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Cutting Edge: Lipopolysaccharide Induces Physical Proximity Between CD14 and Toll-Like Receptor 4 (TLR4) Prior to Nuclear Translocation of NF-κB

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CD14, a GPI-linked protein, plays a pivotal role in LPS-mediated signaling by potentiating leukocyte adherence, activation, and cytokine production. Recent studies have identified the Toll-like receptor 4 (TLR4) as a membrane cofactor in LPS-mediated transmembrane signaling in cytokine induction, although the mechanism responsible for this cooperation is unknown. Using fluorescence resonance energy transfer (RET) techniques, we demonstrate that LPS triggers a physical association between CD14 and TLR4. Because LPS stimulation up-regulates CD14 and TLR4 expression, it was necessary to control for the possibility that these newly expressed molecules were associated with one another independent of LPS stimulation. Although the calcium ionophore A23187 increased the expression of CD14 and TLR4, they did not exhibit energy transfer. However, following A23187 treatment, LPS promoted physical proximity between CD14 and TLR4. Therefore, we suggest that a close interaction between CD14 and TLR4 participates in LPS signaling, leading to nuclear translocation of NF-κB.

Lipopolysaccharide, the primary component of the outer membrane of Gram-negative bacteria, is responsible for the overwhelming innate immune response of the sepsis syndrome. Septicemia leads to multiple organ failure and death in about one-third of the patients. CD14, a GPI-linked cell surface glycoprotein, is the main LPS receptor of leukocytes and contributes to host sensitivity (1). LPS rapidly promotes a large array of leukocyte functions including adherence and reactive oxygen metabolite (ROM) production as well as priming functions such as phagocytosis (2–6). LPS also affects gene expression within hours of receptor engagement by triggering monocytes and macrophages to produce the inflammatory cytokines TNF-α, IL-1, and IL-6 (7, 8). Inasmuch as CD14 lacks transmembrane and intracellular domains, the mechanism(s) of CD14-mediated transmembrane signaling has remained elusive. CD14 physically associates with the leukocyte integrin CR3 (9) and may thus promote cell adherence, binding, and phagocytosis (2–6, 10–12). Recently, Toll-like receptor 4 (TLR4)3 has been identified as another LPS signaling partner. Hyporesponsiveness and hyperresponsiveness to LPS have been traced to TLR4 (13–15). Because LPS and CD14 complexes transiently come into the proximity of CR3 before cell adherence (9), we hypothesized that LPS/CD14 complexes physically associate with TLR4 before the nuclear translocation of NF-κB.

Materials and Methods

Materials

LPS (Escherichia coli serotype 026:B6), lipid A (F583), PMA, polymyxin B, and A23187 were purchased from Sigma Chemical Company (St. Louis, MO). FITC and tetramethylrhodamine isothiocyanate (TRITC) were obtained from Molecular Probes (Eugene, OR). Anti-NF-κB mAbs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). mAbs directed against Mo-5 and CD67 (urokinase receptor) were the generous gifts of Dr. Robert Todd (University of Michigan, Ann Arbor, MI).

Preparation of monocytes

Monocytes were isolated from peripheral blood using Ficoll-Hypaque (Sigma) density gradient centrifugation at 300 × g for 45 min at room temperature. Cells were suspended in pyrogen-free and sterile HBSS containing Ca2+/Mg2+ (Life Technologies, Grand Island, NY) and 50 ng/ml polymyxin B. Cells were allowed to adhere to glass coverslips at 37°C for 15 min then washed with HBSS.

Preparation of FITC- and TRITC-conjugated Abs

F(ab)2 of anti-CD14 (clone 26ic) and mAb for hTLR4 (clone HTA125) were used (16, 17). Abs were dialyzed against 0.15 M carbonate-bicarbonate buffer at pH 9.3 overnight at 4°C. Samples were incubated with dyes at a fluorophore-protein ratio of 40 μg rhodamine or 30 μg FITC per milligram protein at room temperature for 4 h. The fluorescent conjugates were separated from unreacted fluorochromes by Sephadex G-25 (Sigma) column chromatography. Purified conjugates were dialyzed against PBS at pH 7.4 overnight at 4°C.

Abbreviations used in this paper: TLR4, Toll-like receptor 4; RET, resonance energy transfer; TRITC, tetramethylrhodamine isothiocyanate.


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NF-κB nuclear staining

Monocytes were allowed to adhere to glass coverslips for 15 min at 37°C, then treated with LPS as described below. After fixation with methanol at −20°C and being made permeable with 2% Nonidet P-40, cells were blocked with 20% normal goat serum at room temperature for 20 min. Cells were incubated with 1 μg/ml rabbit anti-human NF-κB Ab at room temperature for 60 min, rinsed with PBS, labeled with 1 μg/ml fluorescein-conjugated secondary Ab in the dark at 4°C for 45–60 min, and then washed with buffer.

Fluorescence microscopy

An axiovert-inverted fluorescence microscope with HBO-100 mercury illumination (Carl Zeiss, New York, NY) interfaced to a Dell 410 workstation via Scion SG-7 video card (Vay Tek, Fairfield, IA) was used. The fluorescence images were collected by an intensified charge-coupled device camera, model XC-77 (Hamamatsu, Hamamatsu City, Japan) and processed with ScionImage Software. A narrow bandpass-discriminating filter set was used with excitation at 485DF20 nm and emission of 530DF30 nm for FITC. For rhodamine, an excitation of 540DF20 nm and an emission of 590DF30 nm were used (Omega Optical, Brattleboro, VT). Long-pass dichroic mirrors at 510 and 560 nm were used for FITC and rhodamine, respectively. For energy transfer imaging, the 485DF22, 510LP, and 590DF30 filter combination was used (18).

Single-cell imaging spectrophotometry

Single-cell spectra were obtained using a microscope/imaging spectrophotometer system (18, 19). Labeled cells were illuminated with an excitation filter at 485DF22 nm and a 510LP dichroic mirror for FITC and resonance energy transfer (RET) experiments. For rhodamine emission spectra, excitation was provided with a 540DF20-nm filter and a 560LP dichroic mirror. The emission spectra were obtained with an Acton-150 (Acton, MA) imaging spectrophotometer. The input side of the spectrophotometer was fiberoptically coupled to the microscope. The exit port of the spectrophotometer was attached to a Gen-II intensifier that was coupled with an I-MAX-512 camera (Princeton Instruments, Trenton, NJ). Spectra collection was controlled by a high-speed Princeton ST-133 interface and a Stanford Research Systems (Sunnyvale, CA) DG-535 delay-gate generator. The systems were interfaced to a Dell 410 workstation running WinSpec software (Princeton Instruments) to manage and analyze the data.

Results and Discussion

Previous studies have shown that CD14 is up-regulated by LPS (20, 21). To determine whether TLR4 expression is affected by LPS, adherent monocytes were stimulated with 200 ng/ml LPS or unstimulated for 0 to 30 min, fixed, and then labeled with rhodamine-conjugated TLR4. The results showed that TLR4 was expressed on monocytes, and that the fluorescence of CD14/anti-TLR4 at ratios of 1:5, 1:1, or 5:1. Spectral features are clearly over, RET emission images were only observed on cells treated with LPS, suggesting a role for LPS in promoting the physical proximity between these two molecules.

LPS-induced RET between CD14 and TLR4 was quantitatively studied using spectrophotometry. Cells were labeled with FITC-conjugated anti-CD14, rhodamine-conjugated anti-TLR4, or both reagents. First, we measured the emission spectra of single cells labeled separately with either FITC-conjugated anti-CD14 or rhodamine-conjugated anti-TLR4 (Fig. 2). Compared with that of control cells incubated with only culture medium, the intensities of the fluorescence images showed that anti-CD14 and anti-TLR4 were expressed on monocytes, and that the fluorescence of both receptors was enhanced after LPS stimulation (Fig. 1). More-
labeled CD14 or TLR4 were substantially enhanced on cells stimulated by LPS, as noted above. Spectra were then obtained from cells that were labeled with both FITC-conjugated anti-CD14 and rhodamine-conjugated anti-TLR4. Spectra from control cells showed a small single peak at ~560 nm. In contrast, when cells were incubated with 200 ng/ml LPS, the spectrum showed two peaks at ~560 and ~590 nm, indicating the presence of RET. Moreover, RET was not observed between anti-CD14 and anti-Mo5, anti-Mo1 (CD11b/CD18), or anti-CD87 in the presence and absence of LPS (data not shown). Thus, RET is specific for anti-CD14 and anti-TLR4 in the presence of LPS.

To provide further evidence in support of energy transfer, we tested cells labeled with different ratios of donor and acceptor chromophores. RET emission intensity depends upon the ratio of donor and acceptor chromophores (22). Spectra from cells labeled with different ratios of FITC-conjugated anti-CD14 and rhodamine-conjugated anti-TLR4 reagents are shown in Fig. 2, D–F. As anticipated, emission spectra vary with the donor-to-acceptor ratio. The relative heights of the two peaks vary in a dose-dependent fashion, with the 1:5 donor-acceptor ratio having the greatest relative emission at 590 nm.

Although these data support an LPS-dependent physical association of CD14 and TLR4, they do not discriminate between the possibilities that LPS binding triggers 1) a lateral association of CD14 and TLR4 in the plasma membrane, and 2) the delivery of preassociated CD14 and TLR4 from an intracellular pool to the plasma membrane. To address this issue, we sought to up-regulate CD14 and TLR4 expression in the absence of LPS. Therefore, we treated cells with the calcium ionophore A23187 (2 μM, 20 min, 37°C) in HBSS containing 5 mM CaCl₂. This procedure up-regulated CD14 and TLR4 expression to the same extent as LPS (Fig. 3, A and B). However, CD14-to-TLR4 RET was not observed in these cells (Fig. 3C, A23187 control). Thus, CD14 and TLR4 are not delivered to the plasma membrane in a preassociated form. When LPS (200 ng/ml) is incubated with A23187-treated cells, RET is again observed (Fig. 3C). Hence, the physical proximity of CD14 and TLR4 is induced at the plasma membrane.

Previous studies have shown that the lipid A moiety of LPS is responsible for its key biological features (23). Therefore, we tested the ability of lipid A to promote the molecular proximity of CD14 and TLR4 under conditions identical with those described above for LPS. Again, RET was observed (data not shown). However, a lipid A antagonist does not support CD14-TLR4 RET (Q. Jiang, K. Miyake, S. Qureshi, and H. Petty, unpublished observations). Thus, CD14-TLR4 complex formation parallels certain biological features of the system.

To correlate the physical association of CD14 and TLR4 with downstream elements of signal transduction, we examined NF-κB translocation to the nucleus. In these experiments, monocytes were incubated with 200 ng/ml LPS or culture medium alone for 60 min at 37°C. Cells were fixed as described in Materials and Methods. After blocking with 10% normal goat serum, cells were stained with FITC-conjugated anti-NF-κB mAb. As expected, LPS induced NF-κB staining inside the nucleus (Fig. 1H). In contrast, there was no nuclear staining for cells exposed to HBSS alone (Fig. 1G). RET was observed at the earliest time points tested, whereas roughly 1 h was required to obtain nuclear staining for NF-κB. Thus, physical proximity of CD14 and TLR4 precedes NF-κB nuclear translocation.

In this study we have shown that LPS triggers close physical proximity between CD14 and TLR4. TLR4 is one member of the Toll-like receptor family. TLR4 requires CD14 to participate in the process of LPS-induced signaling, including NF-κB activation (21, 24). The positive RET signal between TLR4 and CD14 indicates that the donor and acceptor chromophores are ≤7 nm apart; on a molecular level, this would roughly correspond to CD14 and TLR4 being nearest protein neighbors. A CD14/LPS/TLR4 complex is consistent with other reports suggesting that LPS is in close physical proximity with TLR4 during signaling, although the binding affinity of LPS for TLR4 might be low (25). We suggest a model wherein LPS induces physical proximity between CD14 and TLR4. LPS is delivered from the serum protein LPS-binding protein to membrane-bound CD14. LPS-charged CD14 has been previously shown to interact with the leukocyte integrin CD11b/CD18 before cell adherence (9); this may contribute to certain immediate responses of leukocytes to LPS. The close physical proximity of CD14 and CD11b/CD18 is lost after adherence. It now appears that LPS-charged CD14 may then promote changes in gene expression by physically interacting with TLR4. We suggest that the LPS/CD14 complex can associate with various membrane partners to elicit different aspects of the inflammatory response. The ability of CD14 to shuttle between CR3 and TLR4 is analogous to urokinase receptor (another GPI-linked protein) shuttle between CR3 and CR4 during neutrophil polarization (26, 27).

We have also shown that the up-regulation of CD14 and TLR4 are very early events in LPS stimulation of human monocytes. Although the up-regulation of CD14 has been previously reported (20, 21), the up-regulation of human TLR4 has not. However, TLR4 expression declines after LPS stimulation in murine peritoneal macrophages (28, 29). It is possible that this difference could be accounted for by species or cell type; differences in human and murine TLR4 responses have been reported (e.g., 25). However, this difference could also be accounted for by the fact that we are studying changes that take place over several minutes, whereas the down-regulation of TLR4 expression occurs over a period of hours and is temporally associated with LPS internalization and changes in TLR4 gene expression.

Although these studies have established a molecular proximity between CD14 and TLR4, they have not established the supramolecular composition of these signaling complexes. That is, there may be other components of the signaling complex. One possibility is CD11b/CD18. However, attempts to measure RET between TLR4 and CD11b/CD18 have not been successful (our unpublished data). It may be that these proteins are part of the same membrane domain or present in an array on a membrane, but too far apart to be detected with RET. However, other microscopic
methods, such as fluctuation correlation spectroscopy, might contribute to further dissecting these signaling complexes within the membranes of intact living cells.

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