

Regulation of cytosolic phospholipase A₂α by hsp90 and a p54 kinase in okadaic acid-stimulated macrophages

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Abstract: In resident mouse peritoneal macrophages, group IVA cytosolic phospholipase A₂ (cPLA₂α) mediates arachidonic acid (AA) release and eicosanoid production in response to diverse agonists such as A23187, phorbol myristate acetate, zymosan, and the enterotoxin, okadaic acid (OA). cPLA₂α is regulated by phosphorylation and by calcium that binds to the C2 domain and induces translocation from the cytosol to membranes. In contrast, OA activates cPLA₂α-induced AA release and translocation to the Golgi in macrophages without an apparent increase in calcium. Inhibitors of heat shock protein 90 (hsp90), geldanamycin, and herbimycin blocked AA release in response to OA but not to A23187, PMA, or zymosan. OA, but not the other agonists, induced activation of a cytosolic serine/threonine 54-kDa kinase (p54), which phosphorylated cPLA₂α in in-gel kinase assays and was associated with cPLA₂α in immunoprecipitates. Activation of the p54 kinase was inhibited by geldanamycin. The kinase coimmunoprecipitated with hsp90 in unstimulated macrophages, and OA induced its loss from hsp90, concomitant with its association with cPLA₂α. The results demonstrate a role for hsp90 in regulating cPLA₂α-mediated AA release that involves association of a p54 kinase with cPLA₂α upon OA stimulation. *J. Leukoc. Biol.* 84: 798–806; 2008.

Key Words: arachidonic acid · heat shock protein 90 · phosphorylation-cytosolic phospholipase A₂

INTRODUCTION

The group IVA cytosolic phospholipase A₂ (cPLA₂α) is a ubiquitous enzyme that supplies arachidonic acid (AA) for eicosanoid production [1–6]. Its essential role in AA release and lipid mediator production has been demonstrated by comparing cells such as macrophages, lung fibroblasts, and mast cells from wild-type and cPLA₂α-deficient mice [3, 7–11]. cPLA₂α is required for production of eicosanoids that regulate normal physiological processes such as parturition and that mediate pathological processes such as neuronal injury in-

duced by ischemia/reperfusion, lung injury induced by sepsis and acid inhalation, type I allergic responses, and pulmonary fibrosis [9, 11–14]. However, the regulatory mechanisms of cPLA₂α remain only partially understood. Post-translational mechanisms are important for acutely regulating cPLA₂α-mediated AA release in agonist-treated cells [1, 4, 10, 14]. Elevated intracellular calcium induces translocation of cPLA₂α to the Golgi apparatus, endoplasmic reticulum, and nuclear envelope [15–18]. This process is required for access to phospholipid substrate and is mediated by an amino-terminal C2 domain on cPLA₂α [18–20].

cPLA₂α is also regulated by phosphorylation on serine residues in the catalytic domain. Phosphorylation of cPLA₂α on Ser-505, Ser-727, and Ser-515 is mediated by MAPK, MAPK-interacting kinase, and calcium- and calmodulin-dependent kinase II, respectively [21–26]. Evidence suggests that phosphorylation of these residues is functionally important, although the mechanism involved in regulating cPLA₂α by phosphorylation is not well understood. Phosphorylation on Ser-505 by MAPKs results in a modest increase of the catalytic activity of cPLA₂α [23, 24, 27]. However, Ser-505 phosphorylation in itself is not sufficient for AA release but is important when combined with transient increases in intracellular calcium [24, 28, 29]. However, the requirement for phosphorylation of cPLA₂α can be overcome under conditions of high, sustained intracellular calcium [23, 30].

Resident peritoneal macrophages have been used extensively as a model to study the regulation of AA release. These cells are enriched in AA and produce eicosanoids from the cyclooxygenase and lipoxygenase pathways. Studies with macrophages from cPLA₂α-deficient mice have demonstrated that cPLA₂α is essential for AA release and eicosanoid production in response to diverse agonists that act through different mechanisms [10, 31–33]. AA release is induced by calcium ionophores, zymosan (yeast cell walls), PMA, and the phosphatase inhibitor okadaic acid (OA). In macrophages, transient increases in calcium and MAPK activation synergistically induce

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Received March 24, 2008; revised May 7, 2008; accepted May 8, 2008.
doi: 10.1189/jlb.0308197

AA release, although neither alone is sufficient [28]. However, OA and PMA trigger AA release without an apparent increase in the concentration of intracellular calcium, although basal calcium levels are required [10, 28]. These tumor promoters preferentially induce the production of prostaglandins in peritoneal macrophages [10, 34, 35]. The lack of leukotriene production is consistent with their poor ability to mobilize calcium, which is required for the activation of 5-lipoxygenase [36, 37]. These observations suggest that the tumor promoters PMA and OA stimulate cPLA₂α-mediated AA release by novel mechanisms.

OA is a natural polyether fatty acid enterotoxin that is produced by dinoflagellates and is responsible for diarrhetic shellfish poisoning in humans [38]. It acts through inhibition of intracellular protein phosphatases, particularly PP2A and -1 [39, 40]. A number of natural product toxins from diverse sources act by inhibiting phosphatases, underscoring the important physiological role played by these enzymes [38]. OA is used extensively to elucidate the involvement of phosphatases in signal transduction and proapoptotic mechanisms [41–43]. By inhibiting serine/threonine phosphatases, OA treatment of cells leads to increased phosphorylation of proteins and activation of kinase cascades. In macrophages and other cells, OA strongly activates MAPK cascades [10, 44, 45]. We have previously reported that *Spodoptera frugiperda* (*Sf9*) cells, expressing cPLA₂α, release AA in response to calcium-mobilizing agonists and to OA and can be used as a model to investigate the regulation of cPLA₂α-mediated AA release [46, 47]. OA induces cPLA₂α translocation to the perinuclear region and triggers AA release in *Sf9* cells without increasing intracellular calcium, as we observed in macrophages. Mutagenesis studies demonstrated that a functional C2 domain is required for translocation and AA release in response to OA. When calcium-mobilizing agonists are used, the C2 domain is necessary and sufficient for translocation of cPLA₂α. In contrast, only full-length cPLA₂α translocates in response to OA, indicating that OA regulates activation of cPLA₂α through a complex mechanism that requires the C2 and the catalytic domains [46].

In this study, we investigated the mechanisms involved in cPLA₂α activation in mouse peritoneal macrophages treated with OA, as our data indicated that it occurs by a novel pathway that does not involve increases in intracellular calcium. We found that heat shock protein 90 (hsp90) is required for cPLA₂α-mediated AA release in OA-stimulated macrophages. This involves phosphorylation of cPLA₂α by a 54-kDa kinase (p54) that associates with cPLA₂α in response to OA and is regulated by hsp90.

MATERIALS AND METHODS

Materials

Pathogen-free ICR female mice were obtained from Harlan Sprague-Dawley (Indianapolis, IN, USA). [5,6,8,9,11,12,14,15-³H]AA (specific activity, 100 Ci/mmol) and [³²P]orthophosphoric acid (9000 Ci/mmol) were from NEN Life Science Products (Boston, MA, USA). Anti-rabbit IgG and anti-mouse IgG HRP-linked F(ab')₂ fragments, [γ-³²P]-ATP (3000 Ci/mmol), and the reagents for ECL detection on immunoblots were from Amersham Pharmacia Biotech

(Little Chalfont, UK). Anti-hsp90 mAb (SPA-830) was from Stressgen (Canada). A phospho-specific antibody against threonine- and tyrosine-phosphorylated p42 and p44 ERKs was purchased from New England Biolabs (Beverly, MA, USA). Human recombinant cPLA₂α was expressed in *Sf9* cells and purified as described previously [47–49]. A23187, zymosan, Tween-40, guanidine hydrochloride, 2-ME, and protein A-Sepharose CL-4B beads were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Zymosan was prepared as described previously [10]. Geldanamycin was generously provided by the National Cancer Institute, Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis (Bethesda, MD, USA). PMA and the potassium salt of OA were from Alexis Corp. (San Diego, CA, USA). DMEM and 10× HBSS were from Whittaker Bioproducts (Norwalk, CT, USA). FBS was from Irvine Scientific (Santa Ana, CA, USA). Human serum albumin (HSA; endotoxin levels lower than 2.0 EU/mg) was purchased from InterGen (Burlington, MA, USA). Nonidet P-40 (NP-40; 10% solution) and reagents for protein determination by the bicinchoninic acid (BCA) method were from Pierce (Rockford, IL, USA).

AA release

Murine resident peritoneal macrophages were isolated as described previously [33]. Macrophages were plated at a density of 0.5×10^6 cells/cm² and incubated overnight in DMEM containing 10% FBS, 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate, 0.29 mg/ml glutamine, and 0.1 μCi/ml [³H]AA. Cells were washed and incubated in serum-free DMEM containing 0.1% HSA and stimulated at 37°C in humidified 5% CO₂ in air. Unless otherwise specified, 0.5 μg/ml A23187, 1 μM OA, 32 nM PMA, or 10 particles zymosan/cell were used as agonists. Radioactive AA released into the medium was measured by scintillation counting and the results expressed as percent of the total radioactivity incorporated (cell-associated plus medium). Background release (typically 3–5% of total arachidonate incorporated) from unstimulated cells treated with vehicle (0.1% DMSO) was subtracted from each experimental point.

Microscopy

Macrophages (5×10^5) were plated on glass-bottomed MatTek dishes in complete media, allowed to adhere, and washed three times. Enhanced cyan fluorescent protein (ECFP)-cPLA₂α was expressed in peritoneal macrophages using recombinant adenovirus as described previously [32, 50]. After 26 h incubation with adenovirus, some cells were treated with geldanamycin for 4 h before stimulation. Macrophages were washed in stimulation media (serum-free DMEM with 0.1% HSA), stimulated with OA for 90 min, and fixed with 3% paraformaldehyde in PBS containing 3% sucrose for 15 min. Cells were permeabilized for 30 min with 0.1% Triton X-100 in PBS and then blocked in 5% FBS in PBS for 1 h. Golgi were labeled using anti-Giantin primary antibody (1:200 in blocking solution, 1 h), followed by Texas Red-conjugated secondary antibody (1:100 in blocking solution, 1 h). Cells were visualized using an inverted Zeiss 200 M microscope with a 175-W xenon lamp and a 63× oil immersion objective with CFP and CY3 filters. Images were collected using a charged-coupled device camera (SensiCam) and software from Intelligent Imaging Innovations, Inc. (Slidebook 4.1, Denver, CO, USA).

Immunoblotting

Macrophage lysates were prepared by scraping cells into ice-cold lysis buffer: 50 mM Hepes, pH 7.4, 137 mM sodium chloride, 10% glycerol, 1% NP-40, 200 μM sodium vanadate, 10 mM tetrasodium pyrophosphate, 100 mM sodium fluoride, 300 nM phospho (p)-nitrophenyl phosphate, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Lysates were centrifuged at 15,000 *g* for 15 min and protein concentration of supernatants determined by the BCA method. Laemmli electrophoresis sample buffer was added to the lysates, and proteins (5–10 μg) were separated by SDS-PAGE. Immunoblotting on nitrocellulose membranes was performed with specific antibodies using the Amersham Biosciences (UK) ECL system for detection. For analysis of the cPLA₂α gel shift, samples were resolved on 10% SDS-PAGE (16 cm long, pH 8.3) with 1% cross-linking (acrylamide:bisacrylamide ratio, 99:1).

In-gel kinase assays

Macrophage lysates (10–15 μg protein) or immunoprecipitated proteins were separated by SDS-PAGE on 0.5 mm-thick minigels. In some cases, 0.1 mg/ml

PLA₂α was included in the gel mixture prior to polymerization. After electrophoresis, gels were incubated sequentially at room temperature, except where otherwise indicated, with the following solutions (25 ml): 20% 2-propanol in 50 mM Tris/HCl, pH 8.0 (two 10-min incubations); 5 mM 2-ME in 50 mM Tris/HCl, pH 8.0 (20 min); 6 M guanidine hydrochloride and 5 mM 2-ME in 50 mM Tris/HCl, pH 8.0 (two 10-min incubations); and 0.04% Tween 40 and 5 mM 2-ME in 50 mM Tris/HCl, pH 8.0 (4°C, overnight, followed by three 20-min incubations at room temperature). Gels were then rinsed for 10 min in 10 mM Hepes, pH 8.0, 2 mM DTT, 0.1 mM EGTA, and 5 mM magnesium chloride. After addition of [γ -³²P]ATP (5 μ M, 5 μ Ci/ml), gels were incubated at room temperature for 1 h, washed extensively (six to eight times) in 40 ml 5% trichloroacetic acid containing 1% tetrasodium pyrophosphate, dried, and analyzed by autoradiography. To confirm that the p54 kinase phosphorylated cPLA₂α in the gels, the p54-kinase band was excised from the gel, eluted as described previously, and subjected to SDS-PAGE and autoradiography [47].

Immunoprecipitation and in vitro kinase assays

Macrophage lysates (50–100 μ g protein) prepared as described above were precleared with nonimmune rabbit serum (1:40 final dilution) and protein A-Sepharose beads (10 μ l packed beads). After incubating for 1 h at 4°C, the beads were centrifuged and discarded. Immunoprecipitation was performed overnight at 4°C with protein A-Sepharose beads (10 μ l) and with anti-cPLA₂α rabbit antiserum (1:40) or anti-hsp90 antibody (4 μ g/ml). For in vitro kinase assays, beads were washed twice with lysis buffer and twice with kinase reaction buffer: 20 mM Hepes, pH 8.0, containing 30 mM β -glycerophosphate, 10 mM p-nitrophenyl phosphate, 10 mM magnesium chloride, 0.5 mM DTT, and 50 μ M sodium orthovanadate. After addition of [γ -³²P]ATP (20 μ Ci), kinase reactions were carried out in a total volume of 50 μ l for 1 h at 30°C with occasional mixing. Reactions were stopped by adding 12.5 μ l 5 \times Laemmli electrophoresis sample buffer and boiling for 4 min. Reaction products were analyzed by SDS-PAGE followed by autoradiography.

Phosphoamino acid analysis

Following in vitro kinase assay and SDS-PAGE, phosphorylated proteins were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes. After excising the p54-kinase band, proteins were hydrolyzed in 6 N HCl at 110°C overnight. HCl was removed by evaporation using a Speed-Vac, and samples were resuspended in 6 μ l first-dimension buffer (7.8% acetic acid, 2.2% formic acid, pH 1.9) containing 400 ng each p-threonine, p-tyrosine, and p-serine standards. After spotting the samples on cellulose plates, phosphoamino acids were resolved by two-dimensional electrophoresis as described previously [48]. Separation along the first dimension was carried out at 1500 V for 20 min. After drying, plates were rotated 90°C, and

second-dimension electrophoresis (in 5% acetic acid, 0.5% pyridine, pH 3.5) was performed at 1300 V for 16 min. [³²P]Phosphoamino acids were visualized by autoradiography, and standards were visualized with ninhydrin (0.25% in acetone). For phosphoamino acid analysis of ³²P-cPLA₂α from unstimulated and OA-treated macrophages, cPLA₂α was immunoprecipitated from macrophages labeled with [³²P]orthophosphate as described previously [28].

RESULTS

OA-induced AA release is blocked by hsp90 inhibitors

OA induces macrophages to release AA through a cPLA₂α-dependent mechanism that is distinct from those used by other agonists. We previously reported that OA does not increase intracellular levels of calcium within the first 5 min of OA exposure [28]. However, there is a 45- to 60-min lag phase before release of AA in response to OA [10]. Because of the delay in cPLA₂α activation in response to OA, intracellular calcium levels were measured for up to 90 min in Fura-2-loaded macrophages as described previously [18]. An increase in intracellular calcium was not observed in response to OA; however, the cells exhibited a robust increase in calcium, when ionomycin was added 90 min after OA treatment (data not shown). Although OA inhibits serine/threonine phosphatases, it triggers activation of many kinase cascades and enhances serine and tyrosine phosphorylation of proteins in cells [45, 51, 52]. In addition to phosphorylation of cPLA₂α on serine residues, there have been reports that cPLA₂α may be tyrosine-phosphorylated [53, 54]. LPS-induced AA release in monocytic cells was reported to be inhibited by herbimycin A, a tyrosine kinase inhibitor [54]. Therefore, we investigated the possibility that tyrosine phosphorylation may be involved in the regulation of OA-induced AA release in macrophages, which when labeled with [³²P]orthophosphate, showed increased phosphorylation of cPLA₂α upon stimulation with OA for 90 min (**Fig. 1A**). Phosphoamino acid analysis revealed increased phos-

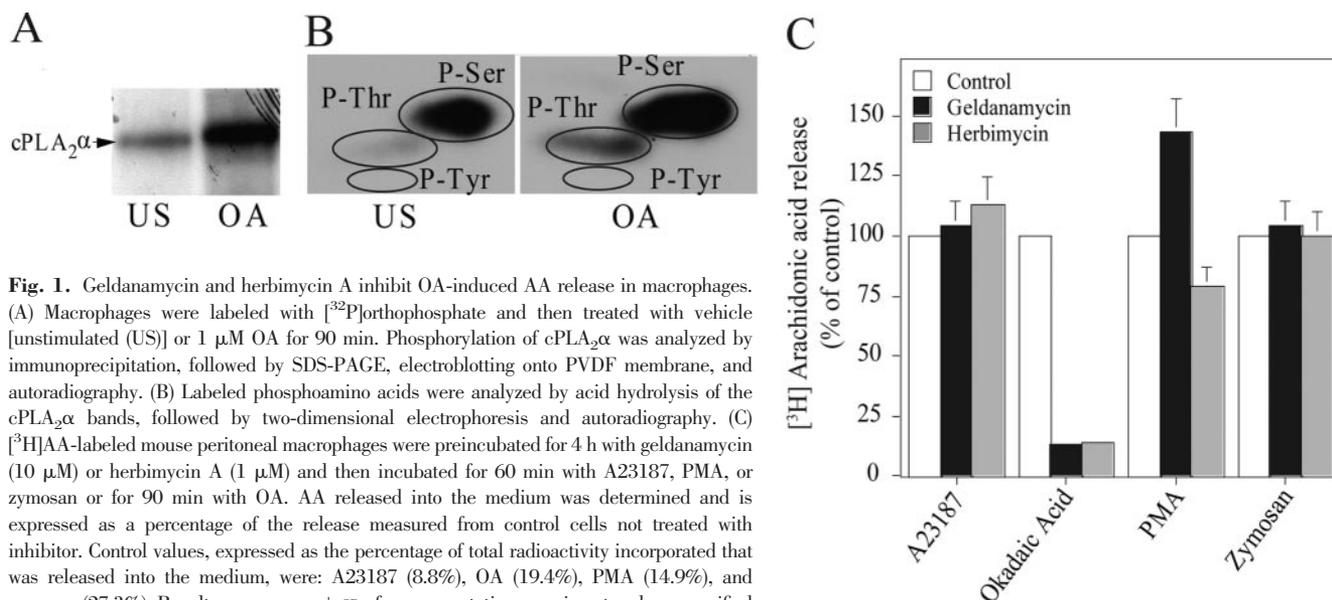


Fig. 1. Geldanamycin and herbimycin A inhibit OA-induced AA release in macrophages. (A) Macrophages were labeled with [³²P]orthophosphate and then treated with vehicle [unstimulated (US)] or 1 μ M OA for 90 min. Phosphorylation of cPLA₂α was analyzed by immunoprecipitation, followed by SDS-PAGE, electroblotting onto PVDF membrane, and autoradiography. (B) Labeled phosphoamino acids were analyzed by acid hydrolysis of the cPLA₂α bands, followed by two-dimensional electrophoresis and autoradiography. (C) [³H]AA-labeled mouse peritoneal macrophages were preincubated for 4 h with geldanamycin (10 μ M) or herbimycin A (1 μ M) and then incubated for 60 min with A23187, PMA, or zymosan or for 90 min with OA. AA released into the medium was determined and is expressed as a percentage of the release measured from control cells not treated with inhibitor. Control values, expressed as the percentage of total radioactivity incorporated that was released into the medium, were: A23187 (8.8%), OA (19.4%), PMA (14.9%), and zymosan (27.3%). Results are average \pm SD of a representative experiment and were verified in two independent experiments.

phorylation of cPLA₂α on serine and threonine residues but not tyrosine residues in response to OA (Fig. 1B). The results suggest that tyrosine phosphorylation of cPLA₂α does not play a role in OA-induced AA release in macrophages. However, we found that herbimycin A inhibited OA-induced AA release (Fig. 1C). Herbimycin A is used as a general tyrosine kinase inhibitor, but this occurs by inhibiting the chaperone protein hsp90 [55]. Another benzoquinone ansamycin inhibitor of hsp90 function, geldanamycin, was also found to inhibit OA-induced AA release by 85–90% (Fig. 1C). The effect of the hsp90 inhibitors was specific for OA, having no significant effect on A23187-, PMA-, or zymosan-induced AA release (Fig. 1C). Hsp90 is required for activation of many kinases including tyrosine kinases of the src family [56, 57], but the src kinase inhibitor PP2 did not block OA-induced AA release in macrophages (data not shown).

Calcium ionophore induces translocation of cPLA₂α to Golgi in peritoneal macrophages [32]; however, the membrane targeted by cPLA₂α in response to OA had not been determined previously. OA stimulated translocation of ECFP-cPLA₂α to Golgi in macrophages, as determined by colocalization with the Golgi marker Giantin (Fig. 2). OA caused an unusual morphological change to the Golgi that appeared as two spherical bodies at separate regions of the cell. OA is known to disrupt Golgi stacks, causing their disintegration into vesicles [58–60], which remain colocalized and form “Golgi clusters”, as we observed in macrophages. Our data show that cPLA₂α associates with disrupted Golgi, as we observed previously with two other Golgi-disrupting agents: nocodazole and brefeldin A [18].

Effect of geldanamycin on OA-induced ERK activation and cPLA₂α phosphorylation on Ser-505

We have shown that activation of the MEK/ERK pathway is essential for OA-induced AA release in mouse peritoneal macrophages [10]. ERKs regulate cPLA₂α by phosphorylation

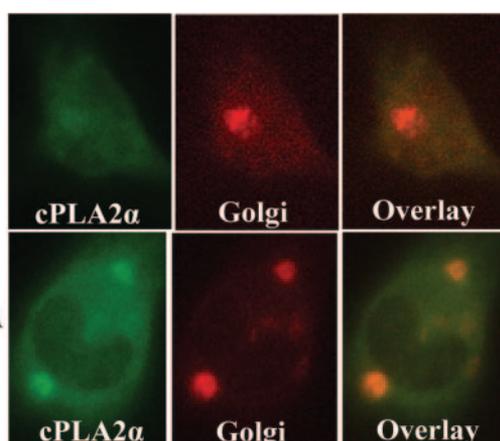


Fig. 2. OA stimulates translocation of cPLA₂α to Golgi. (A) Macrophages expressing ECFP-cPLA₂α were stimulated with 1 μM OA for 90 min or left unstimulated, paraformaldehyde-fixed, permeabilized, and probed with anti-Giantin primary antibody, followed by Texas Red secondary. Images were taken using a 63× oil objective. OA-stimulated ECFP-cPLA₂α translocates to the Golgi in macrophages and colocalizes with Golgi marker Giantin. Images are representative cells of four independent experiments.

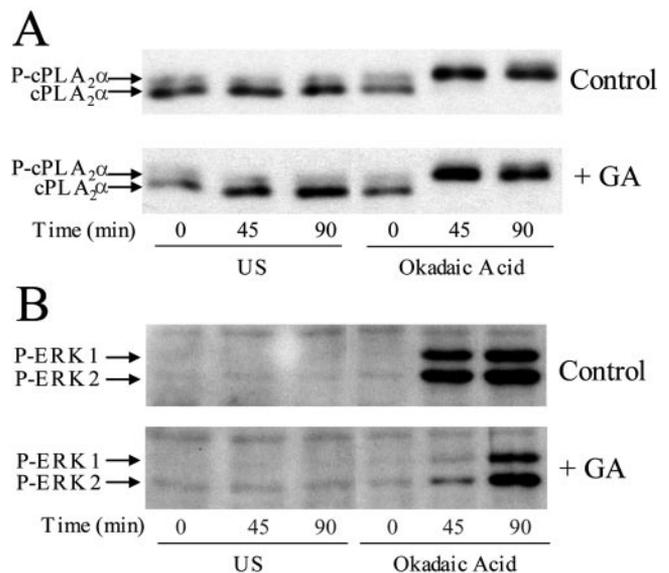


Fig. 3. Effect of geldanamycin on cPLA₂α gel shift and ERK activation. Macrophages were preincubated for 4 h, with or without 10 μM geldanamycin (GA), and then treated with vehicle (unstimulated) or 1 μM OA for the indicated times. Gel shift of cPLA₂α (A) and ERK phosphorylation (B) was determined by SDS-PAGE, followed by immunoblotting using specific antibodies. Results shown are representative of three independent experiments.

on Ser-505 and by an additional mechanism that may involve transcriptional regulation [10]. As hsp90 regulates ERK activation and interacts with upstream activators such as MEK and Raf [61–63], the effect of geldanamycin on OA-induced Ser-505 phosphorylation and ERK activation was investigated. OA induced a retardation in the electrophoretic mobility of cPLA₂α or gel shift, which indicates phosphorylation on Ser-505 (Fig. 3A). This phosphorylation was unaffected by preincubation of macrophages with geldanamycin. Pretreatment of macrophages with geldanamycin resulted in partial inhibition of ERK activation after a 45-min incubation with OA, but by 90 min, ERKs were phosphorylated to a similar extent in control and geldanamycin-treated macrophages (Fig. 3B). We have shown previously that nearly complete inhibition of ERKs 90 min after OA is required for suppression of AA release in macrophages [10]. Therefore, it is unlikely that the inhibition of OA-induced AA release by geldanamycin is caused by its partial inhibitory effect on ERK activation. Hsp90 acts in cells by binding and stabilizing proteins, preventing their degradation [65, 66]. However, geldanamycin did not promote degradation of cPLA₂α, which was expressed at similar levels in unstimulated or OA-stimulated macrophage treated with or without geldanamycin (Fig. 3A).

Detection of kinases activated by OA in macrophages

Because of its ability to inhibit phosphatases, OA triggers the activation of cellular signaling cascades by increasing the phosphorylation and activation of protein kinases. One of the functions of hsp90 in cells is to stabilize protein kinases, allowing for their activation [63, 66]. Thus, the inhibitory effects of geldanamycin and herbimycin on the OA-induced

AA release could be a result of inhibition of a protein kinase involved in cPLA₂α activation. To determine if specific kinases were activated in macrophages treated with OA, macrophage lysates were analyzed by in-gel kinase assays on SDS-PAGE. This detects kinases that autophosphorylate or in gels copolymerized with cPLA₂α, kinases that specifically phosphorylate cPLA₂α. OA treatment resulted in the time-dependent activation of a renaturable kinase, with an apparent molecular weight of ~54 kDa, which phosphorylated cPLA₂α (Fig. 4A). Kinases that phosphorylated cPLA₂α of 42 and 46 kDa were also detected. These kinases were active in untreated cells, and although the activity of the 42-kDa protein did not change significantly during OA treatment, the 46-kDa form was inhibited by OA. An 85-kDa kinase was autophosphorylated in unstimulated and agonist-treated macrophages but did not phosphorylate cPLA₂α, as the signal was not increased in gels containing cPLA₂α (Fig. 4A). Also, a kinase of ~64 kDa was activated by OA. This kinase autophosphorylated and used cPLA₂α as a substrate, as indicated by a more intense band in gels containing cPLA₂α. Activation of the p54 kinase was only observed in lysates from OA-treated cells and not in lysates from macrophages treated with other agonists (A23187, PMA, zymosan) that stimulate AA release (Fig. 4B). The pattern of kinases detected in the in-gel kinase assay was identical in gels containing purified cPLA₂ from unstimulated *Sf9* cells, which is partially (50%) phosphorylated on Ser-505, and gels containing dephosphorylated cPLA₂α (data not shown).

To confirm that the p54 kinase phosphorylated cPLA₂α in the gels, the p54 kinase band was excised from the gel, proteins eluted and subjected to SDS-PAGE and autoradiography. Although a radioactive band appeared at 54 kDa (data not shown), most of the radioactivity appeared in a double band at ~100 kDa, the expected, apparent molecular mass of cPLA₂α (Fig. 4C). This doublet was confirmed to be cPLA₂α

by immunoblotting (data not shown). The cPLA₂α from *Sf9* cells used in the in-gel kinase assays is partially phosphorylated on Ser-505, which induces a decrease in electrophoretic mobility [17, 25, 47, 67]. Phosphorylation of the faster migrating form of cPLA₂α by the p54 kinase suggests that it phosphorylates cPLA₂α on a site other than Ser-505. As the p54 kinase was specifically activated by OA, and the inhibition of AA release by herbimycin and geldanamycin was specific for OA-treated macrophages, the involvement of hsp90 in activation of the p54 kinase was evaluated (Fig. 4, C and D). Pretreatment of macrophages with geldanamycin or herbimycin resulted in partial inhibition of OA-induced p54 kinase phosphorylation of cPLA₂α in the gels. These data suggest that the p54 kinase plays a role in regulating cPLA₂α-mediated AA release by a hsp90-dependent mechanism in response to OA.

OA induces association of the p54 kinase with cPLA₂α

Kinases can exist within cells as part of multi-protein complexes containing substrates, activators, and chaperone molecules such as hsp90 [56, 57]. To provide additional evidence that the p54 kinase regulates cPLA₂α in intact cells, experiments were carried out to determine if it associated with cPLA₂α in macrophages. cPLA₂α was immunoprecipitated from macrophage lysates, and immunoprecipitates were assayed for kinase activities by incubating with radiolabeled ATP to detect activated kinases that autophosphorylated.

A 54-kDa kinase was detected in cPLA₂α immunoprecipitates from OA-stimulated macrophages but not from macrophages treated with PMA or zymosan (Fig. 5A). This kinase activity was not present in control precipitates performed with nonimmune rabbit serum. Moreover, cPLA₂α immunoprecipitates from OA-stimulated macrophages phosphorylated cPLA₂α in an in-gel kinase assay (Fig. 5B). There was less

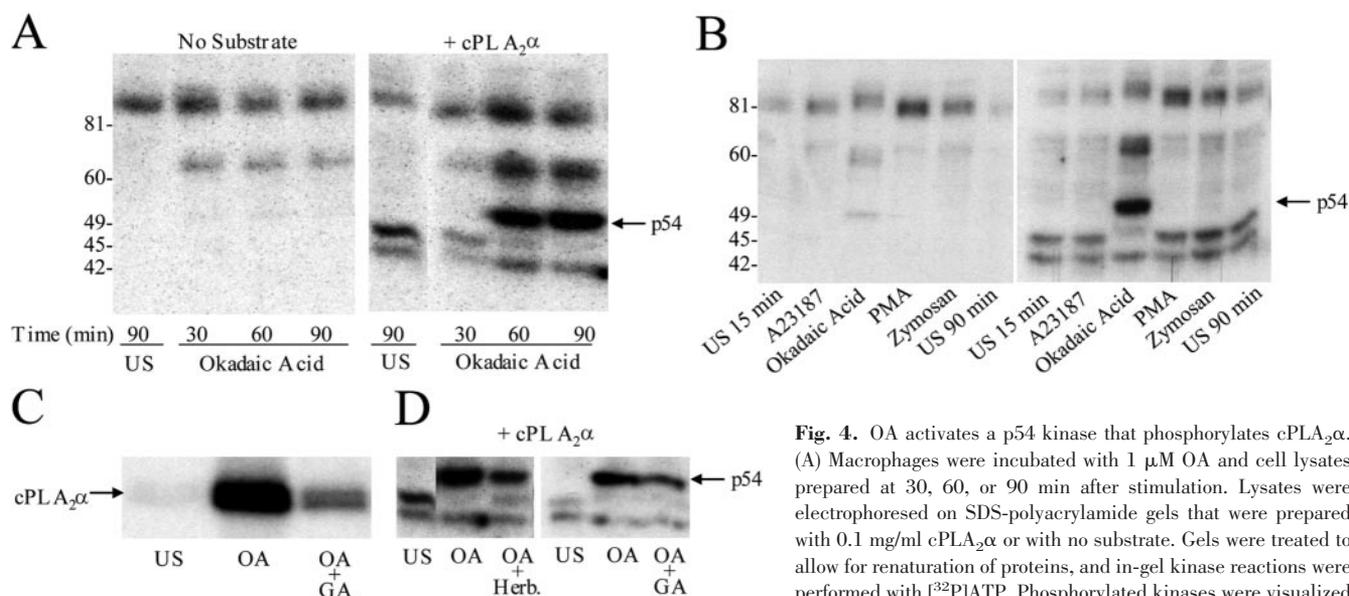


Fig. 4. OA activates a p54 kinase that phosphorylates cPLA₂α. (A) Macrophages were incubated with 1 μM OA and cell lysates prepared at 30, 60, or 90 min after stimulation. Lysates were electrophoresed on SDS-polyacrylamide gels that were prepared with 0.1 mg/ml cPLA₂α or with no substrate. Gels were treated to allow for renaturation of proteins, and in-gel kinase reactions were performed with [³²P]ATP. Phosphorylated kinases were visualized by autoradiography. (B) Macrophages were treated with A23187 (15 min), PMA (15 min), zymosan (30 min), or OA (90 min), and lysates were analyzed for kinase activities as described above. (C) The band corresponding to the p54 kinase was eluted and analyzed by SDS-PAGE and autoradiography. (D) Macrophages were preincubated with 1 μM herbimycin A (Herb.) or 10 μM geldanamycin and then treated for 90 min with OA. Lysates were analyzed for phosphorylated kinases in the presence of cPLA₂α as described above. All results shown are representative of at least three independent experiments.

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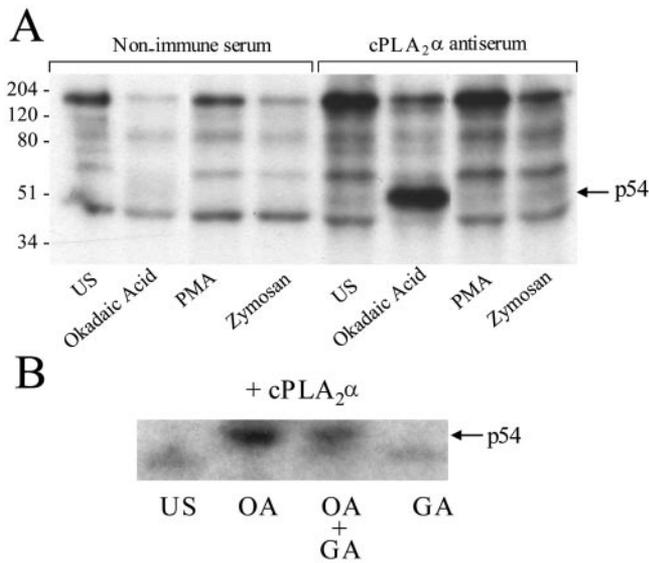


Fig. 5. Coimmunoprecipitation of cPLA₂α and p54 kinase from OA-stimulated macrophages. (A) Macrophages were treated with PMA (15 min), zymosan (30 min), or OA (90 min). After immunoprecipitation of cPLA₂α, in vitro kinase assays were performed in the presence of [³²P]ATP. Phosphoproteins were analyzed by SDS-PAGE and autoradiography. (B) Macrophages were preincubated with or without 10 μM geldanamycin and then treated for 90 min with OA. Immunoprecipitates of cPLA₂α were analyzed by in-gel kinase assay with cPLA₂α copolymerized in the polyacrylamide. All results shown are representative of at least three independent experiments.

phosphorylation of cPLA₂α in the gels using immunoprecipitates of cPLA₂α from OA-stimulated macrophages treated with geldanamycin. The data suggest that the p54 kinase detected by an in-gel kinase assay associates stably with cPLA₂α in OA-treated macrophages.

Association of a kinase with hsp90 in macrophages

The mechanism by which hsp90 regulates the activation of different signaling processes in the cell is not completely

understood but in some cases, involves stabilization of kinases that is necessary for their activation [57, 63, 65, 66]. Experiments were carried out to determine whether the p54 kinase associated with hsp90 in macrophages. Hsp90 immunoprecipitates contained a kinase of ~52 kDa that autophosphorylated in vitro (Fig. 6A). Interestingly, the autophosphorylation occurred in hsp90 immunoprecipitates from unstimulated cells but was undetectable in hsp90 immunoprecipitates from OA-treated macrophages. In contrast, treatment with A23187, PMA, or zymosan did not decrease the autophosphorylation of this kinase in hsp90 immunoprecipitates (Fig. 6A). Analysis of the time course of kinase activity on hsp90 and cPLA₂α immunoprecipitates after addition of OA demonstrated that the appearance of the p54 kinase on cPLA₂α correlated with the disappearance of the 52-kDa kinase activity on hsp90, suggesting that the kinase dissociates from hsp90 and binds to cPLA₂α upon treatment of macrophages with OA (Fig. 6B). Preincubation of macrophages with geldanamycin resulted in inhibition of the p54 kinase on cPLA₂α immunoprecipitates but had no effect on the kinase associated with hsp90 (Fig. 6C).

Phosphoamino acid analysis of autophosphorylated p54 kinase

We observed comparable levels of autophosphorylation of the p52 kinase in hsp90 immunoprecipitates from unstimulated macrophages and the p54 kinase in cPLA₂α immunoprecipitates from OA-stimulated macrophages. However, in-gel kinase assays using lysates from unstimulated cells showed no detectable phosphorylation of cPLA₂α (Fig. 4A). This suggests that the kinase coimmunoprecipitating with hsp90 is able to autophosphorylate but is not able to phosphorylate cPLA₂α. Kinases can autophosphorylate in the basal state but upon activation, are able to autophosphorylate on additional residues and phosphorylate exogenous substrates. To investigate this possibility, phosphoamino acid analysis was carried out on the kinases that coimmunoprecipitated with hsp90 and cPLA₂α from untreated or OA-treated macrophages, respectively. The kinase that coimmunoprecipitated with hsp90 from unstimu-

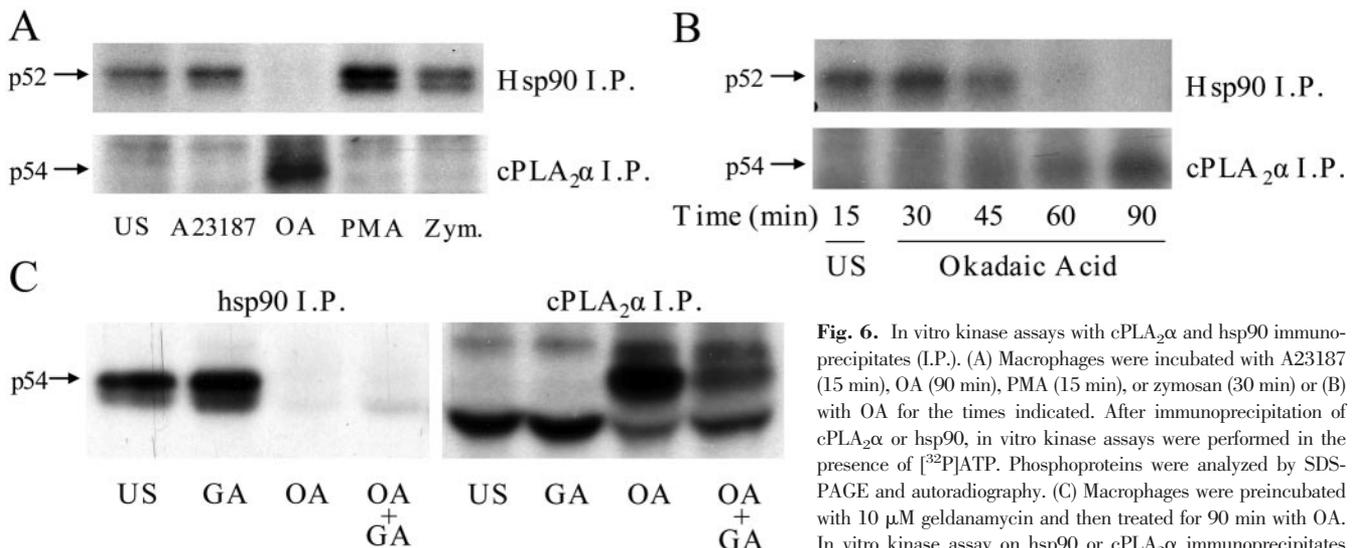


Fig. 6. In vitro kinase assays with cPLA₂α and hsp90 immunoprecipitates (I.P.). (A) Macrophages were incubated with A23187 (15 min), OA (90 min), PMA (15 min), or zymosan (30 min) or (B) with OA for the times indicated. After immunoprecipitation of cPLA₂α or hsp90, in vitro kinase assays were performed in the presence of [³²P]ATP. Phosphoproteins were analyzed by SDS-PAGE and autoradiography. (C) Macrophages were preincubated with 10 μM geldanamycin and then treated for 90 min with OA. In vitro kinase assay on hsp90 or cPLA₂α immunoprecipitates was performed as described above. Results shown are representative of two independent experiments.

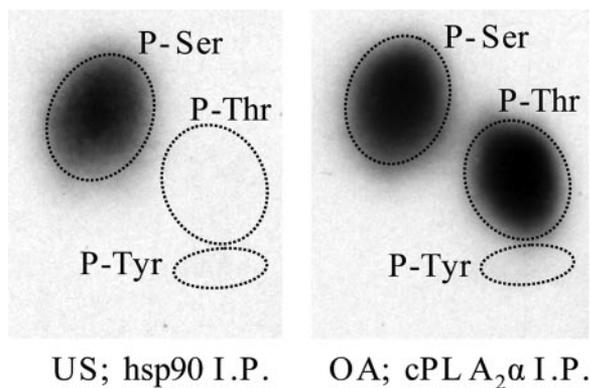


Fig. 7. Phosphoamino acid analysis of p54 kinase. Macrophages were incubated with vehicle (unstimulated) or 1 μ M OA for 90 min, and lysates were immunoprecipitated with antibodies against hsp90 (unstimulated cells) or cPLA₂ α (OA-stimulated cells). Phosphoamino acids were visualized by autoradiography as described in Materials and Methods. The dotted lines contour the position of phosphoamino acid standards included in the samples. Results are representative of three independent experiments.

lated macrophages was autophosphorylated solely on serine residues (**Fig. 7**). In contrast, the p54 kinase associated with cPLA₂ α from OA-stimulated cells autophosphorylated on serine and threonine residues. These data suggest that OA treatment results in activation of the p54 kinase that is then able to autophosphorylate on threonine residues and associate with and phosphorylate cPLA₂ α .

DISCUSSION

cPLA₂ α is a tightly regulated enzyme that is important for controlling levels of AA and downstream lipid metabolites in cells. We have shown previously that A23187, the phagocytic stimulus zymosan, PMA, and the phosphatase inhibitor OA trigger cPLA₂ α -mediated AA release in resident murine peritoneal macrophages through different mechanisms [10, 32, 33]. PMA and OA poorly mobilize calcium in macrophages, although basal levels of calcium are required for AA release [14]. These two tumor promoters also require ERK activation and synthesis of an unknown protein to induce AA release, as pretreatment of macrophages with cycloheximide or actinomycin D inhibits this stimulation [10, 28, 34, 68]. However, the mechanisms by which PMA and OA induce AA release exhibit some differences. Unlike OA stimulation, PMA-induced AA release is dependent on protein kinase C and p38 MAPK activation [10, 44, 69].

Our data suggest that inhibition of OA-stimulated AA release by herbimycin and geldanamycin occurs by blocking hsp90. First, we observe similar effects using two different inhibitors of hsp90. Importantly, the inhibitors do not inhibit AA release from macrophages stimulated with other agonists such as zymosan, A23187, and PMA. Therefore, there is specificity of the inhibitors for the okadaic pathway, which would not be expected if they were acting by nonspecific, “off-target” effects. Also, the observation that the kinase dissociates from hsp90 and associates with cPLA₂ α implicates hsp90 and the p54 kinase in the pathway for cPLA₂ α activation. Our results

demonstrate that tyrosine phosphorylation of cPLA₂ α does not play a role in OA-induced AA release in mouse peritoneal macrophages. Although herbimycin A was described originally and used as a general tyrosine kinase inhibitor, it is now known to target hsp90 function [55]. hsp90 is a ubiquitous chaperone protein that binds to and stabilizes a wide variety of proteins, including tyrosine kinases of the src family, and is a part of large, cytosolic protein complexes involved in various regulatory mechanisms in cells [56, 57]. Unlike other chaperones, hsp90 is generally not involved in nascent protein folding but acts to maintain the activity of signaling proteins, including kinases, as well as to refold proteins that may have denatured under stress conditions [65, 70]. Our results suggest a role for hsp90 in cPLA₂ α activation in response to OA but not zymosan, A23187, or PMA. Although PMA also induces AA release without calcium mobilization, hsp90 is not involved, suggesting that different mechanisms can promote cPLA₂ α activation in the absence of increases in intracellular calcium.

Our experiments show that hsp90 is involved in ERK activation in macrophages, as has been reported for other cell models [61, 71]. hsp90 affects activation of MAPK cascades, primarily by regulating MEK kinases, such as Raf-1 [62, 63]. However, a partial inhibition of ERK activation cannot explain the pronounced inhibition of OA-induced AA release by geldanamycin, which often leads to degradation of proteins that are regulated by hsp90 [63]. cPLA₂ α protein was not degraded during the time-frame of OA-induced AA release in macrophages treated with geldanamycin. This indicates that stabilization of cPLA₂ α by interaction with hsp90 does not explain the dependence of OA-induced AA release on hsp90. Although we did not observe reproducible coimmunoprecipitation of cPLA₂ α and hsp90 in macrophages (data not shown), hsp90 interaction with some proteins is unstable and difficult to detect. The possibility that these two proteins interact in the cytoplasm cannot be ruled out [65, 70].

Our results suggest that hsp90 regulates OA-induced AA release in macrophages through kinase cascades that affect cPLA₂ α activation. Hsp90 is essential for activation of many serine/threonine and tyrosine kinases [56, 57, 65, 70]. The activation of a renaturable p54 kinase that uses cPLA₂ α as a substrate in in-gel kinase assays is unique to OA treatment. Activation of this kinase in response to OA was also observed in human monocyte-derived macrophages and the macrophage-like murine cell line RAW 264.7 cells (data not shown). cPLA₂ α has been shown to interact with other cellular proteins such as annexins, vimentin, and p11, which can regulate its activation. The coimmunoprecipitation data show that the p54 kinase forms a stable complex with cPLA₂ α in cells treated with OA but not with A23187, PMA, or zymosan. The autophosphorylation of the p54 kinase is inhibited by geldanamycin and herbimycin in macrophages, suggesting that it is regulated by hsp90. Phosphatase inhibition by OA leads to activation of many kinase cascades that may be involved in cPLA₂ α regulation. For example, OA stimulates activation of c-Jun N-terminal kinases and MEK4 in peritoneal macrophages, and this is prevented by geldanamycin, suggesting that this pathway may be involved in regulating cPLA₂ α (data not shown). The p54 kinase is also inhibited by the MEK1 inhibitor U0126, suggesting that it is downstream of MAPKs (data

not shown). However, inhibition of AA release by geldanamycin does not correlate with a decrease in phosphorylation of cPLA₂α on Ser-505. It is possible that the p54 kinase phosphorylates a novel residue on cPLA₂α. Consistent with this, our data show for the first time that OA increases threonine phosphorylation of cPLA₂α.

The slightly higher molecular weight of the kinase detected on cPLA₂α immunoprecipitates (54 kDa) than the kinase associated with hsp90 immunoprecipitates (52 kDa) may be explained by gel shift of the kinase upon OA-induced activation. This gel shift could correlate with a change in its catalytic activity, enabling it to autophosphorylate on threonine residues and to phosphorylate cPLA₂α. Our data showing dissociation of the kinase from hsp90 and association with cPLA₂α in response to OA suggest that they are the same kinase; however, identification of the kinase will be necessary for confirmation. Geldanamycin inhibits hsp90 function by inhibiting its ATPase activity [72]. This can result in the dissociation of proteins bound to hsp90 or in the inability of the interacting protein to become activated and dissociate [64]. This may explain the fact that geldanamycin does not affect the kinase activity on hsp90 immunoprecipitates from untreated macrophages, and it inhibits the cPLA₂α-associated activity from OA-treated cells. The time-course data strongly suggest that OA induces dissociation of the kinase from hsp90 and its subsequent association to cPLA₂α. Here, we have identified a novel pathway for cPLA₂α activation by the marine dinoflagellate toxin OA.

ACKNOWLEDGMENT

This work was supported by National Institutes of Health grants HL34303, HL61378, and HL50040.

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