant induced effects on TLR4-mediated signaling (19). Although the mechanisms for these effects are incompletely understood, recent work by Fang and colleagues have suggested that endotoxin-mediated (SH)2-containing inositol 5-phophatase (SHIP) mobilization to lipid rafts is important to the regulation of PI3K and AKT (30). SHIP is an inositol phosphatase that is found within the cytosol during unstimulated conditions. On stimulation, SHIP mobilizes to the lipid raft and serves to dephosphorylate AKT. Exposure to α-tocopherol, on the other hand, attenuates SHIP mobilization, resulting in unabated AKT activation and inhibited macrophage signaling and activation (19).

Thus, these data demonstrate a critical regulatory role of cellular produced oxidants in macrophage activation. Antioxidant exposure results in attenuation of inflammatory mediator production through inhibition of SHIP mobilization to the lipid raft (Fig. 10). These molecular data are intriguing when combined with previous clinical studies that demonstrate a reduction in the development of MODS in critically ill patients treated with antioxidants (83). As a result, it seems that appropriate treatment of critically ill patients with antioxidants may serve to regulate the immune response and potentially prevent the development of dysregulated immune responses.

CONCLUSIONS

Ischemia and reperfusion resulting in the generation of oxidant stress leads to the molecular reprogramming of the tissue-fixed macrophage. As a result of this reprogramming, the macrophage response to subsequent stimuli, such as LPS, results in dysregulated chemokine and cytokine liberation. The mechanism in which this occurs is complex, and appears to require initial alterations in lipid raft annexin VI content, resulting in the cytosolic accumulation of calcium. As a result, calcium-dependent kinases are activated, eventually leading to the generation of ceramide and the alteration in lipid raft protein content. Although these responses are critical to dysregulated immunity, antioxidant exposure results in reversal of these molecular changes and may serve as a means to reverse ischemia- and reperfusion-associated development of MODS.

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ABBREVIATIONS

DAG, diacylglycerol; FcγRII, Fcγ receptor IIA and IIC; GPI, glycosylphosphatidyl-inositol; IL, interleukin; LPS, lipopolysaccharide; LBP, LPS binding protein, MβCD, methyl-β-cyclodextrin; MAPK, mitogen-activated protein kinases; MODS, multiple organ dysfunction syndrome; P-gp, p-glycoprotein; PC, phosphatidylcholine; PC-LPC, phosphatidylcholine-specific phospholipase C; PS, phosphatidylserine; PKC, protein kinase C; SIRS, systemic inflammatory response syndrome; TIR, Toll/interleukin-1 receptor; TLR4, Toll-like receptor 4; TLRs, Toll-like receptors.

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