Differential Role of Cytosolic Phospholipase A₂ in the Invasion of Brain Microvascular Endothelial Cells by Escherichia coli and Listeria monocytogenes

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Invasion of brain microvascular endothelial cells (BMECs) is a key step in the pathogenesis of meningitis due to Escherichia coli and Listeria monocytogenes. Although host cell actin cytoskeletal rearrangements are essential in BMEC invasion by E. coli K1 and L. monocytogenes, the underlying signaling mechanisms remain unclear. This study demonstrates that host cell cytosolic phospholipase A₂ (cPLA₂) contributes to E. coli K1 invasion of BMECs but not to L. monocytogenes invasion of BMECs. This difference was observed with 4-bromo-phenacyl bromide, a nonselective PLA₂ inhibitor, and arachidonyl trifluoromethyl ketone, a selective cPLA₂ inhibitor, and was confirmed with BMEC derived from cPLA₂ knockout mice. Activation of cPLA₂ leads to generation of intracellular arachidonic acid, which is metabolized via cyclooxygenase (COX) and lipo-oxygenase (LOX) pathways into eicosanoids. COX and LOX inhibitors also significantly inhibit E. coli K1 invasion of BMECs.

Neonatal bacterial meningitis occurs in 0.25–1.0 per 1000 live births [1–3] and carries a mortality rate of 15%–40%. More than 50% of survivors have neurologic sequelae [1–3]. Escherichia coli and Listeria monocytogenes rank second and third, respectively, after group B streptococcus, as the common etiologic agents for neonatal bacterial meningitis [1–4]. Most cases result from hematogenous spread, but it is unclear how the circulating bacteria cross the blood-brain barrier (BBB). We previously reported that E. coli K1 invasion of brain microvascular endothelial cells (BMECs) is a key step in the traversal of bacteria across the BBB in vivo [5–7]. We also showed that host cell actin cytoskeletal rearrangements are essential for BMEC invasion by both E. coli K1 and L. monocytogenes [8, 9] and reported a role of focal adhesion kinase and phosphatidylinositol 3-kinase in E. coli K1 invasion of BMECs [10, 11]. However, the specific host cell signaling pathways involved in BMEC invasion by E. coli K1 and L. monocytogenes remain incompletely understood.

While examining a potential role for host cell heterotrimeric G proteins in bacterial invasion of BMECs, we found that isotetrandrine, an inhibitor of G protein activation of phospholipase A₂ (PLA₂) [12, 13], inhibited E. coli K1 invasion of BMECs. Isotetrandrine did not exhibit such inhibitory effect on L. monocytogenes invasion of BMECs. This prompted us to further investigate the role of host cell PLA₂ in invasion of BMECs by these bacteria.

In mammalian cells, PLA₂ is important in several pathophysiologic conditions, by producing precursors of inflammatory molecules from phospholipids—such as platelet-activating factor, prostaglandins, thromboxane, and leukotrienes—which are collectively known as eicosanoids [14]. PLA₂ hydrolyzes phospholipids at their sn-2 position, resulting in the release of lysophospholipids and fatty acids, especially arachidonic acid (AA), which is abundant at the sn-2 position. Previous studies have linked activation of PLA₂ and AA metabolites, generated through the cyclooxygenase (COX) and lipo-oxygenase (LOX) pathways, with actin cytoskeletal rearrangement in eukaryotic cells [15]. PLA₂ activation is also important in Salmonella typhimurium invasion of epithelial cells through an epidermal growth factor (EGF) receptor-mediated pathway, although there was no mention of any specific PLA₂ subclass involvement [16].

PLA₂ can be classified into 12 groups [17], but, conventionally, 3 main forms of PLA₂ are described: Ca²⁺-dependent secretory PLA₂ (sPLA₂) of ~14 kDa in molecular mass, Ca²⁺-independent PLA₂ (iPLA₂), and Ca²⁺-dependent 85-kDa cytosolic PLA₂ (cPLA₂) [14, 18, 19]. Intracellular AA release is mediated primarily by cPLA₂. sPLA₂ has been associated with antibacterial activity against both gram-positive and gram-ne...
Materials and Methods

Reagents. Isotetrandrine (inhibitor of G protein activation of PLA₂), arachidonyl trifluoromethyl ketone (AACOCF₃; selective cPLA₂ inhibitor), bromoeno lactone (BEL; selective iPLA₂ inhibitor), and 12 episcalaradial (selective sPLA₂ inhibitor) were purchased from Biomol Laboratories. We purchased 4-bromophenacyl bromide (BPB; PLA₂ inhibitor) from Sigma Laboratories. We purchased indomethacin, nordihydroguaiaretic acid (NDGA), and NS-398 from Cayman Chemical.

Bacterial strains. E. coli K1 strain E44, a spontaneous rifampicin-resistant mutant of the cerebrospinal fluid isolate RS218 (O18:H11034), was obtained from a neonate with meningitis [9]. The control noninvasive E. coli was laboratory strain HB101 [5]. L. monocytogenes Sv 1/2a EGD wild-type strain and WL-111 non-invasive strain (in-frame intB deletion of strain EGD) were as described elsewhere [8]. E. coli strain E44 and L. monocytogenes strain EGD invade BMECs [5–8]. All bacteria were grown for 14 h at 37°C in brain-heart infusion broth (Difco Laboratories), except E44, which was grown under the same conditions in the presence of rifampin (100 μg/mL).

BMECs. Human BMECs (HBMECs) were isolated from small fragments of cerebral cortex obtained from surgical resections of 4–7-year-old children undergoing neurosurgery for seizure disorders, as described elsewhere [22]. Mouse BMECs (MBMECs) were isolated from 2-month-old C57b6 × Sv129 mice with and without cPLA₂ gene deletion [23], as described elsewhere [22].

BMEC invasion assay. The BMEC invasion assay was performed as described elsewhere [5]. In brief, confluent BMECs in 24-well plates were incubated with 10⁷ E. coli K1 or L. monocytogenes (MOI of 100) in experimental medium (1:1 of M199:Ham F-12 containing 5% heat-inactivated fetal bovine serum) for 1.5 h at 37°C. The monolayers were washed and incubated with experimental medium containing gentamicin (100 μg/mL) for 1 h, to kill extracellular bacteria. The monolayers were washed again and were lysed with 0.5% Triton X-100. Bacterial viability was not affected by treatment with 0.5% Triton X. The released intracellular bacteria were enumerated by plating on sheep blood agar. Bacterial invasion was calculated by the following formula: percentage invasion = (number of bacteria recovered/number of bacteria inoculated) × 100.

Percentage invasions were compared between the invasive wild-type bacteria under different conditions (in the presence of inhibitors or use of cPLA₂ gene-deleted BMECs). The results were expressed as relative invasion, where 100% equaled wild-type bacterial invasion in the absence of either inhibitors or cPLA₂ gene knockout BMECs. The bacterial invasion percentages in the absence of either inhibitors or gene knockout BMECs were 0.1% and 0.08% for E. coli K1 in HBMECs and MBMECs, respectively, and 3.98% and 3.55% for L. monocytogenes in HBMECs and MBMECs, respectively. Each experiment was done in triplicate and was repeated ≥3 times. Where appropriate, the BMEC monolayers were pretreated with inhibitors for 30–60 min before incubation with bacteria.

Thiazolyl blue (MTT) and bactericidal assays. To test for the viability of BMECs in the presence of various inhibitors, we performed the MTT assay, as described in the protocol supplied by the manufacturer (Sigma). Bactericidal assays were done for both E. coli K1 and L. monocytogenes with the various inhibitors mentioned at doses twice those used for the experiments. Experiments with bacteria pretreated for 60 min with maximum doses of inhibitors before incubation with the BMEC monolayers showed no difference in invasion of BMECs by these bacteria, compared with untreated bacteria (data not shown).

Results

Effect of isotetrandrine on invasion of BMECs by E. coli K1 and L. monocytogenes. While investigating the potential role of G proteins in bacterial invasion of BMECs, we observed that E. coli K1 invasion of BMECs was inhibited by isotetrandrine, a known inhibitor of G protein activation of PLA₂, but no such inhibition was seen with L. monocytogenes invasion of BMECs (figure 1). There was a concentration-dependent inhibition of E. coli K1 invasion of BMECs, with 90% inhibition at 5 μM. Inhibition of E. coli K1 was the same whether BMECs were pretreated with isotetrandrine for 1 h or overnight.
inhibition of recombinant sPLA2 is suggested a differential role of host cell cPLA2 in the invasion of BMECs at either dose. These findings suggested iPLA2 and sPLA2 are not important in the invasion of BMECs by these bacteria.

Figure 3. Effect of arachidonyl trifluoromethyl ketone (AA-COCF3), a selective inhibitor of cytosolic phospholipase A2, on the invasion of brain microvascular endothelial cells (BMECs) by Escherichia coli K1 (E44) and Listeria monocytogenes (Listeria). BMECs were pretreated with different concentrations of AA-COCF3 for 60 min before the addition of bacteria. Results are means of ≥ 3 experiments, each done in triplicate. Error bars, SD. *P < .05, vs. control (2-tailed unpaired t test).

(14 h). These results suggested that host cell PLA2 is involved in E. coli K1 invasion of BMECs but not in L. monocytogenes invasion of BMECs.

Effect of COX and LOX pathway inhibitors on E. coli K1 invasion of BMECs. After observing the significant role of host cell cPLA2 only in E. coli K1 invasion of BMECs, we examined further the involvement of downstream signaling molecules linked to cPLA2, such as AA metabolites generated via the COX and LOX pathways, in the invasion of BMECs by E. coli K1. Indomethacin, an inhibitor of COX [30, 31], inhibited E. coli K1 invasion of BMECs in a concentration-dependent manner (figure 6). The maximum inhibition was ∼50% at a concentration of 100 μM, which is twice the IC50 of for BEL and 5 μM for 12-episcalaradial, there was no significant inhibition of invasion of BMECs by these 2 bacteria (figure 4). These findings suggested that iPLA2 and sPLA2 are not important in the invasion of BMECs by these bacteria.

Effect of host cell cPLA2 deficiency on bacterial invasion of BMECs by E. coli K1 and L. monocytogenes. Because the results shown above suggested a role for host cell cPLA2 in E. coli K1 invasion of BMECs but not in L. monocytogenes invasion of BMECs, we then used MBMECs derived from cPLA2 knockout mice to further verify the role of host cell cPLA2. E. coli K1 invasion was significantly less in MBMECs derived from cPLA2 knockout mice than in MBMECs derived from control mice (figure 5). In contrast, L. monocytogenes invasion of MBMECs did not significantly differ between knockout mice and control mice (figure 5). These results corroborate the findings with AA-COCF3, the selective cPLA2 inhibitor. Taken together, they indicate that cPLA2 is involved in E. coli K1 invasion of BMECs but not in L. monocytogenes invasion of BMECs.

Effect of BEL and 12-episcalaradial on BMEC invasion by E. coli K1 and L. monocytogenes. We next investigated the role of different subclasses of PLA2 in E. coli K1 invasion with concomitant comparison of L. monocytogenes invasion in BMECs. AA-COCF3, a selective inhibitor of cPLA2, [25, 26], inhibited E. coli K1 invasion of BMECs, but no such inhibition was noted with L. monocytogenes invasion of BMECs (figure 3). The IC50 of AA-COCF3, for inhibition of cPLA2, is ∼8 μM in other cell types, such as platelets and U937 monocytes [27]. At doses of 10 and 25 μM, there was ∼70% inhibition of E. coli K1 invasion of BMECs. There was no significant inhibition of L. monocytogenes invasion of BMECs at either dose. These findings suggested a differential role of host cell cPLA2 in the invasion of BMECs by these 2 bacteria.

Effect of BEL and 12-episcalaradial on BMEC invasion by E. coli K1 and L. monocytogenes. We then investigated the role of other PLA2 subclasses in bacterial invasion of BMECs, we used selective inhibitors against iPLA2 (BEL), and sPLA2 (12-episcalaradial). The IC50 of BEL for iPLA2 inhibition is ∼1 μM [27, 28], and that for 12-episcalaradial for inhibition of recombinant sPLA2 is ∼5.4 μM [29] in different cell types. These inhibitors exhibit a greater selectivity for their respective PLA2 subclasses at those concentrations. At 10 μM
coli K1 and malian cells have been linked to intracellular signaling via me-
cytoskeletal rearrangements for invasion of BMECs are similar
BMECs by these bacteria [8, 9]. However, it remains unclear
ment of BMECs with cytochalasin D abolished the invasion of
E. coli
rearrangements are prerequisite for invasion of BMECs by
these bacteria. These reagents have an additive effect on inhibition of
E. coli
K1 invasion of BMECs. This concept was further sup-
ported by the demonstration that COX and LOX inhibitors
gested that both COX and LOX pathways are involved in
expression of PLA2 in
E. coli
brain microvascular endothelial cells (BMECs). PLA2 is known to be important for
S. typhimurium
invasion of epithelial cells via the EGF receptor [16], this is the first dem-
onstration of the requirement for host cell PLA2 for E. coli K1
invasion of BMECs.

Further investigation with selective inhibitors of the various
PLA2 subclasses indicated the important role of host cell cPLA2
in E. coli K1 invasion of BMECs. AACOCF3, a selective
inhibitor of cPLA2, inhibited E. coli K1 invasion of BMECs but
not L. monocytogenes invasion of BMECs—further highlighting the requirement
for E. coli K1 invasion of BMECs. Although host cell
PLA2 is known to be important for invasion of these meningeal pathogens. Inhibitors of iPLA2
(BEL) and sPLA2 (12-episcalaradial) are more selective for the
respective subclasses of PLA2 at lower doses [28, 29], on the basis of the IC50 of these reagents. In our study, the lower doses
of these inhibitors exhibited no significant effect on either E. coli K1 or L. monocytogenes invasion of BMECs, which sug-
gests that neither sPLA2 nor iPLA2 has any significant role in
the invasion of BMECs by these bacteria. These reagents have
lower selectivity at higher doses in different cell types [28, 29].
Thus, the inhibition of E. coli K1 invasion of BMECs observed with the higher concentration of the sPLA2 inhibitor (i.e., 25
m
2 [35], was also inhibitory to E. coli K1 invasion of BMECs,
with 50% inhibition at 0.1 m
M (figure 6). These findings sug-
gested that both COX and LOX pathways are involved in
E. coli K1 invasion of BMECs. This concept was further sup-
ported by the demonstration that COX and LOX inhibitors
have an additive effect on inhibition of E. coli K1 invasion of
BMECs (total inhibition, ∼70%) when used in combination
(figure 7).

Discussion

We previously demonstrated that BMEC actin cytoskeletal
rearrangements are prerequisite for invasion of BMECs by E. coli K1 and L. monocytogenes when we showed that pretreat-
ment of BMECs with cytochalasin D abolished the invasion
of BMECs by these bacteria [8, 9]. However, it remains unclear
whether intracellular signaling pathways associated with actin
cytoskeletal rearrangements for invasion of BMECs are similar
for these 2 bacteria. Actin cytoskeletal rearrangements in mammalian cells have been linked to intracellular signaling via me-
tabolites of AA [15]. cPLA2 is an important mediator of intra-
cellular AA generation in mammalian cells through its
preferential hydrolysis of phospholipids at the sn-2 position.
Our findings in this study demonstrate that cPLA2 is important in E. coli K1 invasion of BMECs but not in L. monocytogenes
invasion of BMECs, thereby suggesting diverse intracellular
signaling pathways in BMEC invasion by these bacteria.

Our data with the use of PLA2 inhibitors indicate the impor-
tance of PLA2 in E. coli K1 invasion of BMECs. Isotetrandrine,
an inhibitor of G protein activation of PLA2, inhibited E. coli
K1 invasion of BMECs in a concentration-dependent manner
but not with L. monocytogenes invasion of BMECs. This
distinctive inhibition of E. coli K1 invasion of BMECs by iso-
tetrandrine precluded any nonspecific inhibitory effect on BMECs
by this reagent (e.g., altered permeability of the cells or increased
intracellular penetration of antibiotics). This prompted our fur-
ther investigation into the role of host cell PLA2 in invasion of
BMECs by these bacteria. BPB, a nonselective inhibitor of PLA2
as expected, inhibited E. coli K1 invasion of BMECs but not L.
monocytogenes invasion of BMECs—further highlighting the requirement
for E. coli K1 invasion of BMECs. Although host cell
PLA2 is known to be important for S. typhimurium
invasion of epithelial cells via the EGF receptor [16], this is the first dem-
onstration of the requirement for host cell PLA2 for E. coli K1
invasion of BMECs.

This agent in other cell types (figure 6). BMECs are known to
express COX-2 [32–34]. NS-398, the selective inhibitor of COX-
2 [35], was also inhibitory to E. coli K1 invasion of BMECs,
with 50% inhibition at 0.1 m
M (figure 6). NDGA, an inhibitor
of LOX [36], inhibited E. coli K1 invasion of BMECs in a
concentration-dependent manner, with a maximum inhibition of
50% at the IC50 of ∼10 m
M (figure 7). These findings sug-
gested that both COX and LOX pathways are involved in
E. coli K1 invasion of BMECs.

The higher concentrations of these inhibitors did not
inhibit the invasion of BMECs by these bacteria. These reagents have
lower selectivity at higher doses in different cell types [28, 29].
Thus, the inhibition of E. coli K1 invasion of BMECs observed with the higher concentration of the sPLA2 inhibitor (i.e., 25
m
M 12-episcalaradial) could be explained by possible inhibition
of cPLA2. The higher concentrations of these inhibitors did not
inhibit L. monocytogenes invasion of BMECs, thereby exclud-
ing the possibility of nonspecific effects on BMECs.

Figure 5. Effect of cytosolic phospholipase A2 (cPLA2) deficiency in brain microvascular endothelial cells (BMECs) on Escherichia coli
K1 (E44) and Listeria monocytogenes (Listeria) invasion of BMECs. BMECs were isolated from 2-month-old mice. Results are means of
≥3 different experiments, each done in triplicate. Error bars, SD. *P < .05, vs. control (2-tailed unpaired t test).

Figure 6. Effect of indomethacin (cyclooxygenase inhibitor; A) and NS-398 (cyclooxygenase-2 inhibitor; B) on Escherichia coli K1 invasion
of brain microvascular endothelial cells (BMECs). BMECs were pre-
treated with different concentrations of indomethacin or NS-398 for
60 min before the addition of bacteria. Results are means of ≥3 ex-
periments, each done in triplicate. Error bars, SD. *P < .05, vs. control (2-tailed unpaired t test).
Knockout mice lacking cPLA₂ were used previously to elucidate the role of cPLA₂ in fertility and the pathophysiology of postischemic brain injury [23]. These mice were generated by targeted inactivation of cPLA₂. They develop normally, gain weight at a rate equal to that of wild-type animals, and have life spans >1 year. *E. coli* K1 invasion was significantly decreased in BMECs derived from cPLA₂-deficient mice, compared with MBMECs from control mice. These findings corroborate our observation of the important role of host cell cPLA₂ in *E. coli* K1 invasion of BMECs seen with cPLA₂ inhibitors. Furthermore, the significant decline in *E. coli* K1 invasion in BMECs isolated from cPLA₂ knockout mice excludes the possibility of nonspecific inhibition by AACOCF₃, the selective cPLA₂ inhibitor. In contrast, there was no significant difference in *L. monocytogenes* invasion of control and cPLA₂-deficient MBMECs, which provides further evidence that cPLA₂ does not play a role in *L. monocytogenes* invasion of BMECs. Taken together, these observations indicate a differential role of host cell cPLA₂ in invasion of BMECs by *E. coli* K1 and *L. monocytogenes*.

Activation of cPLA₂ leads to the generation of intracellular AA. AA is the precursor of inflammatory mediators collectively known as eicosanoids (e.g., prostaglandins and leukotrienes). These are generated through either the COX or LOX pathways. Leukotriene synthesis is considered to be necessary for actin polymerization in response to epidermal growth factor, whereas the absence of prostaglandins is detrimental to the breakdown of actin stress fibers in activated cells [15]. We noted ~50% inhibition of *E. coli* K1 invasion of BMECs with the separate use of COX and LOX inhibitors. The absence of a concentration-dependent inhibitory effect with the COX-2 inhibitor NS-398 suggests either a nonspecific response or a high sensitivity of the expressed COX-2 in BMECs to low doses of NS-398. Moreover, there appeared to be an increased inhibitory effect of the combined use of COX and LOX inhibitors on *E. coli* K1 invasion of BMECs. This suggests that AA metabolites generated via both COX and LOX pathways contribute to *E. coli* K1 invasion of BMECs. Further studies are underway to understand the mechanisms involving AA metabolites in *E. coli* K1 invasion of BMECs (e.g., how AA metabolites affect actin cytoskeletal rearrangements in response to *E. coli* K1 invasion of BMECs).

On the basis of our data regarding *E. coli* K1 invasion of BMECs, we propose that activation of host cell cPLA₂ secondary to *E. coli* K1-BMEC interactions leads to the generation of AA metabolites and to *E. coli* K1 invasion of BMECs. Despite the clear demonstration of the importance of host cell cPLA₂ in *E. coli* K1 invasion of BMECs, the lack of complete inhibition of *E. coli* K1 invasion of BMECs by AACOCF₃, and the incomplete abolition of *E. coli* K1 invasion in cPLA₂ knockout BMECs indicate the existence of other host cell signaling molecules and pathways that are important for *E. coli* K1 invasion of BMECs. Further delineation of host cell intracellular signaling associated with *E. coli* K1 invasion of BMECs will enable us to better understand the pathogenesis of *E. coli* K1 meningitis and help in developing novel therapeutic approaches for its prevention and treatment.

References


