Listeria monocytogenes transiently alters mitochondrial dynamics during infection

Fabrizia Stavrakoudi,1,2 Frédéric Bouillaud,3,4 Anna Sartori,5 Daniel Ricquier,3,4 and Pascale Cossart2,1

*Unité des Interactions Bactéries-Cellules, Institut Pasteur; U604, Institut National de la Santé et de la Recherche Médicale and 2USC2020, Institut National de la Recherche Agronomique, 75015 Paris, France; 3Institut Cochin, 4Institut National de la Santé et de la Recherche Médicale U916, and 5Centre National de la Recherche Scientifique Unité Mixte de Recherche 8104, Université Paris Descartes, 75014 Paris, France; and 6Plate-Forme de Microscopie Ultrastructurale, Imagopole, Institut Pasteur, Unité de Recherche Associée 2185; 75015 Paris, France

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Mitochondria are essential and highly dynamic organelles, constantly undergoing fusion and fission. We analyzed mitochondrial dynamics during infection with the human bacterial pathogen Listeria monocytogenes and show that this infection profoundly alters mitochondrial dynamics by causing transient mitochondrial network fragmentation. Mitochondrial fragmentation is specific to pathogenic Listeria monocytogenes, and it is not observed with the nonpathogenic Listeria innocua species or several other intracellular pathogens. Strikingly, the efficiency of Listeria infection is affected in cells where either mitochondrial fusion or fission has been altered by siRNA treatment, highlighting the relevance of mitochondrial dynamics for Listeria infection. We identified the secreted pore-forming toxin listeriolysin O as the bacterial factor mainly responsible for mitochondrial network disruption and mitochondrial function modulation. Together, our results suggest that the transient shutdown of mitochondrial function and dynamics represents a strategy used by Listeria at the onset of infection to interfere with cellular physiology.

Results

Infection with Pathogenic Listeria Induces Mitochondrial Fragmentation. We first investigated the effects of L. monocytogenes infection on mitochondria of epithelial cells by using confocal laser scanning microscopy. Fig. 14 shows L. monocytogenes infection of HeLa cells [1 h, multiplicity of infection (MOI) of 50] inducing strong mitochondrial network fragmentation. Importantly, this network fragmentation is specific to pathogenic L. monocytogenes, because it is not observed with the closely related nonpathogenic species Listeria innocua, even when cells are infected with L. innocua overexpressing the L. monocytogenes invasin Internalin B (InvB) to enter cells [L. innocua(InvB), Fig. 1A]. This finding indicates that mitochondrial fragmentation is not a consequence of stress imposed by the engulfment of bacteria. Furthermore, L. monocytogenes-induced mitochondrial fragmentation is not restricted to HeLa cells, because it also occurs in human placental (Jeg3) and green monkey kidney (Vero) cells (Fig. S14).

To assess whether other invasive pathogens also cause mitochondrial fragmentation, we infected cells with Salmonella enterica serovar Typhimurium, Escherichia coli (Inv) as a model for Yersinia pseudotuberculosis (37), enteropathogenic E. coli (EPEC), and Shigella flexneri. Importantly, infection with either the extracellular pathogen EPEC or with invasive pathogens that remain food-borne disease listeriosis. While listeriosis is a public health issue, L. monocytogenes has been instrumental in elucidating fundamental cell biological questions, e.g., actin polymerization principles (reviewed in refs. 28 and 29).

After cell invasion, L. monocytogenes uses the pore-forming toxin listeriolysin O (LLO) to escape from the phagosome. Although LLO function had been characterized first in Listeria escape from the phagosome under acidic conditions (30), several studies now indicate that this crucial virulence factor also displays activity at neutral pH and acts on cells before bacterial entry (31, 32). Indeed, low levels of LLO secreted before bacterial entry are sufficient to activate prosurvival signaling cascades such as the NFκB and MAPK pathways (33, 34), transcriptionally reprogram host cells (35), and trigger global deSUMOylation (36).

Here we report that L. monocytogenes causes dramatic alterations of mitochondrial dynamics via LLO. Strikingly, mitochondrial fragmentation induced by Listeria infection is a transient phenomenon, indicating that mitochondria are not terminally damaged. We propose that modulation of mitochondrial dynamics and function is a strategy used by pathogenic Listeria at the onset of infection to slow down mitochondrial activity and mitochondria-dependent processes.

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1To whom correspondence may be addressed. E-mail: pcosart@pasteur.fr or fstawr@pasteur.fr.

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confined to a phagocytic vacuole [i.e., *E. coli* (Inv) and *Salmonella enterica* serovar *typhimurium*) did not appear to affect mitochondria (Fig. 1B), even at an MOI of 100 and up to 3 h of infection, supporting the notion that *L. monocytogenes*-induced mitochondrial fragmentation is not a general stress response to bacterial infection. Moreover, mitochondrial fragmentation is not caused by the presence of bacteria in the cytosol, because infection with *Shigella flexneri*, which, like *Listeria*, escapes from the vacuole and polymerizes actin to move intra- and intercellularly, did not cause mitochondrial fragmentation (Fig. 1B).

Correlative light/transmission electron microscopy (TEM) showed that fragmented mitochondria in *Listeria*-infected cells had a disorganized ultrastructure with remodeled cristae compared with mitochondria in noninfected cells (Fig. S1B).

**Impairment of Mitochondrial Dynamics Affects Infection by *Listeria monocytogenes***. Having established that *L. monocytogenes* infection induces mitochondrial fragmentation, we asked whether inhibiting fusion or fission by siRNA would affect early *Listeria* infection stages. Cells depleted of the fusion proteins Mfn1 and Mfn2 (resulting in fissioned mitochondria) or of the fission protein Drp1 (resulting in hyperfused mitochondria) were infected with *L. monocytogenes*. Infection was strongly impaired in cells with fissioned mitochondria, and the strongest inhibition was associated with fragmented mitochondria (Fig. 1C). This result suggests that, for efficient infection, *Listeria* requires the ability to induce mitochondrial fission, because cells with mitochondria that already are fissioned at the onset of infection provide an unfavorable environment for infection. Silencing of proteins regulating mitochondrial dynamics may also affect later stages of infection or have indirect effects on mitochondrial function and infection.

**Listeriolysin O Is Sufficient to Cause Mitochondrial Fragmentation**. Because mitochondria appeared to fragment at early stages of infection, we tested whether a secreted effector of *L. monocytogenes* could cause mitochondrial fragmentation. We first used a noninvasive mutant of *L. monocytogenes* lacking InlB and found that this mutant was still able to induce mitochondrial fragmentation (Fig. 3A). We then asked whether the best-characterized secreted effector of *L. monocytogenes*, the pore-forming toxin LLO, could induce mitochondrial fragmentation. Strikingly, mitochondrial fragmentation was abolished when an LLO-deletion mutant (*L. monocytogenes Δllo*) was used (Fig. 3A), indicating that LLO is required for fragmentation of host-cell mitochondria. Bacteria whose hemolysin (hly) gene carries a point mutation disrupting pore formation (38) did not affect mitochondrial morphology (Fig. 3B), revealing that fragmentation depends on the pore-forming ability of LLO.

LLO appears necessary and sufficient to induce mitochondrial fragmentation, because addition of the purified toxin at nanomolar concentrations [3–6 nM, considered noncytotoxic and not causing lactate dehydrogenase (LDH) release (35)] recapitulated the mitochondrial phenotype observed upon infection (Fig. 3C). Mitochondrial fragmentation was found to occur in a fast, all-or-nothing manner (i.e., within less than 10 min) upon addition of recombinant LLO (Movie S1). Increasing incubation time or LLO concentration resulted in an increased number of cells displaying fragmented mitochondria (Fig. 3D).
Mitochondrial fragmentation could result from enhanced fission or decreased fusion (39). We thus analyzed whether LLO treatment would affect total levels of key mitochondrial dynamics mediators, i.e., Mfn1, Mfn2, and Drp1. Total levels of these proteins did not decrease in cells treated with 3–6 nM recombinant LLO (Fig. 3E). Because LLO forms ion-permeable pores in membranes (30), we hypothesized that LLO might modulate mitochondrial dynamics by inducing ion flux. We tested whether blocking K⁺ efflux or Ca²⁺ influx would prevent LLO-induced mitochondrial fragmentation, given that changes in these ions are known to affect mitochondrial morphology (40, 41). Blocking K⁺ efflux from LLO-treated cells by incubation in high extracellular K⁺ concentrations (135 mM) had no effect (Fig. 4). In contrast, interfering with LLO-induced Ca²⁺ influx by performing LLO treatment in Ca²⁺-free medium strongly prevented mitochondrial fragmentation (Fig. 4). This result suggested that Ca²⁺ influx (rather than K⁺ efflux) through LLO pores is the signal inducing mitochondrial fragmentation.

Fig. 3. LLO induces mitochondrial fragmentation without affecting total levels of key mitochondrial dynamics proteins. (A) HeLa cells infected (1 h, MOI of 50) with a noninvasive L. monocytogenes mutant (ΔinIB) display fragmented mitochondria, indicating bacterial entry is not required. In contrast, no fragmentation occurs with infection by an LLO-deficient mutant (Δhly). Bacteria were stained with anti L. monocytogenes (R11, green), mitochondria were stained with anti-cytocrome c (red), and DNA was stained with DAPI (blue). Insets show 2× enlargements of mitochondria. Arrows point to infected cells. (B) L. monocytogenes carrying a point mutation in the hly gene inactivating its pore-forming ability (W492A) do not cause mitochondrial fragmentation. Mitochondria (red) and bacteria (green) were stained as in A. (C) HeLa cell treatment with recombinant LLO (10 min, 6 nM) was sufficient to induce mitochondrial fragmentation. Mitochondria (red) were stained as in A. (D) HeLa cells were treated with different concentrations of LLO for 10 min or with 6 nM LLO for different periods of time. Morphometric analysis indicated that LLO-induced mitochondrial fragmentation is concentration and time dependent at the cell-population level. The percentage of fragmentation was determined by counting at least 100 cells per data point; data from at least two independent experiments were pooled, and P values were calculated using one-tailed Student’s t test (**P < 0.005, *P < 0.25). (E) Cells treated for 10 min with the indicated amounts of LLO were analyzed by Western blot for Drp1, Mfn1, or Mfn2, showing that LLO treatment does not affect their total levels.

LLO Treatment Causes Mitochondrial Membrane Potential Loss and a Drop in Respiration and Cellular ATP. To investigate the functionality of mitochondria in LLO-treated cells, we measured the mitochondrial membrane potential ΔΨ by assessing release of the ΔΨ-dependent mitochondrial dye tetramethylrhodamine ethyl ester (TMRE) and found that LLO treatment caused a significant drop in ΔΨ compared with untreated cells (Fig. 5A). At the single-cell level, time-lapse microscopy of mitochondria loaded with the TMRE derivative tetramethylrhodamine methyl ester (TMRM) indicated that ΔΨ loss was concomitant with or immediately preceded mitochondrial fragmentation (Fig. 5B).

LLO treatment caused a drop in respiratory activity as measured with an oxygen consumption chamber (Fig. S3). Respiration resumed when the mitochondrial respiratory substrate succinate was added, indicating that no major damage occurred to mitochondria, because the respiratory chain (complex II–complex IV) appeared functional. Furthermore, the resumption of respiration demonstrates that LLO does not form pores in the mitochondrial inner membrane, a finding that is supported further by immunofluorescence analysis, showing plasma membrane rather than mitochondrial localization of LLO (Fig. S4). In contrast, LLO appears to permeabilize the otherwise succinate-impermeable plasma membrane, contributing to mitochondrial depolarization by allowing leakage of bioenergetic substrates out of the cell.

Infection also affected cellular ATP levels in an LLO-dependent manner: Infection with wild-type L. monocytogenes (1 h, MOI of 50) induced a 50% decrease in intracellular ATP levels, but this decrease was not observed with L. innocuaΔinIB) or the L. monocytogenes Δhly mutant (Fig. 5C). Recombinant LLO (6 nM, 10 min) was sufficient to cause an even more pronounced decrease in intracellular ATP levels (Fig. 5C). A similar decrease was obtained by a combination of chemically induced mitochondrial uncoupling with carbonyl cyanide m-chlorophenylhydrazone (CCCP) and Ca²⁺ influx (A23187), although uncoupling or Ca²⁺ influx separately did not lead to a significant difference (Fig. SD). These data strongly suggest that L. monocytogenes infection not only affects mitochondrial dynamics but also interferes with cellular bioenergetics and mitochondrial function.

LLO-Induced Mitochondrial Fragmentation Does Not Correlate with Classical Apoptosis. Mitochondrial fragmentation has been described in apoptotic cells, prompting us to analyze apoptosis markers such as cytochrome c release. Cytochrome c was not released from fragmented mitochondria upon infection or LLO treatment (Fig. 4A and Fig. S5A). In contrast, cytochrome c release was observed in the positive control, i.e., staurosporine-treated cells (Fig. S5A). Interestingly, the observed mitochondrial fragmentation did not depend on mitochondrial transition pore (mTP) opening, because it was not blocked by the mTP inhibitor cyclosporin A. To test further for apoptosis in cells displaying infection-induced mitochondrial fragmentation, we analyzed activated B-cell lymphoma 2 (Bcl2)-associated X (Bax) protein by immunostaining. The proapoptotic Bcl2 protein family member...
Bax is activated by several apoptotic signals and translocates to mitochondria, where it forms pores and participates in fission (42, 43). We could not detect mitochondrial recruitment of activated Bax upon infection or LLO treatment (Fig. S5B).

Together, these experiments suggest that neither L. monocytogenes infection nor treatment with sublytic LLO concentrations causes classical apoptosis in HeLa cells.

**Infection-Induced Mitochondrial Fragmentation Is Transient.** An important question was whether mitochondria that fragment because of LLO produced at initial stages of *Listeria* infection recover their original shape and reform an interconnected network. To this end, we followed recovery by inflicting cells for 1 h and then observing mitochondrial morphology at different time points after bacteria removal. The proportion of cells with fragmented mitochondria decreased steadily (Fig. 6A). Time-lapse imaging indicated that in most cases tubular mitochondrial morphology is recovered within the first few hours (Fig. 6B). In line with these results, we found that intracellular ATP levels recovered by 4 h postinfection (Fig. S6), suggesting that infected cells are not terminally damaged.

**Discussion**

We show here that infection with pathogenic *L. monocytogenes* causes transient mitochondrial network fragmentation and identify the secreted bacterial toxin LLO as the main factor affecting mitochondrial morphology and function at early time points of infection. LLO induces Ca\(^{2+}\)-dependent mitochondrial fragmentation, accompanied by a decrease in the mitochondrial membrane potential \( \Delta \Psi \) and in respiration. As \( \Delta \Psi \) and respiration concomitantly decrease upon LLO treatment, ATP regeneration proceeds inefficiently, contributing to a decrease in intracellular ATP levels. This decrease suggests a transient metabolic “slow-down” of host cells, favoring early stages of infection by interfering with the capacity of the host cell to respond to this event. Such strategy could be common to several bacteria secreting pore-forming toxins, because we found that different recombinant pore-forming toxins had effects comparable to LLO (Fig. S7).

LLO pore formation has been studied at the biophysical and physiological level and induces Ca\(^{2+}\) influx (32, 44). Interestingly, several pathogens manipulate host-cell physiology by inducing Ca\(^{2+}\)-fluxes (45). Importantly, this Ca\(^{2+}\) influx enhances *Listeria* entry into cells (31), suggesting that early LLO action is a crucial step in epithelial cell infection. Ca\(^{2+}\) influx probably represents a first bioenergetic insult to the cell, inducing mitochondrial fragmentation and depolarization as well as blocking mitochondrial movement (Movie S1), although such damage is reversible. While neither uncoupling nor Ca\(^{2+}\) ionophore treatment significantly reduce intracellular ATP levels, such an ATP decrease is reproduced partially by the synergistic action of an uncoupler and a Ca\(^{2+}\) ionophore but these drugs do not cause the dramatic mitochondrial fragmentation observed upon LLO treatment (Fig. S8).

In the case of *Listeria* infection, the bioenergetic crisis is probably aggravated by leakage of small molecules, including respiratory substrates or glycolysis intermediates through LLO pores. LLO-induced \( \Delta \Psi \) decrease also may reflect a host-cell response to prevent mitochondrial Ca\(^{2+}\) accumulation to cytotoxic levels, because mitochondrial Ca\(^{2+}\) uptake is \( \Delta \Psi \) dependent (46). Indeed, several markers of apoptosis were absent in cells with LLO- or infection-induced mitochondrial fragmentation. Our data are consistent with the notion that epithelial cells recover from the attack of pore-forming toxins (including LLO) at sublytic concentrations, regain membrane integrity, and resume the cell cycle (32, 47, 48). The molecular mechanisms underlying LLO recovery are currently unclear: LLO does not colocalize with endocytic markers at early time points (Fig. S4). Infected cells appear to restore their mitochondrial network both morphologically (Fig. 6) and functionally, because intracellular ATP levels recover 4 h postinfection (Fig. S6). Consistent with the view that cytosolic LLO is inactivated rapidly (30), mitochondrial fragmentation is induced only by extracellular LLO, i.e., early during *Listeria* infection; at late time points of infection intracellular bacteria do not affect mitochondrial morphology, even though they produce LLO to escape from the vacuole (Fig. S9). Together, these data indicate that *Listeria* affects host-cell mitochondria only transiently. Permanent impairment of mitochondrial function and dynamics would harm host cells and therefore would be counterselected for, because it would eliminate the bacterial replication niche.

Our data suggest that active induction of mitochondrial fragmentation early during infection is critical for infection. Indeed, infection is impaired in cells with previously fissioned mitochondria and is enhanced in cells with hyperfused mitochondria. Treatments that impair mitochondrial respiration cause fragmentation (49), and, conversely, mitochondrial fragmentation has been shown to limit the spread of incoming Ca\(^{2+}\) across the mitochondrial network (50). Accordingly, fragmentation appears to be an appropriate response to avoid propagation of the consequences of Ca\(^{2+}\) influx to the entire mitochondrial network. A prefusion state would limit the extent of damage caused by LLO action and allow the mitochondrial network to restore cellular bioenergetics more efficiently. Consequently, invading *Listeria* would have less time to take advantage from the transient bioenergetic “slow-down” it induces. The opposite effect would occur in Drp1-knockdown cells with a hyperfused mitochondrial network.

In conclusion, we propose a scenario in which the normal bioenergetic state of the cell represents a barrier to *Listeria* invasion. Consequently, an LLO-induced transient metabolic reprogramming of the cell would promote efficient infection. In
Whether in ionophore A23187 (10.05, one-tailed LLO causes a decrease in the mitochondrial membrane potential ΔΨ values were calculated (**infection can feed -causes the strongest drop in intracellular ATP levels; the effect is more pronounced when cells are incubated with 6 nM LLO becomes apparent. Fragmented mitochondria lose TMRE and retain only the potential-insensitive MitoTracker 488. (C) Intracellular ATP levels were measured 1 h after infection with L. innocua expressing InlB, L. monocytogenes ΔInlB, or wild-type L. monocytogenes. Wild-type L. monocytogenes causes the strongest drop in intracellular ATP levels; the effect is more pronounced when cells are incubated with recombinant LLO (10 min, 6 nM). Cell permeabilization with detergent (0.1% Triton X-100, 10 min) causes complete intracellular ATP release. Experiments were performed three times in duplicate, and mean values (normalized to untreated cells) are shown. Student’s t test). (D) HeLa cells were treated for 10 min with 6 nM LLO or 0.1% Triton X-100, for 30 min with the mitochondrial uncouplers CCCP (100 μM), carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) (1 μM), and valinomycin (100 nM), or with the Ca2+ ionophore A23187 (1 μM). Intracellular ATP levels do not decrease significantly upon Ca2+ influx or uncoupling but do decrease significantly with the combination of both treatments. Statistical analysis was performed as in C. n.s., nonsignificant values.

agreement with this notion, mitochondrial dysfunction has been linked to increased susceptibility to bacterial infection (51, 52). Our work shows that mitochondrial dynamics plays a role in infection with the human pathogen L. monocytogenes. Whether in this case specific signaling cascades are activated downstream of the induced mitochondrial fragmentation and dysfunction is currently unknown, but mitochondrial dynamics and infection do appear to influence each other mutually, because Listeria infection specifically leads to transient disruption of mitochondrial morphology and function, and prior disruption of mitochondrial dynamics by siRNA affects Listeria infection efficiency. Interestingly, the cytomegalovirus protein vMia induces mitochondrial fragmentation and thereby prevents innate immune signaling downstream of mitochondrial antiviral signaling protein (MAVS) (26). Listeria may act similarly, explaining MAVS-independent activation of the innate immune response (53). Given that bioenergetics affect mitochondrial shape, and vice-versa (2, 49), it is likely that mitochondrial localization of innate immunity components serves to coordinate innate immune responses with cellular energy levels via sensing of mitochondrial morphology and function. How innate immunity components and the fission/
fusion machinery sense changes in the bioenergetic status of mitochondria is still unknown. The strong and rapid action of LLO described in our work provides an additional model system to address this question.

Materials and Methods

Reagents, cell lines, and bacterial strains used in this study, detailed experimental protocols, and nonstandard abbreviations are provided in SI Materials and Methods.

10. Alexander C, et al. (2008) OPA1, encoding a dynamin-related GTPase, is mutated in autosomal dominant optic atrophy linked to chromosome 3q28.

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