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## Title
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## Permalink
https://escholarship.org/uc/item/52p686c8

## Journal
Journal of Biological Chemistry, 282(31)

## ISSN
0021-9258

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## Publication Date
2007-08-01

## DOI
10.1074/jbc.m701831200

Peer reviewed

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THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 282, NO. 31, pp. 22834–22847, August 3, 2007
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# TLR-4 and Sustained Calcium Agonists Synergistically Produce Eicosanoids Independent of Protein Synthesis in RAW264.7 Cells*[S]

Received for publication, March 2, 2007, and in revised form, May 21, 2007 Published, JBC Papers in Press, May 29, 2007, DOI 10.1074/jbc.M701831200

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**Arachidonic acid is released by phospholipase $A_2$ and converted into hundreds of distinct bioactive mediators by a variety of cyclooxygenases (COX), lipoxygenases (LO), and cytochrome P450s. Because of the size and diversity of the eicosanoid class of signaling molecules produced, a thorough and systematic investigation of these biological processes requires the simultaneous quantitation of a large number of eicosanoids in a single analysis. We have developed a robust liquid chromatography/tandem mass spectrometry method that can identify and quantitate over 60 different eicosanoids in a single analysis, and we applied it to agonist-stimulated RAW264.7 murine macrophages. Fifteen different eicosanoids produced through COX and 5-LO were detected either intracellularly or in the media following stimulation with 16 different agonists of Toll-like receptors (TLR), G protein-coupled receptors, and purinergic receptors. No significant differences in the COX metabolite profiles were detected using the different agonists; however, we determined that only agonists creating a sustained $Ca^{2+}$ influx were capable of activating the 5-LO pathway in these cells. Synergy between $Ca^{2+}$ and TLR pathways was detected and discovered to be independent of NF-κB-induced protein synthesis. This demonstrates that TLR induction of protein synthesis and priming for enhanced phospholipase $A_2$-mediated eicosanoid production work through two distinct pathways.**

Arachidonic acid (AA)[2] and its eicosanoid metabolites are associated with a variety of different physiological systems, including the central nervous, cardiovascular, gastrointestinal, genitourinary, respiratory, and immune systems. Eicosanoids are formed when phospholipase $A_2$ ($PLA_2$) action liberates AA from the *sn*-2 position of membrane phospholipids (1, 2). Free AA is then converted into potent bioactive mediators by the action of the various cyclooxygenases (COX), lipoxygenases (LO), and cytochrome P450s. The eicosanoid production cascade often works in a nonlinear fashion, with multiple enzymes creating a single product and multiple products acting as a substrate for a single enzyme. COX activity produces prostaglandins (PG) and hydroxyeicosatetraenoic acids (HETEs) (3, 4), whereas lipoxygenases can create leukotrienes (LT), HETEs, and lipoxins (5–8). Cytochrome P450s catalyze the production of HETEs and epoxy-eicosatetraenoic acids, as well as ω-oxidation of various eicosanoids (9). Other enzymes can further act on eicosanoids by catalyzing hydration, dehydration, and β-oxidation reactions (10). Eicosanoid-producing enzymes and their biological receptors are differentially expressed among various cell and tissue types, enhancing signaling specificity (11). This is illustrated by Fitzgerald and co-workers (12), who demonstrated that decreased vascular $PGE_2$ release leads to increased endothelial $PGI_2$ production, impeding the development of atherogenesis.

Early studies of eicosanoid identification and quantification focused on enzyme-linked immunosorbent assays (13, 14). This method is reliable for measuring relative changes in the level of an individual eicosanoid; however, specificity deficiencies and cost limitations reduce their usefulness for studying an array of eicosanoids. Gas chromatography/mass spectrometry (GC/MS) methods were developed that greatly improved upon these limitations (15) and allowed the simultaneous analysis of multiple eicosanoids. One drawback of the GC/MS method is the requirement that eicosanoids be chemically derivatized in

* This work was supported by Grant GM64611 and the LIPID MAPS Large Scale Collaborative Grant GM069338 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1.

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[2] The abbreviations used are: AA, arachidonic acid; ActD, actinomycin D; BAY, BAY 11-7082; C1P, ceramide 1-phosphate; CHX, cycloheximide; COX, cyclooxygenase; ESI, electrospray ionization; FLAP, 5-LO activating protein; FSL-1, *S*-(2,3-bispalmitoyloxypropyl)-Cys-Gly-Asp-Pro-Lys-His-Pro-Ser-Phe; GC, gas chromatography; GPCR, G protein-coupled receptor; HETE, hydroxyeicosatetraenoic acid; HKLM, heat-killed *Listeria monocytogenes*; LC, liquid chromatography; LO, lipoxygenase; 11t $LTC_4$, 11-*trans*-leukotriene $C_4$; LT, leukotriene; LTA, lipoteichoic acid *Staphylococcus aureus*; $LTC_4$, leukotriene $C_4$; MRM, multiple reaction monitoring; MS, mass spectrometry; MS/MS, tandem MS; NF-κB, nuclear factor-κB; ODN1826, synthetic oligodeoxynucleotide 1826; PAF, platelet-activating factor; Pam3SCK4, *N*-palmitoyl-*S*-[2,3-bis(palmitoyloxy)-(2*R*,2*S*)-propyl]-Cys-[*S*]-Ser-[S]-Lys(4) trihydrochloride; PG, prostaglandin; 15d $PGD_2$, 15-deoxy-$\Delta^{12,14}$-$PGD_2$; dhk $PGD_2$, 13,14-dihydro-15-keto $PGD_2$; dhk $PGE_2$, 13,14-dihydro-15-keto $PGE_2$; dhk $PGF_{2\alpha}$, 13,14-dihydro-15-keto $PGF_{2\alpha}$; 15d $PGJ_2$, 15-deoxy-$\Delta^{12,14}$-$PGJ_2$; $PIP_2$, phosphatidylinositol 4,5-bisphosphate; $PLA_2$, phospholipase; $cPLA_2$, group IVA cytosolic $PLA_2$; p-$cPLA_2$ (Ser-505), $cPLA_2$ phosphorylated at serine 505; poly(I-C), polyinosine-polycytidylic acid; TLR, Toll-like receptor; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; HRP, horseradish peroxidase; Kdo, 3-deoxy-D-*manno*-2-octulosonic acid; PPAR, peroxisome proliferator-activated receptor.

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order to be volatile for GC. Although a wide variety of derivatization methods has been developed increasing the number of eicosanoids that can be effectively detected, a single derivatization method is not suitable to prepare all of the diverse eicosanoids for GC volatilization. Furthermore, some eicosanoids are ill-suited for any gas phase analysis (16), making GC/MS insufficient for analysis of the entire class.

The development of electrospray ionization (ESI) eliminated the requirement for derivatization by directly ionizing eicosanoids in biological samples. The carboxylate moiety, a prevalent eicosanoid structural feature, readily ionizes in mass spectrometric analysis using ESI. By coupling ESI with triple quadrupole mass spectrometry, eicosanoids can be ionized, and their molecular precursor ion $[M - H]^-$ can be subjected to collision-induced decomposition to produce a series of distinct product ion fragments. A set of precursor/product ion pairs can be analyzed in a single MS analysis using multiple reaction monitoring (MRM) that, when coupled with high performance liquid chromatography retention times, will uniquely identify a majority of the eicosanoids. ESI-MRM was first employed in this field by Isakson and co-workers in 1996 (17) to quantitate 14 eicosanoids directly from a biological sample. In 2002 the resolving power of LC was coupled with the sensitivity of ESI-MRM to study five eicosanoids from LPS-stimulated synovial cells (18). Recently, Shimizu and co-workers (19) have developed a high throughput method that can detect and quantitate 18 different eicosanoids from biological samples.

These methods detected a limited subset of eicosanoids that was selected based on previous experiments with a given cell type or disease model. However, more than a hundred unique eicosanoids have been discovered to date, with many having unique biological activities. A thorough investigation of these biological processes requires one to simultaneously analyze a large number of eicosanoids in a single analysis. Analogous to gene array analyses in the genomics field, we have created a library of MS/MS spectra for a large number of eicosanoids and have defined a set of MRM precursor/product ion pairs and an LC system that allows for the identification and quantitation of a large number of these compounds in a single LC/MS/MS run. By using this less biased approach, it should be possible to identify novel eicosanoid signaling networks and efficiently translate this methodology across a diverse array of biological models.

This methodology has now been applied to the study of RAW264.7 macrophages. Macrophages express a number of Toll-like receptors (TLR) (20). TLRs comprise a family of receptors that recognize specific structures of microbial pathogens, leading to the induction of numerous pro-inflammatory cytokines, COX-2 up-regulation, and delayed eicosanoid release. Macrophages also express receptors that can induce rapid changes in intracellular $Ca^{2+}$ levels. $Ca^{2+}$ affects a number of processes within the macrophage and specifically binds to group IVA cytosolic $PLA_2$ ($cPLA_2$) and 5-LO promoting their translocation to the membrane phospholipid surface (1, 5). Platelet-activating factor (PAF) and UDP signal through specific G protein-coupled receptors (GPCR) that create a transient $Ca^{2+}$ spike (21–23). Nonreceptor-mediated ionophores such as ionomycin, and purinergic receptor activation by high levels of adenosine triphosphate (ATP), can produce sustained changes in intracellular $Ca^{2+}$ levels that induce eicosanoid production (21, 23, 24). Furthermore, the response to $Ca^{2+}$ agonists has been shown to be synergistically increased by priming cells with a 60-min dose of a TLR activator (21, 25–29), producing more eicosanoid release than the additive amount of TLR and $Ca^{2+}$ stimulation alone. Here we employed LC/MS/MS methodology to specifically investigate these mechanisms of eicosanoid generation, identifying two distinct eicosanoid profiles. We further investigated the synergy between TLR and $Ca^{2+}$ agonists, and we have elucidated details of the mechanism for endotoxin priming and stimulation of eicosanoid production.

## EXPERIMENTAL PROCEDURES

*Materials*—RAW264.7 murine macrophage cells were purchased from American Type Culture Collection (Manassas, VA). LC-grade solvents were purchased from EMD Biosciences. Strata-X solid phase extraction columns were purchased from Phenomenex (Torrance, CA). Phosphate-buffered saline (PBS) was purchased from VWR. Dulbecco's modified Eagle's medium, fetal bovine serum, and broad range DNA Quant-Kit were purchased from Invitrogen. Lipopolysaccharide (LPS), zymosan, ionomycin, ATP, UDP, EGTA, and bovine serum albumin were purchased from Sigma. The TLR agonists *N*-palmitoyl-*S*-[2,3-bis(palmitoyloxy)-(2*R*,2*S*)-propyl]-Cys-[S]-Ser-[S]-Lys (4) trihydrochloride (Pam3SCK4), heat-killed *Listeria monocytogenes* (HKLM), lipoteichoic acid from *Staphylococcus aureus* (LTA), polyinosine-polycytidylic acid (poly(I-C)), flagellin from *Salmonella typhimurium* (flagellin), *S*-(2,3-bispalmitoyloxypropyl)-Cys-Gly-Asp-Pro-Lys-His-Pro-Ser-Phe (FSL-1), imiquimod, gardiquimod, and synthetic oligodeoxynucleotide 1826, 5′-TCCATGACGTTCCTGACGTT-3′ (ODN1826), were purchased from InvivoGen (San Diego, CA). $Kdo_2$-lipid A and 1-palmitoyl-2-arachidonyl phosphatidylcholine was obtained from Avanti Polar Lipids (Alabaster, AL). L-1-Palmitoyl, 2-[$^{14}$C]arachidonyl phosphatidylcholine was purchased from PerkinElmer Life Sciences. All eicosanoids, PAF, and indomethacin were purchased from Cayman Chemicals (Ann Arbor, MI). The $cPLA_2$, p-$cPLA_2$ (Ser-505), anti-rabbit IgG-HRP, and anti-mouse IgG-HRP antibodies were purchased from Cell Signaling (Beverly, MA). The 5-LO, COX-1, COX-2 antibodies, and 5-LO blocking peptide were purchased from AbCam (Cambridge, MA). The glyceraldehyde-3-phosphate dehydrogenase antibody was purchased from HyTest (Turku, Finland). The anti-goat IgG-HRP antibody was purchased from Sigma. Actinomycin D (ActD), cycloheximide (CHX), and BAY 11-7082 (BAY) were purchased from Biomol (Plymouth Meeting, PA). 6-Amino-4-(4-phenoxyphenylethylamino) quinazoline was purchased from Calbiochem. Pyrrophenone was a kind gift from Dr. Kohji Hanasaki (Shionogi Research Laboratories). Zileuton was a kind gift from Prof. Robert C. Murphy (University of Colorado). All other reagents were reagent grade or better.

*Cell Culture and Stimulation Protocol*—The RAW264.7 mouse murine macrophage cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and 100 units/ml penicillin/streptomycin at 37 °C in a humidified 5%

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$CO_2$ atmosphere. Cells were plated in 6-well culture plates with 2 ml of media ($2 \times 10^6$ cells for short term studies, $1 \times 10^6$ cells for long term studies) and allowed to adhere for 24 h, the media were replaced with 1.8 ml of serum-free media and after 1 h were stimulated. Short term stimuli were added for 10 min at the following doses: PAF (100 nM), UDP (25 μM), ionomycin (1 μM), ATP (2 mM). Long term stimuli were added for 12 h at the following doses: Pam3SCK4 (1 μg/ml), HKLM ($10^8$ cells/ml), LTA (1 μg/ml), poly(I-C) (50 μg/ml), LPS (100 ng/ml), $Kdo_2$-lipid A (100 ng/ml), flagellin (50 ng/ml), FSL-1 (1 μg/ml), zymosan (500 ng/ml), imiquimod (5 μg/ml), gardiquimod (1 μg/ml), ODN1826 (1 μM). In priming experiments, cells were incubated with $Kdo_2$-lipid A (100 ng/ml) for 50 min, followed by the addition of vehicle or short term agonist (PAF, UDP, ionomycin, ATP) for 10 min.

*Sample Preparation*—The media were analyzed for extracellular eicosanoid release. After stimulation, the entire 1.8 ml of media was removed, and each sample was supplemented with 100 μl of internal standards (100 pg/μl, EtOH) and 100 μl of EtOH to bring the total volume of EtOH to 10% by volume. Samples were centrifuged for 5 min at 3000 rpm to remove cellular debris, and then purified. Intracellular eicosanoids were analyzed in the remaining adherent cells by scraping them into 500 μl of MeOH and then adding 1000 μl of PBS and 100 μl of internal standards.

Eicosanoids were extracted using Strata-X SPE columns. Columns were washed with 3 ml of MeOH and then 3 ml of $H_2O$. After applying the sample, the columns were washed with 10% MeOH, and the eicosanoids were then eluted with 1 ml of MeOH. The eluant was dried under vacuum and redissolved in 100 μl of LC solvent A (water/acetonitrile/formic acid (63:37:0.02; v/v/v)) for LC/MS/MS analysis.

It was experimentally determined that intracellular samples contained 1–2% of the extracellular media. The remaining 2% of extracellular eicosanoids were not removed with a wash step because it was determined that the process of washing the cells stimulated eicosanoid release. Measurements approaching 2% of the extracellular eicosanoid level were assumed to be due to media carryover.

*Cell Quantitation*—Eicosanoid levels were normalized to cell number using DNA quantitation. After the extracellular media were removed, the cells were scraped in 500 μl of PBS and stored at 4 °C for DNA quantitation using the Broad Range DNA Quant-Kit according to the manufacturer's instructions. Intracellular experiments required scraping cells into pure MeOH for eicosanoid analysis. Because MeOH significantly affected the DNA quantitation assay, we determined DNA levels from a separate well in these experiments. A conversion factor of 664 cells per ng of DNA was experimentally determined by comparison to hemocytometer cell counting.

*LC and Mass Spectrometry*—The analysis of eicosanoids was performed by LC/MS/MS. Eicosanoids were separated by reverse phase LC on a C18 column (2.1 × 250 mm; Grace-Vydac) at a flow rate of 300 μl/min at 25 °C. The column was equilibrated in solvent A (water/acetonitrile/formic acid (63:37:0.02; v/v/v)), and samples were injected using a 50-μl injection loop and eluted with a linear gradient from 0 to 20% solvent B (acetonitrile/isopropyl alcohol (50:50; v/v)) between 0 and 6 min; solvent B was increased to 55% from 6 to 6.5 min and held until 10 min. Solvent B was increased to 100% from 10 to 12 min and held until 13 min; solvent B was dropped to 0% by 13.5 min and held until 16 min.

Eicosanoids were analyzed using a tandem quadrupole mass spectrometer (ABI 4000 Q Trapr®, Applied Biosystems) via multiple reaction monitoring in negative ion mode. The electrospray voltage was −4.5 kV, and the turbo ion spray source temperature was 525 °C. Collisional activation of eicosanoid precursor ions used nitrogen as a collision gas. Supplemental Table 1 lists the precursor → product MRM pairs, the declustering potentials, and collision energies that were used for each analyte, as well as the limit of detection.

Quantitative eicosanoid determination was performed by the stable isotope dilution method, previously described by Hall and Murphy (30). PGs and AA were obtained as precisely weighed quantitative standards from Cayman Chemicals, whereas the concentrations of HETEs and LTs were determined by UV spectroscopy. A standard curve was prepared by adding 10 ng of each internal (deuterated) eicosanoid standard to the following amounts of eicosanoid (nondeuterated) primary standard: 0.3, 1, 3, 10, 30, and 100 ng. Results are reported as nanograms of eicosanoid per million cells (mean ± S.D.).

*Immunoblotting*—Cells were washed twice with cold PBS and scraped into 200 μl of Complete Mini protease mixture solution (Roche Applied Science). Protein concentrations were determined and normalized using the Bio-Rad Protein Assay (Bio-Rad). 15 μg of total protein was loaded onto 4–12% Bis-Tris SDS-polyacrylamide gels, electrophoresed, and transferred onto a nitrocellulose membrane.

For COX-1 or COX-2, the membrane was blocked with 3% bovine serum albumin, 1% casein in Tris-buffered saline buffer containing 0.05% Tween 20 for 1 h, incubated with either 1:100 COX-1 (Cayman Chemicals, Ann Arbor, MI) or 1:100 COX-2 (Cayman Chemicals, Ann Arbor, MI) specific antibody overnight, washed three times in Tris-buffered saline containing 0.05% Tween 20, incubated with 1:2000 anti-rabbit biotinylated IgG secondary antibody (Vector Laboratories, Burlingame, CA) for 30 min, washed three times, and incubated with 1:5000 streptavidin/HRP-conjugated antibody (Vector Laboratories, Burlingame, CA) for 30 min.

For all other proteins, the membrane was blocked with 5% milk protein in PBS buffer containing 0.1% Tween 20 for 1 h, incubated with 1:1000 of the appropriate specific antibody overnight, washed three times in PBS containing 0.1% Tween 20, and incubated with 1:1000 of the appropriate secondary antibody for 1 h. All membranes were washed three times before development using the Western Lightning ECL kit (Amersham Biosciences).

*Group IVA $PLA_2$ Assay*—Group IVA $cPLA_2$ activity was determined by measuring the release of free [$^{14}$C]arachidonic acid. Final assay conditions were as follows: 100 mM HEPES, pH 7.5, 80 μM $CaCl_2$, 0.1 mg/ml bovine serum albumin, 2 mM dithiothreitol, 400 μM Triton X-100, 3 μM phosphatidylinositol 4,5-bisphosphate ($PIP_2$), and 97 μM 1-palmitoyl-2-arachidonylphoshatidylcholine with 100,000 cpm L-1-palmitoyl, 2-[$^{14}$C]arachidonyl phosphatidylcholine. Radiolabeled free fatty acid was separated from the phospholipid substrate via our

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**TABLE 1**
**Major eicosanoids produced by RAW264.7 cells under 21 different agonist conditions**

| Agonist | Receptor | Physiological Response | $PGF_{2\alpha}$ | $PGE_2$ | $PGD_2$ | $PGJ_2$ | 15d $PGD_2$ | 15d $PGJ_2$ | dhk $PGF_{2\alpha}$ | dhk $PGE_2$ | dhk $PGD_2$ | $LTC_4$ | 11t $LTC_4$ | 5-HETE | 11-HETE | 15-HETE | AA |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| KDO | TLR-4 | Toll-like Receptor Agonist | + | + | + | + | + | + | + | + | + | - | - | - | + | + | + |
| Pam3SCK4 | TLR-1/2 | | + | + | + | + | + | + | + | + | + | - | - | - | + | + | + |
| HKLM | TLR-2 | | + | + | + | + | + | + | + | + | + | - | - | - | + | + | + |
| LTA | TLR-2 | | + | + | + | + | + | + | + | + | + | - | - | - | + | + | + |
| Poly(I:C) | TLR-3 | | + | + | + | + | + | + | + | + | + | - | - | - | + | + | + |
| LPS | TLR-4 | | + | + | + | + | + | + | + | + | + | - | - | - | + | + | + |
| Flagellin | TLR-5 | | + | + | + | + | + | + | - | - | + | - | - | - | + | + | + |
| FSL-1 | TLR-6/2 | | + | + | + | + | + | + | + | + | + | - | - | - | + | + | + |
| Zymosan | TLR-6/2 | | + | + | + | + | + | + | + | + | + | - | - | - | + | + | + |
| Imiquimod | TLR-7 | | + | + | + | + | + | + | + | + | + | - | - | - | + | + | + |
| Gardiquimod | TLR-7 | | + | + | + | + | + | + | + | + | + | - | - | - | + | + | + |
| ODN1826 | TLR-9 | | + | + | + | + | + | + | + | + | + | - | - | - | + | + | + |
| KDO | TLR-4 | TLR-priming | + | + | + | + | + | - | - | - | - | - | - | - | + | + | + |
| PAF | PAFr | Transient $Ca^{2+}$ Spike | + | + | + | + | + | - | - | - | - | - | - | - | + | + | + |
| KDO, PAF | TLR-4, PAFr | | + | + | + | + | + | - | - | - | - | - | - | - | + | + | + |
| UDP | P2Y6 | | + | + | + | + | + | - | - | - | - | - | - | - | + | + | + |
| KDO, UDP | TLR-4, P2Y6 | | + | + | + | + | + | - | - | - | - | - | - | - | + | + | + |
| Iono | Ionophore | Sustained $Ca^{2+}$ Modulator | + | + | + | + | + | - | - | - | - | + | + | + | + | + | + |
| KDO, Iono | TLR-4, | | + | + | + | + | + | - | - | - | - | + | + | + | + | + | + |
| ATP | General P2 | | + | + | + | + | + | - | - | - | - | + | + | + | + | + | + |
| KDO, ATP | TLR-4, P2 | | + | + | + | + | + | - | - | - | - | + | + | + | + | + | + |

modified Dole fatty acid extraction procedure (31, 32) and counted in a Packard 1600TR (Packard Instruments).

## RESULTS

*Eicosanoid Production during Long Term TLR Activation*—We examined two factors that modulate the release of eicosanoids in macrophages, TLR activation and changes in intracellular $Ca^{2+}$ levels. TLR activation initiates many changes in the cell, including the induction of gene expression, changes in protein levels, and changes in protein phosphorylation levels. We have measured changes in eicosanoid release when RAW264.7 cells were activated by long term exposure to 12 different TLR agonists, which leads to the induction of a number of pro-inflammatory proteins through the transcription factor NF-$\kappa$B.

We began by qualitatively analyzing the eicosanoids released into the extracellular media of RAW264.7 macrophages when challenged with one of 12 TLR agonists (Table 1). A signal was judged to be significant, and the eicosanoid present (+in shaded cell), if the signal area was three times the noise three standard deviation level. A minus sign indicates that the signal was below this level. In these experiments, we screened all 64 eicosanoids listed in supplemental Table 1. In all, eight different TLRs were examined. We began with $Kdo_2$-lipid A, a nearly homogeneous LPS sub-structure with endotoxin activity equal to that of native LPS. $Kdo_2$-lipid A is a chemically defined LPS, consisting of lipid A and an attached 3-keto-D-*manno*-octulosonic acid disaccharide. The highly variable carbohydrate chains that are present on natural LPS have been removed, and the lipid A portion of the molecule has a fatty acid composition that is greater than 90% homogeneous. $Kdo_2$-lipid A binds to and activates only the TLR-4 receptor (33). Although we looked for each of the 64 different eicosanoids, when RAW264.7 cells were challenged with $Kdo_2$-lipid A for 12 h, only compounds produced by the COX pathway were detected. COX-derived $PGF_{2\alpha}$, $PGE_2$, and $PGD_2$ were released, and prostaglandin $PGD_2$ dehydration metabolites $PGJ_2$, 15-deoxy-$\Delta^{12,14}$-$PGD_2$ (15d $PGD_2$), and 15-deoxy-$\Delta^{12,14}$-$PGJ_2$ (15d $PGJ_2$) were also detected. Additionally, oxidation metabolites 13,14-dihydro-15-keto $PGF_{2\alpha}$(dhk $PGF_{2\alpha}$), 13,14-dihydro-15-keto $PGE_2$ (dhk $PGE_2$), and 13,14-dihydro-15-keto $PGD_{2\alpha}$(dhk $PGD_2$) were identified. The potential side products of COX action on AA,

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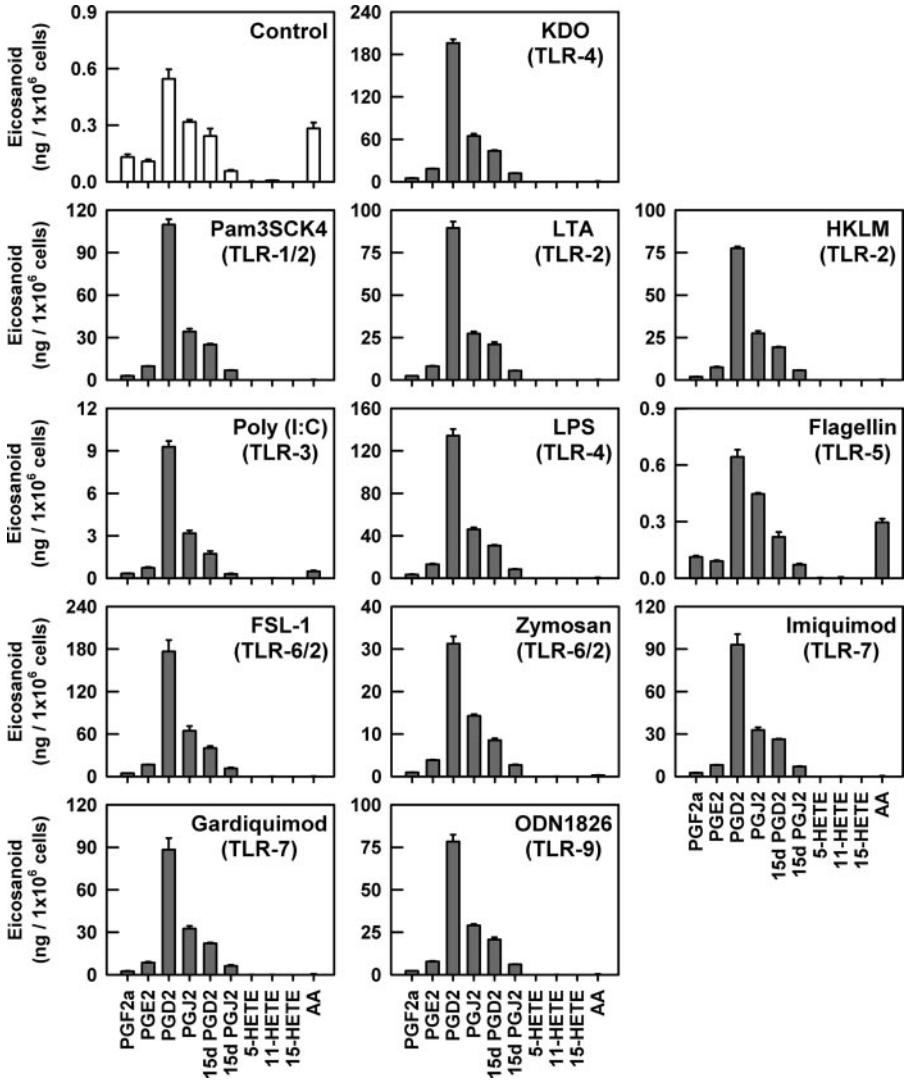


FIGURE 1. **TLR agonist stimulated eicosanoid production profiles.** RAW264.7 cells were stimulated with the following TLR receptor agonists: $Kdo_2$-lipid A (*KDO*, *TLR-4*, 100 ng/ml), Pam3SCK4 (*TLR-1/2*, 1 $\mu$g/ml), LTA (*TLR-2*, 1 $\mu$g/ml), HKLM (*TLR-2*, $10^8$ cells/ml), poly(I-C) (*TLR-3*, 50 $\mu$g/ml), LPS (*TLR-4*, 100 ng/ml), flagellin (*TLR-5*, 50 ng/ml), FSL-1 (*TLR-6/2*, 1 $\mu$g/ml), zymosan (*TLR-6/2*, 500 ng/ml), imiquimod (*TLR-7*, 5 $\mu$g/ml), gardiquimod (*TLR-7*, 1 $\mu$g/ml), ODN1826 (*TLR-9*, 1 $\mu$M). Extracellular media were removed at 12 h and analyzed for eicosanoid levels by mass spectrometry. A representative experiment is shown, and the data are expressed as mean values ± S.D. of three individual replicates.

11-HETE and 15-HETE, were also detected. Their formation was completely inhibited by the COX inhibitor indomethacin (data not shown), confirming that they are being generated by the COX enzymes.

The eicosanoid release profiles of TLRs 1–7 and TLR-9, when stimulated with the appropriate receptor-specific agonists for 12 h, were the same as that seen with $Kdo_2$-lipid A. The one exception was that in response to the TLR-5 agonist, flagellin, dhk $PGF_{2\alpha}$, or dhk $PGE_2$ was not detected. In these experiments, we employed levels of agonist within the range suggested by InvivoGen, but we did not attempt to maximize the response. Therefore, the absolute levels of the response do not necessarily represent maximal responses, and only the relative eicosanoid profiles should be compared.

We then narrowed our focus and repeated these experiments quantitating only those compounds detected in the screen. This examination of long term $Kdo_2$-lipid A stimulation of TLR-4 showed that $PGD_2$ was the most abundant eicosanoid released (Fig. 1). Although not as substantial as $PGD_2$ levels, $PGE_2$ and $PGF_{2\alpha}$ levels were also significantly increased. Metabolites of $PGD_2$ resulting from a single dehydration ($PGJ_2$ and 15d $PGD_2$) were higher than the double dehydration product 15d $PGJ_2$. Activation of TLR-1, -2, -4, -6, -7, and -9 all produced significant levels of PGs, releasing greater than 70 ng/1 × $10^6$ cells of $PGD_2$ in 12 h, whereas TLR-3 activation by poly(I-C) only induced a moderate activation (9 ng/1 × $10^6$ cells $PGD_2$). Although each of these TLR-specific agonists produced different absolute levels of PGs, none altered the relative eicosanoid profile from the one induced by $Kdo_2$-lipid A. Flagellin, the TLR-5 agonist, did not lead to an increase in eicosanoid production over control levels under the conditions employed in RAW264.7 cells.

*Eicosanoid Production during Short Term $Ca^{2+}$ Agonist Activation*—Eicosanoid release is also regulated by increasing intracellular $Ca^{2+}$ levels, which facilitate the translocation of eicosanoid-producing enzymes, such as $cPLA_2$ and 5-LO, to the phospholipid membrane (1, 5) where they can actively generate eicosanoid metabolites. $Ca^{2+}$ fluxes can occur in two distinct modes (21, 23). The release of intracellular $Ca^{2+}$ stores causes a transient spike, which quickly returns to near basal levels, whereas changes in the permeability of the plasma membrane create a sustained $Ca^{2+}$ elevation from the influx of extracellular $Ca^{2+}$. Both of these $Ca^{2+}$ changes occur within seconds of activation. In these studies, four short term stimuli that modified $Ca^{2+}$ levels were examined. PAF and UDP stimulate GPCRs and give rise to a transient $Ca^{2+}$ spike (21, 23), whereas ionomycin and ATP produce sustained increases in $Ca^{2+}$ levels.

The qualitative analysis of PAF and UDP stimulation are shown in Table 1. Following a 10-min stimulation with PAF or UDP, COX-derived $PGF_{2\alpha}$, $PGE_2$, $PGD_2$, 11-HETE, and 15-HETE were released, and $PGD_2$ dehydration metabolites $PGJ_2$ and 15d $PGD_2$ were detected (Table 1). 15d $PGJ_2$ and the three dhk PGs were not detected. 15d $PGJ_2$ was presumably not detected because it requires two separate dehydration steps, which require a longer period of time to occur; even after 60 min of $Kdo_2$-lipid A stimulation, 15d $PGJ_2$ was not detected. Likewise, PG breakdown into dhk $PGF_{2\alpha}$, dhk $PGE_2$, and dhk

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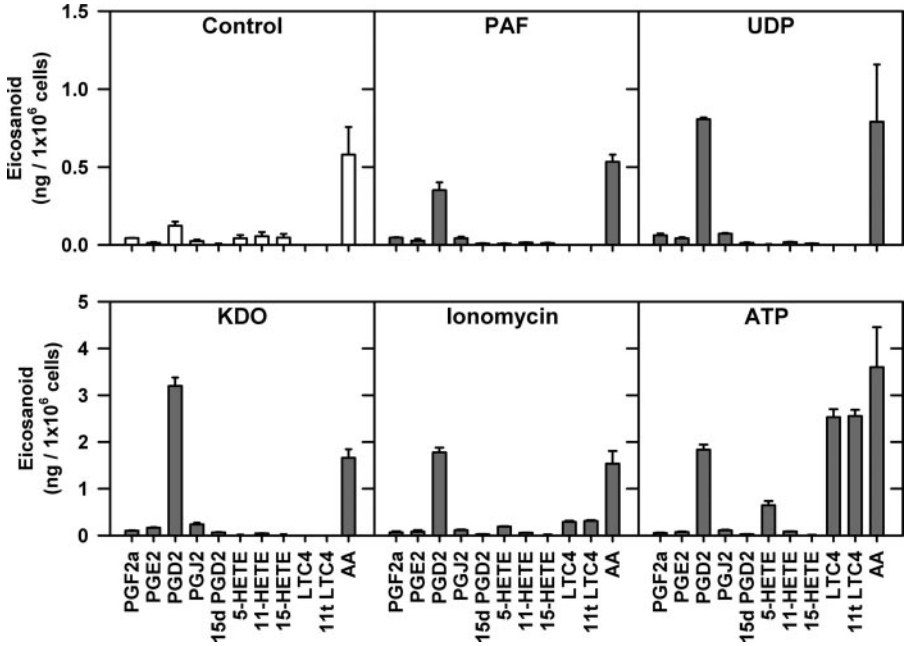


FIGURE 2. **GPCR and sustained $Ca^{2+}$-stimulated eicosanoid production profiles.** RAW264.7 cells were stimulated for 10 min with GPCR agonists (100 nM PAF, 25 $\mu$M UDP) and sustained $Ca^{2+}$ modulators (1 $\mu$M ionomycin, 2 mM ATP). Cells were also stimulated for 60 min with 100 ng/ml $Kdo_2$-lipid A. Extracellular media were removed at the indicated time points and extracted and analyzed for eicosanoid levels by mass spectrometry. A representative experiment is shown, and the data are expressed as mean values ± S.D. of three individual replicates.

$PGD_2$ requires an oxidation and a reduction, and these products were not detected. Again, none of the other 64 eicosanoids were detected. The quantitative analysis demonstrated that the foremost eicosanoids released in these pathways were $PGD_2$ and AA (Fig. 2). The $PGD_2$ levels were between 0.3 ng/1 × $10^6$ cells for PAF and 0.8 ng/1 × $10^6$ cells for UDP. To confirm that extracellular $Ca^{2+}$ is not required for PAF- and UDP-stimulated eicosanoid production, these experiments were performed in the presence of 2 mM EGTA (data not shown). The level of eicosanoid release was unaffected by the removal of extracellular calcium using EGTA, confirming that intracellular calcium stores play a primary role in activating eicosanoid production in response to these two agonists. This supports data by Asmis *et al.* (21), who demonstrated that the calcium spike generated by PAF in murine macrophages does not require extracellular $Ca^{2+}$.

Millimolar concentrations of ATP (23), which activate both P2Y and P2X purinergic receptors, and ionomycin (21), a $Ca^{2+}$ ionophore, both produce sustained $Ca^{2+}$ levels in the cell in addition to inducing a transient $Ca^{2+}$ spike like PAF and UDP. The qualitative screen of RAW264.7 cells challenged by either 2 mM ATP or ionomycin showed that these agonists were capable of generating every eicosanoid produced by PAF or UDP stimulation, and in addition activated the 5-LO pathway. Significant levels of 5-HETE, $LTC_4$, and its heat-induced isomer 11t $LTC_4$ were detected. Although ionomycin produced 2 ng/1 × $10^6$ cells of $PGD_2$ and less than 0.5 ng/1 × $10^6$ cells of the $LTC_4$s, ATP stimulation produced 2.5 ng/1 × $10^6$ cells of both $LTC_4$ and $PGD_2$. $LTC_4$, 11t-LTC4, and 5-HETE production was completely inhibited by the 5-LO inhibitor zileuton (data not shown). Furthermore, when 2 mM EGTA was added extracellularly, it completely blocked the production of 5-LO products without significantly affecting COX activity (data not shown), confirming the role of a sustained $Ca^{2+}$ influx in the activation of this pathway. It also demonstrated that the transient spike of $Ca^{2+}$ from the internal stores was insufficient for this activation.

This is significantly less than the levels of PGs seen in the long term stimulations. Presumably, 12 h of TLR activation allows for more time to release AA and up-regulate eicosanoid-producing proteins such as COX-2. For comparison, a 60-min stimulation of $Kdo_2$-lipid A produced a similar profile to UDP and PAF, generating all of the eicosanoids produced by long term $Kdo_2$-lipid A except 15d $PGJ_2$, dhk $PGF_{2\alpha}$, dhk $PGE_2$, and dhk $PGD_2$. It should be noted that the $PGD_2$ level at 60 min represents a 10-fold activation over control and although this is larger than any of the $Ca^{2+}$ agonists, it pales in comparison to the several hundredfold activation seen in long term TLR stimulations.

*Spatial and Time Dependence of Eicosanoid Production*—The analysis of 16 agonists showed that only millimolar ATP or ionomycin exhibited significant differences in the eicosanoid profile, presumably because of the sustained elevation of $Ca^{2+}$. To explore the nature of these differences, we chose to compare the eicosanoid secretion and degradation over time between short term purinergic activation with ATP and the long term activation with the TLR-4-specific agonist $Kdo_2$-lipid A. In doing so, we also compared the levels of secreted eicosanoids to their levels inside the cell.

$Kdo_2$-lipid A stimulation caused intracellular AA levels to rise dramatically, peaking at 1 h and returning to basal levels by 4 h (Fig. 3); extracellular AA followed the same temporal release pattern. COX-derived 11-HETE, but not lipoxygenase derived 5-HETE, was produced in response to $Kdo_2$-lipid A, peaking at 1 h and slowly returned to basal levels by 12 h. $PGF_{2\alpha}$ and $PGE_2$ were released into the extracellular media at a constant rate over 24 h. Extracellular $PGD_2$, however, increases until 8 h and then began to slowly decline. This decline appears to be due in large part to increases in $PGD_2$ metabolites $PGJ_2$, 15d $PGD_2$, and 15d $PGJ_2$. Significantly elevated levels of $PGJ_2$ were detected after 2 h of stimulation, 15d $PGD_2$ after 4 h, and 15d $PGJ_2$ after 8 h. The sum of the levels of $PGD_2$ and its dehydration metabolites continue to increase similar to $PGE_2$ and $PGF_{2\alpha}$. PGs and their metabolites were not detected intracellularly during the 24-h stimulation period.

ATP activation of purinergic receptors created a sustained $Ca^{2+}$ influx and rapid production of eicosanoids by RAW264.7 cells (Fig. 4). Intracellular AA levels peaked during the first 5

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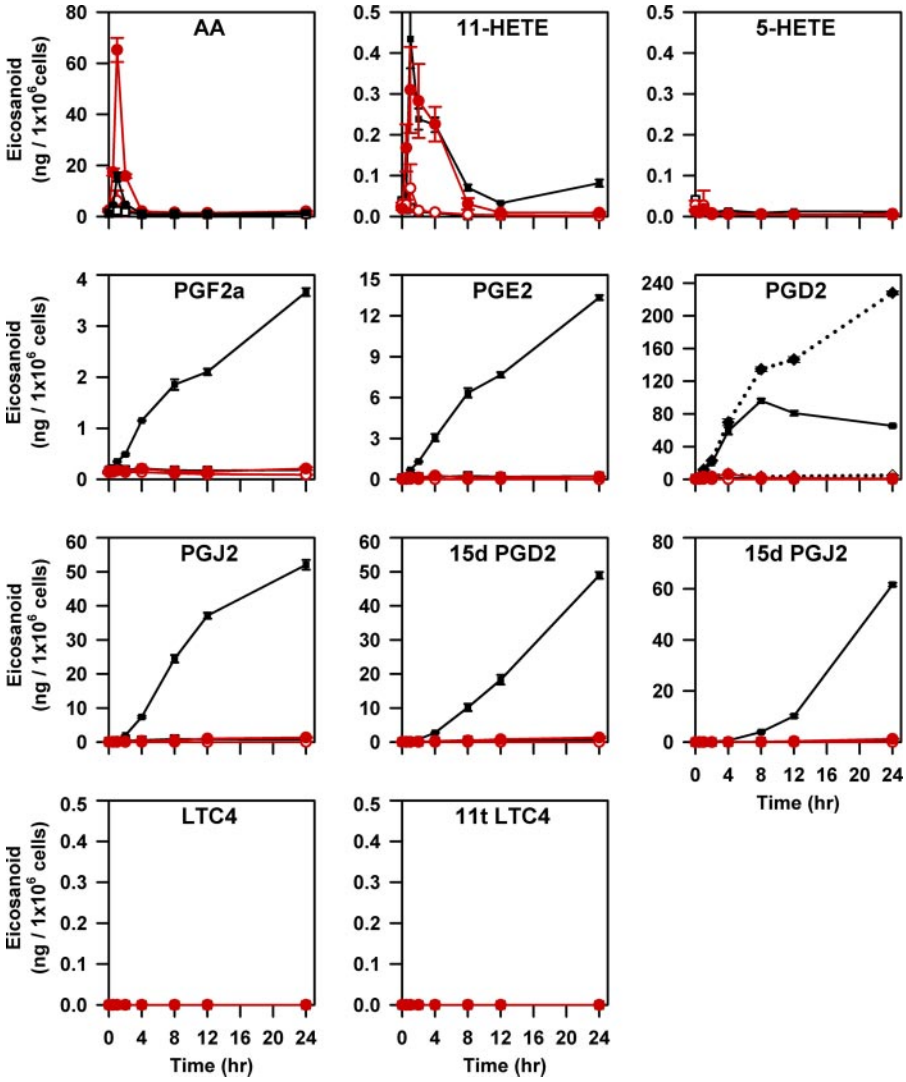


FIGURE 3. **$Kdo_2$-lipid A stimulated intracellular and extracellular eicosanoid production.** RAW264.7 cells were incubated in the absence (*open symbols*) and presence (*closed symbols*) of 100 ng/ml $Kdo_2$-lipid A, and then subsequently extracellular (*black squares*) and intracellular (*red circles*) eicosanoid levels were determined at the indicated times over a 24-h period by mass spectrometry. For $PGD_2$ production, the *dashed line* represents the sum of $PGD_2$, $PGJ_2$, 15d $PGD_2$, and 15d $PGJ_2$ detected at the indicated time point. A representative experiment is shown, and the data are expressed as mean values ± S.D. of three individual replicates.

min of stimulation and returned to near-basal levels by 10 min. AA was also released into the extracellular media during the initial 5 min, and remained elevated throughout the time course. 11-HETE and 5-HETE, products of COX and 5-LO, respectively, were maximally released within minutes of activation. 11-HETE remained stable in the extracellular media, whereas 5-HETE levels slowly dropped over the 60-min time course.

$PGE_2$ and $PGD_2$ were released within minutes of ATP stimulation, and their levels remained constant for the remainder of the 1-h time course, whereas $PGJ_2$ and 15d $PGD_2$ continued to increase. $LTC_4$ showed a biphasic release, with a burst of $LTC_4$ released in the first 5 min, followed by a gradual release after 15 min. However, the second phase of this response was not reproducible. 11t $LTC_4$ increases during the first 15 min, and then remains constant for the remainder of the time course. Small but detectable levels of PG and LT metabolites were detected inside the cell at 5 min, but by 10 min these metabolites were detected only in the extracellular media.

*Synergy between TLR and $Ca^{2+}$ Activations*—Individually, both $Kdo_2$-lipid A and $Ca^{2+}$ agonists can stimulate eicosanoid production in macrophage cells. Previous work has also demonstrated that the level of eicosanoid release can be modulated by adding these agonists together (21, 27–29). Specifically, Aderem *et al.* (25, 26) showed that adding the TLR-4 agonist LPS for 60 min primed murine macrophages for enhanced eicosanoid release by $Ca^{2+}$ agonists.

We studied RAW264.7 cells stimulated with Kdo2-lipid A and $Ca^{2+}$ agonists to determine whether these pathways were overlapping, additive, or synergistic in activating eicosanoid production. To identify the nature of interaction between activation pathways, we calculated a synergistic activation ratio (Equation 1),

$$\text{Synergistic activation} = \frac{[\text{KDO} + \text{Ca}^{2+}\text{agonist}]}{[\text{KDO}] + [\text{Ca}^{2+}\text{agonist}]} \quad \text{(Eq. 1)}$$

where [KDO] is the quantity of eicosanoid produced by KDO alone, [$Ca^{2+}$ agonist] is the quantity of the eicosanoid produced by the $Ca^{2+}$ agonist alone, and [KDO + $Ca^{2+}$ agonist] is the quantity of the eicosanoid produced by stimulation with both agonists. If the agonists act on separate independent pathways, the eicosanoid production would be additive, and the synergistic activation ratio would be 1. If the two agonists activate the same pathway, their outputs would overlap, and the maximum combined output would not be greater that that of either agonist alone. In this case, the ratio would be less than the sum of each pathway separately and would drop below 1. Finally, if these agonists combine to synergistically generate more eicosanoids than the sum of the individual pathways, the activation ratio would be greater than 1 and indicate a synergistic activation.

To this end, RAW264.7 cells were stimulated with $Kdo_2$-lipid A for 50 min followed with a 10-min dose of one of four $Ca^{2+}$ agonists: PAF, UDP, ionomycin, or ATP. The synergistic activation ratios are presented in Fig. 5. Within experimental error, the ratios for the PGs in PAF and UDP were 1. The only exception was that the 11-HETE was elevated with UDP. In contrast,

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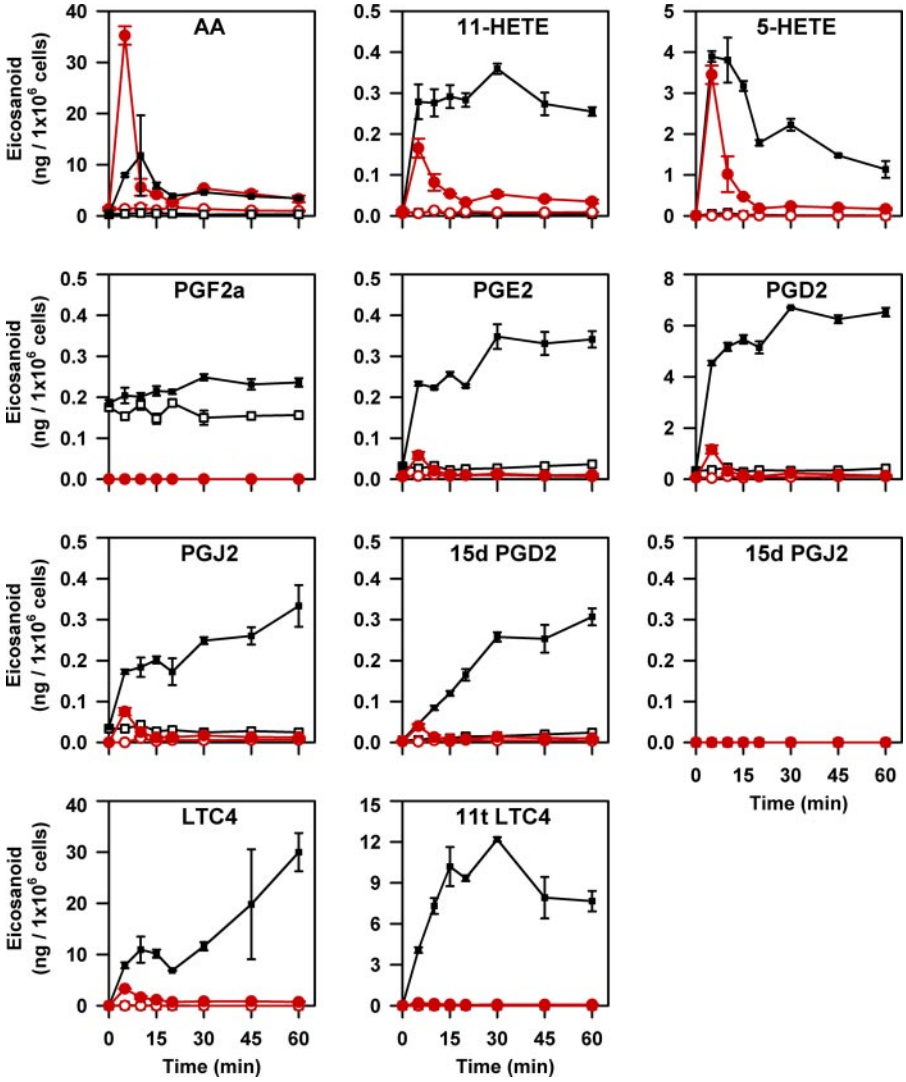


FIGURE 4. **ATP stimulated intracellular and extracellular eicosanoid production.** RAW264.7 cells were incubated in the absence (*open symbols*) and presence (*closed symbols*) of 2 mM ATP, and then subsequently extracellular (*black squares*) and intracellular (*red circles*) eicosanoid levels were determined at the indicated times over a 60-min period by mass spectrometry. A representative experiment is shown, and the data are expressed as mean values ± S.D. of three individual replicates.

$Kdo_2$-lipid A enhanced ionomycin-stimulated $LTC_4$ and AA release by about 8-fold and ATP release of these metabolites by about 3-fold. PG release, however, was not enhanced by priming by either ATP or ionomycin. Again, 11-HETE was the exception, showing some enhanced release that was midway between the 5-LO products and the PGs.

Activation of the TLR-4 receptor is known to activate NF-κB-induced protein synthesis, particularly COX-2, and this has long been thought to be the mechanism that primes macrophages for enhanced eicosanoid production (28, 29, 34). To examine this possibility, protein levels of $cPLA_2$ COX-1, COX-2, 5-LO, and 5-LO-activating protein (FLAP) were assessed by Western blot, and did not noticeably change during the 1st h of $Kdo_2$-lipid A stimulation (Fig. 6*A*). The levels of $cPLA_2$ phosphorylated at serine 505 (p-$cPLA_2$ (Ser-505)), which have been reported to modulate protein activity within the cell (34), also did not change significantly. Cell lysates were further analyzed for $cPLA_2$ activity with our *in vitro* assay (Fig. 6*B*). This assay measures activity under controlled conditions suitable for $cPLA_2$. We did not detect a significant change in the level of $cPLA_2$ activity following stimulation with either $Kdo_2$-lipid A or ATP. Because both the level of protein, detected by Western blot, and the level of $cPLA_2$ activity, analyzed by *in vitro* assay, did not change, it appears that both stimuli lead to activation of the pre-existing $cPLA_2$ but do not increase the level of the enzyme or its phosphorylation state.

This does not eliminate protein synthesis as a mechanism for eicosanoid production by TLR activation during the 1st h. In fact, $Kdo_2$-lipid A activation of TLR-4 directly induced PG synthesis during the 1st h was inhibited by ActD and CHX, which block the transcription and translation of cellular proteins, respectively (Fig. 7*A*). Inhibition of NF-κB with BAY also reduced PG synthesis by 60–90%, implicating this transcription factor in the production of protein required for eicosanoid release in the 1st h of stimulation. On the other hand, ATP stimulation of the purinergic receptors was only slightly blunted (10–30% inhibition) by these inhibitors (Fig. 7*B*), indicating that little or no *de novo* protein synthesis is required for the short term ATP response and that the constitutive enzyme levels are sufficient to produce these eicosanoid levels.

To determine whether protein synthesis was required for the synergistic enhancement of ATP-induced eicosanoid synthesis, RAW264.7 cells were stimulated with $Kdo_2$-lipid A for 50 min and followed with a 10-min dose of ATP in the presence of three inhibitors: ActD, CHX, and BAY (Fig. 8). As expected, these inhibitors reduced the absolute eicosanoid levels produced by combined $Kdo_2$-lipid A and ATP stimulation. Interestingly, the inhibition of protein synthesis did not appear to inhibit $Kdo_2$-lipid A synergistic activation of leukotriene or AA release. Furthermore, direct inhibition of NF-κB activity by BAY also did not affect the synergy between these pathways. To help confirm this finding, a second inhibitor of NF-κB activation, 6-amino-4-(4-phenoxyphenylethylamino) quinazoline, was tested. At a 50 nM dose, this compound did not affect $Kdo_2$-lipid A and ATP synergy (data not shown). Taken together, this demonstrates that, although inhibitors of protein synthesis blocked $Kdo_2$-lipid A-stimulated eicosanoid release and blunted ATP-stimu-

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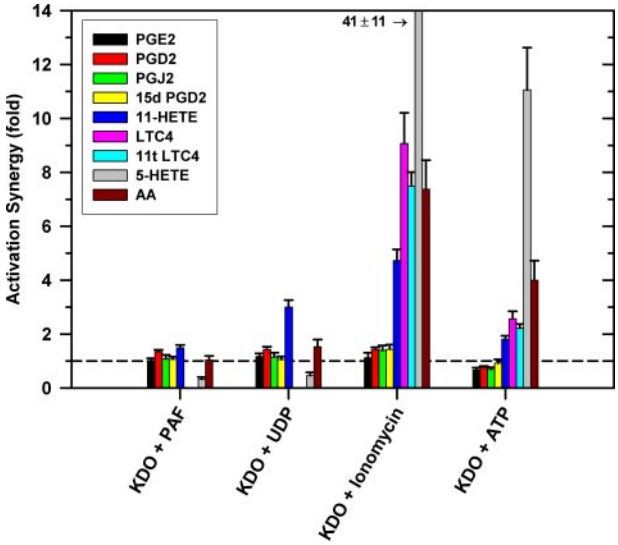


FIGURE 5. **Synergistic activation of eicosanoid release between $Kdo_2$-lipid A and $Ca^{2+}$ agonists.** RAW264.7 cells were incubated with vehicle or 100 ng/ml $Kdo_2$-lipid A for 60 min. During the last 10 min, cells from both conditions were incubated with vehicle, 100 nM PAF, 25 $\mu$M UDP, 1 $\mu$M ionomycin, or 2 mM ATP. Extracellular media were removed and analyzed for eicosanoid levels by mass spectrometry. The synergistic activation ratios were calculated via Equation 1. A representative experiment is shown, and the data are expressed as mean ± S.D. of three individual replicates.

lated release, they did not prevent $Kdo_2$-lipid A from priming for enhanced ATP-stimulated eicosanoid release.

## DISCUSSION

*Eicosanoid Profiles in RAW264.7 Cells*—Macrophages express a large number of distinct TLRs, G protein-coupled receptors, and purinergic receptors. When stimulated, these receptors activate $cPLA_2$ to liberate AA from membrane phospholipids and generate an eicosanoid response by distinctly different pathways. It was anticipated that by using a diverse array of macrophage stimuli, a number of different eicosanoid profiles would be generated that could indicate the presence of agonist-specific responses. To test this hypothesis, we examined the eicosanoid profiles of RAW264.7 cells stimulated with 16 different agonists.

We found that 14 of the 16 agonists produced the same eicosanoid profile. This profile was dominated by COX products, and by far the largest eicosanoid produced was $PGD_2$ and its dehydration products $PGJ_2$, 15d $PGJ_2$, and 15d $PGD_2$. For long term TLR-4 stimulation, this result is consistent with the observations that the levels of COX-2 dramatically increase over this time period, whereas at the same time 5-LO activity is impaired by subsequent nitric oxide production.

It was surprising that the GPCR agonists UDP and PAF exhibited the same profile as TLR agonists because they induce a transient $Ca^{2+}$ spike that activates eicosanoid synthesis within 10 min, well before gene expression and protein synthesis could significantly alter protein levels. Thus, in contrast GPCR agonists should induce eicosanoid synthesis via basally expressed enzymes, which include 5-LO and relatively small amounts of COX. Yet these two agonists induced similar COX-dependent $PGD_2$ dominated profiles as the TLRs.

The uniformity of the eicosanoid profile in response to these agonists was particularly striking in light of the fact that macrophages obtained from *in vivo* sources do yield different eicosanoid profiles, as shown by studies that modulate diet (12, 35) and studies in which different methods of macrophage elicitation are employed (19). Furthermore, a comparative study of human atherosclerotic plaques (36) showed that macrophages in unstable plaques have a significantly higher ratio of $PGE_2$ to $PGD_2$ synthase than stable plaque macrophages. In this study, plaque stability was attributed to changes in the $PGE_2$ and $PGD_2$ synthase levels. The obvious difference between *in vivo* macrophages and the RAW264.7 cells is that the differentiation process occurs in different environments. Thus, it is possible that the eicosanoid profile was set during monocyte to macrophage differentiation when the RAW264.7 cell line was first generated.

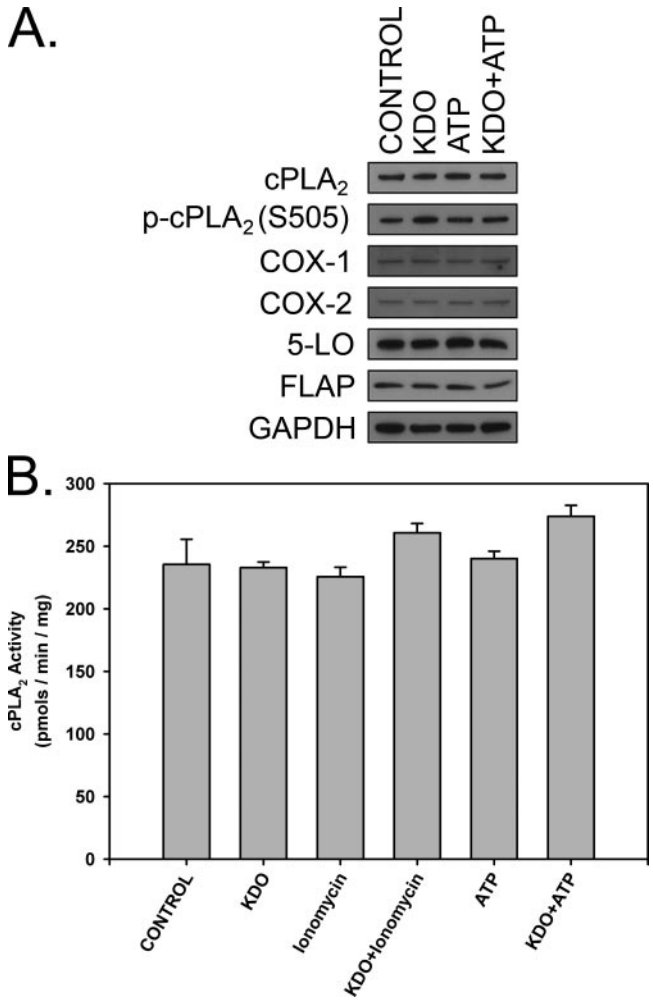


FIGURE 6. **Selected protein levels in $Kdo_2$-lipid A primed RAW264.7 cells.** RAW264.7 cells were incubated with vehicle or 100 ng/ml $Kdo_2$-lipid A for 60 min. During the last 10 min, cells from both conditions were incubated with vehicle or 2 mM ATP. *A,* cell lysates were analyzed by Western blot analysis using antibodies for p-$cPLA_2$ (Ser-505), $cPLA_2$, COX-1, COX-2, 5-LO, FLAP, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). *B,* cell lysates were analyzed for $cPLA_2$ enzyme activity using the group-specific Dole assay.

Only one of the receptor-mediated responses investigated, purinergic activation by millimolar levels of ATP, produced a different eicosanoid profile in RAW264.7 cells. In addition to producing COX-derived eicosanoids previously detected from other short term agonists, ATP activated the 5-LO pathway leading to the production of $LTC_4$. ATP can activate both the

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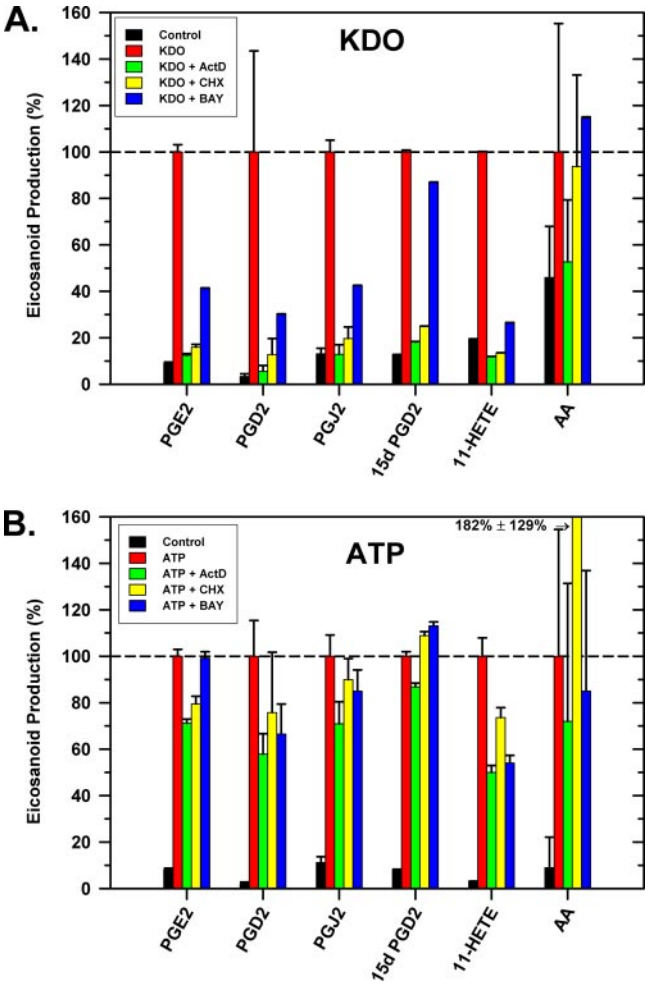


FIGURE 7. **Protein synthesis in discrete $Kdo_2$-lipid A and ATP stimulation.** RAW264.7 cells were incubated with vehicle, 10 $\mu$M ActD, 10 $\mu$M CHX, or 10 $\mu$M BAY for 30 min prior to stimulation. Cells were stimulated with 100 ng/ml $Kdo_2$-lipid A for 60 min (*A*) or 2 mM ATP for 10 min (*B*). Extracellular media were removed at the indicated time points, extracted, and analyzed for eicosanoid levels by mass spectrometry. Eicosanoids were expressed as relative amounts to stimulation in the absence of inhibitor. A representative experiment is shown, and the data are expressed as mean values ± S.D. of three individual replicates.

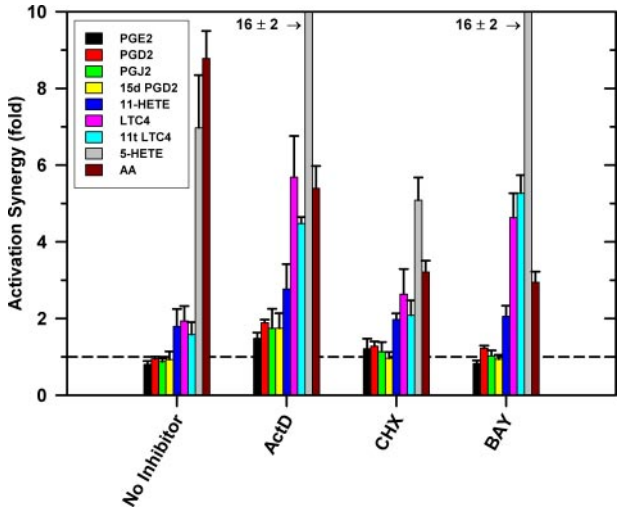


FIGURE 8. **Protein synthesis in $Kdo_2$-lipid A and ATP synergistic activation.** The synergistic activation ratios for $Kdo_2$-lipid A and ATP were analyzed in the presence of vehicle, 10 $\mu$M ActD, 10 $\mu$M CHX, or 10 $\mu$M BAY and incubated for 30 min prior to stimulation. RAW264.7 cells were incubated with either vehicle or 100 ng/ml $Kdo_2$-lipid A for 60 min; during the last 10 min, cells from both conditions were incubated with either vehicle or 2 mM ATP. Extracellular media were removed and analyzed for eicosanoid levels by mass spectrometry, and the synergistic activation ratios were calculated via Equation 1. A representative experiment is shown, and the data are expressed as means ± S.D. of three individual replicates.

P2Y GPCRs to produce a transient $Ca^{2+}$ spike and P2X cation channels to generate sustained elevated $Ca^{2+}$ levels (23). However, of all the purinergic receptors, only the $P2X_7$ requires millimolar levels of ATP for activation (37, 38). This receptor is expressed and functional in RAW264.7 cells, and Balboa *et al.* (24) have shown that this receptor is responsible for the majority of AA-derived metabolite release in response to 2 mM ATP stimulation in murine macrophages. Peripheral tissue cells contain millimolar levels of ATP in the cytosol, and release cytosolic ATP during cellular stress and nonphysiological necrosis (39, 40).

The eicosanoid profile generated by ATP stimulation was also seen with ionomycin stimulation. Ionomycin is a $Ca^{2+}$ ionophore that also creates a sustained $Ca^{2+}$ mobilization in murine macrophage cells (21). In both cases, the emergence of 5-LO products was accompanied by higher levels (3–4-fold) of PG release when compared with PAF and UDP. In the case of ATP, the 5-LO products were produced in equivalent amounts to the PGs. Thus, ATP stimulation not only turns on the 5-LO pathway but also enhances the total eicosanoid production. This response is presumably because of the extended elevated $Ca^{2+}$ levels, which are required for 5-LO activity in this model. These levels would keep $cPLA_2$ at the membrane longer and would last long enough to see the activation of the 5-LO.

*Intracellular Eicosanoid Levels*—During these studies, we have also examined the eicosanoid levels inside the cell. $Kdo_2$-lipid A induced an intracellular AA release, which peaked at 60 min and receded as extracellular prostaglandin levels began to rise. The AA inside the cell was ~4 times greater than the amount detected outside. Given the large difference between the volume of the cell and volume of extracellular media, the intracellular concentration of AA would be significant. 11-HETE was also detected inside the cell at a level roughly equal to the extracellular level. These findings confirm the observations of Balsinde and co-workers (41), who also saw increases in intracellular AA-derived compounds following [$^3$H]AA labeling of phospholipids and stimulation of $P388D_1$ murine macrophages. Significant levels of prostaglandins and their metabolites were not detected intracellularly during long term $Kdo_2$-lipid A stimulation.

Similar results were observed for the short term $Ca^{2+}$-based stimulation. Within 5 min of stimulation, ATP generated a spike of free AA that dissipated by 10 min. The AA peak coincided with the burst of PGs, LTs and HETEs that were also released in the first 5 min of ATP stimulation. Similar to $Kdo_2$-lipid A, AA spiked inside the cell ~4 times higher than secreted levels. In addition to AA, small but detectable levels of PGs and LT could be detected intracellularly at 5 min after stimulation with ATP. By 10 min, eicosanoid levels returned to those found in the controls. Taken together, this suggests that the eicosanoids do not build up in RAW264.7 cells but instead are rapidly secreted.

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It has been reported that the $PGD_2$ metabolite 15d $PGJ_2$ could be detected intracellularly using a monoclonal antibody (10), and suggested that it initiates an anti-inflammatory response through the transcription factor PPAR-λ. We were unable to detect significant levels of 15d $PGJ_2$ in the cells at any time during our studies, indicating that at least 98% of this metabolite remained outside the cell. If 15d $PGJ_2$ is binding to PPAR-γ in RAW264.7 cells, it is either being reabsorbed or the levels needed to activate PPAR-γ are so low that they are below our 5 pg detection limit.

*TLR and $Ca^{2+}$ Synergy*—We have shown previously that incubating macrophages with LPS acts to prime them for enhanced eicosanoid production in response to the $Ca^{2+}$ agonist PAF (21, 28, 29). In that work, $P388D_1$ cells were primed with LPS prior to activation with PAF, creating an enhanced release of [$^3$H]AA metabolites that was decreased in the presence of protein synthesis inhibitors ActD and CHX. This led to the hypothesis that TLR-4-induced protein synthesis, presumably COX-2, was responsible for priming the cells for a $Ca^{2+}$ burst, which would activate $cPLA_2$ to synthesize PGs.

In this study, we investigated TLR priming in RAW264.7 cells. Although the previous studies investigated either $PGE_2$ or total radiolabel release, we have now examined the complete eicosanoid spectrum. When primed with $Kdo_2$-lipid A, GPCR agonists PAF and UDP stimulated eicosanoid production that was not enhanced. The transient $Ca^{2+}$ spike was sufficient to activate eicosanoid production, presumably through COX-1, but this pathway was additive with the TLR-4 pathway. On the other hand, $Kdo_2$-lipid A appears to synergistically enhance the release of specific eicosanoids produced by ATP and ionomycin. Although the majority of PGs showed no significant change, the release of AA, COX-derived 11-HETE, and 5-LO products increased dramatically. The 5-LO product 5-HETE was enhanced by 20–40-fold, whereas the total AA released, *i.e.* the sum of all AA and eicosanoids, was enhanced 3–5-fold. This indicates that both $cPLA_2$ mediated AA release and 5-LO mediated products were enhanced.

Our results differ from the previous published work in that here we found no enhancement of PG production. This difference from $P388D_1$ cells is due in part to the fact that in the previous studies the enhancement was calculated under the assumption that TLR activation did not generate significant levels of $PGE_2$ within 1 h of activation. However, our results clearly demonstrate that in RAW264.7 cells TLR-4 activation leads to eicosanoid generation within this time frame, and if this is taken into account there is no significant enhancement of the PGs.

Furthermore, when ActD and CHX were used to inhibit protein synthesis, or BAY was used to inhibit NF-κB specifically, eicosanoid production via $Kdo_2$-lipid A was completely shut down during the 1st h. However, the levels of AA were not affected. This confirms that eicosanoid production during this period is dependent upon new protein synthesis, presumably COX-2, and that this enzyme production requires NF-κB. Apparently the AA produced in response to $Kdo_2$-lipid A activation cannot reach the existing pools of COX but can be used by newly synthesized COX. This could be due to the fact that without a $Ca^{2+}$ spike, $cPLA_2$ is brought to the membrane via $PIP_2$ (42, 43) or ceramide 1-phosphate (C1P) (44, 45), which is located in specific subcellular organelles, whereas a $Ca^{2+}$ spike allows $cPLA_2$ to bind nonspecifically or to a different set of membranes.

In contrast to 60 min of $Kdo_2$-lipid A, stimulation with ATP alone in the presence of protein synthesis inhibitors had only a small effect on eicosanoid release. Inhibition of NF-κB also had no significant effect on eicosanoid synthesis. Because ATP-induced eicosanoid production was complete within 10 min, it is not expected that protein synthesis would play a significant role in this pathway.

The total eicosanoid release of ATP-activated cells that were primed with $Kdo_2$-lipid A was also diminished by the presence of ActD, CHX, and BAY. This was not surprising, in light of the fact that these inhibitors blunted ATP-stimulated eicosanoid release and completely inhibited $Kdo_2$-lipid A eicosanoid production. However, the synergistic activation ratio was unchanged by these inhibitors, as well as by the NF-κB activation inhibitor 6-amino-4-(4-phenoxyphenylethylamino) quinazoline. This indicates that TLR-4 priming does not require the NF-κB-induced protein synthesis triggered by TLR-4 activation.

The separation of eicosanoid release enhancement from protein synthesis is bolstered by the results shown in Fig. 6. The levels of $cPLA_2$, COXs, 5-LO, and FLAP do not change significantly during this time frame. The enhancement effect appears to be due to synergistic activation of $cPLA_2$ and the subsequent release of AA and LTs, whereas protein synthesis only affects COX metabolites. Because the phosphorylation levels and *in vitro* activity of $cPLA_2$ are not noticeably altered, changes in the components that translocate $cPLA_2$ to its membrane substrate are likely to play a significant role.

*Current Model of Eicosanoid Activation*—We have developed a working model that explains the eicosanoid profiles and priming characteristics demonstrated in RAW264.7 cells (Fig. 9). This model accounts for the data through the differential regulation of $cPLA_2$, COX, and 5-LO.

The activity of $cPLA_2$ can be controlled by three possible mechanisms. First, the enzyme levels in the cell could be increased by gene up-regulation. There is evidence that this does not occur for $cPLA_2$ in macrophages (46, 47), and in our hands the level of this enzyme does not appear to change in response to various agonists. Second, the activity could be increased by phosphorylation of the enzyme. Phosphorylation of Ser-505 has been shown to increase $cPLA_2$ activity when studied *in vitro* (48); however, there is some question whether this mechanism is utilized *in vivo* to regulate activity, because in resting cells the enzyme exhibits some basal phosphorylation (46, 49). In this regard, our data demonstrate that Ser-505 is highly phosphorylated on $cPLA_2$ in basal RAW264.7 cells, and neither $Kdo_2$-lipid A, ionomycin, nor ATP affects the *in vitro* activity. The third and most likely mechanism for macrophage regulation of $cPLA_2$ is through control of its translocation to membranes.

Under resting conditions, $cPLA_2$ is a soluble enzyme and must translocate to the membrane to reach its substrate and to produce AA. To date, translocation has been shown to be

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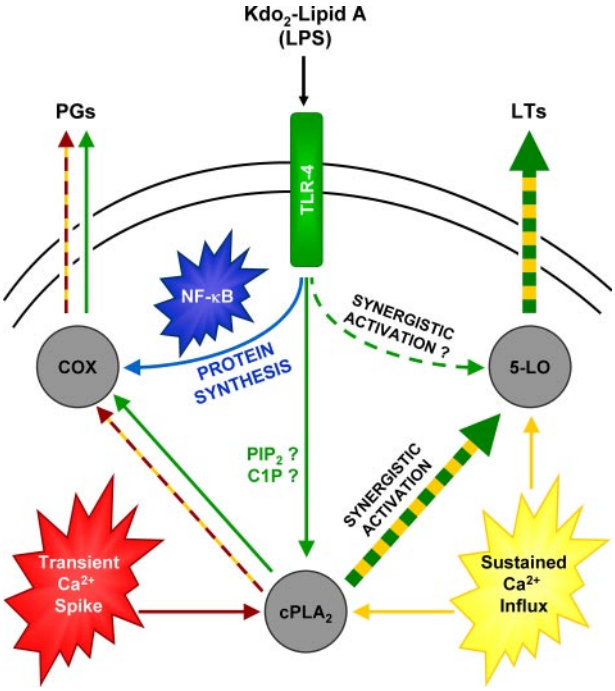


FIGURE 9. **Model for TLR and $Ca^{2+}$ activation of eicosanoid production.** Eicosanoid production pathways stimulated by transient $Ca^{2+}$ agonists (*red*), sustained $Ca^{2+}$ agonists (*yellow*), and TLR agonists (*green*) are indicated by color.

increased by both changes in $Ca^{2+}$ and phospholipids. Increases in cytosolic $Ca^{2+}$ facilitate $cPLA_2$ binding to phospholipids in membranes via its C-2 domain. Translocation can also be accomplished in a $Ca^{2+}$-independent fashion through an increase in specific membrane phospholipids. Six and co-workers (42, 43) have shown that $PIP_2$ increases $cPLA_2$ membrane affinity and activity. In addition to membrane binding affects, they also identified a potential $PIP_2$ conformational change that increases specific activity of the enzyme. $PIP_2$ generation has recently been linked with TLR activation (50, 51). Murayama and co-workers (44) have recently demonstrated a similar *in vitro* activation effect with C1P. Additionally, ceramide (52, 53) and diacylglycerol (54) have also been shown to increase $cPLA_2$ membrane affinity. Localized concentrations of a number of specific phospholipid species could realistically facilitate translocation, and thus increase the activity of the $cPLA_2$ enzyme.

The activities of the COX enzymes appear to be primarily controlled by expression. COX-1 is usually expressed constitutively, whereas COX-2 is typically found at low levels in resting cells but can be up-regulated by several hundredfold in response to certain agonists. Once expressed, both enzymes do not require $Ca^{2+}$ or phosphorylation for activity. However, the enzymatic mechanism requires a tyrosine radical for activity that is easily quenched (3, 4), leading to its inactivation after a relatively small number of turnovers. This rapid inactivation naturally limits PG production in the absence of continual expression.

Similar to $cPLA_2$, 5-LO appears to be controlled by a complex set of mechanisms. It contains a similar C-2 domain to $cPLA_2$ that requires significant levels of $Ca^{2+}$ for translocation to the membrane. This enzyme can also be activated by phosphorylation (1, 48). Additionally, the presence of FLAP is essential for productive *in vivo* activity, although its exact role remains unclear (5). The catalytic activity of 5-LO can be inhibited by nitric oxide produced by long term TLR activation (55). Using this information regarding $cPLA_2$, COX, and 5-LO regulation, we shall describe our results parting terms of an integrated model for regulating eicosanoid production.

In response to PAF or UDP, a burst of $Ca^{2+}$, but no sustained change, is generated. This leads to a transient release of AA, as $cPLA_2$ temporarily moves to the membrane in response to $Ca^{2+}$. Because this occurs within minutes, little or no protein synthesis occurs. Thus, released AA must be converted to eicosanoids by basally expressed enzymes. PG production may be further limited by the lack of COX-2 expression, as constitutive COX enzyme becomes rapidly suicide-inactivated. The transient $Ca^{2+}$ changes are insufficient to activate 5-LO, thus leading to very low levels of predominantly PG production.

Ionomycin and ATP produce a significant, sustained $Ca^{2+}$ elevation that leads to a more robust, longer lasting translocation of $cPLA_2$ to the membrane. This is reflected by a 2–4-fold higher release of total AA-derived metabolites by ionomycin and ATP when compared with GPCR receptor agonists. In addition to increased $cPLA_2$ activity, a sustained $Ca^{2+}$ flux is sufficient to additionally activate 5-LO. In this case, the COX and 5-LO pathways are both active and generate PGs and LTs, respectively.

Short term $Kdo_2$-lipid A stimulation activates protein synthesis that begins to increase COX levels in 60 min. Because $Kdo_2$-lipid A does not induce $Ca^{2+}$ release, it must activate $cPLA_2$ by an alternative mechanism. Presumably this occurs by increasing the levels of $PIP_2$ or C1P that sequester the enzyme to the membrane. Again, the 5-LO arm is not active because there is no $Ca^{2+}$ change. This stimulation pathway produces a moderate level of primarily PG production.

The level of total eicosanoid production by either transient or sustained $Ca^{2+}$ activation pales in comparison to long term $Kdo_2$-lipid A stimulation. Long term stimulation with $Kdo_2$-lipid A leads to significant protein synthesis, in particular COX-2; it would also allow for the significant changes in the levels of $PIP_2$ or C1P, which could explain the increase in $cPLA_2$ activity. Both of these factors would also explain the significant increase in the levels of PG released. Again, the lack of a $Ca^{2+}$ increase means that only the PG arm of the pathway is active and thus the PG dominated eicosanoid profile.

$Kdo_2$-lipid A priming causes two dramatic changes in the RAW264.7 cell response to ATP. First, the total level of AA released, *i.e.* the sum of AA and all of the eicosanoids detected increase considerably. Second, PG levels are not enhanced, whereas leukotrienes are significantly enhanced. An increase in total eicosanoid production, including arachidonic acid, implies a corresponding change in $cPLA_2$ activity. We have shown that the levels of $cPLA_2$ and its *in vitro* activity do not seem to change during priming. TLR-4 activation also increases the mitogen-activated protein kinase pathways. However, we could not detect an increase in $cPLA_2$ phosphorylation. Of course, some other mechanism

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for $cPLA_2$ activation may be involved. It is possible that the $Ca^{2+}$ could be acting synergistically with $PIP_2$ or C1P to increase the levels of $cPLA_2$ at the surface. *In vitro* studies have shown that $PIP_2$ increases the specific activity of $cPLA_2$, which could indicate a potential mechanism for synergy.

The activation ratio of the 5-HETE is significantly greater than that for total AA production. This implies that the 5-LO activity is synergistically enhanced as well. However, whereas 5-LO has a $Ca^{2+}$ requirement and similar C-2 binding domain as $cPLA_2$, we have no evidence indicating the precise mechanism for synergistic activation of 5-LO. Enhanced 5-LO activity could pull the bulk of the increased AA release through the 5-LO pathway. This would account for the lack of enhancement in the PG levels; it is also possible that the levels of COX are limiting over the 60-min incubation.

The fact that the UDP and PAF agonists did not show enhancement with $Kdo_2$-lipid A priming reinforces the idea that synergy requires a sustained increase in $Ca^{2+}$ coupled to the non-protein synthesis portions of the $Kdo_2$-lipid A priming. The lack of synergy with UDP and PAF could also be explained if the COX levels are limited.

In summary, we have found that it is critical to track all eicosanoid products when characterizing agonist-induced cellular responses. This has allowed us to refine our understanding of the $Kdo_2$-lipid A priming of RAW264.7 cells and to discover that TLR-4 priming involves a synergistic activation of $cPLA_2$, and possibly 5-LO, that does not requires protein synthesis. Further studies are required to identify the precise mechanism of this synergy.

*Acknowledgments—We thank Dr. Richard Harkewicz for discussions regarding mass spectrometry and Faith E. Jacobsen for assistance preparing the manuscript.*

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# TLR-4 and Sustained Calcium Agonists Synergistically Produce Eicosanoids Independent of Protein Synthesis in RAW264.7 Cells

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*J. Biol. Chem. 2007, 282:22834-22847.*
*doi: 10.1074/jbc.M701831200 originally published online May 29, 2007*

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