Suppression of PLC β 2 by Endotoxin Plays a Role in the Adenosine A_{2A} Receptor-Mediated Switch of Macrophages from an Inflammatory to an Angiogenic Phenotype

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Toll-like receptor (TLR) 2, 4, 7, and 9 agonists, together with adenosine A_{2A} receptor (A_{2A}R) agonists, switch macrophages from an inflammatory (M1) to an angiogenic (M2-like) phenotype. This switch involves induction of A_{2A}Rs by TLR agonists, down-regulation of tumor necrosis factor α (TNF α) and interleukin-12, and up-regulation of vascular endothelial growth factor (VEGF) and interleukin-10 expression. We show here that the TLR4 agonist lipopolysaccharide (LPS) induces rapid and specific post-transcriptional down-regulation of phospholipase C(PLC) β 1 and β 2 expression in macrophages by destabilizing their mRNAs. The PLC β inhibitor U73122 down-regulates TNF α expression by macrophages, and in the presence of A_{2A}R agonists, up-regulates VEGF, mimicking the synergistic action of LPS with A_{2A}R agonists. Selective down-regulation of PLC β 2, but not PLC β 1, using small-interfering RNA resulted in increased VEGF expression in response to A_{2A}R agonists, but did not suppress TNF α expression. Macrophages from PLC β 2^{-/-} mice also expressed increased VEGF in response to A_{2A}R agonists. LPS-mediated suppression of PLC β 1 and β 2 is MyD88-dependent. In a model of endotoxic shock, LPS (35 μ g/mouse, i.p.) suppressed PLC β 1 and β 2 expression in spleen, liver, and lung of wild-type but not MyD88^{-/-} mice. These studies indicate that LPS suppresses PLC β 1 and β 2 expression in macrophages *in vitro* and in several tissues *in vivo*. These results

suggest that suppression of PLC β 2 plays an important role in switching M1 macrophages into an M2-like state. (Am J Pathol 2009, 175:2439–2453; DOI: 10.2353/ajpath.2009.090290)

Lipopolysaccharide (LPS), a component of the cell walls of Gram-negative bacteria, is a major activator of innate immune responses, and a potent inducer of the expression of cytokines and inflammatory mediators by macrophages.^{1–3} LPS, acting through Toll-like receptor-4 (TLR4), initiates a signal transduction pathway that activates transcription factors such as nuclear factor κ B (NF- κ B), AP1, and CREB.^{4,5} These transcription factors translocate to the nucleus, bind to *cis*-acting elements in the promoters of a variety of genes, and strongly induce their expression. LPS is a major contributor to the symptoms of Gram-negative sepsis in humans, and insights into the mechanisms by which LPS activates macrophages are of major significance for our understanding of innate immunity and disease.^{1,6}

Macrophages play key roles in the regulation of inflammation and wound healing, and are exquisitely sensitive to their microenvironment. Depending on particular exogenous or endogenous stimuli, they modulate their expression of an array of cytokines and growth factors,⁷ and can be induced to differentiate into cells that either exacerbate or inhibit inflammation. Unstimulated macrophages do not produce tumor necrosis factor α (TNF α) and make only low levels of vascular endothelial growth factor (VEGF).⁸ On stimulation by interferon- γ and patho-

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gen associated molecular patterns such as LPS, double-stranded RNA, and lipoteichoic acid, or endogenous signals, such as high mobility group box-1 proteins, hyaluronan and heparan sulfate proteoglycans, macrophages acquire a pro-inflammatory phenotype.^{9–12} This pathway of activation is termed the “classically activated” or M1 phenotype. As a result, macrophages express high levels of interleukin-1, interleukin-6, interleukin-12, TNF α , and inducible nitric oxide synthase (iNOS), thereby exhibiting increased cytotoxic and inflammatory activities.^{13–15} Resolution of inflammation is achieved through the development of anti-inflammatory mechanisms. Macrophages can be induced to develop an anti-inflammatory phenotype, termed the M2 or “alternatively activated macrophage” by Gordon et al,¹⁶ who reported that treatment of unstimulated macrophages with interleukin-4, interleukin-13, or glucocorticoids induces their expression of cytokines, such as interleukin-10, interleukin-4, interleukin-13, and transforming growth factor- β , and up-regulates their expression of interleukin-4R α and CD206.^{16–19} Unlike classically activated M1 macrophages that produce copious amounts of pro-inflammatory cytokines and exhibit potent cytotoxic activity for numerous microorganisms, M2-macrophages are anti-inflammatory, and promote angiogenesis, tissue remodeling, and repair.^{7,9,14,16,20–23} We have shown previously that while the TLR4 agonist LPS, and agonists of TLR2, 7, and 9, strongly induce expression of the inflammatory cytokine TNF α by macrophages, the retaliatory metabolite adenosine strongly inhibits this induction, while simultaneously up-regulating the expression of the angiogenic growth factor VEGF.^{8,20,24,25} Adenosine A_{2A} receptors (A_{2A}Rs) were shown to play a key role in this phenotypic switch of macrophages from an inflammatory (M1) to an angiogenic (M2-like) phenotype, and expression of A_{2A}Rs is strongly induced by LPS. This pathway provides an interleukin-4 and interleukin-13 independent means of converting inflammatory M1 macrophages to an M2-like phenotype, which is induced by elevated microenvironmental levels of adenosine. Adenosine is a metabolite produced by breakdown of ATP that is normally present extracellularly at low levels, but that rapidly accumulates under conditions of stress, such as hypoxia, ischemia, or inflammation.^{26,27}

In our investigations of the signaling pathways involved in this switch of macrophages from an M1 to an M2-like phenotype, we tested the effects of various inhibitors of lipid-mediated signaling. The selective phospholipase C (PLC) β inhibitor U73122 was found to strongly down-regulate LPS-induced expression of TNF α by macrophages, while synergizing with A_{2A}R agonists to up-regulate VEGF expression. In this article, we report that signaling through the phosphatidyl-inositol-specific phospholipase C β 2 (PLC β 2) isoform plays a key role in this regulation of macrophage activation, and the surprising observation that LPS rapidly and selectively down-regulates the expression of PLC β 1 and PLC β 2 isoforms both *in vitro* in macrophages, and *in vivo* in several tissues.

Materials and Methods

Chemicals and Reagents

The specific A_{2A}R agonists 2-[p-(2-carboxyethyl)-phenylethyl amino]-5'-N-ethyl carboxamidoadenosine (CGS-21680) and the nonspecific AR agonist 5'-N-ethylcarboxamidoadenosine (NECA) were purchased from Sigma Chemical Co. (St. Louis, MO). The PLC inhibitor 1-(6-[17 β -3-methoxyestra-1,3,5-(10)triene-17-yl]amino)hexyl) 1H-pyrroledione (U73122) and its inactive analog 1-[6-[17 β -3-methoxyestra-3,5-(10)triene-17-yl]amino)hexyl) 2,5-pyrroledione (U73433) were purchased from BioMol International (Plymouth Meeting, PA). Phenol-extracted *Escherichia coli* LPS (a TLR4 agonist free of TLR2 agonists) was kindly provided by Dr. Stefanie Vogel (University of Maryland). Antibodies to PLC β 1 (sc-205), β 2 (sc-206), β 4 (sc-404), PLC γ 1 (sc-7290) and PLC γ 2 (sc-407), PLC δ 1 (sc-30062), as well as secondary antibodies and small-interfering RNA (siRNA) oligonucleotides (sc-36267 for PLC β 1 siRNA and sc-36271 for PLC β 2 siRNA) were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Antibodies to nucleophosmin (no. 3542) and PLC β 3 (no. 2482) were purchased from Cell Signaling Technology, Inc (Danvers, MA), and an antibody to α -tubulin (no. T6074) was purchased from Sigma-Aldrich, Inc (St. Louis, MO).

Animals

C57Bl/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME) and housed in the New Jersey Medical School animal facility. Mice on the C57Bl/6J background with a specific deletion of the MyD88 gene (MyD88^{-/-} mice) were kindly provided by Dr. Shizuo Akira (Osaka University, Japan). Heterozygous mice (MyD88^{+/-}) were bred to provide MyD88^{-/-} and MyD88^{+/+} mice as litter mate controls. MyD88 mice were maintained in a barrier facility under specific pathogen-free conditions. Mice with a targeted disruption of the PLC β 2 gene (PLC β 2^{-/-} mice) were developed by Dr. D. Wu, and were provided courtesy of the University of Rochester. These mice were also bred on a C57Bl/6J background. All animal procedures were reviewed and approved by the New Jersey Medical School Institutional Animal Care and Use Committee.

Preparation of Macrophages

Mouse peritoneal macrophages were harvested as previously described⁸ with some modifications. Seven to eight-week-old mice were intraperitoneally injected with 2.5 ml of thioglycolate broth (DIFCO, Detroit, MI; 4% w/v). Four days later, mice were sacrificed by cervical dislocation and peritoneal macrophages were harvested and cultured as a monolayer in RPMI 1640 medium (Cellgro, Mediatech, Inc, Herndon, VA), supplemented with 10% fetal bovine serum (FBS; Gemini Bio-Products, Calabasas, CA), 2 mmol/L L-glutamine, 100 IU/ml Penicillin and 100 μ g/ml Streptomycin (Irvine Scientific, Santa Ana, CA). The cultures were found to be >98% pure, as as-

Table 1. Sequences of Anti-PLC β 1 and Anti-PLC β 2 siRNAs

PLC β 2 siRNA (sc-36271)*	PLC β 1 siRNA (sc-36267)*
Sense strand (A) 5'-CCACUGAAUCACUAAUUUCA-3'	Sense strand (A) 5'-CCAAGUGUUGAUUGAGAAA-3'
Scrambled control 5'-GCUUUCAUCCCAAUAAA-3'	Scrambled control 5'-GAGCAGAUUACGUUAGUA3'
Sense strand (B) 5'-CCACAGAUUCUUGUUCAA-3'	Sense strand (B) 5'-CCACAGAAGAGAUGUCUAA-3'
Scrambled control 5'-GCAUAUCUCCUAGUACA-3'	Scrambled control 5'-GCAAAUCUAAGGCAUAGCA-3'
Sense strand (C) 5'-GCAAGAUCCUAAUAGAA-3'	Sense strand (C) 5'-CCAGAGCGUGUUAACAGAA-3'
Scrambled control 5'-GUGCCUAAACCUGAAA-3'	Scrambled control 5'-GGCUUACCGACAGCUAAA-3'

*The siRNAs were purchased from Santa Cruz Biotechnology and consisted of a pool of three separate complementary strands combined together to produce double-stranded RNA.

sessed by nonspecific esterase staining and staining with the macrophage specific F4/80 mAb.

Cell Culture

RAW264.7 cells were obtained from American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 medium, supplemented as described above. The cells were grown at 37°C in a humidified incubator in 5% CO₂ and 95% air. All experiments were performed at a concentration of 1×10^6 cells/ml of medium. The cells were plated 20 to 24 hours before stimulation.

Cytotoxicity Assay

To assess the cytotoxic effects of various treatments on macrophage viability, an MTT assay was used.²⁸ Media was removed from each well at the end of the incubation period and replaced with methyl thiazole tetrazolium (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) in RPMI 1640 without serum and phenol red (50 mg/ml MTT). The plates were then incubated at 37°C for 2 hours. Medium was then removed, cells were washed with PBS, and 250 μ l of dimethyl sulfoxide was added for 5 minutes to dissolve the blue formazan product. An aliquot (100 μ l) of the solution from each well was then transferred to a 96-well plate, and absorption was read at 550 nm with a 690 nm reference wavelength.

siRNA Transfection of RAW264.7 Cells

Electroporation was used to transfect cells with anti-PLC β 2 and anti-PLC β 1 siRNA oligonucleotides. Since primary macrophages are resistant to transfections, we used the macrophage-like RAW264.7 cell line, which is more readily transfected. Both anti-PLC β 2 and anti-PLC β 1 siRNAs were comprised of a pool of three separate complementary strands combined together and annealed to produce a double-stranded RNA. Compositions of these sense strands, as well as those of corresponding negative controls (scrambled siRNAs with the same nucleotide composition, but no significant sequence homology to the mouse genome) are listed in

Table 1. Cells were transfected by using an Amaxa Nucleofector (Walkersville, MD) following Amaxa Protocol no. D032 optimized for RAW264.7 cells. Following transfection, cells were transferred into 60 mm culture dishes or 6-well plates and incubated overnight before further treatment.

Isolation of RNA for Quantitative RT-PCR Analysis

Mouse peritoneal macrophages were plated in 60 mm dishes (4×10^6 cells/dish). Following overnight incubation, cells were treated with various reagents as indicated in the figure legends. RNA was isolated by using TRIzol reagent (Invitrogen Corporation, Carlsbad, CA) according to the manufacturer's protocol and quantified by using SYBR Green II (Sigma-Aldrich). Integrity of the RNA was analyzed by electrophoresis on formaldehyde agarose gels.

cDNA

cDNA was synthesized from total RNA by using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA) following the manufacturer's instructions. Real-time quantitative PCRs were performed by means of TaqMan technology by using ABI 7500 Real-time PCR System (Applied Biosystems). PLC β 1, PLC β 2, and PLC β 3 primers and probes were designed by using Applied Biosystems Primer Express 2.0 software and synthesized at the University of Medicine and Dentistry of New Jersey Molecular Resource Facility. 5'-ends of the probes were labeled with the fluorescent dye 6-carboxy fluorescein and 3'-ends were coupled to the quencher molecule Black Hole Quencher™ dye-1. The sequences of the primers and probes are listed in Table 2. For each sample, mRNA levels were normalized to that of endogenous cyclophilin D (Applied Biosystems' Assay identification no. Mm00835365_g1). Cyclophilin D was used as a housekeeping gene, since it was determined in our prior studies that its expression does not change significantly as a result of the treatments used in these experiments.

Table 2. Sequences of Primers and Probes for Q-RT-PCR Analyses of mRNA Levels*

mRNA levels	Sequence
PLC β 1	Forward primer: 5' -TGCACGCCTTGCAACTCA-3' Reverse primer: 5' -CATCCCACTTGACGAATTGG-3' Probe: 5' -FAM - CCGTGTGCGTGTCCGACAGCC -BHQ-3'
PLC β 2	Forward primer: 5' -CTGCCCTCGGTGCTTGTC-3' Reverse primer: 5' -TTGTACACGGATTCTGGGAAGTC-3' Probe: 5' -FAM - TTGCAAAAGGCAAAATGATGTATCAACC -BHQ-3'
PLC β 3	Forward primer: 5' -GAACGCCTCCCGGAACA-3' Reverse primer: 5' -ATCCGTCCATCCTGGTTCAC-3' Probe: 5' -FAM - CTTCTCGCGAAAGCATACACGAAGC -BHQ-3'

*Primers and probes were designed by using Applied Biosystems' Primer Express 2.0 software and synthesized at the University of Medicine and Dentistry of New Jersey Molecular Resource Facility. 5'-endsof the probes were labeled with the fluorescent dye 6-carboxy fluorescein (FAM), and 3'-ends were coupled to the quencher molecule, Black HoleQuencherTM dye-1 (BHQ-1).

Immunoprecipitation and Western Blot Analyses

After the various treatments, cells were lysed by adding radioimmunoprecipitation assay (RIPA) buffer containing complete protease inhibitor cocktail (sc-29130, Santa Cruz). The contents were scraped into a microcentrifuge tube and passed through a 21-gauge needle to fragment the DNA. Samples were centrifuged at 10,000 $\times$ G for 10 minutes and an aliquot was used for a Bradford-based protein determination. Cell lysates were boiled for 5 minutes with SDS-Laemmli buffer and aliquots containing 50 μ g of protein were loaded onto 10% SDS-polyacrylamide gels for electrophoresis. Following electrophoresis, proteins were transferred to nitrocellulose membranes (Protran, Whatman, Dassel, Germany) by using a Bio-Rad semidry transfer system, according to the manufacturer's instructions. The membranes were then blocked with 5% milk in Tris-buffered saline with 0.1% Tween 20 for 1 hour, washed, and then incubated with primary antibodies at dilutions of 1:500 to 1:2000 overnight. The blots were then washed with Tris-buffered saline with 0.1% Tween 20 and incubated for 1 hour with alkaline phosphatase-conjugated secondary IgG antibody (1:5000 to 1:20,000). Immunoreactive bands were developed by using a chemiluminescent substrate ECL Plus (GE Health care, Piscataway, NJ) and an autoradiograph was obtained by using a Typhoon 9410 analyzer (GE Health Care). For analysis of mouse tissues, 100 mg liver, lung, and spleen were homogenized and extracted with RIPA buffer containing complete protease inhibitor cocktail, and processed as described above. Lysates containing 100 μ g protein in 500 μ l were precleared by adding control IgG, together with 20 μ l Protein A/G-Agarose (Santa Cruz). The lysates were incubated at 4°C for 30 minutes, and the beads were pelleted by centrifugation at 1000 $\times$ g for 5 minutes at 4°C. The supernatants were transferred to a fresh microcentrifuge tube, 1 μ g primary antibody was added, and the samples were incubated overnight at 4°C. Immunoprecipitates were collected by centrifugation at 1000 $\times$ g for 5 minutes at 4°C. The pellets were washed ($\times$ 2) with RIPA buffer, dissolved in 40 μ l SDS-Laemmli sample buffer, and analyzed by electrophoresis and Western blotting as described above.

VEGF and TNF α Assays

VEGF and TNF α levels in macrophage-conditioned media were assayed by using a corresponding sandwich enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN), according to the manufacturer's protocol. Each sample was assayed in duplicate and results were presented as means $\pm$ SD.

Plasmids and Constructs

PCR was used to amplify a cDNA fragment of 3986 bp, encompassing the whole mouse PLC β 2 promoter region (−3946 to +38.). Forward primer was designed with a site for the restriction enzyme Mlu I and a 5' terminal GC-clamp: 5'-GGCGC-ACGCGT-GCACCTGGTCCG-TATTTTGT-3'. Reverse primer also had a 5'-terminal GC clamp, followed by the restriction site for XhoI: 5'-GGCCG-CTCGAG-CACGTTAGGGGGCAATAGAA −3'. The PCR amplicon was cloned into the pGL3 basic (promoterless) luciferase vector (Promega Corporation, Madison, WI) to generate a reporter plasmid for the PLC β 2 promoter. The cloned PCR product was sequenced, and confirmed to be identical to the published sequence of the mouse PLC β 2 5' upstream region.

Transient Transfections and Reporter Assays

Plasmids were prepared by using Endofree plasmid maxiprep kit (Sigma-Aldrich). Transient transfections were performed by using Superfect (Qiagen, Inc, Valencia, CA), following the manufacturer's guidelines. Cells were co-transfected with the Renilla luciferase vector, phRL-TK (Promega), to normalize for transfection efficiency. After 18 hours incubation, the transfectants were resuspended in fresh medium and allowed to adhere for 6 to 7 hours at 37°C. The medium was then changed to RPMI 1640 with 1% FBS, and the cells were stimulated as follows: LPS (100 ng/ml); NECA (1 μ mol/L); LPS with NECA, or control medium—1% FBS in RPMI. After 22 hours incubation, cells were lysed with passive lysis buffer (Promega) and luciferase assays were performed by using the Dual luciferase Assay Kit (Promega) following the manufactur-

er's protocol. Luciferase light units were measured by using Lmax Luminescence Microplate Reader (Molecular Devices, Sunnyvale, CA) using dual injector system. The results reported are representative of at least three independent experiments. An NF- κ B promoter luciferase reporter plasmid (pNF κ B-Luc, CLONTECH, San Diego, CA) that responds to LPS was used as a positive control for LPS treatment. This NF- κ B luciferase vector contains four tandem copies of the NF- κ B consensus sequence fused to a TATA-like promoter region from the herpes simplex virus thymidine kinase promoter. pTAL-Luc (the enhancerless reporter vector lacking the NF- κ B promoter elements) was used as a negative control.

Nuclear Run-On Assay

Nuclear run-on analysis was performed by using a modification of the protocol described by Greenberg and Bender,²⁹ as follows: 5×10^7 RAW264.7 cells were harvested and rinsed ($\times 2$) with ice-cold PBS. The cell pellet was resuspended in 10 ml of sucrose buffer I (0.32 M sucrose with 0.5% v/v Nonidet NP40 and 1 mmol/L DTT). Cells were disrupted by using a Dounce homogenizer to release intact nuclei. To collect nuclei, 10 ml of sucrose buffer II (2 M sucrose) were added to the homogenized cells and mixed well. The homogenate was then layered on a sucrose cushion (10 ml of sucrose buffer II), and centrifuged for 45 minutes at 30,000 g at 4°C. The supernatant was removed, and the nuclear pellet was gently resuspended in 200 μ l ice-cold glycerol storage buffer. The nuclei were then aliquoted into chilled 1.5-ml microcentrifuge tubes and stored at -80°C before use in the transcriptional run-on assay.

For the analysis of PLC β 2 transcription, a 141 bp region in the vicinity of third PLC β 2 exon (Transcript Accession no. NM_177568, Region 349-489) was cloned into the pPCR-Script plasmid (Stratagene, La Jolla, CA). pPCR-Script with a β -actin insert was used as a positive control, and the empty pPCR-Script vector was used as a control for hybridization specificity. Two hundred fifty micrograms of each plasmid were linearized with XhoI, denatured at room temperature by addition of 1M NaOH, and neutralized by addition of 6 $\times$ standard saline citrate. Ten micrograms of each linearized plasmid was applied to a 0.45 μ BioBond nylon membrane (Sigma-Aldrich), using a slot blot apparatus. The membrane was air dried overnight and baked for 20 minutes at 80°C in a vacuum oven. For the transcriptional run-on assay, 200 μ l of nuclei were used for each treatment. Two hundred microliters of 2 $\times$ reaction buffer with nucleotides plus 10 μ l [α -³²P]-UTP (10 mCi/ml, Perkin Elmer, Waltham, MA) was added, and incubated for 30 minutes at 30°C with gentle shaking. RNase-free DNase I (Qiagen) and high salt buffer were added to the labeled nuclei and mixed thoroughly. After a short incubation at room temperature, SDS/Tris buffer and proteinase K were added and incubated for 30 minutes at 42°C. TRIzol reagent (Invitrogen) was then used to extract and precipitate RNA according to the manufacturer's instructions. RNA was re-dissolved in 50 μ l 50% formamide in diethylpyrocarbonate-treated

water. A 5- μ l aliquot of each sample was counted and determined to have more than 5×10^6 cpm/ml. The labeled RNAs were then mixed with an equal volume of 10mM N-tris(hydroxymethyl) methyl-2-aminoethanesulfonic acid, pH7.4, 10mM EDTA, 0.2% (w/v) SDS/NaCl solution, and incubated with the cDNA plasmids immobilized on the nylon membranes for 36 hours at 65°C with shaking in a hybridization oven. After hybridization, the membrane strips were washed with 2 $\times$ standard saline citrate, digested with RNase A, and washed again in 2 $\times$ standard saline citrate. Finally, membranes were air-dried, and analyzed by using a Typhoon 9410 Phosphorimager (GE Health care).

In Vivo Model of Acute Endotoxemia

C57Bl/6J wild-type and MyD88^{-/-} mice were injected i.p. with LPS (1.4 μ g/g body weight in PBS). Additional mice were injected with PBS alone as controls. Mice were sacrificed at various time points (0 to 18 hours) following injection, and liver, lung, and spleen samples were flash frozen in liquid nitrogen. The tissues were then homogenized and extracted with TRIzol (Invitrogen) for the preparation of total RNA, or with RIPA buffer containing complete protease inhibitor cocktail for the preparation of protein extracts. RNA extracts were processed for quantitative RT-PCR (Q-RT-PCR) as described above. Protein extracts were processed by immuno-precipitation and Western blot analysis as described above.

Statistical Analysis

Differences between groups were analyzed by the unpaired Student's *t*-test or one-way analysis of variance followed by Dunnett's test, as appropriate, by using the statistical analysis programs in KaleidaGraph software (Synergy Software, Reading, PA). All data are presented as mean $\pm$ SEM unless otherwise indicated. *P* values <0.05 are considered significant.

Results

U73122, A Selective PLC β Inhibitor, Modulates VEGF and TNF α Expression by Macrophages

U73122 is an antagonist of phosphoinositide-specific PLC.^{30,31} Macrophages were pretreated for 30 minutes with U73122 (0 to 10 μ mol/L), and then incubated for 18 hours in the presence of this compound with LPS, NECA, CGS-21680, or LPS with NECA, or LPS with CGS-21680, as described above. As shown in Figure 1, U73122 alone had little effect on VEGF production by control or LPS-treated macrophages. However, in the presence of NECA, U73122 induced a dose-dependent increase in VEGF production (half maximal inhibitory concentration = ~ 2 μ mol/L). On the other hand, U73122 inhibited LPS-induced TNF α production in a dose-dependent manner, (IC_{50} = ~ 2 μ mol/L). The concentration of U73122 that induced VEGF expression was similar to that

Figure 1. U73122, a selective PLC β inhibitor, modulates VEGF and TNF α expression by macrophages. Mouse peritoneal macrophages from C57Bl/6J mice were pretreated for 30 minutes with U73122 (0, 0.4, 2.5, and 10 μ mol/L). LPS (100 ng/ml), NECA (1 μ mol/L), or LPS with NECA were then added. Conditioned media were harvested after 18 hours incubation and assayed for VEGF (left) and TNF α (right) content by enzyme-linked immunosorbent assay. Data are the mean $\pm$ SEM of triplicates from an experiment that was repeated at least three times with similar results.

for the inhibition of TNF α production. U73122 was not toxic to the cells at concentrations up to 10 μ mol/L. The maximum increase in VEGF production was close to that observed by the combined action of LPS with NECA, but U73122 together with LPS/NECA did not augment VEGF production beyond the increase induced by LPS with these adenosine receptor agonists alone. Similar results were obtained by using the more specific A_{2A}R agonist CGS21680 (data not shown). U73443, a relatively inactive analog of U73122, had only minor effects on TNF α and VEGF expression. Also, similar effects of U73122 were observed by using RAW264.7 cells (data not shown).

LPS Suppresses the Expression of Select PLC Protein and mRNA Isoforms

To determine whether LPS treatment of macrophages affects the level of PLC isoforms, macrophages from C57Bl/6J mice were treated for 24 hours with LPS, NECA, a combination of LPS and NECA in RPMI-1% FBS, or with RPMI-1% FBS alone as control, followed by lysis and Western blot analysis as described above. As shown in Figure 2, A and B, levels of PLC β 1 and PLC β 2 were markedly reduced by LPS, while levels of PLC β 3, PLC β 4, PLC γ 1, and PLC δ 1 were not affected. Reduction of PLC β 2 protein levels was apparent by as early as 8 hours following LPS treatment. NECA treatment did not affect the levels of PLC isoforms, and the suppression of PLC β 1 and PLC β 2 by LPS was not affected by NECA treatment. LPS treatment of RAW264.7 cells induced a similar suppression of PLC β 1 and PLC β 2 isoforms (data not shown).

To determine whether LPS alters the steady state mRNA levels of the various PLC isoforms, RNA was ex-

Figure 2. LPS suppresses the expression of select PLC isoforms at both the protein and mRNA levels in macrophages. Murine peritoneal macrophages from C57Bl/6J mice were treated with LPS (100 ng/ml) (L), NECA (1 μ mol/L) (N), a combination of LPS and NECA (LN), or with RPMI-1% FBS alone as control (C). **A:** Following treatment for 24 hours, media were removed, cells were solubilized in SDS-polyacrylamide gel electrophoresis sample buffer and lysates (50 μ g protein/sample) were Western-blotted with the indicated antibodies. Nucleophosmin (NPM) was used as a control house-keeping gene. Similar results were obtained in at least three independent experiments. **B:** Band intensities of PLC β 1, PLC β 2, and PLC β 3 Western blots were quantified by using the ImageQuant analysis program (GE Health care). * P < 0.01 (L or LN different from C). **C:** Following treatment of macrophages for 2, 4, and 8 hours, RNA was extracted from each sample, and analyzed by Q-RT-PCR. For each sample, mRNA levels were normalized to those of endogenous cyclophilin D as a house-keeping gene. Graphs show relative levels of PLC β 1 (left) and PLC β 2 (right) mRNAs. * P < 0.01 (LPS or NECA different from Control).

tracted from control, LPS, NECA, or LPS/NECA-treated macrophages and Q-RT-PCR was performed. Markedly reduced levels of mRNA for both PLC β 1 and PLC β 2 were observed within 2 hours of treatment, with suppression of

Figure 3. LPS-induced down-regulation of PLC β 1 and PLC β 2 expression in macrophages is post-transcriptional. To determine the effects of LPS on the stability of PLC β 1 and PLC β 2 mRNAs, murine peritoneal macrophages from C57Bl/6J mice were treated with actinomycin D (10 μ g/ml) for 2 hours, followed by either LPS (100 ng/ml) or control medium (1% FBS/RPMI) in the presence of actinomycin D for 0, 1, 3, 5, and 8 hours. Total RNA was isolated and mRNA levels were quantitated by using Q-RT-PCR. For each sample, mRNA levels were normalized to that of endogenous cyclophilin D as a house-keeping gene. PLC β 1 (**left**) and PLC β 2 (**right**) mRNA levels are shown in **A**. The graphs show representative time courses of three separate experiments. **B:** To analyze the effects of LPS on PLC β 2 transcription, a PLC β 2 promoter luciferase reporter plasmid (pPLC β 2-Luc) was constructed by using the pGL3 basic vector. RAW264.7 cells were co-transfected with pPLC β 2-Luc or pGL3 together with a Renilla luciferase plasmid and incubated for 24 hours. The cells were then treated with LPS (100 ng/ml), NECA (1 μ mol/L), LPS with NECA, or medium control for 18 hours, lysed and assayed for Renilla and firefly luciferase activity. **C:** RAW264.7 cells were co-transfected with an NF- κ B promoter luciferase reporter plasmid (pNF κ B-Luc) and the Renilla luciferase plasmid, as a positive control for LPS treatment. pTAL-Luc (the enhancerless reporter vector lacking the NF- κ B promoter elements) was used as a negative control. **D:** A nuclear run-on assay was performed for a direct analysis of transcriptional regulation. RAW264.7 cells were treated with either LPS (100 ng/ml) or control media for 2 hours and intact nuclei were isolated. Nuclei were then labeled *in vitro* with 32 P-UTP to allow the completion of transcription of initiated transcripts. These *in vitro*-labeled RNAs were then isolated and hybridized to a nylon membrane preloaded with linearized plasmids containing either a PLC β 2 cDNA probe, a β -actin probe (housekeeping gene), or the empty pPCR-Script plasmid. **E:** Quantified results of the nuclear run-on hybridization for PLC β 2 of untreated and LPS-treated RAW264.7 cells. Results shown are mean $\pm$ SEM for triplicate analyses. Values are normalized to the corresponding levels of β -actin.

mRNA being apparent for at least several hours (Figure 2 C.) In contrast to the reduced levels of PLC β 1 and PLC β 2, mRNA steady state levels of PLC β 3 were slightly increased by LPS (data not shown).

LPS-induced Down-Regulation of PLC β 1 and PLC β 2 Expression Is Post-Transcriptional

Suppression of PLC β 1 and PLC β 2 gene expression could be regulated transcriptionally or post-transcriptionally. To investigate the mechanism of PLC β down-regulation by LPS, we examined the effects of LPS on the stability of the transcribed mRNAs and on the transcription of the PLC β 1 and PLC β 2 genes. To investigate whether the LPS-induced down-regulation is mediated by modulation of the stability of mRNA, we performed an mRNA stability assay.³² Mouse peritoneal macrophages were treated with Actinomycin D (10 μ g/ml) for 2 hours to block transcription. The cells were then treated with either LPS (100 ng/ml) or control (1% FBS/RPMI) for various times. Total RNA was isolated and mRNA levels of PLC β 1 and PLC β 2 isoforms were quantified by using Q-RT-PCR, to determine the rate of degradation of mRNA. The results of these experiments are shown in Figure 3A. PLC β 1 and PLC β 2 mRNAs were extremely stable in control cells (half life >12 hours), but in LPS-treated cells the mRNAs were markedly destabilized (half life $\sim$ 2.5 hours). Both peritoneal macrophages and RAW 264.7 cells (data not shown)

showed the destabilization of PLC β 2 and PLC β 1 mRNAs by LPS treatment. These results strongly suggest that regulation of mRNA stability plays an important role in the LPS-mediated down-regulation of PLC β 1 and PLC β 2 expression.

To determine whether LPS also affects the expression of PLC β 2 at the transcriptional level, a PLC β 2 promoter construct was engineered and transfected into RAW264.7 cells. To generate the reporter construct of the PLC β 2 promoter region, the sequence of the open reading frame of PLC β 2 gene was identified in GenBank (GeneID: 18796). The sequence upstream of the PLC β 2 open reading frame was then selected, including the transcriptional start site and 5'untranslated region, plus an additional $\sim$ 4 kb of upstream sequence. This region has been reported previously to incorporate the PLC β 2 promoter.³³ This region was amplified by PCR, and cloned into the pGL3 basic (promoterless) luciferase vector, creating pPLC β 2-Luc construct. RAW 264.7 cells were then transiently co-transfected with pPLC β 2-Luc together with a Renilla luciferase plasmid. These transiently transfected cells were then treated with LPS, NECA, or both, lysed and assayed for Renilla and firefly luciferase activity to determine whether PLC β 2 promoter activity is affected by LPS treatment. A pNF κ B-Luc plasmid was used as a positive control for LPS treatment, and pTAL-Luc was used as a negative control. As shown in Figure 3B, RAW264.7 cells transfected with the pPLC β 2-

Luc construct expressed high levels of luciferase in comparison with cells transfected with the pGL3-Luc control plasmid. LPS or NECA did not induce any significant changes in the expression of luciferase activity as compared with the controls. Efficacy of LPS in this system was apparent by the induction of luciferase expression from the pNF κ B-Luc vector (Figure 3C). These results suggest that LPS is not transcriptionally down-regulating PLC β 2 expression by affecting promoter activity.

To confirm the results of the PLC β 2 promoter analysis regarding the mode of down-regulation of PLC β 2 in response to LPS treatment in a more direct manner, we performed a nuclear run-on experiment. For this assay, nuclei of RAW264.7 cells treated with either LPS or control media were labeled *in vitro* with 32 P-UTP to complete the transcription of all initiated gene transcripts. The labeled *in vitro* transcribed RNAs were isolated and hybridized to a membrane preloaded with linearized plasmids containing either a PLC β 2 cDNA probe, a β -actin probe (housekeeping gene), or no insert (hybridization control). Specific binding of radio-labeled RNA was quantified by using the ImageQuant analysis program (GE Health care). This assay measures the level of transcription of the PLC β 2 gene induced by the relevant stimulus. Figure 3D shows the nuclear run-on hybridization analysis for PLC β 2 for untreated RAW264.7 cells and for cells treated with LPS for 2 hours before isolation of nuclei. For each group, the labeling of PLC β 2 was normalized to the level of β -actin. The quantified levels of PLC β 2 transcripts are shown in Figure 3E. Clearly, significant alteration of PLC β 2 transcription was not induced by LPS treatment. Similar results were obtained following 4 hours of LPS-treatment (data not shown). Thus, the results of the nuclear run-on experiment confirm those of the PLC β 2 promoter analysis described above, and strongly suggest that the down-regulation of PLC β 2 expression induced by LPS is mediated post-transcriptionally.

Selective siRNA-mediated Depletion of PLC β 2, but not of PLC β 1, Results in Increased VEGF Expression by RAW264.7 Cells in Response to A $_{2A}$ R Ligation

The results presented above suggest that inhibition of PLC β 2 using a specific inhibitor induces a change in macrophages that enables them to respond to the treatment with adenosine A $_{2A}$ receptor agonists by up-regulating VEGF expression. To test this hypothesis, we transfected siRNAs into RAW264.7 cells to selectively down-regulate the expression of either the PLC β 1 or PLC β 2 isoforms. siRNAs were electroporated into RAW264.7 cells as described above. Controls included blank transfections and transfections with scrambled siRNAs. A strong down-regulation of the expression of PLC β 1, but not of PLC β 2, was induced by the PLC β 1 siRNA transfection at both the mRNA and protein levels. In a similar manner, a strong down-regulation of PLC β 2 but not of PLC β 1 was induced by PLC β 2 siRNA transfection. Figure

4A, shows Western blot analyses of the expression of PLC β 1 and PLC β 2 18 hours following transfection with corresponding siRNAs and controls. Figure 4B, shows the corresponding Q-RT-PCR analyses of PLC β 2 and PLC β 1 mRNA levels 18 hours following siRNA treatment. A clear reduction in the level of both protein and mRNA was induced by the siRNAs in a dose dependent manner. Once specific siRNA-mediated depletion was achieved, transfected cells were treated for 18 hours with LPS, NECA, or LPS/NECA as described earlier, to determine the effects of the depletion on production of TNF α and VEGF in response to LPS and A $_{2A}$ R agonists.

As shown in Figure 4C, siRNA suppression of PLC β 1 resulted in the production of increased amounts of TNF α with increasing amounts of transfected PLC β 1 siRNA. In these transfected cells, NECA induced a similar suppression of LPS-induced TNF α production as in control cells, suggesting that PLC β 1 is not involved in the A $_{2A}$ R-mediated suppression of TNF α expression. Similarly, siRNA down-regulation of PLC β 2 also resulted in increased expression of TNF α in response to LPS, and did not block the down-regulation of LPS-induced TNF α expression by NECA (Figure 4D). Scrambled siRNAs for both PLC β 1 and PLC β 2 did not up-regulate TNF α expression, suggesting the effects of the siRNAs are specific.

In contrast to control cells, which show little response to NECA, strong induction of VEGF expression was induced by NECA in cells treated with PLC β 2 siRNA, but not in PLC β 1 siRNA-treated cells (Figure 4E and F). Sham-transfected cells and cells transfected with scrambled siRNAs responded in a manner similar to nontransfected cells. It is notable that strong up-regulation of VEGF expression was induced even at the level of 1- μ g PLC β 2 siRNA, where depletion of PLC β 2 mRNA was not yet apparent. siRNA mediates its effects both by suppressing translation, as well as by promoting degradation of its target mRNA. As PLC β 2 protein was clearly reduced at this siRNA dose (Figure 4A, bottom), it is likely that the lower dose of siRNA used here is functioning to block translation, while degradation is only induced at the higher doses.

Overall, these results suggest that PLC β 2, but not PLC β 1, plays an important role in regulating the response of macrophages to A $_{2A}$ R stimulation. The response of PLC β 2 depleted cells to NECA in terms of the production of VEGF expression matches that observed in response to the PLC β 2 inhibitor U73122. However, these results suggest that U73122, unlike the siRNAs that specifically target PLC β 2, does not limit its pharmacological action to PLC β 2, as LPS-induced TNF α expression was suppressed by U73122, while PLC β 2-specific siRNAs increased TNF α expression.

Expression of TNF α and VEGF by Macrophages from Mice Lacking the PLC β 2 Gene (PLC β 2 $^{-/-}$ Mice)

PLC β 2 expression by macrophages from PLC β 2 $^{-/-}$ mice was undetectable, by both RT-PCR and Western

Figure 4. siRNA-mediated depletion of PLC β 2, but not of PLC β 1, results in increased VEGF expression by RAW264.7 cells in response to A $_2$ AR ligation. PLC β 1 and PLC β 2 siRNAs (along with corresponding scrambled siRNAs) were electroporated into RAW264.7 cells by using Amaxa nucleofection. **A:** Western blot analyses of the expression of PLC β 1 (**top**) and PLC β 2 (**bottom**) 18 hours following transfection with increasing concentrations of corresponding siRNAs and scrambled siRNA controls (Sc). RAW264.7 cells were also treated in parallel with LPS (100 ng/ml) (L), NECA (1 mmol/L) (N), LPS with NECA (LN), or control medium (C). **B:** The corresponding Q-RT-PCR analyses of PLC β 1 and PLC β 2 mRNA levels 18 hours following siRNA treatment. Negative controls included nucleofected scrambled siRNAs and a "No zap" control, where the cells were incubated with siRNAs without nucleofection. Untransfected cells were also treated in parallel with LPS (100 ng/ml) as a control for the down-regulation of PLC β 1 (**left**) and PLC β 2 (**right**) expression. **C-F:** siRNA-transfected cells were incubated for 18 hours with LPS (100 ng/ml), NECA (1 μ mol/L), LPS with NECA, or control medium. **C and D:** TNF α levels produced by PLC β 1 and PLC β 2 siRNA-treated cells, respectively (* P < 0.01 for LPS-induced TNF α production in siRNA-treated versus untreated cells). **E and F:** VEGF levels secreted by PLC β 1 and PLC β 2 siRNA-treated cells, respectively (* P < 0.01 for NECA-induced VEGF production in siRNA-treated versus untreated cells). Data are the mean $\pm$ SEM of triplicates from an experiment that was repeated at least three times with similar results.

analyses (data not shown). Macrophages obtained from knockout and wild-type mice were treated in parallel with LPS, or with various concentrations of NECA (100 nmol/L to 5 μ mol/L). As shown in Figure 5A, NECA stimulated a marked increase in VEGF secretion by macrophages from PLC β 2 $^{-/-}$ mice while having only a minor effect in macrophages from wild-type mice. Similar results were obtained by using CGS21680 (data not shown). VEGF expression in response to LPS and NECA was strongly up-regulated in wild-type and PLC β 2 $^{-/-}$ macrophages to a similar extent. To compare the effects of NECA on the inhibition of TNF α production in macrophages from PLC β 2 $^{-/-}$ mice versus the wild-type macrophages, cells were treated with various concentrations of LPS (0 to 100 ng/ml) in the presence or absence of NECA (1 μ mol/L). As shown in Figure 5B, TNF α production by macrophages from PLC β 2 $^{-/-}$ mice in response to LPS was similar to that of macrophages from wild-type mice, and the down-regulation of TNF α expression by NECA in PLC β 2 $^{-/-}$ macrophages was also similar to that in wild-type mice.

MyD88 is an Important Adaptor Required for the Suppression of PLC β 1 and PLC β 2 Expression by LPS in Macrophages

MyD88 is a key adapter molecule involved in mediating signaling by most TLRs.^{34–36} Signaling through TLR4, which is a critical receptor for LPS, is transmitted through a MyD88-dependent pathway, as well as through a MyD88-independent pathway that involves adaptors such as TRIF.^{36,37} To determine whether the down-regulation of PLC β 1 and β 2 isoforms requires LPS signaling through MyD88, or whether this effect is mediated in a MyD88-independent manner, expression of these PLC β isoforms in response to LPS was determined in macrophages from MyD88 knockout mice. Figure 6A, shows the mRNA levels of PLC β 1 and β 2 in wild-type versus MyD88 $^{-/-}$ macrophages; Figure 6B, shows Western blot analyses of PLC β 1 and PLC β 2 expression in wild-type versus MyD88 $^{-/-}$ macrophages. While LPS caused a strong suppression of PLC β 1 and PLC β 2 expression at both the mRNA and protein levels in wild-type macrophages, no suppres-

Figure 5. Expression of TNFα and VEGF by macrophages from mice lacking the PLCβ2 gene (PLCβ2^{-/-} mice). Macrophages obtained from mice specifically engineered to lack the PLCβ2 gene (PLCβ2^{-/-}) and C57Bl/6J wild-type (WT) mice were treated in parallel with the indicated concentrations of LPS (1 to 100 ng/ml) or NECA (100 nmol/L to 5 μmol/L). **A:** A comparison of VEGF levels secreted by wild-type versus PLCβ2^{-/-} macrophages in response to increasing concentrations of NECA (N). **B:** A comparison of TNFα production by macrophages from wild-type and PLCβ2^{-/-} mice in response to increasing concentration of LPS. Data are the mean ± SEM of triplicates from an experiment that was repeated at least three times with similar results. **P* < 0.01 (PLCβ2^{-/-} versus wild-type).

sion of these isoforms was seen in the MyD88^{-/-} macrophages. These results clearly indicate that MyD88-dependent signaling is required for the suppression of PLCβ1 and PLCβ2 expression by LPS.

LPS Administration to Mice *in vivo* in a Model of Acute Endotoxemia Suppresses the Expression of PLCβ1 and PLCβ2

To study whether LPS administration *in vivo* might modulate the expression of PLC isoforms, C57Bl/6 wild-type

and MyD88^{-/-} mice were injected i.p. with LPS (1.4 μg/g body weight in PBS) or PBS alone. LPS induced a rapid and time-dependent suppression of both PLCβ1 and PLCβ2 mRNAs in liver, lung, and spleen of wild-type mice (Figure 7, A–F). This suppression was apparent by 2 hours, and reached a maximum of >80% by 4 to 5 hours. PLCβ1 and PLCβ2 protein levels analyzed by immunoprecipitation were also decreased 18 hours following treatment with LPS. Western blots for PLCβ2 are shown in Figure 8, A and B.

To determine whether the LPS-mediated down-regulation of PLCβ1 and β2 *in vivo* was MyD88 dependent, the effect of LPS administration to MyD88^{-/-} mice was compared with that in wild-type mice. In contrast to its effects in wild-type mice, LPS did not induce significant suppression of PLCβ1 and PLCβ2 mRNAs in MyD88^{-/-} mice (Figure 9, A and B). These results indicate that the LPS-mediated suppression of PLCβ1 and PLCβ2 that we observed in macrophages *in vitro* is mirrored in an *in vivo* model of LPS-induced acute endotoxemia.

Discussion

Phosphatidylinositol-specific PLC plays a crucial role in the initiation of receptor mediated signal transduction by hydrolyzing the highly phosphorylated lipid phosphatidylinositol 4,5-bisphosphate [PI(4,5)P₂], generating two intracellular products: inositol 1,3,4-trisphosphate—a universal calcium-mobilizing second messenger; and diacylglycerol—an activator of protein kinase C.^{38,39} At least 13 different isoforms of mammalian PLC isozymes have been discovered, including PLCβ1–4, PLCγ1, PLCγ2, PLCσ1, PLCε, and others, with molecular weights ranging from 85 to 165 kDa.^{38–40} There is tissue-specific distribution of these isoforms, but all are involved in generation of lipid second messengers in response to stimuli in a receptor-mediated manner.⁴¹ While PLC activation generally occurs at the plasma membrane, certain PLC isoforms have also been shown in the Golgi apparatus and nucleus.^{42,43} Phosphoinositide signaling disorders

Figure 6. MyD88 is an important adaptor required for the suppression of PLCβ1 and PLCβ2 expression by LPS in macrophages. Macrophages from MyD88^{-/-} mice and C57Bl/6J wild-type (WT) controls were treated with LPS (100 ng/ml) or medium control. mRNA was isolated from the cells after 0, 2, 4, and 8 hours. Total protein was isolated after 18 hours incubation. **A:** The quantified relative mRNA levels of PLCβ1 (left) and β2 (right) in wild-type versus MyD88^{-/-} macrophages. Data are the mean ± SEM of triplicates from an experiment that was repeated at least three times with similar results. **B:** Typical Western blot analyses for PLCβ1 (top) and PLCβ2 (bottom) protein isoforms, respectively, in lysates of wild-type and MyD88^{-/-} macrophages treated with LPS (L) or control medium (C) for 18 hours.

Figure 7. LPS administration to mice *in vivo* in a model of acute endotoxemia suppresses the expression of PLC β 1 and PLC β 2 mRNA. C57Bl/6 wild-type mice were injected i.p. with LPS (1.4 μ g/g body weight in PBS) or with PBS alone as controls. Mice were sacrificed at various time points (0 to 5 hours) following injection, and their liver, lungs, and spleen were flash frozen in liquid nitrogen. The tissues were homogenized and extracted with TRIzol (Invitrogen) for the preparation of total RNA. The RNA samples were then processed for Q-RT-PCR, and the levels of PLC β 1 and PLC β 2 mRNAs were determined. Cyclophilin D mRNA levels (house-keeping gene) were also determined. **A, C, E.** The levels of PLC β 1 mRNA normalized to cyclophilin D over the 5-hour time course in liver, lung, and spleen following LPS or PBS injection. **B, D, F.** The levels of PLC β 2 mRNA. Data are the mean $\pm$ SEM of triplicates from an experiment that was repeated at least three times with similar results.

have been implicated in the pathogenesis of human diseases ranging from cancer to type 2 diabetes.⁴⁴

PLC β isoforms are activated by heterotrimeric GTP-binding proteins linked to various cell surface receptors. All PLC β isoforms can be activated by members of the Gq family of α subunits, while PLC β 2 and PLC β 3 can also be potentially activated by G $\beta\gamma$.³⁹ PLC β s are key signaling proteins in the cellular action of many hormones, neurotransmitters, growth factors, and other extracellular stimuli.⁴⁵ PLC β isozymes share many of the structural features present in other members of the PLC family, including conserved catalytic X and Y domains, two membrane-phospholipid binding regions and the PH and C2 domains. PLC- β isozymes, however, are distin-

Figure 8. LPS administration to mice *in vivo* in a model of acute endotoxemia suppresses the expression of PLC β 2 protein in liver, lung, and spleen. C57Bl/6 wild-type (WT) mice were injected i.p. with LPS (1.4 μ g/g body weight in PBS) or with PBS alone as control. Mice were sacrificed at 18 hours following injection, and their liver, lungs, and spleen were flash frozen in liquid nitrogen. The tissues were homogenized and extracted with RIPA buffer containing protease inhibitor cocktail. The samples were then immunoprecipitated with an anti-PLC β 2 antibody, electrophoresed on SDS-polyacrylamide gel electrophoresis, and Western blotted. **A:** Typical immunoblots comparing control and LPS-treated PLC β 2 levels. **B:** The quantitated relative band intensities of the PLC β 2 bands. Data are the mean $\pm$ SEM of triplicates from an experiment that was repeated three times with similar results.

guished by the presence of an elongated C-terminus, which contains many of the determinants for interaction with Gq as well as for other functions such as membrane binding and nuclear localization.³⁹

We and others have shown previously that macrophages play an important role in regulating angiogenic responses by producing the angiogenic growth factor VEGF.^{8,20,24,46–48} Unstimulated macrophages do not produce VEGF, and activation of macrophages by exposure to LPS induces the expression of inflammatory mediators such as TNF α , interleukin-12, and iNOS, but not of VEGF or interleukin-10.^{20,49} Macrophages activated by LPS have been termed M1 or “classically activated” macrophages.^{7,50} We have shown recently that treatment of M1 macrophages with adenosine or adenosine A_{2A}R agonists results in a switch of these macrophages from an M1 to an M2-like phenotype, characterized by the down-regulation of TNF α , interleukin-12, and iNOS, and the up-regulation of VEGF and interleukin-10 expression.^{8,20,25} This switch requires the up-regulation of A_{2A}R expression by LPS in a MyD88, IRAK-4, TRAF6, and NF- κ B-dependent manner.²⁰ LPS also transcriptionally up-regulates HIF1 α expression, which is then stabilized in an A_{2A}R-dependent manner under normoxic conditions, resulting in the transcriptional up-regulation of VEGF expression through activation of the Hypoxia Response Element in the VEGF promoter.²⁵ In our studies of

Figure 9. LPS-mediated down-regulation of PLCβ1 and β2 *in vivo* is MyD88-dependent. The effects of LPS administration to MyD88^{-/-} mice on the levels of PLCβ1 and PLCβ2 were compared with those in C57Bl/6J (WT) mice *in vivo*. As described in Figure 7, LPS or PBS was injected i.p. into wild-type and MyD88^{-/-} mice. Four hours following injection, mice were sacrificed and their liver, lungs, and spleen were flash frozen in liquid nitrogen. The tissues were then processed as described in Figure 7, and levels of PLCβ1 and PLCβ2 mRNA were determined by using Q-RT-PCR and normalized to those of cyclophilin-D. **A** and **B:** The quantified relative levels of PLCβ1 and PLCβ2 mRNA, respectively. Data are the mean ± SEM of triplicates from an experiment that was repeated at least three times with similar results.

the signaling pathways involved in A_{2A}R-dependent induction of VEGF expression, we examined the effects of several inhibitors of various signaling pathways in macrophages. Of interest in these studies was the observation that U73122, an inhibitor of PLC that has been reported to selectively inhibit PLCβ2,³¹ induced a dose-dependent increase in VEGF production by macrophages in the presence of A_{2A}R agonists such as CGS 21680 or NECA. It appeared from this study that U73122 was mimicking the effects of LPS in terms of its ability to synergize with A_{2A}R agonists to stimulate VEGF expression, thus suggesting that LPS might act to alter the activity or expression of PLCs as part of its effects on macrophage function.

In the studies reported here, we investigated the effects of LPS on the expression of PLC isoforms in macrophages. LPS was found to induce a strong and sustained suppression of PLCβ1 and PLCβ2 at both the mRNA and protein levels, while mildly increasing expression of PLCβ3 mRNA. Expression of several other PLC isoforms (PLCβ4, PLCγ1, PLCδ1) was not affected. This suppression of PLCβ1 and β2 was apparent at the protein level within 8 hours of LPS challenge, and at the mRNA level within 2 hours. The suppression of PLCβ1 and PLCβ2 was shown to be mediated largely at the level of mRNA stability, rather than at the transcriptional level. While mRNA for these PLC isoforms under nonstimulated conditions, was extremely stable ($t_{1/2} > 12$ hours), the stability of these mRNAs was dramatically shortened in LPS-treated cells ($t_{1/2} = \sim 2.5$ hours). RAW264.7 cells transfected with a luciferase reporter plasmid for the

PLCβ2 promoter (pPLCβ2-Luc) did not show significant modulation of luciferase expression in response to LPS, suggesting that LPS does not modulate PLCβ1 and PLCβ2 expression at the transcriptional level. In addition, a nuclear run-on analysis did not show altered levels of PLCβ1 or PLCβ2 transcripts in response to LPS challenge, directly supporting the conclusion that LPS does not regulate PLCβ1 and PLCβ2 gene transcription, but rather regulates their levels by de-stabilizing their mRNAs. MyD88 signaling is required for this suppression, as it is not observed in macrophages from MyD88^{-/-} mice.

We have shown previously that RAW264.7 cells mimic macrophages in their response to LPS and A_{2A}R agonists.⁸ To determine the functional significance of the LPS-mediated down-regulation of PLCβ1 and PLCβ2, RAW264.7 cells were selectively depleted of these isoforms by using siRNA. While down-regulation of PLCβ1 had no significant effect on NECA-induced VEGF expression, down-regulation of PLCβ2 resulted in greatly increased expression of VEGF in response to NECA. These results indicate that suppression of PLCβ2 plays an important role in the A_{2A}R-mediated induction of VEGF expression in macrophages. However, TNFα expression in response to LPS treatment was not altered, and suppression of TNFα expression induced by A_{2A}R agonists was similar to that in control cells. This suggests that PLCβ2 is involved in only part of the “angiogenic switch” of macrophages, namely, that which results in the up-regulation of VEGF expression, but not in the A_{2A}R-dependent suppression of TNFα expression. The role of PLCβ2 suppression in the regulation of VEGF was confirmed by using macrophages from mice specifically engineered to lack the PLCβ2 gene.⁵¹ While the absence of PLCβ2 in macrophages results in their increased response to NECA in the absence of LPS, this effect is not as profound as that observed in siRNA-treated RAW264.7 cells. Since these mice lack PLCβ2 throughout their development and growth, it is likely that compensatory mechanisms have developed in the PLCβ2^{-/-} mice that are not present in the siRNA-treated cells, resulting in the blunting of their response to NECA. TNFα expression by PLCβ2^{-/-} macrophages in response to LPS is similar to that of macrophages from wild-type mice. This again indicates that the effects of the pharmacological inhibitor U73122 are not limited to PLCβ2, as U73122 down-regulates LPS-induced TNFα expression. Why PLCβ2-specific siRNAs up-regulate TNFα expression in response to LPS is not clear. However, it could be, for example, that these siRNAs might indirectly affect the expression levels of TLR4, thus magnifying the effect of LPS on TNFα expression, an effect that would not exist in the PLCβ2^{-/-} mice. Further studies will be required to clarify this observation.

While LPS has been shown in numerous studies to strongly up-regulate the expression of an array of genes that play important roles in inflammation and repair, there have been only occasional reports of LPS suppressing gene expression. For example, LPS-induced down-regulation of Signal Regulatory Protein-α, an essential regulatory protein of LPS-mediated innate immune activation, has been reported.⁵² Similarly, LPS-mediated down-regulation of Iron Regulatory Protein-1 (IRP1), Elk-3, scavenger

ger receptor B1, and the ATP binding cassette transporter A1 has been reported.^{52–57} The down-regulation of Iron Regulatory Protein-1 was shown to be dependent on the LPS-dependent induction of nitric oxide by the inducible nitric oxide synthase.⁵⁴ The LPS-induced suppression of PLC β 2 reported here is mediated through a MyD88-dependent pathway, but could also require the induction of a down-stream mediator such as nitric oxide or TNF α . The LPS-induced suppression of PLC β 2 reported here plays a key role in the response of macrophages to stimulation through A_{2A}Rs, and modifies the expression of VEGF by these cells. PLC β 2 thus occupies an important position as a mediator of the switch of macrophages from an M1 to an M2-like phenotype.

To determine whether the LPS-mediated suppression of PLC β 1 and PLC β 2 observed *in vitro* in macrophages also occurs *in vivo*, we used an *in vivo* model of LPS-induced acute endotoxemia. LPS induced a rapid and sustained down-regulation of PLC β 1 and PLC β 2 in liver, spleen, and lung. In mice lacking the MyD88 gene (MyD88^{−/−} mice), LPS did not induce the strong depletion of PLC β 1 and PLC β 2 observed in wild-type mice, indicating that the suppression observed *in vivo* is MyD88-dependent, in a manner analogous to that observed *in vitro* in macrophages. While liver, lung, and spleen all contain high numbers of resident macrophages, we have not yet analyzed which cell types within these tissues show the LPS-mediated suppression. The functional significance *in vivo* of the LPS-induced suppression also remains to be determined.

Several prior studies have suggested that PLC activities are involved in the activation of macrophages and other cell types by LPS.^{58–60} The molecular mechanisms of LPS-induced macrophage activation have been shown previously to involve several signal transduction pathways. Among them, protein kinase C, protein kinase A, src-related tyrosine kinases, and mitogen-activated protein kinases such as ERK, JNK, and p38, are especially important in the induction of pro-inflammatory gene expression.⁵⁹ Pittner and Spitzer⁶¹ showed that LPS inhibits PI-PLC but not PC-PLD in liver parenchymal cells. PI-PLC and PC-PLC activities were shown to be involved in LPS-induced iNOS expression in RAW264.7 cells.⁶² LPS stimulation of PC-PLC resulted in activation of p42/44 mitogen-activated protein kinases in human alveolar macrophages.⁶³ Recent studies in mice lacking PLC β 2, PLC β 3, or both, have shown that chemokine-induced G-protein-coupled PLC activation is greatly reduced, with neutrophils and macrophages exhibiting defective Ca⁺⁺ efflux in response to chemoattractants including fMLP, interleukin-8, and several other G-protein coupled ligands.^{51,64,65} Nevertheless, despite this defect, no significant chemotactic defects were observed in either neutrophils or macrophages from these PLC β null mice. Similarly, adhesive capacity, phagocytosis, and uptake of oxLDL and apoptotic cells were unaffected. While the viability of macrophages from these mice was similar to that of controls, macrophages from PLC β 3-null mice have been shown to exhibit hypersensitivity to several inducers of apoptosis.⁶⁶ This has led to the proposal that PLC β 3 plays a role in enhancing macrophage sur-

vival. In an atherosclerosis model, mice null for PLC β 3 and ApoE showed increased apoptosis and decreased survival of macrophages in developing atherosclerotic lesions, and reduced atherosclerotic lesion size compared with ApoE null controls.⁶⁶ Our results presented here indicate that PLC β 2 plays a novel and key role in regulating the switch of macrophages from an inflammatory (M1) phenotype to an angiogenic (M2-like) phenotype, suggesting that regulation of this pathway might provide an additional target for the regulation of inflammation, wound healing, and fibroproliferative responses that form the basis of many chronic inflammatory diseases.

In summary, we have found that U73122, a selective PLC β 2 inhibitor, sensitizes macrophages to stimulation through A_{2A}Rs, and results in increased production of VEGF by macrophages, as well as suppression of TNF α production in response to LPS. LPS was found to strongly suppress PLC β 1 and PLC β 2 expression in macrophages, but did not suppress other PLC isoforms. This suppression was mediated post-transcriptionally by destabilization of mRNA, and requires signaling through a MyD88-dependent pathway. Selective depletion of PLC β 1 and PLC β 2 by using siRNA showed that PLC β 2, but not PLC β 1, is involved in the A_{2A}R-dependent signaling pathway that mediates the induction of VEGF expression in macrophages. This was confirmed by using macrophages from mice lacking PLC β 2. Finally, LPS administration to wild-type, but not MyD88^{−/−} mice, results in the down-regulation of PLC β 1 and PLC β 2 in liver, spleen, and lung, mimicking the effects of LPS on macrophages *in vitro*. These results suggest an important role for PLC β 2 as a modulator of LPS and adenosine signaling in innate immunity, by regulating the M1/M2 phenotype of macrophages, and provide a potential target for the pharmacological modulation of macrophages in the context of inflammatory diseases.

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