Lipopolysaccharide Internalization Activates Endotoxin-Dependent Signal Transduction in Cardiomyocytes

Douglas B. Cowan, Sabrena Noria, Christof Stamm, Lina M. Garcia, Dimitrios N. Poutias, Pedro J. del Nido, Francis X. McGowan, Jr

Abstract—We tested the hypothesis that bacterial lipopolysaccharide (LPS) must be internalized to facilitate endotoxin-dependent signal activation in cardiac myocytes. Fluorescently labeled LPS was used to treat primary cardiomyocyte cultures, perfused heart preparations, and the RAW264.7 macrophage cell line. Using confocal microscopy and spectrofluorometry, we found that LPS was rapidly internalized in cardiomyocyte cultures and Langendorff-perfused hearts. Although LPS uptake was also observed in macrophages, only a fraction of these cells were found to internalize endotoxin to the extent seen in cardiomyocytes. Colocalization experiments with organelle or structure-specific fluorophores showed that LPS was concentrated in the Golgi apparatus, lysosomes, and sarcomeres. Similar intracellular localization was demonstrated in cardiomyocytes by transmission electron microscopy using gold-labeled LPS. The internalization of LPS was dependent on endosomal trafficking, because an inhibitor of microfilament reorganization prevented uptake in both cardiomyocytes and whole hearts. Inhibition of endocytosis specifically restricted early activation of extracellular signal–regulated kinase proteins and nuclear factor-κB as well as later tumor necrosis factor-α production and inducible nitric oxide synthase expression. In conclusion, we have demonstrated that bacterial endotoxin is internalized and transported to specific intracellular sites in heart cells and that these events are obligatory for activation of LPS-dependent signal transduction.

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Key Words: Golgi apparatus ■ microfilaments ■ endocytosis ■ lysosomes ■ signal transduction

Endotoxin or lipopolysaccharide (LPS) from the outer membrane of Gram-negative bacteria is the primary trigger of the systemic inflammatory response in sepsis.1 Sepsis or septic shock develops in >500 000 patients each year in the United States alone, of which nearly half die.2 The clinical manifestations of sepsis are in large part attributable to LPS-induced signal transduction and gene expression in both myeloid cells (eg, macrophages) and nonmyeloid cells (eg, endothelial cells and cardiomyocytes). These events are the primary cause of myocardial dysfunction in sepsis, which is an important determinant of patient outcome.

In the heart, substantial evidence has been collected that indicates LPS exerts its effects in 2 overlapping phases. Reduced systolic function and contractile reserve occur within minutes to hours of LPS exposure.3 These phenomena occur in the absence of systemic acidosis, hypotension, or decreased coronary perfusion. Early myocardial dysfunction has been related to direct LPS effects and rapid LPS-stimulated production of proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α).4,5 Additional elaboration of proinflammatory cytokines and other mediators in response to the LPS signal results in injury from a variety of mechanisms that include free radical production, nitric oxide generation, and arachidonic acid metabolite release. These events result in progressive contractile dysfunction, diminished β-adrenergic responsiveness, impaired oxidative metabolism, and cell death. Therefore, defining how the LPS signal is transduced in the heart is relevant to understanding the pathophysiology of myocardial dysfunction during sepsis.

The delayed effects of LPS in the heart have been well studied; however, little is known about the process of early LPS signaling or injury. Our laboratory has previously shown that LPS rapidly activates members of the extracellular signal–regulated kinase (ERK), signal transducer and activator of transcription (STAT), and nuclear factor-κB (NF-κB) signal transduction cascades in cardiomyocytes.6 Unlike cells of reticuloendothelial origin, signaling through these pathways seems to be receptor-mediated but independent of the glycosyl phosphatidylinositol-linked receptor CD14 and lipopolysaccharide-binding protein.6 The signaling events that precede activation of these pathways in cardiac cells, however, have not been thoroughly studied.

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From the Departments of Anesthesia (D.B.C., D.N.P., F.X.M.) and Cardiac Surgery (L.M.G., C.S., P.J.D.), Children’s Hospital and Harvard Medical School, Boston, Mass, and the Department of Laboratory Medicine and Pathobiology (S.N.), University of Toronto, Toronto, Ontario, Canada.

Correspondence to Douglas B. Cowan, PhD, Department of Anesthesia, Enders Room 1355, Children’s Hospital, 300 Longwood Ave, Boston, MA 02115. E-mail douglas.cowan@tch.harvard.edu

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Recently, a number of laboratories have shown that LPS is internalized by several cell types, but the role of LPS uptake in activating signal transduction remains controversial. Poly morphonuclear leukocytes and HeLa cells endocytically transport monomeric LPS to the Golgi apparatus. Although CD14 may be important in monomeric LPS recognition and transfer to either a coreceptor or directly into the plasma membrane in myeloid cells, the intracellular trafficking of LPS to the Golgi appears independent of this receptor. On the other hand, LPS aggregates are likely transported in macrophages to lysosomes in conjunction with CD14, where acyloxyacyl hydrolase deacylates LPS.

The latter function is ostensibly more relevant to the detoxification and clearance of endotoxin rather than the initiation of signaling.

LPS-mediated signal transduction is presently believed to be governed by the TLR4 transmembrane receptor in both cardiac and myeloid cells. Because endocytosis of ligand-activated cell-surface receptors often regulates signal transduction and gene expression, we have studied the relationship between LPS intracellular trafficking and signal activation in the heart. Given the complexity and diversity of the intracellular trafficking and signal activation, it is evident that identifying the early mechanisms of endotoxin signaling in the myocardium is essential for developing novel and specific strategies to prevent cardiac dysfunction during sepsis.

In the present study, we found that LPS was rapidly internalized in both cardiomyocytes and perfused whole hearts. In cardiomyocytes, LPS was sorted through an endosomal pathway to the Golgi complex, lysosomes, and contractile apparatus. This process was linked with the immediate activation of ERK and NF-κB signaling pathways and the later production of TNF-α and nitric oxide. Our findings indicate that endocytic membrane trafficking and retrograde transport of LPS regulates endotoxin-dependent signal transduction in cardiac muscle.

Materials and Methods

Cell Culture and Isolated Perfused Heart Preparations

Animal procedures received institutional approval and were conducted according to National Institutes of Health guidelines (publication No. 85-23, 1985). Wistar rat (Charles River Laboratories, Portage, Michigan, Canada) cardiomyocytes and the RAW264.7 mouse macrophage cell line (American Type Culture Collection) were cultured as described earlier. Adult rat hearts were Langendorff-perfused with Krebs-Henseleit buffer at 10 mL/min constant flow essentially as described. A spectrophotometer (SLM-Aminco) measured real-time emission light from hearts at 550 to 650 nm after excitation at 540 nm. Optical sections (0.5 μm) were merged and projected with the BioRad software.

Fluorescent and Gold Labeling of LPS

Salmonella typhosa LPS (Sigma) was labeled with BODIPY FL (4,4-difluoro-4-bora-3a, 4a-diaza-s-indacene fluorocene), Oregon Green 488, or Texas Red X succinimidyl ester derivatives (Molecular Probes), as described earlier. LPS was also labeled with 1.4-nm-diameter mono-Sulfo-N-hydroxysuccinimide ester Nanogold particles (Nanoprobes) and purified by gel filtration. The labeling efficiency for each of the succinimidyl ester compounds was calculated to be 82.6% to 93.5% on the basis of absorbance measurements and known extinction coefficients. In addition, both fluorophore and gold-labeled LPS (LPS-Au) stimulated TNF-α secretion and nitrite production in cardiomyocytes and RAW264.7 cells to the same extent as unlabeled LPS.

Treatments and Staining

Cells were treated with 0.01 to 1 μg/mL labeled LPS. Some cells were treated with 50 nmol/L LysoTracker Red DND-99, 50 nmol/L BODIPY TR C6-ceramide, or 500 nmol/L MitoTracker CMX Ros (Molecular Probes) along with BODIPY FL–labeled LPS (BODIPY FL–LPS). Others were pretreated for 30 minutes with 1, 10, or 100 μmol/L cytochalasin D (Sigma) before LPS or 50 and 500 μmol/L H2O2 treatment in combination with cytochalasin. For staining, cultured treated were fixed and mounted directly or stained with either TRITC-phallolidin (Sigma) or Texas Red X-phallolidin (Molecular Probes). Perfused hearts were treated with Texas Red X-LPS±10 μmol/L cytochalasin D and then fixed, paraffin-embedded, and mounted for visualization.

Confocal Laser Microscopy

Slides were visualized using a BioRad MRC1024 confocal microscope with a Nikon ×60 oil immersion objective, NA=1.60±0.17. BODIPY FL was excited at 488 nm and detected between 506 to 538 nm. TRITC and Texas Red X were excited at 568 nm and detected between 589 and 621 nm. Optical sections (0.5 μm) were merged and projected with the BioRad software.

Transmission Electron Microscopy

LPS-Au–treated cells were fixed in 2.5% glutaraldehyde (Sigma) and silver (Ag)-enhanced (Nanoprobes) before staining with 0.25% uranyl acetate and 0.5% osmium tetroxide (Electron Microscopy Sciences). Sections (60 nm) were cut with a Reichert Ultracut-S ultramicrotome and mounted on copper grids (200 mesh) for electron microscopy.

Immunoblot Analyses

Immunoblotting was performed as described earlier. The anti-ERK1 (K-23) antibody (Santa Cruz) was used at 0.1 μg/mL to detect ERK1/2, whereas the antiphospho-p44/42 mitogen-activated protein kinase (MAPK) E10 antibody (New England Biolabs) was diluted to 0.05 μg/mL to detect phosphorylated (active) forms of ERK1/ERK2. The anti-inducible nitric oxide synthase (iNOS; NOS type II) antibody (Transduction Laboratories) was diluted to 0.1 μg/mL.

Electrophoretic Mobility Shift Assays

Nuclear extracts were isolated from LPS-treated cardiomyocytes using the method of Andrews and Faller and gel shift assay reactions were carried out as described previously.

Measurement of TNF-α and Nitrite Levels

Rat TNF-α levels were determined in culture media samples using the Quantikine (R&D Systems) sandwich ELISA. Nitric oxide production was determined in culture media using Greiss reagent. Comparisons of TNF-α and nitrite production were made using an ANOVA followed by the Tukey-Kramer test.

Results

LPS Is Internalized in Cardiomyocyte Cultures and Whole Hearts

To determine whether BODIPY FL–LPS was internalized in cardiomyocyte cultures, we incubated cells with 1 μg/mL labeled LPS for various times (Figures 1A through 1D). Fixed cardiomyocytes were stained for filamentous actin (F-actin) (red). A diffuse distribution of LPS (green) was apparent in cardiomyocytes by 30 minutes, with more intense globular staining at perinuclear locations at 60 minutes and 24 hours. Labeled LPS was also observed to localize to a region consistent with either the A- or H-band of the sarcomere.
Serial 0.5-μm mid (Z)-plane optical sections of cardiomyocytes treated with labeled LPS for 60 minutes are shown in Figure 2 (top). An intense punctate staining pattern was observed in the perinuclear region in addition to staining in the contractile apparatus. For comparison, BODIPY FL-LPS staining of RAW264.7 macrophages indicated that 15% of the macrophages avidly internalized LPS whereas the remaining cells exhibited relatively low levels of uptake (bottom). Despite variability in the degree of internalization, all macrophages were found to contain labeled LPS.

To establish that LPS internalization was not attributable to the BODIPY FL compound, we labeled endotoxin with 2 structurally different fluorophores. LPS labeled with either Oregon Green 488 or Texas Red X succinimidyl esters exhibited a similar pattern of staining as demonstrated for BODIPY FL-LPS in both cardiac myocytes and RAW264.7 cells (not shown). Furthermore, unreacted BODIPY FL isolated from the gel filtration column used to purify labeled LPS exhibited no internalization. Similar levels of internalization were seen in both cell types at LPS concentrations ranging from 0.1 to 10 μg/mL; however, detection of fluorescent LPS at concentrations ≤0.01 μg/mL was more difficult than that observed at higher concentrations.

Langendorff-perfused whole hearts were also found to internalize LPS. Perfused hearts were fixed and sectioned to confirm the intracellular localization of Texas Red X-LPS (Figures 3A through 3C). Figure 3A shows the level of autofluorescence and background fluorescence in a control heart perfused with unreacted fluorophore alone, whereas

Figures 3B and 3C demonstrate endotoxin uptake in hearts perfused with Texas Red X-LPS. Figure 3D shows the rate of Texas Red X-LPS uptake in a working heart model. Texas Red X-LPS uptake occurred within 10 minutes. To demonstrate that LPS was localized to an intracellular space, we perfused hearts for 8.5 minutes with labeled LPS followed by a washout period using perfusate alone (Figure 3E). A drop in fluorescent emission was seen between 8.5 and 10 minutes, indicating that labeled LPS was eliminated from the vascular lumen. After the initial decline in signal intensity, cardiac fluorescent emission stabilized (10 to 30 minutes), demonstrating that Texas Red X-LPS remained within heart cells.

LPS Is Transported to the Golgi Complex and Lysosomes

The intracellular location of LPS was investigated using 2 strategies. In the first, cardiomyocyte cultures were treated with 0.1 μg/mL BODIPY FL-LPS for 60 minutes. Simultaneously, the cells were exposed to 3 different organelle-specific stains (BODIPY TR C₃-ceramide, Golgi apparatus; LysoTracker Red DND-99, lysosomes; and MitoTracker CMX Ros, mitochondria) and then visualized on a confocal microscope. The second approach used an electron microscope and LPS labeled with 1.4-nm-diameter gold particles.

Figure 4 shows typical results from the experiments using confocal microscopy. For simplicity, cardiomyocytes with a single nuclei are depicted; however, binucleated cells exhibited comparable staining patterns. The left column of the photomicrographs (panels A, D, and G) shows the perinuclear...
staining by LPS, as noted in Figures 1 and 2. The center column (panels B, E, and H) shows the organelle-specific stains for the Golgi complex (top), lysosomes (middle), and mitochondria (bottom). The right column (panels C, F, and I) reveals the merged images from the corresponding left and center columns. Any overlap in the areas stained with LPS (shown in green) and the organelle-specific stains (shown in red) appear as yellow in the right column. As demonstrated in panels C and F, there is considerable overlap between LPS and Golgi or lysosome-specific stains. The yellow staining seen in panel I is bleed-through between red and green fluorescent channels and is not considered colocalization. This assertion was confirmed when individual 0.5-μm optical sections were examined rather than images of the projected series shown in Figure 4 (not shown). The greatest degree of LPS colocalization was seen with the Golgi-specific stain, with nearly all of the spherical bodies observed in panel C appearing yellow. The merged image in panel F shows that a majority of the lysosomes colocalize with LPS; however, individual green and red areas of staining are also apparent (particularly when discrete optical sections were examined), indicative of a less-uniform distribution of LPS in the lysosomes compared with that of the Golgi.

For transmission electron microscopy, LPS was labeled with NanoGold particles rather than a gold colloid to minimize the size of the attached label and ensure uniformity of particle size. LPS-Au-Ag associated with structures, consistent with the interior of endosomes or early lysosomes (Figures 5A through 5C). In most cases, LPS-Au-Ag was associated with membranous regions within these vesicles, similar to the findings of Kreigsmann et al.28 It was also observed that some LPS-Au-Ag was associated with the plasma membrane (Figure 5A); however, surface staining was never seen at sites consistent with caveolae or clathrin-coated pits. A longer treatment (60 minutes) resulted in localization of LPS-Au-Ag in lysosomes (arrows in Figures 5D and E) or compact vesicular structures surrounding the Golgi complex (Figure 5F). These vesicles were primarily located near trans-face and alongside the Golgi. Labeled LPS was not found associated with the cisternae of the Golgi despite the high level of colocalization demonstrated in Figure 4.
Activation of Signal Transduction by Lipopolysaccharide Is Dependent on Uptake

To ascertain whether LPS internalization and intracellular trafficking are required for activation of signal transduction in cardiomyocytes, we inhibited endocytosis with cytochalasin D. Figure 6 demonstrates that in perfused whole hearts, 10 μmol/L cytochalasin D completely blocked the internalization of Texas Red X-LPS. Similarly, BODIPY FL-LPS was not internalized in cardiomyocyte cultures treated with cytochalasin D (not shown).

We investigated whether cytochalasin D blocked early (≤60 minutes), intermediate (4 hours), or late (24 hours) signaling in cardiomyocytes. Figure 7A shows the activity of ERK proteins at 10 minutes after LPS or H2O2 treatment in the presence of 1, 10, and 100 μmol/L cytochalasin D. ERK was maximally phosphorylated 10 minutes after LPS exposure, as shown in lanes 1 (untreated) and 2 (0.1 μg/mL LPS), in agreement with our previous observations.6 Increasing doses of cytochalasin D (lanes 3 through 5) attenuated ERK phosphorylation, whereas cytochalasin D alone had no effect on ERK activity (not shown). To demonstrate that cytochalasin was inhibiting ERK phosphorylation specifically through prevention of endocytosis and not via inhibition of the MAPK pathway, 50 μmol/L hydrogen peroxide (H2O2) plus cytochalasin D was used to treat cardiomyocytes. ERK proteins were activated by H2O2 in the presence of 10 μmol/L cytochalasin D (lane 6).

Moreover, the ability of nuclear proteins to specifically bind a NF-κB consensus binding sequence after 60 minutes of LPS treatment (Figure 7B; compare lanes 1 and 2) was reduced in the presence of escalating concentrations of cytochalasin (lanes 3 through 5). Inhibitor alone had no effect.
on NF-κB binding (lane 6). The specificity of the DNA protein interaction was established in lanes 7 and 8. A 50-fold molar excess of identical unlabeled binding site abolished the shifted complex (lane 7), whereas an unrelated but similarly sized sequence had no effect on complex formation at a 500-fold molar excess (lane 8). The composition of the NF-κB complex was investigated by preincubating nuclear extracts with antibodies directed against either NF-κB p50 or p65 subunits (lanes 9 and 10, respectively). Only the anti-p65 antibody efficiently supershifted the complex, indicating that either the p50 antibody did not bind to its target antigen well or that p50 was not a part of the NF-κB complex. The latter possibility would indicate that other NF-κB subunits (ie, p52) might be involved in orchestrating LPS-induced gene expression. In addition, 500 μmol/L H₂O₂ was found to cause specific binding of nuclear proteins to the NF-κB consensus-binding sequence in the presence of 10 μmol/L cytochalasin D (not shown).

In Figure 8A, the effect of inhibiting LPS uptake on TNF-α production from cardiomyocyte cultures treated for 4 hours was established. Concentrations of cytochalasin D (1, 10, and 100 μmol/L) decrease LPS-induced TNF-α production. This effect was the result of inhibiting LPS internalization and did not result from preventing TNF-α secretion, because there was no detectable accumulation of intracellular TNF-α in cell lysates from these cultures (not shown). Untreated cells and cultures treated with only cytochalasin D were not significantly different (lanes 1 and 6). The expression and activity of iNOS 24 hours after 0.1 μg/mL LPS administration in the presence of 0, 1, 10, and 100 μmol/L cytochalasin D is shown in Figures 8B and 8C (lanes 2 through 5).

### Discussion

We have provided evidence that LPS is internalized in both cardiomyocyte cultures and the cells of Langendorff-perfused hearts. In addition, intracellular LPS was localized to the cardiomyocyte Golgi complex, lysosomes, and contractile apparatus. It is likely that the intracellular transport of LPS depends on its molecular composition at the plasma membrane. In other cell types, it has been shown recently that monomeric LPS is transported to the Golgi apparatus, whereas aggregates move into lysosomal compartments. Although LPS in solution would presumably exist as an aggregate, we found endotoxin in both the Golgi complex and lysosomes (Figures 5 and 6), indicating that LPS may be internalized in both monomeric and aggregate form in cardiomyocytes.

Selective intracellular sorting of endotoxin may depend on molecular weight or conformation, because labeling compounds that vary greatly in size, to a certain extent, affected the intracellular localization of LPS. Fluorescently labeled LPS was found largely in the Golgi complex with less in the lysosomal compartments (Figure 4). LPS-Au, on the other hand, was concentrated in small vesicles surrounding, but not within, the cisternae of the Golgi, in addition to being within lysosomes and endosomes (Figure 5). The large size of the NanoGold conjugate (15 000 M₉) may cause LPS to be trafficked within the cardiomyocyte in a manner typical of endotoxin aggregates. By comparison, BODIPY FL (≥500 M₀) may sort in a manner more representative of LPS monomers.
LPS cannot be metabolized in the Golgi complex, a situation reminiscent of the endoplasmic reticulum (ER)-overload response may be occurring.\textsuperscript{5,31} In the ER-stress response, the NF-κB signaling pathway is activated because of an accumulation of proteins in the ER. This could occur because of a backlog from the Golgi complex as the result of LPS deposition in that organelle. Our present and earlier\textsuperscript{6} results are consistent with this phenomenon but do not exclude other possibilities.

LPS was also internalized in the cardiomyocytes and vascular cells of perfused whole hearts (Figure 3). This finding represents the first demonstration of LPS internalization in a solid organ. The spectrofluorometer used for some of these studies measured output signal from a section of the left ventricular free wall. The effective excitation light penetration from the 400-W xenon lamp used in these experiments was \(\approx4\) mm, and the resultant emission light was estimated to be unaffected by tissue absorbance.\textsuperscript{24} Microscopic examination of tissue sections from the perfused hearts verified that fluorescent LPS was evenly distributed intracellularly throughout the ventricular wall. A longer wavelength fluorophore (Texas Red X) was used to avoid the large amount of autofluorescence attributable to myoglobin that is observed near the peak emission wavelength for BODIPY FL or Oregon Green 488.

We observed that uptake of LPS can be completely prevented by treating the perfused hearts with cytochalasin D just before and during the administration of Texas Red X-LPS (Figure 6). There are several studies in the literature that support our finding that LPS internalization is dependent on microfilament reorganization.\textsuperscript{12,13} Although cytochalasins have been used to block LPS uptake in several cell types, this molecule has also been shown to prevent downstream signaling as a consequence of LPS exposure.\textsuperscript{32–34} Interestingly, Poussin et al\textsuperscript{12} showed that cytochalasin D did not prevent LPS-dependent p38 MAPK and NF-κB activation in THP-1 cells. In their study, cytochalasin D actually increased interleukin-8 secretion after LPS treatment.\textsuperscript{15}

Additionally, we have demonstrated that cytochalasin D treatment of cardiomyocytes, in a dose-dependent manner, prevented the immediate activation of ERK and NF-κB signaling pathways (Figure 7) and the delayed production of TNF-α and nitric oxide (Figure 8). Activation of these signaling proteins was specifically attributable to the prevention of internalization of LPS and not the result of direct inhibition of the ERK and NF-κB pathways, because \(\text{H}_2\text{O}_2\) could stimulate these signaling cascades in the presence of cytochalasin. Hydrogen peroxide has previously been proven to stimulate both of these pathways in cardiomyocytes,\textsuperscript{35,36} likely through a receptor-independent means.

On the basis of the assumption that in cardiac muscle cells TNF-α secretion leads to inducible nitric oxide synthase gene expression,\textsuperscript{37} it is not surprising that microfilament disruption prevents nitric oxide production, because TNF-α is regulated by the NF-κB and ERK pathways.\textsuperscript{6} Consequently, neither pathway can be activated in response to LPS, when internalization is blocked (Figure 7).

There is presently insufficient evidence to conclude that LPS signaling occurs as a direct result of concentration in the

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**Figure 8.** A, TNF-α production in cardiomyocytes treated with BODIPY FL-LPS and cytochalasin D (CD). Cells were pretreated for 30 minutes with 1, 10, and 100 \(\mu\)g/mL cytochalasin D (bars 3 through 5, respectively) before being exposed to 0.1 \(\mu\)g/mL labeled LPS for 4 hours (bars 2 through 5) in the presence (bars 3 through 5) or absence (bar 2) of cytochalasin. After treatments, culture media were analyzed for TNF-α, and untreated cardiomyocyte culture media were used as a negative control (bar 1). Results are presented as pg/mL TNF-α (mean±SD), where \(n=4\) and all experiments had an equal number of cells. There was a significant increase (*\(P<0.001\)) in TNF-α secretion in cells treated with LPS alone (bar 2) compared with untreated cardiomyocytes (bar 1), whereas increasing concentrations of cytochalasin D (bars 3 through 5) significantly reduced (\(*P<0.001\)) TNF-α production. Cytochalasin D alone (bar 6) did not significantly alter TNF-α secretion compared with the control (bar 1). B, Immunoblot analysis of iNOS. C, Nitrite production in cardiomyocyte cultures treated with BODIPY FL-LPS and cytochalasin for 24 hours. Samples were run in the same order (ie, 1 to 6) as described for TNF-α. A significant increase (\(*P<0.001\)) was demonstrated in nitrite accumulation in cells treated with LPS alone (bar 2) compared with untreated cardiomyocytes (bar 1). Increasing concentrations of cytochalasin D (bars 3 through 5) significantly reduced (\(*P<0.001\)) nitric oxide production. Results are presented as \(\mu\)mol/L nitrite (mean±SD), where \(n=4\) and values have been adjusted for total cardiomyocyte protein.
Golgi apparatus or other cellular compartments. On the basis of the rapidity of some responses, it is probable that signals are generated from an intermediate structure like the endosome. This supposition is supported by the observation that although LPS-Au was biologically active (as measured by the ability to stimulate TNF-α secretion and nitric oxide production), it was not localized within the Golgi complex. Whether LPS is receptor associated (eg, TLR4) within cellular compartments or integrated into an intracellular membrane also remains to be elucidated. Internalization of ligand-activated receptors is a well-recognized means of modulating signal transduction, with most examples of signaling from within endosomes indicating that ligand receptor complexes are associated with caveolae or clathrin-coated pits.23 We did not observe LPS associated with either type of structure (Figure 5), suggesting that other mechanisms may be involved.

In conclusion, we have shown that LPS is internalized and sorted to specific locations in cardiomyocytes and that these events are required for endotoxin-dependent signal activation. A complete understanding of the initial events that result in the development of contractile function may lead to the development of therapies designed to protect the heart from endotoxin exposure.

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