TLR3-Dependent Induction of Nitric Oxide Synthase in RAW 264.7 Macrophage-Like Cells via a Cytosolic Phospholipase A_2/Cyclooxygenase-2 Pathway[1]

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dsRNA is a by-product of viral replication capable of inducing an inflammatory response when recognized by phagocyte cells. In this study, we identify group IVA cytosolic phospholipase A_2 ($cPLA_2\alpha$) as an effector of the antiviral response. Treatment of RAW 264.7 murine macrophage-like cells with the dsRNA analog polyinosinic:polycytidylic acid (poly-IC) promotes the release of free arachidonic acid that is subsequently converted into PGE_2 by the de novo-synthesized cyclooxygenase-2 (COX-2) enzyme. These processes are blocked by the selective $cPLA_2\alpha$ inhibitor pyrrophenone, pointing out to $cPLA_2\alpha$ as the effector involved. In keeping with this observation, the $cPLA_2\alpha$ phosphorylation state increases after cellular treatment with poly-IC. Inhibition of $cPLA_2\alpha$ expression and activity by either small interfering RNA (siRNA) or pyrrophenone leads to inhibition of the expression of the inducible NO synthase (iNOS) gene. Moreover, COX-2-derived PGE_2 production appears to participate in iNOS expression, because siRNA inhibition of COX-2 also leads to inhibition of iNOS, the latter of which is restored by exogenous addition of PGE_2. Finally, cellular depletion of TLR3 by siRNA inhibits COX-2 expression, PGE_2 generation, and iNOS induction by poly-IC. Collectively, these findings suggest a model for macrophage activation in response to dsRNA, whereby engagement of TLR3 leads to $cPLA_2\alpha$-mediated arachidonic acid mobilization and COX-2-mediated PGE_2 production, which cooperate to induce the expression of iNOS. *The Journal of Immunology,* **2007, 179: 4821–4828.**

Cells from the innate immune system are capable of recognizing a variety of chemical structures from invading microorganisms, generating immune and inflammatory responses to terminate the infection. TLRs present in these cells recognize proteins, carbohydrates, lipids, and nucleic acids from invading entities with high specificity, which leads to the activation of a number of intracellular signaling cascades. So far, 11 members of the TLR family have been identified in mammals (1, 2), of which 4 are implicated in the recognition of nucleic acids (3, 4). One of these is TLR3, which specifically recognizes the dsRNA that is produced in some instances during viral replication (3, 4). TLR3, like the rest of the TLRs that interact with nucleic acids, is an intracellular receptor localized in endosomal membranes of cells. The N-terminal part of the receptor binds to its ligand, the dsRNA, in the lumen of the endosome, while the C-terminal part is cytoplasmic and has a Toll-IL-1R domain to interact with the adaptor protein Toll-IL-1R domain-containing adaptor inducing IFN-β and initiate signaling cascades (4, 5). TLR3 appears to be the only TLR that does not recruit the adaptor protein MyD88 (5). The mechanism by which viral dsRNA is transported to the endosomal compartment is not well-understood, but the involvement of CD14 as a mediator of the internalization of dsRNA analogs such as polyinosinic:polycytidylic acid (poly-IC)[3] has been described (6).

Other intracellular receptors that can bind dsRNA, include the retinoic acid inducible gene-1 (*RIG-1*), MDA-5 (Helicard) (7). Both RIG-1 and MDA-5 recruit an adaptor protein, Cardif, a mitochondria-anchored protein, to transmit downstream signaling. The main difference between these receptors and the TLRs is that TLRs usually recognize dsRNA in an endosome environment, while RIG-1 and MDA-5 recognize it within the cytoplasm (7).

Phospholipase A_2 (PLA_2) constitutes a superfamily of enzymes with key roles in inflammation and immunity due to its ability to generate free arachidonic acid (AA), the first committed step in the synthesis of pro- and anti-inflammatory eicosanoids (8–11). At present, 15 different PLA_2 groups have been described (12). Mammalian PLA_2s include secreted ($sPLA_2$), low m.w. forms with millimolar requirements for calcium ions, and cytosolic, high m.w. forms. Of all, only one, the group IVA PLA_2—also known as cytosolic phospholipase $A_2\alpha$ ($cPLA_2\alpha$), selectively cleaves phospholipids containing AA (13). $cPLA_2\alpha$ is regulated in cells by several factors, including the intracellular calcium levels and phosphorylation reactions (13), ceramide-1-phosphate (14, 15), and polyphosphoinositides (16, 17). $cPLA_2\alpha$ is activated by a variety of cellular ligands acting on immune surface receptors such as FcRs (18, 19) and β-glucan receptors like Dectin-1 (20) or TLR4 (21). In some systems, $cPLA_2\alpha$ activation regulates the transcriptional events that culminate in the induction of cyclooxygenase-2 (COX-2), the downstream enzyme that metabolizes AA (22–24). The role of this enzyme during immune processes has been widely

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Received for publication April 18, 2007. Accepted for publication July 24, 2007.

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[1] This work was supported by the Spanish Ministry of Education and Science (Grants BFU2004-01886/BMC and SAF2004-04676), the Fundación La Caixa (Grant BM05-248-0), and the Spanish Ministry of Health (ISCIII-RETIC RD06).

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[3] Abbreviations used in this paper: poly-IC, polyinosinic:polycytidylic acid; PLA_2, phospholipase A_2; AA, arachidonic acid; $sPLA_2$, secreted PLA_2; $cPLA_2$, cytosolic PLA_2; $iPLA_2$, calcium-independent PLA_2; $cPLA_2\alpha$, group IVA cytosolic PLA_2; COX-2, cyclooxygenase-2; iNOS, inducible NO synthase; LTB_4, leukotriene B_4; siRNA, small interfering RNA.

studied, not only in cellular models, but also in knockout animals (25). cPLA$_2\alpha$-null mice are resistant to many inflammation-related alterations like ischemia-reperfusion injury, anaphylactic responses, autoimmune arthritis, or allergic encephalomyelitis (25).

In the current work, we have investigated the activation and signaling role of cPLA$_2\alpha$ during stimulation by a viral infection. Our results support a key regulatory role for this enzyme in the signaling events that ultimately lead to the expression of inducible NO synthase (iNOS), an important effector enzyme. Roles for COX-2-derived PGE$_2$ and TLR3 within the cPLA$_2\alpha$-driven signaling cascade have also been unveiled.

Materials and Methods

Reagents

RPMI 1640 and FCS were obtained from Invitrogen Life Technologies. [5,6,8,9,11,12,14,15-^{3}H]AA (200 Ci/mmol) was purchased from Amersham Ibérica. Anti-COX-2 Abs were obtained from Cayman Chemical. Pyrrophenone was a gift from Dr. T. Ono (Shionogi, Osaka, Japan). Anti-cPLA$_2\alpha$ and anti-phospho-cPLA$_2$ (Ser^{505}) rabbit polyclonal Abs were obtained from Cell Signaling Technology. Anti-iNOS polyclonal Ab was purchased from BD Transduction Laboratories. All other reagents were obtained from Sigma-Aldrich.

Cells

RAW 264.7 macrophage-like cells were cultured in RPMI 1640 medium supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine at 37°C in a 5% CO_2 humidified incubator.

Assay for AA release

Cells (1×10^6) were labeled with 0.5 μCi/ml [^{3}H]AA overnight. Nonincorporated label was eliminated by washing three times with serum-free medium containing 0.1 mg/ml albumin. Cells were then stimulated with 25 μg/ml poly-IC in the same medium. When inhibitors were used, the cells were pretreated for 20–30 min prior to exposure to poly-IC. Supernatants were then collected, centrifuged at $13{,}000 \times g$ for 10 min, and assayed for radioactivity by liquid scintillation counting. The cell monolayers were scraped and total radioactivity was measured by liquid scintillation counting.

Intracellular Ca^{2+} measurements

RAW 264.7 cells were loaded with 3 μM Fluo-3-AM for 30 min in medium with 10% serum at 37°C in a 5% CO_2 incubator. Cells were then washed and incubated with HBSS containing 10 mM HEPES and 1.3 mM $CaCl_2$. Fluorescence was monitored under a Bio-Rad laser scanning Radiance 2100 system connected to a Nikon microscope, at 488 laser excitation and the combination of a HQ500 long-band pass and a HQ560 short-band pass-blocking filters, with the iris totally open. At the end of each experiment, calibration was done by adding 2 mM $MnCl_2$ plus 10 μM ionophore A23187, and lysing with 40 μM saponin to obtain the background signal (17).

Immunoblot assays

After stimulation, supernatants were discarded, and cells (1×10^6) were lysed with a buffer consisting of 20 mM Tris (pH 7.4), 150 mM NaCl, 0.5% Triton X-100, 1 mM Na_3VO_4, 1 mM PMSF, and a protease inhibitor mixture (Sigma-Aldrich), at 4°C. Homogenates were then clarified by centrifugation at $13{,}000 \times g$ for 10 min. Protein from the supernatants was quantified by the Bradford protein assay kit (Bio-Rad), and 50 μg of protein was analyzed by immunoblot using Abs against cPLA$_2\alpha$, iNOS, COX-2, or actin as described elsewhere (26, 27). Detection of immunoreactive bands was conducted by using the Supersignal West Pico Chemiluminescent Substrate (Pierce) in a Bio-Rad VersaDoc 5000 system. The resulting digital images were analyzed for quantitative band densitometry at different time exposures, within the linear response defined by Quantity One software (version 4.5.2; Bio-Rad).

Small interfering RNA (siRNA) transfection

The siRNAs directed against mouse TLR3 (5′-AAG GAU GUU UUC GGG CCG CCU (dTdT)-3′) (45), COX-2 (5′-UAA GGA GCU UCC UGA UUC A (dTdT)-3′), or cPLA$_2\alpha$ (5′-CGA GAC ACU UCA AUA AUG ATT (dTdT)-3′), were obtained from MWG Biotech. Cells (1×10^6) were transiently transfected with oligonucleotide (20 nM) in the presence of 10 μg/ml lipofectamine (Invitrogen Life Technologies) under serum-free conditions for 6 h. Afterward, 5% serum was added and the cells were maintained at normal culture conditions for 20 h (TLR3 siRNA) or 48 h (cPLA$_2$ siRNA). Then, the cells were used for experiments as described above. For COX-2 siRNA, stimuli were added 1 h after the siRNA, and were allowed to act for the indicated periods of time without any further serum addition. A scrambled siRNA was used as a negative control. When siRNA-transfected cells were labeled with [^{3}H]AA, the label was added to the cells for the last 12 h of transfection. By using a negative fluorescent siRNA, we observed that under our experimental conditions 95% of the cells were transfected. Also, the siRNA used produced a 70–90% reduction in the expression of the corresponding targets. None of the siRNAs used had any significant effect on cell viability.

PGE$_2$ production

Supernatants from stimulated cells (1×10^6) were collected and assayed for PGE$_2$ using ELISA kits from Cayman Chemical or Sapphire Bioscience, following the manufacturers' instructions. Both kits produced results completely comparable in terms of sensitivity and reproducibility.

Assay for leukotriene B$_4$ (LTB$_4$)

Supernatants from cells (1×10^6) were collected and assayed for LTB$_4$ using the ELISA kit from Assay Designs, following the manufacturer's instructions.

RT-PCR and quantitative real-time PCR

RNA was extracted by the TRIzol reagent method (Invitrogen Life Technologies), following the manufacturer's instructions. First-strand cDNA was synthesized from total RNA using Moloney's murine leukemia virus reverse transcriptase (Promega) with random primers. cDNAs were then amplified by PCR using two different pairs of primers for mouse iNOS: 1 (PrimerBank ID 6754872a1), forward 5′-GTT CTC AGC CCA ACA ATA CAA GA-3′ and reverse 5′-GTG GAC GGG TCG ATG TCA C-3′; and 2 (PrimerBank ID 6754872a2), forward 5′-ACA TCG ACC CGT CCA CAG TAT-3′ and reverse 5′-CAG AGG GGT AGG CTT GTC TC-3′ (28). The primers for mouse TLR3 were: forward 5′-CCC CCT TTG AAC TCC TCT TC-3′ and reverse 5′-TTT CGG CTT CTT TTG ATG CT-3′. Also, primers for mouse β-actin (forward 5′-GGC ATT GTT ACC AAC TGG GAC GAC-3′ and reverse 5′-CCA GAG GCA TAC AGG GAC AGC ACA G-3′) were used as a reference gene for amplification. For quantitative real-time RT-PCR, the cDNA was amplified by real-time PCR using the Absolute QPCR SYBR Green Mix from ABgen and a Chromo4 Detector PTI-200 (MJ Research). Thermal cycles were set at 95°C for 15 min, followed by 35 cycles comprising each a denaturation step at 95°C for 30 s, an annealing step at 60°C (iNOS) or 55°C (TLR3) for 30 s, and an extension step at 72°C for 30 s. Amplification of the appropriate product was verified in all reactions by analyzing the dissociation curves that were obtained after PCR by increasing the temperature from 70 to 90°C and reading every 2°C. The expression level for iNOS or TLR3 with respect to β-actin was calculated using the method $2^{-\Delta\Delta Ct}$ as described elsewhere, where Ct is the cycle threshold (29).

Results

Characterization of the AA mobilization response of macrophages stimulated by dsRNA

Macrophages respond to stimulants of the innate immune response by mobilizing AA via a PLA$_2$-mediated mechanism that may proceed in two temporally distinct manners, namely an immediate response that occurs immediately after the cell is exposed to the stimulant (30–33), and a delayed response that takes several hours to develop and requires de novo protein synthesis (34, 35). Because AA mobilization in response to dsRNA has not been characterized in terms of effectors involved, we began the current study by determining whether poly-IC, a dsRNA analog that mimics a viral infection (3, 6, 36), was able to activate the PLA$_2$-mediated release of AA to the extracellular medium. To this end, RAW 264.7 macrophages, labeled with 0.5 μCi/ml [^{3}H]AA, were exposed to poly-IC for various periods of time. As shown in Fig. 1*A*, after a lag of ~30 min, poly-IC did induce a rapid time-dependent release of [^{3}H]AA from the cells. AA release proceeded linearly up to ~2 h, after which it reached a plateau. Complete inhibition of the AA release response to poly-IC was achieved with a pyrrophenone, a selective cPLA$_2\alpha$ inhibitor (37), at concentrations as low as 0.5 μM (Fig. 1*B*). Moreover, cPLA$_2\alpha$ phosphorylation after

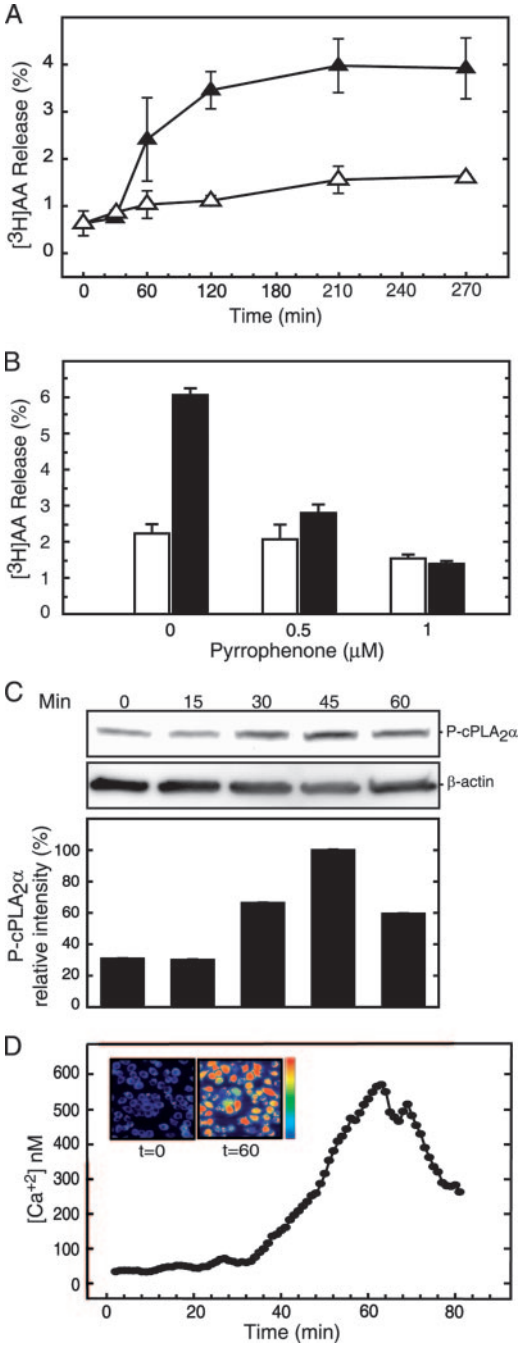

FIGURE 1. Characterization of AA release in RAW 264.7 cells. *A*, RAW 264.7 cells were labeled with [^{3}H]AA and assayed for AA release as indicated in *Materials and Methods* in the presence (closed symbols) or absence (open symbols) of 25 μg/ml poly-IC for the indicated periods of time. *B*, RAW 264.7 cells were labeled with [^{3}H]AA and assayed for AA release in the presence (■) or absence (□) of 25 μg/ml poly-IC for 2 h and in the absence or presence of the indicated concentrations of pyrrophenone. *C*, Homogenates from cells treated with poly-IC for different periods of time were subjected to immunoblot using Abs against phospho-cPLA$_2\alpha$ and β-actin. Quantification of the cPLA$_2\alpha$ bands is shown in the *lower panel*, normalized with respect to the β-actin content. *D*, Fluo-3-labeled cells were stimulated with 25 μg/ml poly-IC and fluorescence changes were recorded by microscopy. *Insets*, Pictures of the cells before ($t = 0$) and after ($t = 60$) application of poly-IC. Results are representative of three independent experiments.

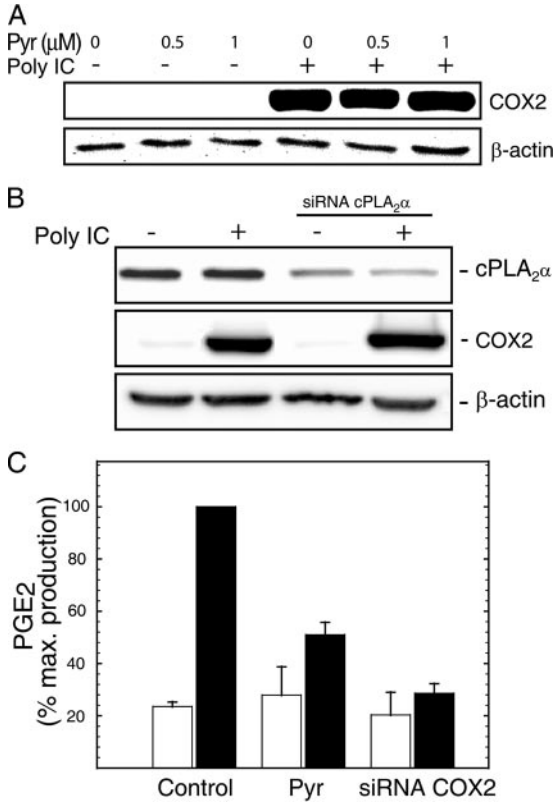

FIGURE 2. Induction of COX-2 by dsRNA does not depend on cPLA$_2\alpha$ activation. *A*, Homogenates from RAW 264.7 cells were treated with or without 25 μg/ml poly-IC for 18 h in the presence or absence of pyrrophenone and analyzed by immunoblot using Abs against COX-2. Actin was used as a control for loading. *B*, Control cells or cells transfected with 20 nM siRNA against cPLA$_2\alpha$ for 48 h were stimulated with 25 μg/ml poly-IC for 18 h, and homogenates were analyzed by immunoblot with Abs against cPLA$_2\alpha$, COX-2, or actin as a loading control. *C*, PGE$_2$ quantification by ELISA. Control cells or cells pretreated with 1 μM pyrrophenone for 30 min, or transfected with 20 nM siRNA against COX-2, were incubated with (■) or without (□) 25 μg/ml poly-IC for 18 h. The results are shown as means ± SEM of three independent determinations.

stimulation of the cells with poly-IC was increased (Fig. 1*C*), indicating that at least part of the requirements for activation of the enzyme are fulfilled (13). Another key regulator of cPLA$_2\alpha$ in activated cells is an increase in the intracellular Ca^{2+} concentration (13). Therefore, the ability of poly-IC to increase the intracellular Ca^{2+} level was studied next. Fluo-3-labeled macrophages were treated with poly-IC, and cellular fluorescence was measured by live cell microscopy. The results, as shown in Fig. 1*D*, indicate that poly-IC raised the intracellular Ca^{2+} concentration after a 30–40 min lag, which correlated well with the kinetics of AA release under these conditions (cf. Fig. 1, *A* and *D*). Collectively, these results clearly suggest that cPLA$_2\alpha$ is the relevant enzyme for AA release in poly-IC-treated cells.

Role of cPLA$_2\alpha$ in COX-2 expression and PGE$_2$ production in dsRNA-treated macrophages

cPLA$_2\alpha$ has been described to regulate the induction of COX-2 expression in some cell systems (22–24), and COX-2 expression is known to be up-regulated by dsRNA in macrophages (38). Thus, we studied next whether cPLA$_2\alpha$ is involved in COX-2 induction in the dsRNA-treated cells. To this end, RAW 264.7 macrophages were stimulated by poly-IC in the presence of pyrrophenone, and COX-2 expression was analyzed by immunoblot. Under these conditions, no effect of cPLA$_2\alpha$ inhibition on COX-2 expression levels could be ascertained (Fig. 2*A*). To further confirm this result, cPLA$_2\alpha$ was knocked down by siRNA technology. This treatment

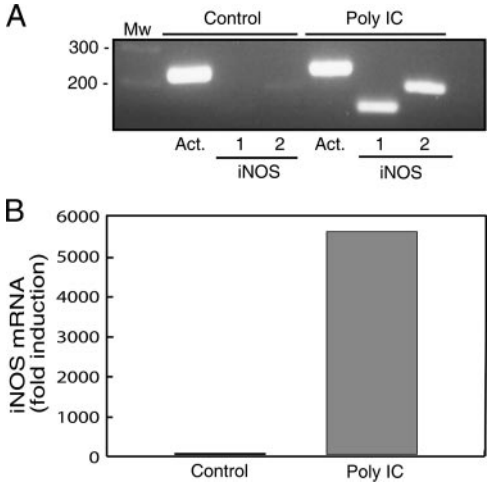

FIGURE 3. iNOS mRNA expression in RAW 264.7 cells treated with poly-IC. mRNA from control cells or cells stimulated with 25 μg/ml poly-IC was analyzed for iNOS and β-actin expression by RT-PCR. For iNOS mRNA, two different sets of primers were used (see *Materials and Methods*) (*A*). The iNOS mRNA was also analyzed by quantitative real-time PCR. In this case, only primer set 2 was used to study iNOS expression (*B*).

strongly blunted cPLA$_2\alpha$ expression levels, yet COX-2 expression was unaffected (Fig. 2*B*). Collectively, these results indicate that COX-2 expression by dsRNA stimulation in RAW 264.7 macrophages is independent of cPLA$_2\alpha$. However, PGE_2 production by dsRNA was blunted by both pyrrophenone and siRNA against COX-2, suggesting that cPLA$_2\alpha$ and COX-2 act coordinately to generate PGE_2 under these conditions (Fig. 2*C*).

Role of cPLA$_2\alpha$ in iNOS expression in dsRNA-treated cells

To further characterize the role of cPLA$_2\alpha$ in the signaling events triggered by dsRNA in macrophages, we studied the expression of another important proinflammatory enzyme, iNOS. By analyzing mRNA for iNOS by RT-PCR using two different sets of primers (Fig. 3*A*), or by quantitative real-time PCR (Fig. 3*B*), a marked increase in the mRNA levels for iNOS was detected after cellular treatment with poly-IC. This finding correlated with the induction of protein observed by immunoblot (Fig. 4*A*). Importantly, when the cells were treated with pyrrophenone, a marked reduction of iNOS expression was observed, suggesting a mediator role for cPLA$_2\alpha$ in this process (Fig. 4*B*). To further confirm this pharmacological finding, cells made deficient in cPLA$_2\alpha$ by treatment with siRNA targeting cPLA$_2\alpha$ were used next. Under these conditions, iNOS expression was strongly inhibited, confirming an upstream regulatory role for cPLA$_2\alpha$. (Fig. 4*C*). Of note, the expression of COX-2 was analyzed as well in the same experiments, and did not change in the cPLA$_2\alpha$-deficient cells (Fig. 4, *B* and *C*). This stresses the selectivity of action of cPLA$_2\alpha$ toward iNOS induction.

Identification of the lipid metabolite that mediates iNOS expression in dsRNA-treated RAW 264.7 macrophage-like cells

Fig. 5*A* shows that the selective COX-2 inhibitor NS-398 markedly inhibited iNOS expression by dsRNA-treated RAW 264.7 macrophage-like cells. This suggested the possible implication of a COX product in iNOS expression. To further explore this possibility, siRNA technology was used to knockdown COX-2 levels in dsRNA-treated macrophages. Under our conditions, a ~70% decrease in the amount of COX-2 protein was achieved (Fig. 5*B*). Importantly, iNOS expression in the COX-2-deficient cells was markedly blunted as well (Fig. 5*B*), thus strengthening the existence of a functional link between COX-2 activity and iNOS expression.

The possible involvement of a lipoxygenase metabolite of AA on iNOS induction by dsRNA was initially studied by analyzing the effect of the 5-lipoxygenase activating-protein inhibitor MK886. This inhibitor, when used at various concentrations up to 25 μM, exerted no significant effect on the response (data not shown), suggesting the lack of a role for AA lipoxygenation reactions on dsRNA-induced iNOS expression. Because LTB_4 generation has been reported in LPS- and calcium ionophore-treated RAW 264.7 cells (39), we studied whether LTB_4 production in response to poly-IC could induce the production of LTB4. However, by using a commercial-specific ELISA, we could not detect significant LTB_4 production in cells treated with poly-IC at 3 or 24 h (data not shown). In contrast, significant levels of LTB_4 were detected when the calcium ionophore A23187 was used as a positive control (39). In the light of these negative data, studies on the lipoxygenase pathways were not pursued further.

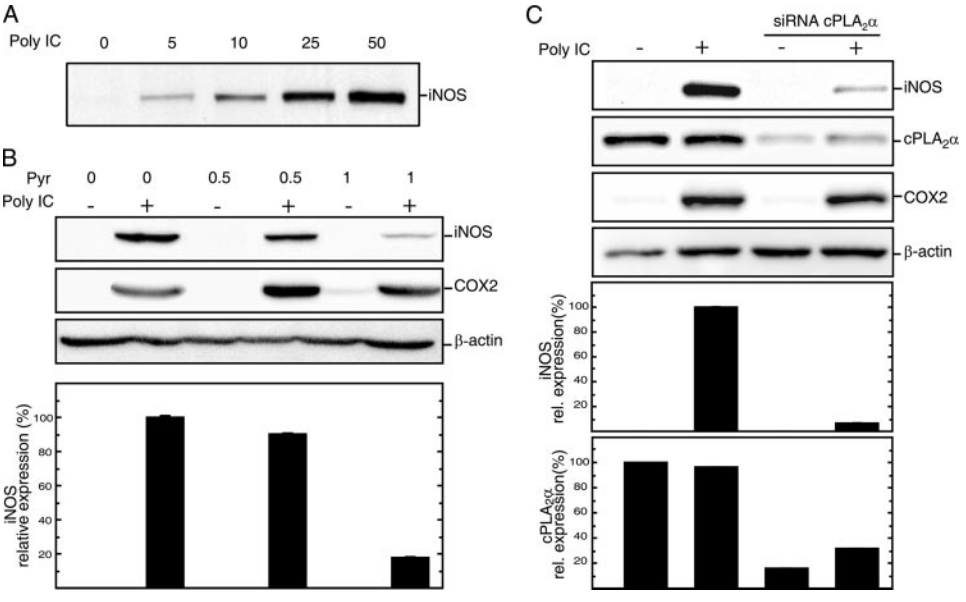

FIGURE 4. Involvement of cPLA$_2\alpha$ in the regulation of iNOS expression by dsRNA. *A*, Homogenates from RAW 264.7 cells treated with different concentrations of poly-IC for 18 h were analyzed by immunoblot with Abs anti iNOS. *B* and *C*, Homogenates from control cells, or cells pretreated with 1 μM pyrrophenone for 30 min (*B*), or transfected with 20 nM siRNA against cPLA$_2\alpha$ (*C*) were treated with or without 25 μg/ml poly-IC for 18 h and analyzed by immunoblot using Abs against iNOS, COX-2, cPLA$_2\alpha$, or β-actin as a loading control. *Lower panels* in *B* and *C* show the quantification of iNOS and cPLA$_2\alpha$ bands normalized for the actin signal. Experiments shown are representative of at least three independent determinations.

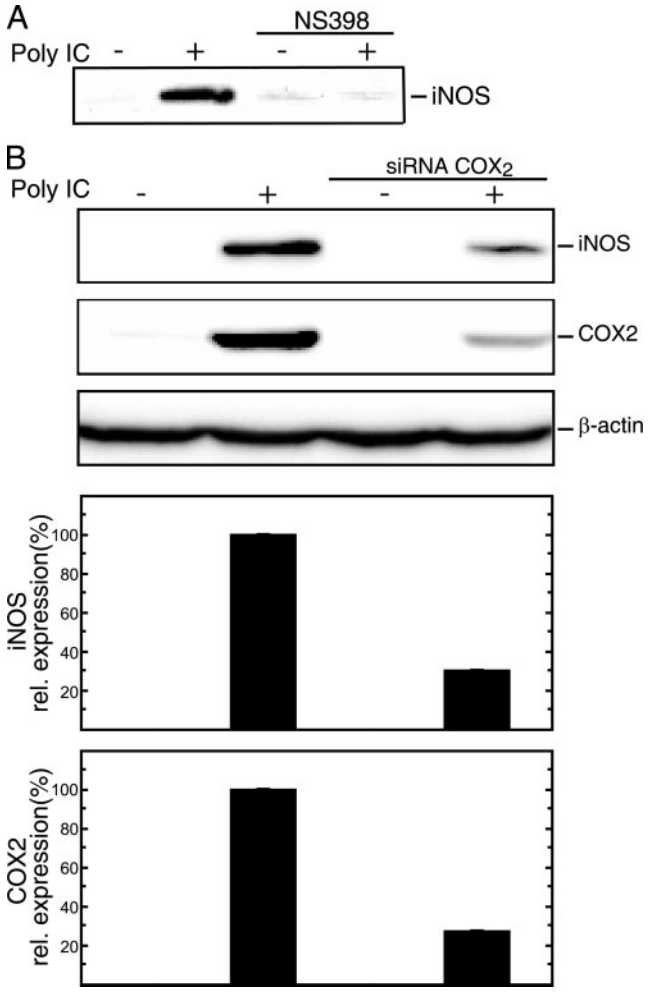

FIGURE 5. Effect of COX-2 inhibition on iNOS expression. *A*, RAW 264.7 cells were stimulated with 25 μg/ml poly-IC in the presence or absence of 5 μM NS398. *B*, Cells were transfected with 20 nM siRNA against COX-2 and treated or not with 25 μg/ml poly-IC, as indicated. Homogenates were analyzed by immunoblot using Abs anti-iNOS (*A* and *B*) COX-2 (*B*), and actin as loading control (*B*). *Lower panels*, The quantification of iNOS and COX-2 bands normalized for the actin signal. Experiments shown are representative of at least three independent determinations.

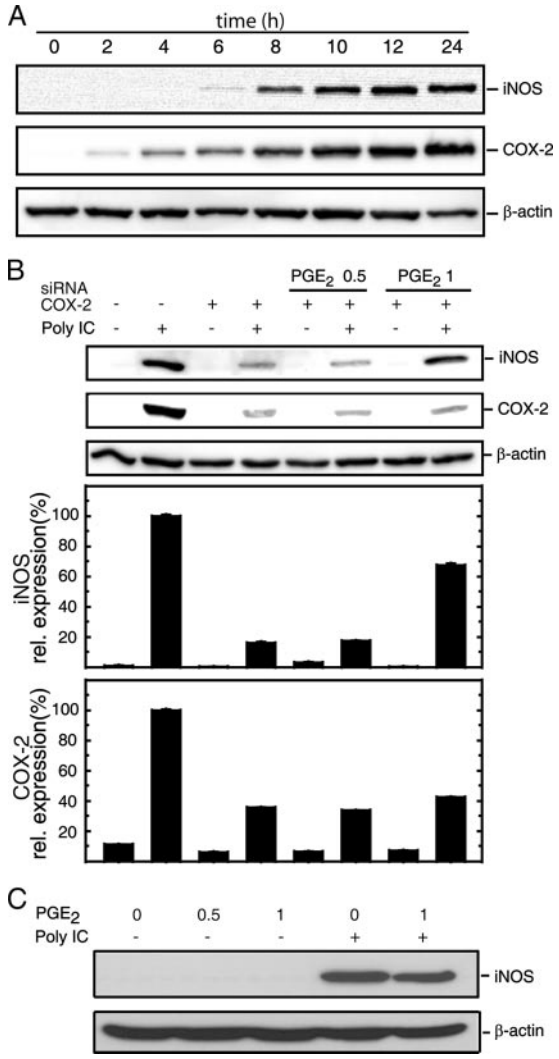

FIGURE 6. PGE_2 restores the expression of iNOS in COX-2-depleted cells. *A*, RAW 264.7 cells were stimulated with 25 μg/ml poly-IC for the indicated times. Homogenates were analyzed by immunoblot using Abs to iNOS, COX-2, and β-actin as a loading control. *B*, The cells were either transfected or not with 20 nM siRNA against COX-2, stimulated with 25 μg/ml poly-IC, and treated as well with 0.5 or 1 μM PGE_2, as indicated. After 18 h, homogenates were analyzed by immunoblot with Abs to iNOS and COX-2. *Lower panels* in *B* show the quantification of iNOS and COX-2 bands normalized for the β-actin signal. *C*, Homogenates from control cells or cells treated with 0.5 or 1 μM PGE_2, 25 μg/ml poly-IC, or poly-IC plus 1 μM PGE_2 for 18 h were analyzed by Western blot for iNOS and β-actin expression. The data are representative of at least three independent determinations.

Consistent with the possibility that a COX-2 metabolite is involved in iNOS expression, COX-2 synthesis occurred much earlier than iNOS. Fig. 6*A* shows the time course of appearance of COX-2 and iNOS proteins in poly-IC-treated RAW 246.7 macrophage-like cells. The former was already observed 2 h after poly-IC addition to the cells, while clear induction of iNOS was only observed 6 h after application of the stimulus.

PGE_2 is a major product of the metabolism of AA via the COX pathway in macrophages exposed to stimuli that elicit immediate responses (40). Using exogenous PGE_2, it was possible to greatly overcome the reduction of iNOS expression in COX-2-deficient cells. As shown in Fig. 6*B*, PGE_2 at a concentration of 1 μM overcame the inhibition of iNOS expression in COX-2-deficient cells by ~70–80%. Of note, PGE_2 alone did not have any stimulatory effect, nor did it enhance the poly-IC-induced iNOS production in cells expressing normal COX-2 levels (Fig. 6*C*). This indicates that exogenous PGE_2 restored iNOS expression not by acting as a stimulant on its own, but by restituting the signaling that was lost with COX-2 reduction. Moreover, the finding that PGE_2 alone does not induce iNOS indicates the existence of signal(s) for iNOS induction that are independent of the $cPLA_2$/COX-2 pathway. Collectively, the data are consistent with a scenario whereby dsRNA-induced COX-2 is fed by $cPLA_2\alpha$ to generate PGE_2 which in turn helps regulate iNOS expression.

Role of TLR3 on COX-2 and iNOS induction by dsRNA in macrophages

The intracellular receptor TLR3 is potentially capable of recognizing dsRNA and initiate an inflammatory response (3). To study the possible implication of TLR3 in the signaling pathway followed by poly-IC to activate $cPLA_2\alpha$ and induce COX-2 and iNOS, we set out to knock down the expression of this receptor in RAW 264.7 macrophage-like cells by siRNA technology. The effect of such strategy on the TLR3 mRNA is shown in Fig. 7*A*. The

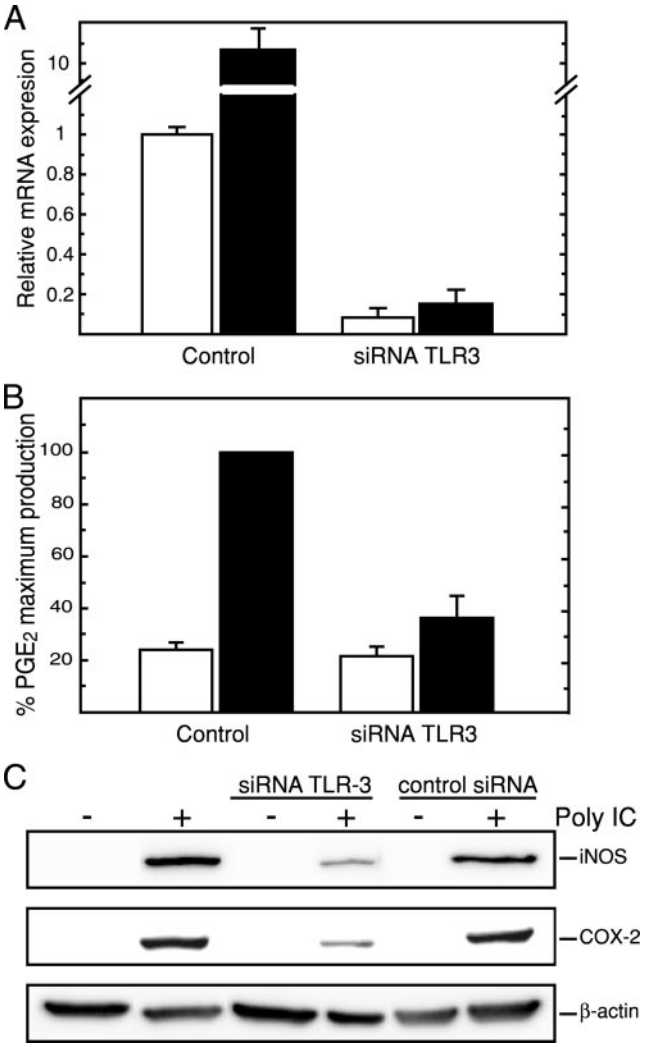

FIGURE 7. Involvement of TLR3 on iNOS and COX-2 expression by dsRNA. RAW 264.7 cells were transfected or not with 20 nM siRNA against TLR3 and stimulated (■) or not (□) with 25 μg/ml poly-IC for 18 h. *A*, mRNA was extracted and TLR3 expression was analyzed by quantitative real-time PCR. *B*, Cellular supernatants were used for PGE_2 quantification using an ELISA kit. *C*, Homogenates were analyzed by Western blot with Abs to iNOS, COX-2, and β-actin as a loading control. Experiments shown are representative of at least three independent determinations.

siRNA not only reduced the TLR3 mRNA content of unstimulated cells, but also the induction of newly synthesized TLR3 by poly-IC (Fig. 7*A*). Under these conditions, PGE_2 production was markedly decreased (Fig. 7*B*). The expression of COX-2 and iNOS were severely impaired as well (Fig. 7*C*). These results strongly suggest that TLR3 is a receptor to which poly-IC binds to initiate an intracellular signaling cascade that includes the following sequential steps: 1) activation of cPLA$_2\alpha$, 2) COX-2 induction, 3) metabolism of free AA to PGE_2, and 4) induction of iNOS.

Discussion

In the present study, we have evaluated cPLA$_2\alpha$ activation by dsRNA in the RAW 264.7 macrophage-like cell line and its implication in downstream gene expression. We have found that inhibition of cPLA$_2\alpha$ by selective pharmacological strategies dramatically reduces phospholipid hydrolysis induced by the dsRNA analog poly-IC. Moreover, poly-IC induces cPLA$_2\alpha$ phosphorylation which is an important requisite for enzyme activation (13). Phosphorylation of cPLA$_2\alpha$ is conducted by MAPKs (13), and these kinases have recently been reported to be activated by poly-IC in macrophages (38, 41). Another important regulatory factor for cPLA$_2\alpha$ activation is the elevation of the intracellular Ca^{2+} concentration, which we have also detected upon poly-IC stimulation of the macrophages.

The release of AA induced by dsRNA characteristically shows a 30-min lag. This feature is not observed when macrophages are challenged by other innate stimuli that elicit immediate responses via surface receptors such as yeast-derived zymosan (30, 33) or platelet-activating factor (31, 32). It has been shown that poly-IC has to be internalized by the cell for it to reach the intracellular compartments where its receptors are localized (e.g., TLR3), and that CD14 is the surface receptor that regulates poly-IC internalization (6). Thus, the existence of a time lag in the AA response of macrophages to poly-IC could be easily explained by the necessity of the stimulant to reach its intracellular receptor before any AA release may occur.

We have detected that RAW 264.7 cells also express multiple sPLA$_2$ proteins, namely those of groups IB, IID, IIE, V, and XII by PCR (Y. Sáez, J. Balsinde, and M. A. Balboa, unpublished data). Whether any of these isoforms have any role in poly-IC-induced AA mobilization and PGE_2 production by RAW264.7 macrophage-like cells is uncertain at present. We have used cell-impermeable and permeable sPLA$_2$ inhibitors, namely LY311727 (42) and scalaradial (43–45), but neither of these produced inhibitory effects on AA release (J. Pindado, V. Ruipérez, J. Balsinde, and M. A. Balboa, unpublished data). Although in vitro measurements using cell extracts confirmed that, at the concentrations used in these experiments, both LY311727 and scalaradial quantitatively inhibit the sPLA$_2$ activity of RAW 264.7 cell homogenates without having any effect on cPLA$_2$ or calcium-independent PLA$_2$ (iPLA$_2$) activities (46–51), no LY311727- or scalaradial-inhibitable responses have been described in intact RAW 264.7 cells. Thus, a positive control for these experiments is lacking. It could be that the failure of the inhibitors to blunt AA release in our experiments was due to the inhibitors not readily reaching their targets, or being rapidly degraded. Whether sPLA$_2$s may serve roles against a viral infection other than substrate providers for eicosanoid-synthesizing enzymes under our conditions is also unknown at present. Interestingly, certain sPLA$_2$s have been reported to block HIV-type 1 entrance into the host cells (52) as well as to neutralize the virus via degradation of the viral membrane (53).

We have also detected the expression of the calcium-independent group VIA PLA$_2$ (also know as iPLA$_2$) by PCR (data not shown). Previous studies using the iPLA$_2$ inhibitor bromoenol lactone have suggested a role for iPLA$_2$ enzymes in the cellular response to poly-IC leading to iNOS induction (41, 54). In our hands, however, treating the RAW 264.7 cells with bromoenol lactone concentrations as low as 10 μM leads to cell death, as measured by permeabilization to propidium iodide (J. Pindado, J. Balsinde, and M. A. Balboa, results not shown) (55–57), thus preventing us from carrying out further analyses in this regard. Nevertheless, our results, together with studies by others (41, 54) highlight the existence of two independent lipid-regulated pathways leading to iNOS expression. The first one involves the generation of iPLA$_2$-derived lysolipids (41), and the one unveiled in this study involves the tight coupling of cPLA$_2\alpha$ and COX-2 to generate PGE_2. Whether the two routes operate in parallel under the same stimulatory conditions appears likely, and this would confer on the system greater versatility for the cellular regulation of iNOS. Clearly, additional studies will be needed to assess the contribution of each PLA$_2$-mediated pathway to overall regulation of iNOS expression.

A striking feature of our work is the finding that COX-2 induction in the dsRNA-treated cells occurs rather early, i.e., protein is clearly observable after 2 h of stimulation, and induction does not depend on the activity of the cPLA$_2\alpha$. However, cPLA$_2\alpha$ still feeds COX-2 with the AA substrate that is needed for generation of PGE$_2$, the eicosanoid that we found here to mediate iNOS induction in response to poly-IC. It has been described that RAW 264.7 cells express three PGE$_2$ receptor types, namely EP2, EP3, and EP4 (58). Thus, it is likely that the PGE$_2$ produced by dsRNA via a cPLA$_2\alpha$/COX-2 pathway, acting in an autocrine manner, binds to its surface receptors to induce iNOS expression. It is interesting to note here that the experiments where cells were challenged with PGE$_2$ alone, showed the inability of this eicosanoid to induce iNOS (Fig. 6*C*). There are at least two possible explanations for this finding. First, the concentration of PGE$_2$ receptors in the cell surface under resting conditions could be too low to provide a sustained signal for the induction of iNOS. Only after cellular stimulation may PGE$_2$ receptors reach the appropriate concentration to produce an adequate signal for gene induction. In support of this possibility, it has been shown that RAW 264.7 cells increase their EP2 and EP4 mRNA content after LPS stimulation (58). The second possibility is that other signals provided by poly-IC independently of the PLA$_2$/COX-2 pathway are also involved in the induction of iNOS. It is intriguing to speculate whether the lysolipids generated by the iPLA$_2$ pathway discussed above could serve this role (41, 54).

Our data show that knockdown of TLR3 by siRNA technology prevents PGE$_2$ generation, and COX-2 and iNOS induction in response to poly-IC. These results clearly suggest that TLR3 is a receptor through which poly-IC exerts its actions on RAW 264.7 macrophage-like cells. Our data are in agreement with those of Flavell and colleagues (3) who used cells from mice deficient in TLR3 by genetic ablation to demonstrate that TLR3 couples poly-IC stimulation to activation of NF-κB. In contrast, Steer et al. (38) also used cells from TLR3 knockout mice, but found no evidence for the involvement of TLR3 in poly-IC signaling leading to COX-2 induction or peroxynitrite production. However, in the later studies, IFN-γ was added along with poly-IC during cellular stimulation, raising the possibility that other signaling pathways may have become activated, and compensated for the lack of TLR3 signaling (38).

Our results suggesting that double-stranded viruses may induce both COX-2 and iNOS in immunoinflammatory cells raise the question of whether single-stranded viruses produce the same effects by activating similar intracellular signaling. For instance, the hepatitis C virus has been shown to induce COX-2 in liver cells (59). This could be biomedically important because single-stranded viruses are responsible for the majority of clinically relevant human diseases. Moreover, our results could help clarify the mechanisms through which viral infections can exacerbate certain illnesses. For example, it is known that viral infection of the pulmonary tract can exacerbate both asthma and pulmonary obstructive disease, causing much of the morbidity and mortality associated with these disorders (60). Based on our data, a scenario could be envisioned where TLR3-expressing cells in the pulmonary tract respond to viral dsRNA by generating PGs (through cPLA$_2\alpha$ and COX-2) and NO (through iNOS) that increase inflammation. Moreover, our finding that TLR3-mediated signaling centrally involves cPLA$_2\alpha$ activation may be important not only during viral infection but also in those settings where dsRNA is present, e.g., when RNA is released from necrotic cells (61). Accumulation of RNA has also been detected in the neurofibrillary tangles and neuritic plaques found in Alzheimer's disease patients, an illness with a major inflammatory component (62, 63). Thus, it is intriguing to speculate that the RNA from the plaques is recognized by microglia through TLR3, this leading to activation of cPLA$_2\alpha$ which in turn exacerbates the inflammatory response.

Acknowledgments

We thank Montse Duque and Yolanda Sáez for expert technical assistance.

Disclosures

The authors have no financial conflict of interest.

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