Biochemical Pharmacology 79 (2010) 1149–1155

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

# Anthrax lethal toxin down-regulates type-IIA secreted phospholipase $A_2$ expression through MAPK/NF-κB inactivation

Benoit Raymond [a,b,1], Lucas Ravaux [c,1], Sylvie Mémet [d,e], YongZheng Wu [a,b], Aude Sturny-Leclère [d,e], Dominique Leduc [a,b], Chantal Denoyelle [c], Pierre L. Goossens [f,g], Miguel Payá [h], Michel Raymondjean [c], Lhousseine Touqui [a,b,*]

[a] Institut Pasteur, Unité de Défense Innée et Inflammation, Paris, France
[b] Inserm, U874, Paris, France
[c] Université Pierre et Marie Curie, UR4 Physiologie, Physiopathologie et Vieillissement, Paris, France
[d] Institut Pasteur, Unité de Mycologie Moléculaire, France
[e] CNRS URA3012, Paris, France
[f] Institut Pasteur, Unité des Toxines et Pathogénie Bactérienne, Paris, France
[g] CNRS, URA-2172, Paris, France
[h] Departamento de Farmacología, Universitat of València, Spain

ARTICLE INFO

*Article history:*
Received 23 September 2009
Accepted 30 November 2009

*Keywords:*
Anthrax
Phospholipase A2
NF-κB
MAPK kinase
Macrophages

ABSTRACT

*Bacillus anthracis*, the etiological agent of anthrax, produces lethal toxin (LT) that displays a metalloproteolytic activity toward the *N*-terminus of the MAPK-kinases. We have previously shown that secreted type-IIA phospholipase $A_2$ (sPLA$_2$-IIA) exhibits potent anthracidal activity. *In vitro* expression of sPLA$_2$-IIA in guinea pig alveolar macrophages (AMs), the major source of this enzyme in lung tissues, is inhibited by LT. Here, we examined the mechanisms involved in sPLA$_2$-IIA inhibition by LT. We first showed that chemical inhibitors of p38 and ERK MAPKs reduced sPLA$_2$-IIA expression in AMs indicating that these kinases play a role in sPLA$_2$-IIA expression. LT inhibited IL-1β-induced p38 phosphorylation as well as sPLA$_2$-IIA promoter activity in CHO cells. Inhibition of sPLA$_2$-IIA promoter activity was mimicked by co-transfection with dominant negative construct of p38 (DN-p38) and reversed by the active form of p38-MAPK (AC-p38). Both LT and DN-p38 decreased IL-1β-induced NF-κB luciferase activity. This contrasted with the effect of AC-p38, which enhanced this activity. However, neither LT nor specific p-38 inhibitor interfered with LPS-induced IκBα degradation or NF-κB nuclear translocation in AMs. Subcutaneous administration of LT to guinea pig before LPS challenge reduced sPLA$_2$-IIA levels in broncho-alveolar lavages and ears. We conclude that sPLA$_2$-IIA expression is induced *via* a sequential MAPK-NF-κB activation and that LT inhibits this expression likely by interfering with the transactivation of NF-κB in the nucleus. This inhibition, which is operating both *in vitro* and *in vivo*, may represent a mechanism by which *B. anthracis* subvert host defense.



## 1. Introduction

Anthrax is primarily a disease of herbivores due to *Bacillus anthracis* infection, but humans are also susceptible [1]. *B. anthracis*, the etiological agent of anthrax disease, is a Gram-positive, spore-forming bacterium. Although infection by this bacterium frequently occurs *via* the cutaneous route, both gastrointestinal and pulmonary infections cause the highest mortality in human. Macrophages and/or dendritic cells take up inhaled spores leading to their germination. This is followed by the migration of these cells to the draining lymph nodes [2]. Encapsulated bacilli then enter the blood compartment and disseminate in the whole organism. Despite appropriate therapy, all these forms of infection may progress to fatal systemic anthrax, leading to shock-like symptoms and respiratory failure [3].

Studies have suggested that type-IIA secreted phospholipase $A_2$ (sPLA$_2$-IIA) may represent an effector involved in host defense against invading bacteria. Indeed, sPLA$_2$-IIA is a highly bactericidal enzyme, especially toward Gram-positive bacteria [4–6] and is found at high levels in airways of humans [7,8] and various animal species [9–11]. sPLA$_2$-IIA belongs to a family of enzymes that catalyzes the hydrolysis of phospholipids leading to the generation of lysophospholipids and free fatty acids [12]. We have recently shown that sPLA$_2$-IIA is highly bactericidal towards *B. anthracis*

* Corresponding author at: Institut Pasteur, Unité de Défense Innée et Inflammation, 25, rue Dr. Roux, 75015 Paris, France.
Tel.: +33 1 45 68 86 84; fax: +33 1 45 68 87 03.
*E-mail address:* touqui@pasteur.fr (L. Touqui).
[1] These authors contributed equally to this work.

0006-2952/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2009.11.023

[13] and protects infected mice against *B. anthracis* infection [14]. These findings suggest that $sPLA_2$-IIA may represent an efficient weapon participating in host defense against anthrax.

However, a number of studies have reported the ability of *B. anthracis* to subvert immune response [15–18]. This inhibition mainly results from the action of specific *B. anthracis* toxins. Indeed, *B. anthracis* carry two plasmids, pXO1 [19] and pXO2 [20], which encode the primary virulence factors: lethal and edema toxins, and the proteins required for capsule synthesis, respectively. The toxins are composed of three secreted proteins: protective antigen (PA), lethal factor (LF) and edema factor (EF). These proteins act in pairs [21] leading to the lethal toxin (LT) and the edema toxin (ET), composed of the association of LF/PA and EF/PA respectively. Uptake of LT by the target cells is followed by the release of LF within the cytoplasm of these cells. There, LT displays its metallo-proteolytic activity toward the *N*-terminus of the MAPK-kinase (MAPKK), thereby interfering with the MAPK pathway [22]. We have recently reported that LT was able to inhibit $sPLA_2$-IIA expression by guinea pig AM [13], the main source of pulmonary $sPLA_2$-IIA in guinea pig [9]. However, the mechanisms involved in this inhibition have not been examined. The present study has been addressed to identify the signaling pathways involved in this modulation and whether LT is able to interfere with $sPLA_2$-IIA production *in vivo*.

## 2. Materials and methods

### 2.1. Animals and reagents

Male Hartley guinea pigs were purchased from Charles River Laboratories (L'Arbresle, France). RPMI 1640 cell culture medium was obtained from Invitrogen (Cergy-Pontoise, France) and Fetal calf serum (FCS) from Hyclone (Logan, USA). Chinese hamster ovary (CHO) cells were purchased from ATCC (Manassas, USA). SB 203580 and PD 98059 were purchased from Biomol (Le Perray-en-Yvelines, France). MG-132 and *Pseudomonas aeruginosa* LPS were purchased from Sigma Aldrich (St. Louis, MO, USA). Antibodies directed against, IκBα (sc-371), MEK-3 (sc-959) and β-actin (sc-81178) were from Santa Cruz Biotechnology (Santa Cruz, USA). Antibodies directed against p38-MAPK (9212), phospho-p38-MAPK (p38pT180-Y182, 9211), ERK (9102) and phospho-ERK (ERKpT202-Y204, 9101) were from Cell Signaling (Danvers, USA). Recombinant LF and PA from *B. anthracis* were purchased from List Biological Laboratory (Campbell, USA). Dominant negative and active form constructs of p38-MAPK are a gift of Dr. Jacques Pouyssegur (Institute of Developmental Biology and Cancer, CNRS, Nice, France).

### 2.2. Preparation of AMs and CHO cells and incubation procedures

Guinea pig broncho-alveolar lavages (BALs) were performed with PBS and AMs were isolated as previously described [23]. AMs, adjusted at $2 \times 10^6$ cells/mL in RPMI 1640 with 3% FCS, were pretreated either with LT, MAPK inhibitors or NF-κB inhibitor 1 h before LPS stimulation. Chinese hamster ovary (CHO) adjusted at $2 \times 10^4$ cells/mL (24-well plates) in HAM F12 with 10% FCS, 4 mM glutamine, 100 units/mL penicillin and 100 mg/mL streptomycin were incubated with LT for 1 h before stimulation with IL-1β. After stimulation, media and cell Lysates were collected for $sPLA_2$-IIA activity, EMSA and Western blot analyses were performed as described below.

### 2.3. Assay of $sPLA_2$–IIA activity

$sPLA_2$-IIA activity was assayed using [$^3$H]-oleic acid-labeled membranes of *Escherichia coli*, following the method of Franson et al. [24] modified by Paya et al. [25]. Briefly, the *E. coli* strain CECT 101 was seeded in medium containing 1% tryptone, 0.5% yeast extract, 0.5% NaCl and 0.6% sodium dihydrogen orthophosphate, pH 5.0 and grown for 6–8 h at 37 °C in the presence of 5 μCi/mL [$^3$H]-oleic acid (specific activity 10 Ci/mmol) until growth approached the end of the logarithmic phase. After centrifugation at 1800 × *g* for 10 min at 4 °C, the membranes were washed in buffer (0.7 M Tris–HCl, 10 mM $CaCl_2$, 0.1% bovine serum albumin, pH 8.0), re-suspended in saline and autoclaved for 30–45 min. The membranes were then washed, centrifuged again and frozen at −70 °C. At least, 95% of the radioactivity was incorporated into the phospholipid fraction.

Aliquots (5–20 μL) of AM supernatants, broncho-alveolar lavage fluids (BALF) or ear homogenates were incubated with 250 μL of buffer A (100 mM Tris–HCl, 1 mM $CaCl_2$, pH 7.5), for 15 min 37 °C in the presence of 10 μL autoclaved [$^3$H]-oleic acid-labeled membranes. The reactions were stopped by addition of 100 μL ice-cold stop solution (0.25% BSA in 100 mM Tris–HCl, 1 mM $CaCl_2$, pH 7.5). After centrifugation at 1800 × *g* for 10 min at 4 °C, the radioactivity in the supernatants was determined by liquid scintillation counting. BALF and ear homogenates were treated with the $sPLA_2$-IIA inhibitor, LY311727 to check that hydrolysis is due to $sPLA_2$-IIA activity. The latter was expressed as picomoles hydrolyzed substrate per minute per milligram ear homogenates or per milliter BALF or AM supernatants.

### 2.4. Nuclear protein extraction and electrophoretic mobility shift assays

Nuclear proteins were extracted from AMs as previously described [26]. The NF-κB double-stranded oligonucleotides (Santa Cruz Biotechnology, Santa Cruz, USA) consensus sequence 5′-AGT TGA GGG GAC TTTT CCC AGG C-3′ was γ-$^{32}$P-labeled with T4 polynucleotide kinase (New England Biolabs, Ipswich, USA) on the overhanging ends. Protein concentrations were determined by using Nanodrop spectrophotometer (Nyxor Biotech, Paris, France). Binding reactions were performed as follow: briefly, incubations were performed by adding 5 μg of nuclear extract, 10 μL of 2× binding buffer (40 mM HEPES, pH 7, 140 mM KCl, 4 mM DTT, 0.02% Nonidet P-40, 8% Ficoll, 200 μg/mL BSA, 1 μg of poly(dI:dC), and 1 μL of γ-$^{32}$P-labeled probe, in a total volume of 20 μL for 20 min at room temperature. The reaction mixtures were separated on a 5% polyacrylamide gel in 0.5% Tris/borate/EDTA buffer at 150 V for 2 h. Gels were dried and exposed for 2 to 12 h. We have previously shown, using supershift analysis, that antibodies directed against NF-κB's p50 and p65 subunits shift the NF-κB band in LPS-stimulated AMs, thus confirming that the observed complexes belong to the NF-κB family [27].

### 2.5. Protein extraction and Western blot analyses

Proteins from AMs and CHO cells were extracted in lysis buffer (10 mM Tris–HCl, 150 mM NaCl, 3 mM EDTA, 100 μM leupeptin, 100 μM aprotinin, 1 μM soybean trypsin inhibitor, 5 mM NEM, 1 mM PMSF, 5 mM benzamidine and 1% Triton X-100, pH 7.4) and electrophoresed under reducing conditions. Semidry transferred proteins were applied onto polyvinylidene difluoride membranes. Nonpecific binding sites were blocked overnight with 5% BSA in 20 mM Tris–HCl, pH 7.6, 140 mM NaCl and 0.1% Tween 20. Blots were probed for 1 h with indicated antibodies. After washing, the immunoreactive bands were visualized using the specifics peroxidase-conjugated anti-IgG and the ECL Plus Western Blotting Detecting System (Amersham Biosciences, Orsay, France).

### 2.6. Plasmid constructions and transfection

CHO cells, adjusted at $2 \times 10^4$ cells/mL (24-well plates) in HAM F12 with 10% FCS, 4 mM glutamine, 100 units/mL penicillin and