Mycobacterial Lysocardiolipin Is Exported from Phagosomes upon Cleavage of Cardiolipin by a Macrophage-Derived Lysosomal Phospholipase A2

Karsten Fischer, Delphi Chatterjee, Jordi Torrelles, Patrick J. Brennan, Stefan H. E. Kaufmann and Ulrich E. Schaible

*J Immunol 2001; 167:2187-2192; doi: 10.4049/jimmunol.167.4.2187*

http://www.jimmunol.org/content/167/4/2187
Mycobacterial Lysocardiolipin Is Exported from Phagosomes upon Cleavage of Cardiolipin by a Macrophage-Derived Lysosomal Phospholipase A$_2$

Karsten Fischer, Delphi Chatterjee, Jordi Torrelles, Patrick J. Brennan, Stefan H. E. Kaufmann, and Ulrich E. Schaible

Pathogenic mycobacteria are able to survive and proliferate in phagosomes within host macrophages (Mφ). This capability has been attributed in part to their cell wall, which consists of various unique lipids. Some of these are important in the host-pathogen interaction, such as resistance against microbial effector mechanisms and modulation of host cell functions, and/or are present as Ags to T cells. Here we show that two lipids are released from the mycobacterial cell wall within the phagosome of infected Mφ and transported out of this compartment into intracellular vesicles. One of these lipids was identified as lysocardiolipin. Lysocardiolipin was generated through cleavage of mycobacterial cardiolipin by a Ca$^{2+}$-independent phospholipase A$_2$, present in Mφ lysosomes. This result indicates that lysosomal host cell enzymes can interact with released mycobacterial lipids to generate new products with a different intracellular distribution. This represents a novel pathway for the modification of bacterial lipid Ags. 


Tuberculosis remains one of the leading causes of human mortality from infection (1). In general, mycobacteria are resistant to most common chemotherapeutic agents, as well as to simple disinfectants (2). This resistance is mediated in part by the unique cell wall, dominated by the extremely hydrophobic esterified mycolic acids, resulting not only in the characteristic acid-fastness, but also in an extreme degree of hydrophobicity and impermeability to hydrophilic solutes (3). The mycobacterial cell wall also contributes to bacterial survival in the host through modulation of the antimicrobial function of macrophages (Mφ) as well as the activation of lymphocytes (4, 5). Lipoarabinomannan (LAM) suppresses immune responses by various mechanisms including impaired T cell activation, inhibition of IFN-γ-mediated Mφ activation, scavenging of free oxygen radicals, and inhibition of protein kinase C activity (4, 6). Phosphatidylinositolmannosides (PIMs) inhibit Ag-specific proliferation of peripheral blood monocytes (7). Pthiocerol dimycocerosate and its functional localization in the mycobacterial cell wall were shown to be crucial for survival of Mycobacterium tuberculosis in the lungs of mice (8). Mycobacterial phospholipids have also been implicated in immunomodulation; human blood monocytes infected with M. tuberculosis release phosphatidylethanolamine and phosphatidylglycerol as mycobacterial origin, which activate suppressor lymphocytes (9). Lipids of mycobacteria can be recognized by the immune system as Ags. In humans, T cells specific for mycobacterial glycolipids are considered part of the immune response against M. tuberculosis (10). Mycobacterial lipids of various complexities, such as LAM, PIM$_X$, mycolic acid, glucose monomycolate, and isoprenoid glycolipids, are presented to T cells by CD1 molecules leading to lysis of infected Mφs and killing of M. tuberculosis (11, 12). It is not known yet whether mycobacterial lipids need to be processed by host cells to become antigenic although it was recently reported that galactosylceramide derivatives require an enzymatic cleavage before they are recognized by NK T cells in the context of CD1d (13). Our experiments show that mycobacterial lipids are released from mycobacteria, transported out of the phagosome, and altered by host cell enzymes. In the case of cardiolipin, its cleavage by a lysosomal-type Ca$^{2+}$-independent phospholipase A$_2$ occurs before the transfer of lysocardiolipin out of the phagosome. We postulate that this processing pathway can generate biologically active lipid derivatives that play a role during infection by and persistence of mycobacteria within their host.

Materials and Methods

**Chemicals**

All reagents were purchased from Sigma (Deisenhofen, Germany), if not indicated otherwise.

**Cell culture**

Mφs from 6- to 8-wk-old C57BL/6 mice were differentiated in tissue culture medium containing 20% L929 cell supernatant (American Type Culture Collection, Manassas, VA). To label Mφs lipids radioactively, 10$^7$ Mφs were incubated for 12 h in the presence of 10 μCi $[^{14}C]$palmitic acid, 50 Ci/ mmol (Hartmann, Braunschweig, Germany).
M. bovis bacillus Calmette-Guérin (BCG; Copenhagen strain) was cultured in 7H9 Middlebrook medium (Difco, Detroit, MI) supplemented with albumin dextrose catalase (Difco), and harvested during logarithmic growth phase. Bacterial lipids were radioactively labeled by incubating 10^9 M. bovis BCG for 12 h in the presence of 10 μCi [14C]palmitic acid. MΦ were infected at a 10:1 multiplicity of infection.

**Subcellular fractionation**

Mycobacteria containing phagosomes were prepared using a 12/50% sucrose step gradient as previously described (14). The top layer, containing intracellular vesicles, and the 12/50% interface consisting predominantly of mycobacterial phagosomes were collected. The supernatant from the washes containing the remaining bacteria. To recover all intracellular vesicles, the supernatant was further centrifuged at 100,000 g for 1 h. No bacteria were observed in this preparation (data not shown). The 12/50% interface was washed once in PBS, and the mycobacteria were stripped off the phagosomal membranes by several Nonidet P-40 washes. The resulting pellet contained the mycobacteria (data not shown). The supernatants from the washes contained the phagosomal membrane and were combined and lyophilized.

To obtain lysosomes, noninfected MΦ were lysed and the lysate was layered on top of a 20% Percoll gradient (Biochrom, Berlin, Germany) in triethanolamine buffer (pH 6.5) and centrifuged for 21 min at 25,000 × g. The lysosome-enriched fractions were separated from the Percoll by centrifugation for 1 h at 100,000 × g. These fractions were highly enriched in enzyme activity per milligram of protein for the lysosomal marker enzyme β-galactosidase, and the proteins lysosome-associated membrane protein-1 and cathepsin D as determined by Western blot (data not shown). For cleavage experiments, lysosomes derived from 5 × 10^6 MΦ were disrupted in 1% Triton X-100, 50 mM acetate, pH 4, before the addition of purified mycobacterial lipids. Mixtures were incubated at 37°C for 2 h. Reaction was stopped by freezing, and samples were lyophilized, extracted, and analyzed by high performance thin layer chromatography (HPTLC).

**Lipid extraction and HPTLC analysis**

Lipids were extracted overnight in chloroform/methanol (2:1; v/v). Samples were spotted in one corner of a 10 × 10 cm HPTLC plate (Merck, Darmstadt, Germany). The HPTLC plate was developed in the first dimension in chloroform/methanol/ammonium hydroxide (80:20:2; v/v/v) to 8 cm, and, in the second dimension, in propanol/water/acetic acid (80:10:10; v/v/v) to 8 cm. If one-dimensional analysis was performed, chloroform/methanol/water (65:25:4; v/v/v) was used as solvent system. Lipids were analyzed using the following spray reagents: 0.2% ninhydrin in ethanol, a phosphorus dip (Dittmers reagent), and 1% α-naphthol in 5% H2SO4/ethanol (15). Radioactive lipids were visualized and quantified using a phosphoimager (FUJIX BAS 1000; FUJIX, Tokyo, Japan). For the analysis of single lipids, the radioactive lipids were scraped off the HPTLC plate and re-extracted, and radioactivity was measured.

**Purification of lipids**

Lipids from 20 g lysylized M. bovis BCG were extracted as described above. The total lipid extract was chromatographed by multiple silica gel chromatography, applying chloroform/methanol/acetone gradient in 10% steps (16). The fraction containing the highest concentration of lipid 4, which is cleaved by lysosomal enzymes, was further separated on C18 Sep-Pak cartridge (Waters; Millipore, Bedford, MA). In the last step this lipid was cleaved by a lysosomal phospholipase A2. The resulting methylesters were analyzed by gas chromatography-mass spectrometry (GC-MS; Hewlett-Packard, Palo Alto, CA). The resulting methylesters were analyzed by HPTLC.

**Lipid identification**

To identify fatty acids, 200 μg lipid was dried and methanolyzed as described (17). The resulting methyl esters were analyzed by gas chromatography-mass spectrometry (GC-MS; Hewlett-Packard, Palo Alto, CA). The sample was analyzed by 1H- and 13C-nuclear magnetic resonance (NMR) (300 MHz; Varian NMR; Varian Associates, Palo Alto, CA), matrix-associated laser desorption ionization time of flight mass spectrometry (MALDI-MS) (Voyager Elite; PerSeptive Diagnostics, Cambridge, MA; 2, 5 dihydroxybenzoic acid was used as matrix), and electron spray ionization mass spectrometry (ESI-MS) (V6 Quattro-SQ; Fisons Instruments, Loughborough, U.K.).

**Incubation of mycobacterial cardiolipin with phospholipase A2**

[14C]-labeled cardiolipin was lyophilized and resuspended in 0.1% Triton X-100 in PBS (pH 8.9) followed by the addition of 10 U phospholipase A2 (porcine pancreas), and the mixture incubated at 25°C for 1 h. The lipids were extracted and analyzed by HPTLC.

**Results**

Here we demonstrate by using radioactively labeled mycobacteria that, in infected MΦ, cardiolipin is released from the bacteria and subsequently cleaved by a lysosomal phospholipase A2. The resulting lysocardiolipin could be detected within intracellular vesicles distinct from phagosomes.

**Mycobacterial lipid content of different cellular compartments of infected MΦ**

MΦ infected with [14C]palmitic acid-labeled mycobacteria were lysed at different time points after infection (2, 8, and 48 h), fractionated into mycobacteria, phagosomal membranes, and other intracellular vesicles, and analyzed for their lipid content by HPTLC. For both fractions, the number of different lipid species, as well as the total amount of radioactivity, increased. Thus, the total radioactivity recovered from the vesicle fraction at 48 h postinfection was 14 times higher than the one measured at 2 h.

**Export of two mycobacterial lipids from the phagosome**

Lipid patterns of radioactively labeled MΦ infected with [14C]palmitic acid-labeled M. bovis BCG for 48 h and the respective M. bovis BCG isolated (Fig. 2). The lipid pattern of M. bovis BCG was much more complex that than of the MΦ, which consisted of 17 major lipids compared with >30 in the case of M. bovis BCG. Comparison between M. bovis BCG grown in culture (B) and intracellularly (C) revealed that the relative amount of different lipid species varied between these two conditions, e.g., the amount of TDM was higher in intracellularly grown mycobacteria. The pattern of the vesicles of the infected MΦ roughly resembled that of uninfected MΦ, indicating that either mycobacterial lipids were metabolized by MΦ, or dominant lipid compounds transported out of the phagosome were identical in both MΦ and mycobacteria. Two lipids (3a and 2) present in the vesicle fraction were not detectable in uninfected MΦ, indicating that they were specific for mycobacteria (Rf 3a: 0.17/0.86; 2: 0.73/0.98). Lipid 2 is probably TDM-related and currently analyzed in detail. Lipid 3a was not identical with lipid 1 as revealed by different Rf values (Fig. 2) and comparative HPTLC (data not shown). Furthermore, lipid 3a was...
not present in the *M. bovis* BCG pattern; therefore, it represents a cleavage product modified after its release from the bacteria and before its transport to other vesicles.

**FIGURE 2.** Export of mycobacterial lipids from the phagosome. Two lipid species found in the lipid pattern of vesicles from infected cells were absent from the Mφ lipid pattern (3a, position indicated by a circle; 2) and one was found in the mycobacterial lipid pattern (2). a, Mφ; b, *M. bovis* BCG labeled with 10 μCi [14C]palmitic acid for 12 h. Lipids were extracted from dried cells in CHCl₃/CH₃OH/H₂O (16:6:1, v/v/v) for 12 h. c, Forty-eight-hour time point of vesicles from [14C]*M. bovis* BCG-infected Mφ; d, Isolated *M. bovis* BCG from Fig. 1 shown. Lipids were compared with respective standards (data not shown). Cer, Ceramide; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; SM, sphingomyeline; MycB, mycoside B; TMM, trehalose monomycocytate.

**FIGURE 3.** Lysosomal enzymes cleave lipid 4 into two new products (3b and 2). One of the resulting products (3b) had the same *R*ₚ value as 3a found in vesicles of infected Mφ (3b = 0.170.85; 3a = 0.170.86). [14C]-labeled lipids were separated on HPTLC plates, and the single lipids were re-extracted from the plate. The lipids were resuspended in 1% Triton X-100 in 10 mM HEPES pH 4.5 and incubated at 37°C for 2 h in presence or absence of purified Mφ lysosomes. The lipid content was re-extracted and separated by HPTLC in two dimensions.

Lysoosomal enzymes of Mφ can modify mycobacterial lipids

To study processing, radioactively labeled mycobacterial lipids were isolated by HPTLC and incubated with purified Mφ lysosomes. Although most of the lipids were not affected by this treatment (data not shown), lipid 4 was cleaved, resulting in two new products (Fig. 3), termed 3b and 5. Thus, lysoosomal Mφ enzymes can modify mycobacterial lipids into distinct products.

**Identification of lipid 4 as cardiolipin**

The characteristics of lipid 4 were examined using three different spray reagents revealing that it did not contain amino or carbohydrate groups, but did contain phosphorous (Fig. 4a). After methanolyzing, the lipid was analyzed by GC-MS to define its fatty acid content (Fig. 4b). The two major fatty acids were identified as hexadecanoic acid (C16) and octadecenoic acid (C18:1). 1H-NMR analysis confirmed that one of the fatty acids was unsaturated (5005 ppm), whereas the characteristic signals of carbohydrates or
Hydrin stain was negative, and phosphorus. Lipid 4 was separated by HPTLC and contained amino or carbohydrate groups, but does contain phosphorus. Lipid 4 was separated by HPTLC and analyzed using the following spray reagents: the ninhydrin stain was negative, α-naphthol stain was red, but not blue, therefore indicating the absence of carbohydrates, the Dittmers reagent stained the phosphorous-containing compound. The fatty acid content of lipid 4 is mainly C16 and C18:1. The sample was methanolyzed and analyzed by GC-MS. c. MALDI-MS analysis: lipid 4 has a molecular mass of 1404 Da, indicating its identity with cardiolipin. d. Comparative HPTLC with a cardiolipin standard verified that lipid 4 is cardiolipin. e. The pattern of lipids extracted from CSN of radioactively labeled M. bovis BCG and separated by HPTLC revealed that cardiolipin is released from in vitro grown mycobacteria. The CSN pattern was visualized by phosphoinager, and cardiolipin was spray detected.

Carbohydrys were not detected (data not shown). The ESI-MS spectrum confirmed a high purity of the product (>99%) because no mass peaks were observed between 350 and 650 m/z (data not shown). The major peak at 701 m/z was double-nosed, suggesting that the analyzed lipid is double-negatively charged and probably has a molecular mass of 1402 Da (M^-2). MALDI-MS analysis verified the molecular mass (M^+) of 1403 Da (Fig. 4c). From the combined data obtained we propose that the lipid represents a phospholipid with C16 and C18:1 fatty acids and a molecular mass of 1404 Da. The only known mycobacterial phospholipid with such properties is diphosphatidylglycerol (cardiolipin), which is one of the major mycobacterial phospholipids (19). The identification was confirmed by comparative HPTLC using a cardiolipin standard derived from bovine heart revealing identical Rf values (Fig. 4d). Analysis of the standard cardiolipin by ESI-MS gave a similar double-nosed peak, but at 723 Da, which is due to the different fatty acid content and higher unsaturation of the fatty acids of cardiolipin derived from heart (data not shown).

**Cardiolipin is released from mycobacteria grown in vitro**

To see whether cardiolipin is released from mycobacteria, radioactively labeled M. bovis BCG were cultured in Middlebrook medium for 24 h, and the culture supernatant (CSN) was collected, lyophilized, extracted, and analyzed by HPTLC. Although the HPTLC pattern of CSN lipids reveals a number of lipid species also present in the cellular pattern, only certain lipids are released into the CSN, suggesting that this represents rather a selective process and is not just an effect of cell lysis (data not shown). Among these lipids a significant amount of cardiolipin is detected in the CSN (Fig. 4e).

**Cardiolipin is hydrolyzed by lysosomal-type Ca^2+ -independent phospholipase A_2**

Radioactively labeled mycobacterial cardiolipin was incubated in vitro with various phospholipases. Only in the case of phospholipase A_2, the resulting cleavage products were identical with the products derived by incubation with lysosomes (Fig. 5a). Comparison of the migration properties of the cleavage products with known cardiolipin catabolites and HPTLC analysis with the respective standards indicate that lipid 3c was identical with lysocardiolipin and lipid 5 to di-lysocardiolipin (data not shown). Lipid 3a from a vesicle preparation, lipid 3b from incubation of cardiolipin with phospholipase A_2 were re-extracted and developed on a HPTLC. Lipid 3a had an Rf value identical with the products of cardiolipin cleaved by either purified lysosomes or phospholipase A_2 (Fig. 5b). This shows that Mø lysosomes contain a phospholipase A_2 activity that can cleave mycobacterial cardiolipin released from intracellular bacteria into lysocardiolipin and di-lysocardiolipin. To characterize the type of phospholipase activity present in Mø lysosomes, the phospholipase A_2 substrate [3H]-labeled phosphatidylycholine was incubated with purified lysosomes under various conditions (Fig. 6). The activity of the lysosomal phospholipase A_2 was calcium-independent because depletion of calcium with EGTA had no effect. The phospholipase A_2 activity was optimal at pH 4, reduced by 66% at pH 6.2, and

**FIGURE 4.** Analysis of lipid 4. a. Lipid 4 does not contain amino or carbohydrate groups, but does contain phosphorus. Lipid 4 was separated by HPTLC and analyzed using the following spray reagents: the ninhydrin stain was negative, α-naphthol stain was red, but not blue, therefore indicating the absence of carbohydrates, the Dittmers reagent stained the phosphorous-containing compound. b. The fatty acid content of lipid 4 is mainly C16 and C18:1. The sample was methanolyzed and analyzed by GC-MS. c. MALDI-MS analysis: lipid 4 has a molecular mass of 1404 Da, indicating its identity with cardiolipin. d. Comparative HPTLC with a cardiolipin standard verified that lipid 4 is cardiolipin. e. The pattern of lipids extracted from CSN of radioactively labeled M. bovis BCG and separated by HPTLC revealed that cardiolipin is released from in vitro grown mycobacteria. The CSN pattern was visualized by phosphoinager, and cardiolipin was spray detected.

**FIGURE 5.** The enzyme that cleaves cardiolipin is a phospholipase A_2. Cardiolipin was dissolved in 0.1% Triton X-100 in PBS (pH 8.9) and incubated at 25°C for 1 h in the presence or absence of 10 U phospholipase A_2 from porcine pancreas. a. The lipid content was re-extracted and separated by HPTLC. b. To confirm that lipid 3a found in vesicles is identical with the cleavage product of cardiolipin derived by incubation with lysosomes (3b) and phospholipase A_2 (3c), comparative HPTLC was performed. The respective lipids were re-extracted from HPTLC and separated in one dimension.
The extraordinary array of lipids, glycolipids, and lipoconjugates in the mycobacterial cell wall plays an important role in tuberculosis as well as in other mycobacterial infections. Here we report that mycobacterial lipids, notably lycocardiolipin, are transferred out of phagosomes into lysosomes and that lycocardiolipin is derived from the cleavage of cardiolipin by a lysosomal type Ca\(^{2+}\)-independent phospholipase A\(_2\) that can cleave mycobacterial cardiolipin allowing subsequent detection of lysocardiolipin outside of the phagosome.

**Discussion**

The extraordinary array of lipids, glycolipids, and lipoconjugates in the mycobacterial cell wall plays an important role in tuberculosis as well as in other mycobacterial infections. Here we report that mycobacterial lipids, notably lycocardiolipin, are transferred out of phagosomes into lysosomes and that lycocardiolipin is derived from the cleavage of cardiolipin by a lysosomal type Ca\(^{2+}\)-independent phospholipase A\(_2\).

In earlier studies from our laboratory, confocal microscopy of infected murine M\(_\phi\) revealed that large quantities of mycobacterial cell wall material are transferred from the phagosome and accumulate in late endosomes/lysosomes. LAM and phosphatidylinositol-tetraammonoside (PIM\(_4\)) have been determined as the main translocate in late endosomes/lysosomes. LAM and phosphatidylinositol-phosphatidylethanolamine, and phosphatidyglycerol (data not shown). This observation is in contrast to earlier statements claiming that mycobacterial lipids are highly resistant to degradation by the host cell.

We have shown that mycobacterial lipids are transported to late endosomes/lysosomes containing several glycolipid-degrading enzymes such as lipases and glycosidases. However, the use of purified lysosomes and individual lipids revealed that lysosomal enzymes did not alter the majority of the mycobacterial lipids investigated. In contrast, cardiolipin, an abundant and integral compound of the mycobacterial envelope, was cleaved by a lysosomal phospholipase A\(_2\) into lycocardiolipin and di-lyso-cardiolipin. Cardiolipin is present in the inner membrane of prokaryotes as well as in mitochondria of eukaryotic cells, most prominently in the heart muscle of mammals. Cardiolipin itself was associated with the mycobacteria during the entire infection period of 48 h, whereas lycocardiolipin was transferred out of the phagosome into other vesicles, indicating that cleavage of cardiolipin occurred in the phagosome. Interestingly, cardiolipin was released from intracellular mycobacteria, suggesting that within the phagosome, released cardiolipin is cleaved immediately. The phospholipase activity responsible for the cleavage is of host cell origin, because mycobacteria do not express surface-exposed phospholipase A\(_2\). Furthermore, lycocardiolipin was not found in the CSN. A lysosomal-type Ca\(^{2+}\)-independent phospholipase A\(_2\) is responsible in the degradation of mycobacterial cardiolipin, which is distinct from the groups of the secreted or cytoplasmic phospholipases A\(_2\). Evidence that this type of phospholipase interacts with mycobacterial lipids indicates a new function for these lysosomal enzymes, which could be important during mycobacterial infection. Enzyme activity was detected at pH 6.2, which is the pH value of intact mycobacterial phagosomes, but optimal activity was observed at low pH (26). This indicates that the cleavage could occur at a pH of 6.2 in the phagosome or more effectively in the phagolysosome with its lower pH. Acidified phagolysosomes contain most probably nonviable bacteria (14). One possible function for this phospholipase could be processing of potential lipid Ags, such as the B cell Ag cardiolipin, suggesting a similar role as lysosomal proteases in Ag processing for MHC class II presentation. Moreover, host cells up-regulate their lysosomal phospholipase A activity following mycobacterial infection (27). This may hint toward a bactericidal activity of this enzyme as shown for other phospholipase A\(_2\) types (28). It may also be a direct response to the increased amount of phospholipids in the phagosome, facilitating degradation of cardiolipin, and release of lycocardiolipin.
It has been shown that lysophospholipids can suppress IL-2 secretion of activated CD4 T cells and inhibit membrane fusion (29–31). Pathogenic mycobacteria survive in Mφ and reside within phagosomes that do not fuse with lysosomes (26). Therefore, lysocardiolipin may be a candidate lipid involved in preventing phagolysosome fusion or suppression of CD4 T cell functions (26, 31). Furthermore, lysophospholipids can influence signal transduction pathways, e.g., by stimulating arachidonic acid release and activating protein kinase C (32, 33).

The ability of Mφ to modify mycobacterial cardiolipin, which subsequently leads to an altered intracellular distribution of lysocardiolipin, could be crucial for the biological effects of cardiolipin. We envisage this novel processing pathway as a general mechanism of how infected cells alter lipids released from intracellular pathogens. The resulting lysophospholipids are likely to have functions that influence the outcome of mycobacterial infections.

Acknowledgments
We thank Dr. Helen Collins and Lucia Lom-Terborg for critically reading our manuscript and Dr. Robert Hurwitz for technical advice.

References