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Adenovirus Type 5 Exerts Multiple Effects on the Expression and Activity of Cytosolic Phospholipase A_2, Cyclooxygenase-2, and Prostaglandin Synthesis[1]

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In this study, we examine how infection of murine and human fibroblasts by adenovirus (Ad) serotype 5 (Ad5) affects the expression and activity of cytosolic phospholipase A_2 (cPLA$_2$), cyclooxygenase-2 (COX-2), and production of PGs. Our experiments showed that infection with Ad5 is accompanied by the rapid activation of cPLA$_2$ and the cPLA$_2$-dependent release of [^{3}H]arachidonic acid ([^{3}H]AA). Increased expression of COX-2 was also observed after Ad infection, as was production of PGE_2 and PGI_2. Later, however, as the infection progressed, release of [^{3}H]AA and production of PGs stopped. Late-stage Ad5-infected cells also did not release [^{3}H]AA or PGs following treatment with a panel of biologically diverse agents. Experiments with UV-inactivated virus confirmed that Ad infection is accompanied by the activation of a host-dependent response that is later inhibited by the virus. Investigations of the mechanism of suppression of the PG pathway by Ad5 did not reveal major effects on the expression or activity of cPLA$_2$ or COX-2. We did note a change in the intracellular position of cPLA$_2$ and found that cPLA$_2$ did not translocate normally in infected cells, raising the possibility that Ad5 interferes with the PG pathway by interfering with the intracellular movement of cPLA$_2$. Taken together, these data reveal dynamic interactions between Ad5 and the lipid mediator pathways of the host and highlight a novel mechanism by which Ad5 evades the host immune response. In addition, our results offer insight into the inflammatory response induced by many Ad vectors lacking early region gene products. ***The Journal of Immunology,*** **2007, 179: 4170–4179.**

Prostaglandins are lipid mediators whose activities are critical to a number of biological processes. PG synthesis begins with the phospholipase A_2 (PLA_2)[3]-dependent release of arachidonic acid (AA) from membrane phospholipids. Many different PLA_2 can participate in this process, although cytosolic PLA_2 (cPLA$_2$) is the only PLA_2 that displays selectivity for AA, and therefore its activity is most likely critical to PG synthesis (1, 2). The cyclooxygenase (COX) enzymes sequentially convert the AA to PGG_2 and PGH_2 and, finally, a series of PG synthase enzymes convert PGH_2 to the biologically active PGs, such as PGE_2, PGI_2, and PGJ_2. PGs exert their effects by binding to cell surface or intracellular receptors. PGE_2, for example, can bind to four different receptors referred to as EP_{1-4}. These are rhodopsin-type receptors with seven membrane-spanning regions that can activate a variety of intracellular signaling pathways (3).

Through these receptors, PGs participate in a wide variety of biological responses. PGE_2, for example, can suppress cytokine (4, 5) and chemokine (6) production by activated macrophages. In the brain, PGE_2 can promote fever and vessel dilation (7). PGE_2 can also exert multiple effects on dendritic cells, such as promoting survival (8), enhancing maturation (9), modulating rates of migration (9, 10), and regulating Th1 vs Th2 differentiation (11). PGD_2 (12) and PDJ_2 (13) also regulate dendritic cell function, indicating that PGs represent a critical link between the innate and adaptive immune response. PGs can also regulate B (14) and T (15) cell functions, and the antiviral activities of PGs have been well documented (16). Of course, overproduction of PGs can have damaging consequences to the host. For example, the degeneration of neurons that occurs during Alzheimer's disease has been linked to the up-regulation of cPLA$_2$ and overproduction of eicosanoids (17), as has the pathogenesis of experimental autoimmune encephalitis (17, 18).

The human adenovirus (Ad) is a nonenveloped, dsDNA virus. Over 50 serotypes of Ad have been reported, with Ad serotype 5 (Ad5) being one of the most common in the human population. Ads typically cause mild respiratory disease or conjunctivitis in healthy humans, although more severe infections have been reported in immunocompromised individuals, including those recovering from bone marrow transplants or infected with HIV (19). Ads can also establish long-term persistent infections in the tonsils (20). Decades of research with these viruses have revealed extensive interference with the host immune response. Several immunomodulatory genes are located in the E3 transcription unit, including the following: E3 19K, which causes MHC class I molecules to be retained in the endoplasmic reticulum (21); E3 14.7K, which prevents TNF from triggering apoptosis in infected cells (22); and E3 10.4K and E3 14.5K, whose products together cause internalization of TNFRs and Fas (23). Several anti-immune factors are also encoded outside the E3 region, including E1B 19K and VA RNA1 (24). Ads are also important for their potential use as live vaccine vectors and as vectors for therapeutic genes. Various replication-competent and replication-incompetent Ad vectors

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[3] Abbreviations used in this paper: PLA_2, phospholipase A_2; AA, arachidonic acid; Ad, adenovirus; Ad5, Ad serotype 5; COX, cyclooxygenase; cPLA$_2$, cytosolic PLA_2; p.i., postinfection.

have been developed (25). Many contain early region deletions to enhance the carrying capacity of the vector and to reduce the inflammatory response in the host.

In this study, we examine the impact of Ad5 on the production of PGs and key enzymes in the PG pathway. Our results show that Ad5-infected cells initially produce PGs, but later, once viral gene expression occurs, PG production ceases and infected cells also become refractory to stimulation by external ligands. Release of [^{3}H]AA follows the same pattern, and we have correlated these effects with changes in the intracellular position of cPLA$_2$.

Materials and Methods

Reagents

All medium and chemicals were purchased from Sigma-Aldrich, unless otherwise stated. The phospho-specific cPLA$_2$ (p505/cPLA$_2$) Ab was obtained from Cell Signaling Technology. The murine cPLA$_2$ mAb (sc-454) was purchased from Santa Cruz Biotechnology. The murine COX-1 mAb and the rabbit polyclonal COX-2 Ab were purchased from Cayman Chemical. The Ad E1A murine mAb (MS-588-P1) was purchased from Labvision. HRP-conjugated goat anti-rabbit and goat anti-mouse secondary Abs, as well as tetramethylrhodamine isothiocyanate-conjugated goat anti-mouse and FITC-conjugated goat anti-rabbit were obtained from Sigma-Aldrich. Radiolabeled compounds were purchased from DuPont NEN. PMA was purchased from Biomol International, and LPS was purchased from List Biological Laboratories. The cPLA$_2$ inhibitor was purchased from Calbiochem.

Cell culture

Murine cell lines were cultured in DMEM supplemented with 10% FCS and maintained at 37°C and 8% CO_2. IMR-90 and HEL 299 human lung fibroblasts were purchased from American Type Culture Collection; cultured in MEM (Eagle) with 2 mM L-glutamine and Earle's balanced salt solution adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate, and supplemented with 10% FCS; and maintained at 37°C and 5% CO_2.

Viruses and infection

Construction of the mutant Ad *dl309* and *dl758* has been described previously (26, 27). The mutant *dl309* was derived from Ad5 and lacks the 10.4K, 14.5K, and 14.7K genes of the E3 transcription unit. The mutant *dl758* was derived from a full-length Ad5-Ad serotype 2-Ad5 recombinant virus (*rec700*) and lacks the E3 14.7k gene. The Ad5 and *dl309* viruses were originally provided by L. Gooding (Emory University, Atlanta, GA), whereas *rec700* and *dl758* were originally provided by W. Wold (St. Louis University, St. Louis, MO). For these experiments, virus stocks were prepared using standard methods with A549 cells. For virus infections, cells were plated overnight, washed, and incubated with 10 PFU/cell for 2 h in medium containing 2% FCS. The cells were returned to medium with 10% FCS and incubated for indicated times. Infection rates were monitored by immunofluorescence with an anti-E1A antiserum and were typically 85–90%. UV inactivation was performed by exposing a thin film of virus stock to UV light, 2 cm from a UVG-11 UV lamp (Ultraviolet Products) for 20 min. UV-inactivated virus displayed no growth on permissive A549 cells.

Quantitative PG immunoassays

Production of PGE$_2$ and 6-keto-PGF$_{1\alpha}$ was determined using specific immunoassays purchased from R&D Systems. Briefly, 2.5×10^4 C3HA cells or 4×10^4 HEL 299 cells were plated into 24-well flat-bottom tissue culture plates (Fisher Scientific) and allowed to adhere overnight. Virus infections and treatments were performed as indicated, samples were collected, and analysis was performed, according to the manufacturer's instructions. OD was determined using a PolarStar microplate reader (BMG Labtechnologies).

Immunoblotting

Cell monolayers were washed twice with cold PBS, solubilized in lysis buffer (50 mM HEPES (pH 7.4), 1 mM EGTA, 1 mM EDTA, 0.2 mM sodium orthovanadate, 1 mM PMSF, 0.2 mM leupeptin, and 0.5% SDS), and collected by scraping. The protein concentration for each sample lysate was determined using the Pierce bicinchoninic acid system (Pierce). Equal protein samples (15–30 μg) were loaded on 8% Tris-glycine gels and subjected to electrophoresis using the Novex MiniCell System (Invitrogen Life Technologies). Following transfer, blocking, and probing, bands were visualized using the SuperSignal Chemiluminescent system (Pierce).

[^{3}H]AA release assays

A total of 2.5×10^4 cells was plated into 24-well flat-bottom tissue culture plates (Fisher Scientific) and labeled overnight with 0.1 μCi/ml [^{3}H]AA. The following morning, the cells were washed twice with HBSS, allowed to recover for an additional 2 h, and washed again before treatment. At indicated time points, 275-μl aliquots of medium were removed from the wells and centrifuged to remove debris. A total of 200 μl of the supernatant was removed for scintillation counting (Beckman Coulter model LS 5801), and total [^{3}H]AA release was calculated by multiplying by a factor of 2. Each point was performed in triplicate, and maximum radiolabel incorporation was determined by lysing untreated controls with 0.01% SDS and counting the total volume.

cPLA$_2$ enzyme assay

Lysates were prepared by sonication in 20 mM Tris (pH 8.0), 100 mM NaCl, 1 mM EDTA, 50 mM NaF, 30 mM sodium pyrophosphate, and 0.2 mM Na_3VO_4, and cleared by ultracentrifugation ($100{,}000 \times g$ for 1 h at 4°C). The activity of cPLA$_2$ was measured in vitro using vesicles of [^{14}C]arachidonyl phosphatidylethanolamine and dioleoylglycerol, as described previously (28, 29). The [^{14}C]arachidonyl phosphatidylethanolamine and dioleoylglycerol were mixed, dried under nitrogen, and resuspended in 50 mM HEPES (pH 7.4). The solution was sonicated on ice for 10 s, quick frozen in liquid nitrogen, and resonicated for 1 min. Enzymatic reactions were initiated by adding 10 μl of freshly prepared substrate to assay tubes containing 90 μl of buffer (50 mM HEPES, 150 mM sodium chloride, 2 mM 2-ME, and 1 mM calcium chloride (pH 7.4), with 1 mg/ml BSA) and test protein. The final concentration of substrate was 2 μM [^{14}C]arachidonyl phosphatidylethanolamine and 1 μM dioleoylglycerol. Reaction tubes were incubated at 37°C for 1 h, and the reaction was terminated with EDTA. To extract free fatty acids, 2 ml of isopropanol/heptane/sulfuric acid (90:10:1) was added to each tube; the tubes were vortexed; and 750 μl of water and 1.2 ml of heptane were added, followed by revortexing. The phases were separated by low speed centrifugation, and 1 ml of the heptane phase was transferred to a fresh tube containing 2 ml of heptane and 100 mg of silicic acid. After mixing, the tubes were centrifuged, and 1 ml of heptane was removed for scintillation counting. All points were performed in triplicate.

Immunofluorescence microscopy

Cells were plated on 8-well glass chamberslides (Nalge Nunc International), incubated overnight to achieve 80% confluence ($1–4 \times 10^4$ cells/well depending upon cell type), infected with Ad5 for indicated time point and/or treated with 10 ng/ml PMA, washed two times with PBS, and fixed for 20 min in 10% formaldehyde in PBS. The cells were permeabilized with 0.1% saponin and incubated with primary Ab in 2% BSA for 45 min. Cells were washed with PBS and incubated with secondary Abs for 45 min. For calcium experiments, 15 min before the end of treatment time, Fluo-4 AM was added to each well for a final concentration of 5 μM. Cells were washed twice with PBS and fixed with 10% formaldehyde in PBS for 15 min. Cells were washed twice and mounted in 10% glycerol. Microscopy was conducted on a Zeiss Axioscop 2 Plus, and images were captured and processed by a Spot charge-coupled device camera and software (Diagnostic Instruments).

Results

Infection of C3HA murine fibroblasts with Ad5

The murine, 3T3-like cell line, C3HA, was used for many of the experiments in this study. We chose these cells because they strongly express Ad early gene products and have been used previously to study the effects of Ad on host cell function (22, 30–32). (Note that because C3HA cells are murine in origin, Ad late genes are not expressed and the cells are nonpermissive for Ad replication.) C3HA cells were also selected because they express high levels of cPLA$_2$ (29), and the regulation of cPLA$_2$ activity in these cells has been characterized previously (29, 31–33). The progress of Ad infections in C3HA cells was monitored with an Ab to the Ad5 E1A gene product, the major *trans*-activating gene product of Ad. As shown in Fig. 1, the Ad5 E1A gene product was first detected in the cytosol 6 h postinfection (p.i.) (Fig. 1*B*), whereas strong cytosolic expression of E1A occurred 8 h p.i. (Fig. 1*C*). By

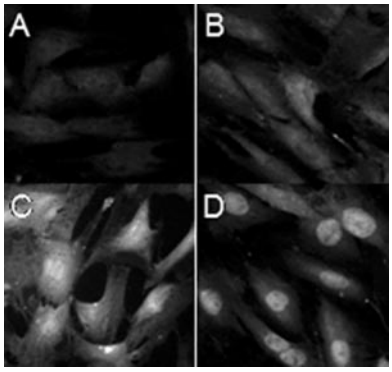

FIGURE 1. Time course of Ad5 infection in murine fibroblasts. C3HA fibroblasts were infected with 10 PFU/cell Ad5 and then stained for E1A using a commercial mouse mAb and fluorescent secondary Ab. *A*, Mock infected. *B*, Infected for 6 h. *C*, Infected for 8 h. *D*, Infected for 16 h. When tested on its own, the tetramethylrhodamine isothiocyanate-coupled goat anti-mouse secondary did not display any reactivity with Ad5-infected cells.

16–18 h p.i. (Fig. 1*D*), 85–90% of the cells displayed strong, uniform nuclear staining for E1A.

Infection with Ad5 induces rapid release of [^{3}H]AA and rapid activation of cPLA$_2$

As shown in Fig. 2*A*, we found that infection with Ad5 was accompanied by the rapid release of [^{3}H]AA, which continued linearly for 6 h and then ceased. Note that the break in the *x*-axis shown in Fig. 2*A* represents the point in the infection protocol in which the inoculating medium containing 2% FCS was removed and replaced with fresh medium with 10% FCS. Fig. 2*A* also shows that mock-infected cells released [^{3}H]AA. The release of [^{3}H]AA from mock-infected cells occurred later (2–4 h p.i.) and was typically one-third the magnitude seen with Ad5 infection. Our experiments showed that the release of [^{3}H]AA from mock-infected cells stemmed from the increase in FCS concentration that occurred when the inoculating medium was removed and replaced with fresh medium (data not shown). Our experiments also showed that the release of [^{3}H]AA from C3HA cells infected with Ad5 was dependent on the activity of cPLA$_2$. We found that the Ad5-induced release of [^{3}H]AA could be blocked completely by cPLA$_2\alpha$ inhibitor (Calbiochem), an *N*,*N*-disubstituted 4-aminopyrrolidine compound (34) (Fig. 2*B*). We also investigated whether phosphorylation of serine residue 505 is important in this situation for cPLA$_2$ activation. As shown in Fig. 2*C*, we found that infection with Ad5 resulted in the rapid phosphorylation of cPLA$_2$ at serine 505 (35), suggesting that phosphorylation at this residue may indeed be important for activation of cPLA$_2$ and initiation of [^{3}H]AA release. In contrast, whereas [^{3}H]AA release continued for 6 h p.i., phosphorylation of serine 505 returned to control levels by 2 h p.i. Long-term experiments did not reveal any rephosphorylation at this site (Fig. 2*D*), making it unlikely that phosphorylation at serine 505 is responsible for the sustained activity of cPLA$_2$ throughout the entire 6-h time period. We did not observe any changes in the total amount of cPLA$_2$ throughout the experimental period (Fig. 2, *C* and *D*).

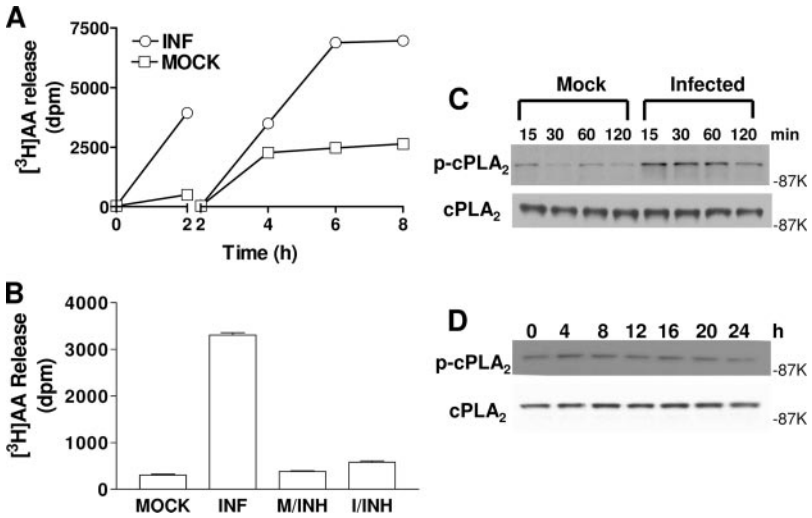

FIGURE 2. Ad5 infection induces rapid release of [^{3}H]AA and phosphorylation of cPLA$_2$. *A*, Release of [^{3}H]AA from C3HA fibroblasts infected with Ad5. Cells were labeled overnight with [^{3}H]AA and infected the next day with Ad5 (10 PFU/cell) or mock infected, and supernatants were collected at the indicated time points. Radioactivity in supernatants was then determined by liquid scintillation counting. All points were performed in triplicate, and values shown are means ± SEM from a representative experiment. Error bars are less than symbol size where not shown. The first 2-h time point on the *x*-axis represents the point in the protocol where the virus inoculum (in medium with 2% FCS) was removed and replaced with medium containing 10% FCS. Fresh medium with 10% FCS was then added, the *y*-axis value reset to zero (which we confirmed), and the infection allowed to proceed. *B*, The cPLA$_2$ inhibitor (*N*-((2*S*,4*R*)-4-(biphenyl-2-ylmethyl-isobutyl-amino)-1-(2-(2,4-difluorobenzoyl)-benzoyl)-pyrrolidin-2-ylmethyl)-3-(4-(2,4-dioxothiazolidin-5-ylidenemethyl)-phenyl)acrylamide, HCl) inhibits the Ad5-dependent release of [^{3}H]AA. The inhibitor was added with Ad5 (10 PFU/ml) during the 2-h inoculation time period, supernatants were collected, and radioactivity was determined by liquid scintillation counting. All points were performed in triplicate, and values shown are means ± SEM from a representative experiment. Error bars are less than symbol size where not shown. In parallel cultures, after 16 h, staining with anti-E1A Ab did not reveal any effect of the inhibitor on Ad5 infectivity. *C* and *D*, Western blots examining the expression of cPLA$_2$ and phosphorylation of cPLA$_2$ at serine 505. Cells were infected with Ad5 (10 PFU/cell) for the indicated times, and lysates were prepared. Following SDS-PAGE and transfer, membranes were probed with indicated Abs. Results shown are representative of three experiments. *C*, Densitometry revealed 2.5-, 7.6-, 3.2-, and 2.5-fold increases in phospho-cPLA$_2$ vs mock infected at 15, 30, 60, and 120 min, respectively. Also in *C*, densitometry revealed 0.92-, 0.99-, 1.0-, and 1.0-fold changes in cPLA$_2$ vs mock infected at 15, 30, 60, and 120 min, respectively. *D*, Fold changes in phospho-cPLA$_2$ vs zero time at 4, 8, 12, 16, 20, and 24 h were 1.2, 1.1, 1.1, 1.1, 1.0, and −0.9, respectively. Also in *D*, fold changes in cPLA$_2$ vs zero time at 4, 8, 12, 16, 20, and 24 h were 1.1, 1.1, 1.0, 1.0, 1.0, and −0.9, respectively.

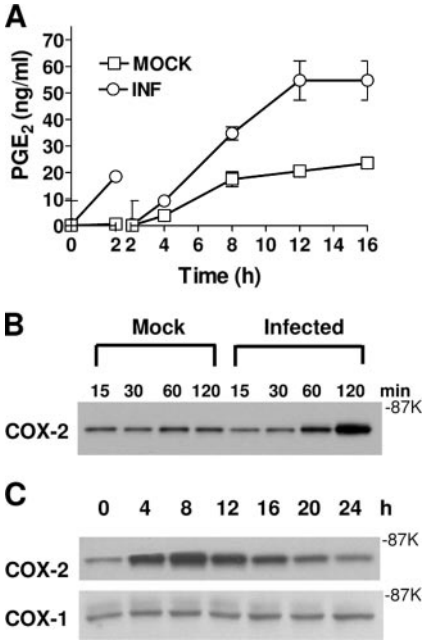

FIGURE 3. Ad5 infection induces rapid production of PGE_2 and expression of COX-2. *A*, Production of PGE_2 by C3HA cells infected with Ad5. Cells were infected with Ad5 (10 PFU/cell) or mock infected, and levels of PGE_2 were measured in supernatants at indicated time points by commercial ELISA. All points were performed in duplicate, and values shown are means ± SEM from a representative experiment. Where not shown, error bars are less than symbol size. The procedures used to generate the break in the *x*-axis at the 2-h time point are described in the legend to Fig. 2. *B* and *C*, Western blots examining the expression of COX-1 and COX-2. Infections were performed for the indicated times, and lysates were separated by SDS-PAGE. Following transfer, membranes were probed with indicated Abs. *B*, Densitometry revealed −1.5-, −1.1-, 1.4-, and 2.5-fold changes in the expression of COX-2 vs mock-infected samples at 15, 30, 60, and 120 min, respectively. *C*, Densitometry revealed 3.5-, 4.3-, 3.6-, 2.8-, 1.9-, and 1.3-fold increases in the expression of COX-2 vs the zero time point at 4, 8, 12, 16, 20, and 24 h, respectively. Also in *C*, densitometry revealed 1.1-, 1.2-, 1.0-, 1.0-, −0.9-, and −0.9-fold changes in the expression of COX-1 vs the zero time point at 4, 8, 12, 16, 20, and 24 h, respectively. Results shown are representative of at least two independent experiments.

Ad5 infection induces production of PGE_2 and expression of COX-2

As shown in Fig. 3*A*, infection of C3HA cells by Ad5 was also accompanied by production of PGE_2. PGE_2 was detected 2 h p.i., and levels continued to increase until ~12 h p.i. Again, switching the cells from 2 to 10% FCS-containing medium also induced a response. In this case, PGE_2 production by mock-infected cells occurred 4–8 h p.i. and reached levels approximately one-third of the Ad5-infected cultures. As shown in Fig. 3*B*, infection by Ad5 was also accompanied by the rapid induction of COX-2. Levels of COX-2 increased above constitutive levels 1 h p.i. (Fig. 3*B*) and peaked 8 h p.i. (Fig. 3*C*). In contrast, the expression of COX-1 did not change following Ad5 infection (Fig. 3*C*). We did not observe increases in the expression of COX-2 in mock-infected cells (Fig. 3, *B* and *C*), suggesting that constitutive levels of COX-1 and COX-2 are sufficient for PGE_2 production in mock-infected cells.

Ad5 infection inhibits the release of [^{3}H]AA and PGE_2 in response to external stimuli

The experiments in Figs. 2 and 3 showed that although Ad5 induced release of [^{3}H]AA and PGE_2, these processes also stopped after 6 and 12 h, respectively. In our experience, release of [^{3}H]AA or production of PGE_2 from C3HA cells can continue in a linear manner for at least 24 h following treatment with ligands such as PMA or LPS. We hypothesized, therefore, that Ad5 was exerting a suppressive effect on these processes. To test this hypothesis, late-stage Ad5-infected cells were treated with a panel of biologically diverse ligands. As shown in Fig. 4*A*, we found that release of [^{3}H]AA from late-stage Ad5-infected cells was greatly reduced following stimulation with fibroblast growth factor, PMA, A23187, IL-1, and LPS, in which levels of suppression were 76, 77, 76, 100, and 100%, respectively. In addition, we found that production of PGE_2 was greatly suppressed in late-stage Ad5-infected cells that were treated with external stimuli. As shown in Fig. 4*B*, production of PGE_2 was greatly reduced in C3HA cells that were infected by Ad5 for 18 h and then treated with PMA or LPS.

Ad5 gene expression is required for suppression of [^{3}H]AA release, but not for early release of [^{3}H]AA

Experiments were next performed with UV-inactivated Ad5 to test whether Ad5 gene expression is indeed required for late-stage suppression of [^{3}H]AA release. As shown in Fig. 4*C*, UV inactivation completely abrogated the ability of Ad5 to suppress the PMA-induced release of [^{3}H]AA, indicating that transcriptionally active virus is required for this effect. The time required for suppression of [^{3}H]AA release to appear in Ad5-infected cells is also consistent with the need for Ad5 gene expression. As shown in Fig. 4*D*, inhibition of the PMA response was first detected 6 h after the infection was initiated, the time point at which Ad5 E1A protein is first detected in infected cells (Fig. 1). Infection by Ad5 for 18 h was required to observe maximum inhibition of this response (Fig. 4*D*), which correlates with the time required for uniform nuclear expression of E1A in the culture (Fig. 1). In contrast, as shown in Fig. 4*E*, UV-inactivated virus was capable of inducing release of [^{3}H]AA at levels similar to live virus, indicating that viral gene expression is not required for this response.

Suppression of [^{3}H]AA release in late-stage Ad-infected cells is independent of E3 region gene products

Several Ad5 genes whose products are responsible for suppressing host immune function are located in the E3 transcription unit. We tested, therefore, whether E3 products are responsible for the suppression of [^{3}H]AA release seen in late-stage Ad-infected cells. As shown in Fig. 4*F*, we found that Ad deletion mutants lacking either the E3 14.7K gene (*dl758*) or the entire E3 transcription unit (*dl309*) suppressed PMA-stimulated release of [^{3}H]AA as effectively as wild-type Ad5 or the full-length Ad5/Ad serotype 2 recombinant *rec700*. We conclude, therefore, that the ability of Ad5 to prevent $cPLA_2$ activation is a novel activity independent of E3-encoded gene products.

Inhibition of [^{3}H]AA release is not due to lack of signaling or inactivation of $cPLA_2$

A series of experiments were next performed to understand the mechanism of Ad5-mediated suppression of [^{3}H]AA and PGE_2 release. The *inset* in Fig. 4*B* shows that although Ad5 infection prevented induction of additional COX-2 by PMA, levels of COX-2 remained elevated from the early stages of infection. This result suggested to us that levels of COX-2 were not limiting production of PGE_2 and, therefore, we focused our experiments upstream on $cPLA_2$. Initially, we examined the signals responsible for $cPLA_2$ activation. PMA was used as the ligand because activation of $cPLA_2$ by PMA is well defined and dependent on the sequential activation of protein kinase C and p42/44 MAPK (36).

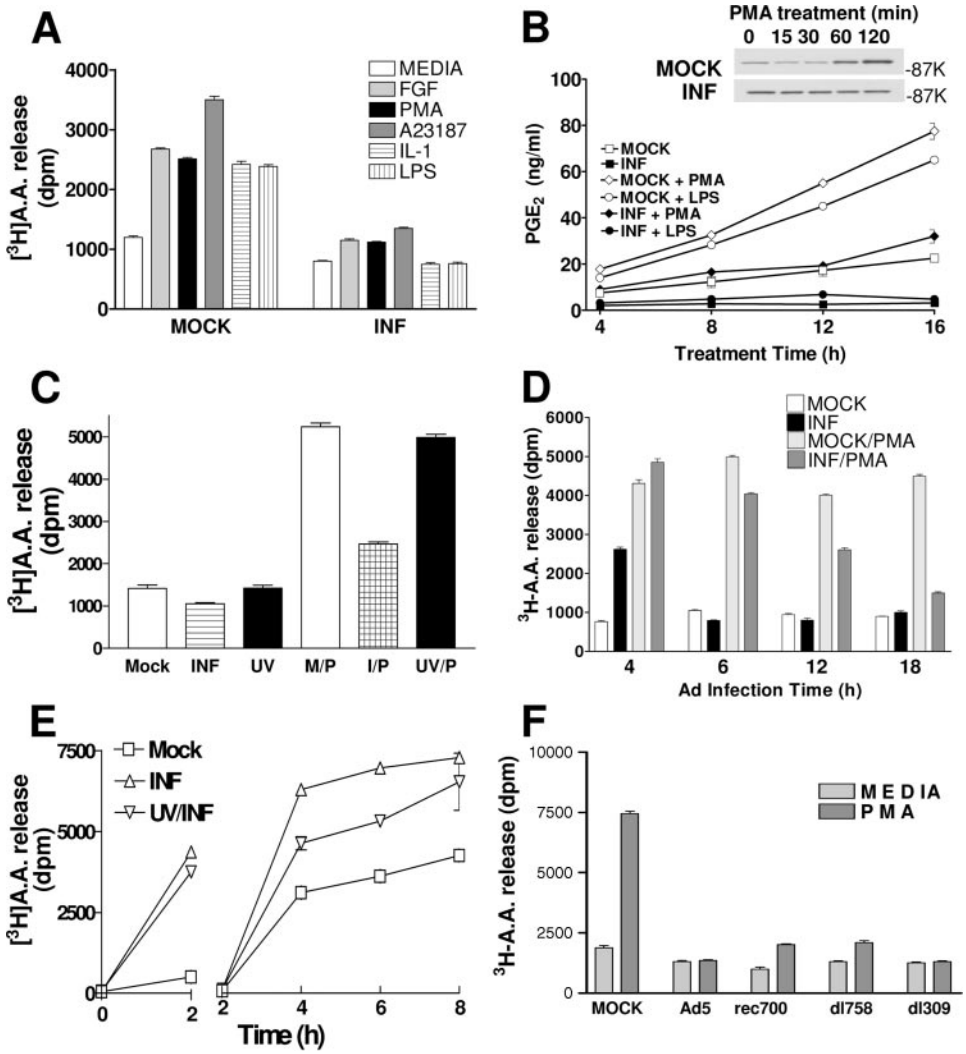

FIGURE 4. Characterization of the effects of Ad5 on the production of PGE_2 and release of [^{3}H]AA. *A*, Release of [^{3}H]AA by late-stage Ad5-infected cells following exogenous stimulation. C3HA cells were either mock infected or infected with Ad5 (10 PFU/cell) for 18 h, then treated with fibroblast growth factor (10 ng/ml), PMA (10 ng/ml), A23187 (10 μM), IL-1β (10 ng/ml), or LPS (1 μg/ml) for 2 h. *B*, Production of PGE_2 following exogenous stimulation in late-stage Ad5-infected cells. C3HA cells were either mock infected or infected with Ad5 (10 PFU/cell) for 18 h and then treated with PMA (10 ng/ml) or LPS (1 μg/ml) for the indicated times. Levels of PGE_2 in culture supernatants were quantified by ELISA. Values shown are means ± SEM from a representative experiment, and all points were performed in duplicate. The PMA-induced expression of COX-2 in late-stage Ad5-infected cells is shown in the *inset* in *B*. C3HA cells were either mock infected or infected by Ad5 for 18 h and treated with PMA (10 ng/ml) for the indicated times, and Western blots were performed with COX-2-specific Ab. Densitometry revealed −0.3-, −0.2-, 1.9-, and 2.7-fold changes in mock-infected cells treated with PMA vs time zero for 15, 30, 60, and 120 min, respectively. Densitometry for Ad5-infected cells showed −0.04-, −0.04-, −0.07-, and 0.08-fold changes following treatment with PMA vs time zero for 15, 30, 60, and 120 min, respectively. *C*, UV-inactivated Ad5 fails to inhibit the PMA-induced release of [^{3}H]AA. C3HA cells were either mock infected or infected with Ad5 or UV-inactivated Ad5 for 18 h, and then treated with PMA (10 ng/ml) for 2 h. *D*, Full suppression of PMA responsiveness by Ad5 requires overnight infection. C3HA cells were labeled overnight with [^{3}H]AA, infected with Ad5 or mock infected for varying times, and then stimulated with PMA (10 ng/ml) for 2 h. *E*, UV-inactivated Ad5 stimulates early release of [^{3}H]AA. C3HA cells were either mock infected or infected with Ad5 or UV-inactivated Ad5 for the indicated times. *F*, E3 deletion mutants suppress the PMA-induced release of [^{3}H]AA. C3HA cells were infected with the indicated full-length or deletion mutant Ads overnight, and then treated with PMA (10 ng/ml) for 2 h. *A* and *C–F*, Culture supernatants were collected and radioactive content was determined by scintillation counting. Values shown are means ± SEM from representative experiments, and all points were performed in triplicate.

As shown in Fig. 5*A*, following treatment with PMA, we observed a 2-fold increase in phosphorylation of serine 505 that was not inhibited by infection with Ad5, suggesting that kinase-dependent phosphorylation of $cPLA_2$ is normal in Ad5-infected cells. Western blots examining the phosphorylation of p42/44 MAPK also revealed normal phosphorylation in PMA-treated, Ad5-infected cells (data not shown). To confirm that phosphorylation was indeed causing activation of $cPLA_2$, lysates from control and infected cells were assayed for enzymatic activity in vitro using a mixed micelle assay (29, 32). As shown in Fig. 5*B*, lysates from mock or infected cells treated with PMA displayed similar levels of enhanced enzymatic activity. Apparently, the phosphorylation of $cPLA_2$ in Ad-infected cells is indeed resulting in a normally activated enzyme. Another important signal for the activation of $cPLA_2$ is calcium (37). We hypothesized that if Ad5-infected cells were depleted of calcium, $cPLA_2$ could be inhibited from properly binding to and inserting in membranes. As shown in Fig. 5*C*, Ad5 infection did not cause calcium levels to decrease. Rather, we found that Ad5 infection was accompanied by a 50% increase in intracellular calcium 6–8 h after the infection was initiated

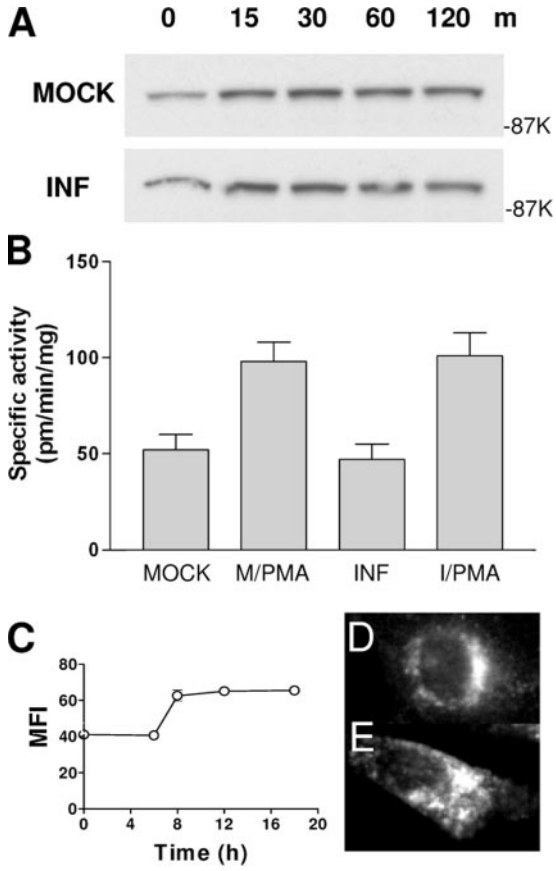

FIGURE 5. Infection by Ad5 does not suppress signaling to $cPLA_2$. *A*, The PMA-induced phosphorylation of $cPLA_2$ at serine 505 following infection with Ad5. C3HA cells were either mock infected or infected for 18 h with Ad5, and then treated with 10 ng/ml PMA for the indicated times. Lysates were separated by SDS-PAGE, and Western blots were performed using a serine 505-phosphospecific rabbit polyclonal antiserum. Densitometry revealed 2.0-, 2.1-, 2.0-, and 1.8-fold increases for mock-infected cells vs time zero at 15, 30, 60, and 120 min, respectively. Ad5-infected cells showed 2.1-, 2.0-, 1.9-, and 1.8-fold increases vs time zero at 15, 30, 60, and 120 min, respectively. *B*, PMA-dependent increase in enzyme activity was not inhibited by infection with Ad5. C3HA cells were mock infected or infected with Ad5 for 18 h, and then treated with PMA (10 ng/ml) for 2 h. Lysates were prepared and analyzed for $cPLA_2$ activity, as described in *Materials and Methods*. All points were performed in duplicate, and values shown are means ± SEM from a representative experiment. *C*, Infection by Ad5 does not deplete intracellular calcium. C3HA cells were infected with Ad5 for the indicated times, and levels of calcium were visualized by fluorescence microscopy following staining with Fluo-4 AM. Fluo-4 AM was added to the cultures 15 min before end of each time period. Fluorescence intensity was determined using Photoshop software, and values shown are means ± SEM for 30 cells at each time point. *D* and *E*, Representative images of intracellular calcium at 6 and 8 h p.i. with Ad5.

(Fig. 5*C*). Increased levels of calcium were generally seen in the cytosol and perinuclear region of the cell (Fig. 5, compare *D* and *E*). In summary, whereas $cPLA_2$ in infected cells is inhibited from releasing [^{3}H]AA following treatment with PMA, Ad5 infection did not inhibit kinase-dependent signaling to $cPLA_2$, nor were Ad5-infected cells depleted of intracellular calcium.

Infection with Ad5 alters the intracellular position of $cPLA_2$

$cPLA_2$ is typically found in the cytosol, and upon stimulation, the enzyme translocates to internal membranes. Fig. 6*A* shows the somewhat punctate, cytosolic staining pattern typically observed in

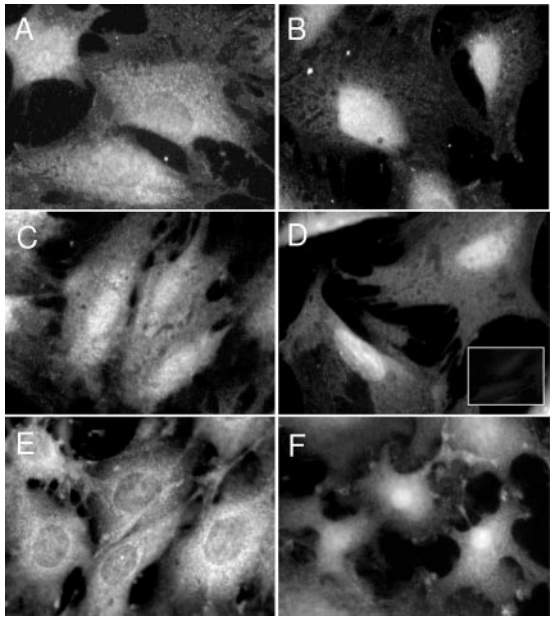

FIGURE 6. Infection by Ad5 triggers changes in the intracellular position of $cPLA_2$. The intracellular distribution of $cPLA_2$ in mock- and Ad5-infected cells visualized by fluorescence microscopy using a $cPLA_2$-specific mAb. *A*, $cPLA_2$ staining in mock-infected cells. *B–D*, $cPLA_2$ staining following infection with Ad5 for 6, 8, and 18 h, respectively. *E*, $cPLA_2$ staining in mock-infected cells treated with PMA (10 ng/ml) for 1 h. *F*, $cPLA_2$ staining in C3HA cells infected by Ad5 for 18 h and then treated for 1 h with PMA (10 ng/ml). Nonspecific staining, using only the secondary Ab, in C3HA cells infected with Ad5 for 18 h is shown in the *inset* in *D*.

unstimulated C3HA cells. This pattern changed dramatically in cells infected by Ad5. We found that at 6 h p.i., $cPLA_2$ staining was observed almost exclusively in the nuclear/perinuclear region of the cell (Fig. 6*B*). Later, 10–12 h p.i., this pattern changed to

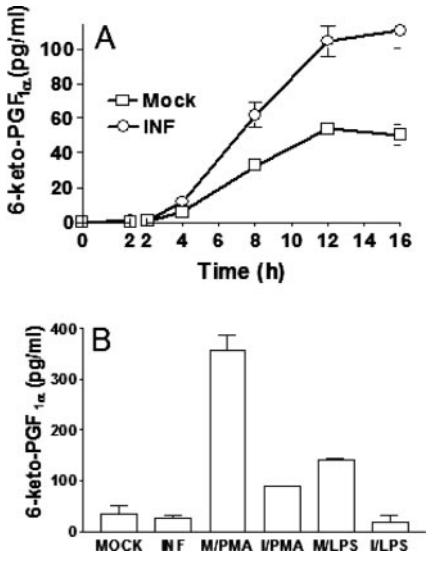

FIGURE 7. Characterization of the effects of Ad5 on the production of 6-keto-$PGF_{1\alpha}$. The effects of Ad5 on the production of PGI_2 were estimated from effects on production of the stable hydration product 6-keto-$PGF_{1\alpha}$. *A*, C3HA cells were mock infected or infected with Ad5 (10 PFU/cell) for the indicated times. *B*, C3HA cells were mock infected or infected with Ad5 for 18 h, and then treated with PMA (10 ng/ml) or LPS (1 μg/ml) for an additional 16–18 h. In both panels, culture supernatants were collected and analyzed for 6-keto-$PGF_{1\alpha}$ by ELISA.

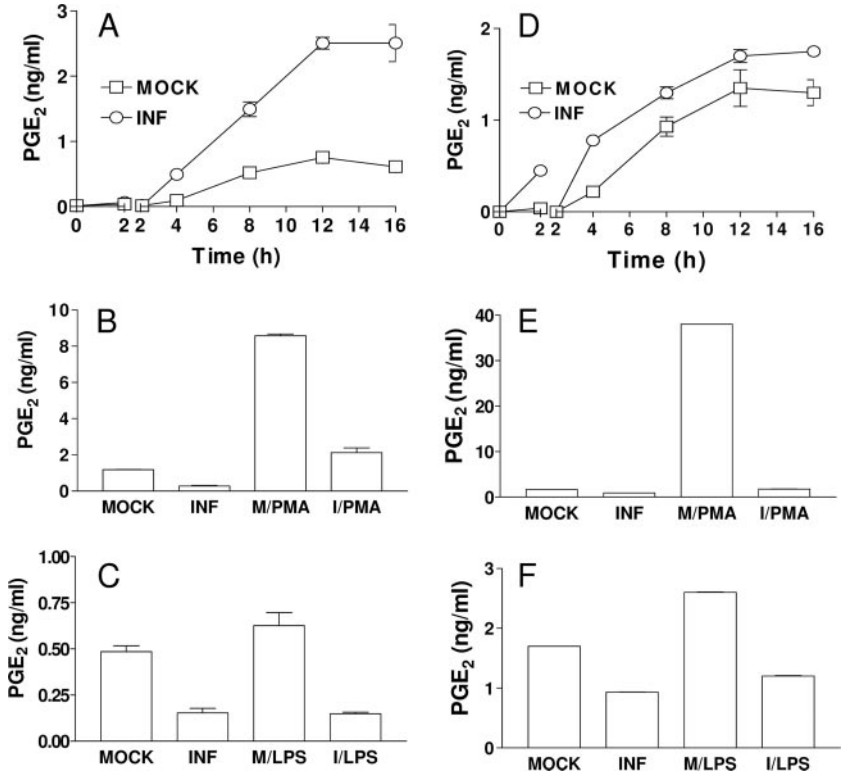

FIGURE 8. The effects of Ad5 on PGE_2 production are also seen with human fibroblasts. Human fibroblasts were infected with Ad5, and levels of PGE_2 were determined by ELISA. *A*, IMR-90 cells were mock infected or infected with Ad5, and culture supernatants were collected at the indicated times. *B*, IMR-90 cells were mock infected or infected with Ad5 and then treated with PMA. *C*, IMR-90 cells were mock infected or infected with Ad5 and then treated with LPS. *D*, HEL299 cells were mock infected or infected with Ad5, and culture supernatants were collected at the indicated times. *E*, HEL299 cells were mock infected or infected with Ad5 and then treated with PMA. *F*, HEL299 cells were mock infected or infected with Ad5 and then treated with LPS. For all panels, samples were assayed in duplicate and representative experiments are shown. All infections were for 18 h with 10 PFU/cell. Treatments with PMA (10 ng/ml) and LPS (1 μg/ml) were for an additional 16–18 h. Levels of PGE_2 were determined by ELISA, and values shown are means ± SEM, and, where not shown, error bars are less than symbol size.

some extent, and we observed both concentrated nuclear/perinuclear staining and diffuse cytosolic staining (Fig. 6*C*). Finally, 16–18 p.i., another change occurred and we again noted only nuclear/perinuclear $cPLA_2$ staining (Fig. 6*D*). We questioned, therefore, whether the altered position of $cPLA_2$ in Ad5-infected cells could account for the inability of $cPLA_2$ to respond to PMA. As shown in Fig. 6*E*, treatment of mock-infected C3HA cells with PMA caused a ring of perinuclear staining to appear in virtually every cell. In contrast, the $cPLA_2$ in Ad5-infected cells did not translocate similarly. Instead, Ad5-infected cells that were stimulated with PMA retained the pattern of nuclear/perinuclear staining seen with only Ad infection (Fig. 6*F*). We did note that the treatment of Ad5-infected cells with PMA caused the cells to shrink and retract from the substrate. This change in morphology was not seen following the PMA treatment of mock-infected cells.

Infection with Ad5 exerts similar effects on the production of PGI_2

PGI_2, prostacyclin, is another prostanoid that can exert effects on immunity and inflammation (38). PGI_2 is unstable, but its levels can be estimated by measuring amounts of its reaction product, 6-keto-$PGF_{1\alpha}$. As shown in Fig. 7*A*, we found that infection by Ad5 resulted in the production of 6-keto-$PGF_{1\alpha}$, which ceased after ~12 h. Fig. 7*B* shows that C3HA cells produce 6-keto-$PGF_{1\alpha}$ in response to PMA or LPS, and that infection by Ad5 inhibits these responses. From these results, we conclude that the effects of Ad5 on the PG biosynthetic pathway are not specific for PGE_2 and most likely apply to all of the PGs that can be produced by these cells.

Infection with Ad5 exerts similar effects on PG production in human lung fibroblasts

Ad5 is a human virus, and, therefore, experiments were performed to test whether Ad5 influences production of PGE_2 in human fibroblasts as it does in murine fibroblasts. IMR90 cells are normal human lung fibroblasts that have been used extensively in studies with Ad (39–41). As shown in Fig. 8*A*, these cells produced PGE_2 rapidly following infection with Ad5, and PGE_2 production by IMR90 cells ceased ~12 p.i. IMR90 cells infected with Ad5 also did not respond to PMA (Fig. 8*B*) or LPS (Fig. 8*C*). A similar pattern was noted with HEL299 cells, human embryonic lung fibroblasts that have also been used to study Ad gene expression (42). As shown in Fig. 8*D*, in these cells we found rapid production of PGE_2 after infection with Ad5, which continued for ~4 h, after which both mock- and Ad5-infected cells released similar levels of PGE_2. Overnight infection of these cells with Ad5 results in near complete inhibition of PGE_2 production in response to both PMA (Fig. 8*E*) and LPS (Fig. 8*F*).

Discussion

In these experiments, we have attempted to characterize the effects of infection by Ad5 on the production of PGs and several key enzymes in the PG biosynthetic pathway. Our experiments revealed two phases to this response, as follows: an early stimulatory phase, followed later by an inhibitory phase. Because our laboratory is particularly interested in identifying new methods for inhibiting the activity of $cPLA_2$, we focused our mechanism studies on the later inhibitory phase. Our experiments did not reveal an effect of Ad5 on the expression or activity of $cPLA_2$, nor on signals reaching $cPLA_2$. We did note an effect of Ad5 on the intracellular position of $cPLA_2$ and its ability to translocate, raising the hypothesis that Ad5 interferes with the activity of this enzyme by altering its intracellular mobility.

Crofford et al. (43) have reported that infection of synoviocytes with the vector Ad/RSVlacZ, which lacks E1A, E1B, and a portion of E3, results in rapid expression of COX-2 and production of PGE_2. UV-psoralen treatment of the virus did not block this effect, indicating that this response is most likely a component of the host innate response triggered when the virus binds to the cell surface. Hirschowitz et al. (44, 45) have also reported PG production in

small cell lung cancer cells following infection with E1/E3-deleted Ad vectors. These reports did not, however, examine the breadth of this response. Our results show that infection by Ad5 induced the production of both PGE_2 and PGI_2, raising the possibility that Ad infection results in broad activation of the PG biosynthetic machinery and production of several different PGs. There are several potential reasons that cells might want to produce PGs following infection by Ad5. PGE_2, for example, can have a direct benefit to host cells and actually inhibit Ad replication (46). PGE_2 might also be beneficial to the host because of its ability to drive the immune response toward a Th2 phenotype (47). Ab responses are well known to be effective against this virus (48). Finally, PGs could be beneficial to the host for their distant effects on a variety of organs and physiological processes (7).

Our experiments did, for the first time, reveal that infection by Ad5 also triggers the release of [^{3}H]AA. A $cPLA_2$ inhibitor that we tested blocked this response, suggesting that the Ad5-dependent release of [^{3}H]AA is dependent on the activity of $cPLA_2$. We also observed the rapid phosphorylation of $cPLA_2$ at serine 505, suggesting that this may be the activating signal responsible for the Ad5-dependent activation of $cPLA_2$. Phosphorylation of $cPLA_2$ at this residue is p42/44 MAPK dependent (35), and Ad-dependent activation of MAPKs has been reported previously (43). In contrast, phosphorylation of serine 505 was transient, whereas release of [^{3}H]AA continued for several hours. It is possible, therefore, that additional signals are involved, or that additional PLA_2 play a role in this response. Sequential activation of different PLA_2 isoforms has been reported previously (49–52) and will clearly need to be evaluated in this system.

The activation of $cPLA_2$, induction of COX-2, and ultimately PG production all occurred rapidly, before Ad5 gene expression occurred. Because viruses often carry genes to counteract the host immune response, we were interested in knowing whether Ad5 made any attempt to silence this response. Suppressing PG production might permit the virus to replicate to higher levels. It is also possible that suppression of PG production results in a Th1-type response that would be less effective against this virus. Preventing a highly effective immune response may be part of the mechanism that allows Ad to form persistent infections. Our evidence suggests that Ad exerts multiple effects on the PG pathway. We were not surprised to find the PMA-induced expression of COX-2 blocked in late-stage Ad-infected cells. Ad exerts broad effects on host cell transcription and translation, and, in addition, Ad E1A inhibits the translocation of NF-κB (53), which can be critical for expression of COX-2 (54). In contrast, late-stage Ad5-infected cells still retained COX-2 from the early phase of the infection, making it unlikely that the failure of late-stage cells to produce PGs results from a lack of COX-2. Instead, our experiments pointed to an effect on $cPLA_2$ that ultimately limits PG production. We hypothesize that sufficient AA is released in the early phase of infection to enable COX-2 and downstream PG synthases to continue PG production for 12 h. Beyond that, because of the inhibitory effects of Ad5 on $cPLA_2$, free AA becomes limiting and PG production ceases. Clearly, confirmation of this hypothesis will require a more complete analysis of the activity of COX-2 and downstream PG synthases during the later stages of infection.

A number of experiments were performed to understand the basis for inhibition of $cPLA_2$ activity in late-stage Ad5-infected cells. These cells did not respond to any of the ligands that we tested, which represented a broad range of biologically active compounds, indicating to us that suppression was unlikely mediated through effects on receptors or upstream signaling. In the absence of any effect of Ad5 on the expression of $cPLA_2$, we hypothesized unresponsiveness might arise from disruption of the terminal kinases responsible for phosphorylation of $cPLA_2$. We focused our experiments on the response to PMA because this pathway has been well defined (35). Our data did not support that hypothesis; the PMA-induced phosphorylation and activation of $cPLA_2$ were normal, as was phosphorylation of p42/44 MAPK (data not shown). Instead, we correlated the unresponsiveness of $cPLA_2$ to changes in its intracellular position and ability to translocate. We found that after 6–8 h of infection, about the time Ad5 proteins began appearing, $cPLA_2$ became located in the nuclear/perinuclear region of the cell. Although some diffuse, cytosolic $cPLA_2$ staining was noted later in the response, the bulk of the $cPLA_2$ was found in the nuclear/perinuclear region of Ad5-infected cells. The nuclear/perinuclear translocation of $cPLA_2$ has been noted in a number of cell types following ATP depletion (55), or treatment with norepinephrine (56) and histamine (57). We have also noted the nuclear/perinuclear translocation of $cPLA_2$ in C3HA cells treated with TNF and cycloheximide to induce apoptosis (33). Experiments with calcium ionophores (58–61) suggest that this is the normal site for $cPLA_2$ translocation following elevation of intracellular calcium. However, these translocation events have been associated with activation of $cPLA_2$ and release of AA. We did not observe translocation to this location early in response to Ad5 (when [^{3}H]AA release was occurring), nor when Ad5-infected cells were activated with PMA, raising the possibility that Ad5 is trapping $cPLA_2$ in an inappropriate location that, regardless of signaling, prevents its normal translocation and membrane binding.

There are several potential mechanisms by which Ad5 gene products could influence the intracellular position of $cPLA_2$. As mentioned above, the perinuclear/nuclear translocation of $cPLA_2$ has been associated with elevated intracellular calcium, and we did note an increase in levels of intracellular Ca^{2+} about this time, possibly indicating a role for Ca^{2+} in this repositioning process. Alternatively, $cPLA_2$ can bind the nuclear oncogene B-*myb*, which in turn can carry it into the nucleus (62). Because the Ad E1A gene product and B-*myb* share homology (63), it is possible that E1A itself may be responsible for the late-stage movement of $cPLA_2$ into the nuclear/perinuclear region. It is also possible that Ad5 influences the movement of $cPLA_2$ through effects on the cytoskeleton. Ad E1A has been shown to exert effects on both vimentin-containing (64) and actin-containing filaments (65, 66), both of which are important for the intracellular position and translocation of $cPLA_2$ (67, 68). It is also possible that Ad5 influences the position and translocation of $cPLA_2$ through effects on the ceramide biosynthetic pathway. Ceramide can directly bind to the CaLB domain of $cPLA_2$, facilitating membrane docking and subsequent AA release (69), and recent studies indicate that Ad infection results in ceramide accumulation (70). Ceramide-1-phosphate (71–73) and sphingosine-1-phosphate (72, 73) can also influence movement of $cPLA_2$ and membrane binding, although the effects of Ad infection on these molecules have not been tested. It is likely the Ad5 gene(s) responsible for this effect is located in the E1, E2, or E4 transcription units. Ad late genes are not expressed in murine cells, and our experiments with viruses lacking the E3 region rule out this section of the genome.

Ad-derived vectors have been used extensively to deliver foreign gene products. In many cases, these vectors provoke a strong inflammatory response that can limit replication and expression of foreign Ags (25, 74). Although it is clear that cytokine production is elevated in these situations (25), the role of PGE_2 and other lipid mediators has not been examined. Because these vectors often lack several or all Ad early gene products, it is likely that the vectors are unable to suppress production of PGE_2 once it has been initiated

by the host. It is possible, therefore, that excess PG production contributes to the vector-dependent inflammatory response and that higher levels of Ag production could be achieved by controlling PG production. In addition, because of the effects of PGs on dendritic cells, the immune response to vectored Ags may, a priori, be biased toward a Th2 phenotype. Further study in this area will undoubtedly improve our understanding of the immune response to Ad-derived vectors. Overall, whereas a handful of viruses have been examined for their effects on the production of lipid mediators (75, 76), information in this area is highly limited.

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Disclosures

The authors have no financial conflict of interest.

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