Cytosolic phospholipase A2α (cPLA2α) is the rate-limiting key enzyme that cleaves arachidonic acid (AA) from membrane phospholipids for the biosynthesis of eicosanoids, including prostaglandin E2 (PGE2), a key lipid mediator involved in inflammation and carcinogenesis. Here we show that cPLA2α protein is S-nitrosylated, and its activity is enhanced by nitric oxide (NO). Forced expression of inducible nitric-oxide synthase (iNOS) in human epithelial cells induced cPLA2α S-nitrosylation, enhanced its catalytic activity, and increased AA release. The iNOS-induced cPLA2α activation is blocked by the specific iNOS inhibitor, 1400W. The addition of the NO donor, L-arginine and NO donor significantly increased cPLA2α activity and AA release. These findings demonstrate that S-nitrosothiol forms derived NO S-nitrosylates and activates cPLA2α.

Site-directed mutagenesis revealed that Cys-152 of cPLA2α is critical for S-nitrosylation. Furthermore, COX-2 induction or -nitrosylation is mediated at least in part by specific iNOS binding complex, it appears that COX-2-induced augmentation of cPLA2α S-nitrosylation of cPLA2α in vitro. Incubation of cultured cells with the iNOS substrate L-arginine and NO donor significantly increased cPLA2α activity and AA release. These findings demonstrate that S-nitrosothiol forms derived NO S-nitrosylates and activates cPLA2α.

These lipid products function as local hormones through binding to other membrane receptors to mediate a myriad of biological functions, such as inflammation and carcinogenesis. In normal physiological regulation of cPLA2α activity and imbalance of AA release has been implicated in the pathogenesis of inflammatory and neurodegenerative disorders, such as allergic reactions, arthritis, acute lung injury, autoimmune disease, and several types of cancers, some of which are accompanied by PGE2 accumulation (7–20).

Recent evidence suggests that functional coupling between cPLA2α and COX-2 represents an important mechanism for prostaglandin synthesis (5, 21–24). COX-1 and COX-2 have a similar Km for arachidonic acid, but COX-2 metabolizes the AA cleaved by activated cPLA2α (5, 21, 22). The exact molecular mechanism for the coupling between cPLA2α and COX-2 remains to be further defined, although possible explanations include colocalization of the enzymes in certain cellular compartments and/or involvement of unknown binding proteins (5).

The biosynthesis of prostaglandins is regulated by nitric oxide (NO) in various cell types (25). This phenomenon was first reported by Salvemini et al. (26), who showed that NO activated cyclooxygenase and enhanced PG synthesis in the mouse macrophage cell line RAW264.7. Since then, this observation was confirmed and extended by other investigators in various cellular systems and animal models (for a review, see Ref. 25). Therefore, COX enzymes may represent important endogenous targets for modulating the multifaceted roles of PGE2 and other eicosanoids.
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NO. Kim et al. (27) reported that iNOS-derived NO S-nitrosylates COX-2 and thus enhances COX-2 catalytic activity in macrophages, which provides mechanistic explanation for NO-induced COX-2 activation. Recently, some NO donors combined with several nonsteroidal anti-inflammatory drugs (e.g., nabumetone and indomethacin) have been synthesized and used in the treatment of inflammatory and noninflammatory disorders, suggesting the possible beneficial effect of drugs acting on NO and PGs simultaneously. On the other hand, fatty acid has been shown to act as the storage of NO in lipid bilayers and micelles and as the regulator of NO release (28). In human blood plasma and urine, nitrofatty acids are abundantly present (29, 30), and nitroproline is a potent endogenous ligand for peroxisome proliferator-activated receptor-γ (31). Despite these achievements, however, the potential effect of NO on the regulation of PLA$_2$ has not been studied.

This study was designed to investigate whether nitric oxide signaling might modulate cPLA$_2$α activation. Our data indicate that cPLA$_2$α is S-nitrosylated and activated by iNOS-derived NO in human epithelial cells. This effect is mediated by Cys-152 of cPLA$_2$α. Thus, S-nitrosylation of cPLA$_2$α may represent an important mechanism for regulation of AA release and eicosanoid biosynthesis. Furthermore, we show that COX-2 facilitates the association between iNOS and cPLA$_2$α, thereby dramatically enhancing iNOS/NO-induced cPLA$_2$α S-nitrosylation and PG synthesis. These results reveal a novel iNOS-mediated functional coupling between cPLA$_2$α and COX-2 in NO-mediated PG synthesis in human cells.

EXPERIMENTAL PROCEDURES

Materials—The protein A-Sepharose and protein G-agarose assay System (catalog number 17-0610-02) were from GE Healthcare. [5,6,8,9,11,12,14,15-3H(N)] Arachidonic acid (NET-298) was from PerkinElmer Life Sciences (Waltham, MA). Recombinant human interferon-γ, 1-arginine, 1-aspartic acid, 70–80% confluence were from Sigma (St. Louis, MO). Recombinant human IL-1β and TNF-α were from Peprotech (Rocky Hill, NJ). Recombinant human IL-6 and IL-8 were from R&D Systems (Minneapolis, MN). Recombinant human IL-1β, human IL-6, S-methyl methanethiosulfonate solution, Triton X-100, sodium 1-ascorbate, and neomycin were from Sigma (St. Louis, MO). N$_2$-Nitrosoglutethimide (GSNO), 1400W, NOC-18, anti-human COX-2 antibody, and the cPLA$_2$ assay kit (catalog number 765021) were provided by Cayman Chemical (Ann Arbor, MI). N$_6$-(6-(biotinamido)hexyl)propyridylthiono-(2’-pyridyl)dithio)propionamidine and immobilized Streptavidin were from Pierce (Rockford, IL). Micro Bio-Spin Chromatography Column was from Bio-Rad. Anti-human iNOS and cPLA$_2$α antibody were from Santa Cruz Biototechnology (Santa Cruz, CA). The cell culture medium and serum were from Invitrogen.

Cell Culture, Transfection, and Transduction—Four types of human epithelial cells were utilized in this study (liver epithelia-derived SG231 and CCLP1, lung epithelia-derived A549, and kidney epithelia-derived HEK293). SG231 cells were cultured in α-minimal essential medium supplemented with 10% fetal bovine serum (FBS) as we described (32, 33). CCLP1 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS (32, 33). A549 cells (obtained from ATCC) were cultured in Dulbecco’s modified Eagle’s medium containing 10% FBS. HEK293 cells (obtained from ATCC) were cultured in Eagle’s minimum essential medium containing 10% FBS. All of the cells were cultured at 37 °C in a humidified CO$_2$ incubator. For overexpression of iNOS in these cells, the iNOS virus or control Y5 virus (provided by the University of Pittsburgh Vector Core Facility) at different titers were added into the culture medium without FBS for 2 h, followed by 5% FBS overnight. The cPLA$_2$α expression plasmid was transiently transfected into the cells with low expression of endogenous cPLA$_2$α (CCLP and HEK293). For this purpose, the cells were seeded on the 6-well plate with 10% FBS the day before transfection. On the following day, the cells in each well (80% confluence) were transfected with 1 µg of cPLA$_2$α or control MT2 plasmid using Lipofectamine Plus reagent (6 µl of Plus reagent, 4 µl of Lipofectamine) in serum-free medium. For CCLP1 cells, the transfection medium was replaced with culture medium containing 5% FBS 3 h later. For HEK293 cells, the transfection reagents were removed after 2 h and replaced with regular medium with 5% FBS. After an additional 16-h incubation, the culture media were collected for PGE$_2$ production assay, whereas the cells were washed twice with cold PBS and subjected to further analysis as described under “Results.”

In Vitro cPLA$_2$ Activity Assay—2-Deoxy-2-thioarachidonoylphosphatidylcholine was used as the substrate to measure cPLA$_2$ activity in vitro. The assay was performed by using the cPLA$_2$ assay kit purchased from Cayman Chemical (catalog number 765021). The secretary PLA$_2$ and calcium-independent PLA$_2$ were excluded or deactivated from the whole cell lysate to improve the assay specificity. All procedures were performed according to the manufacturer’s instructions with minor modifications. In brief, after transfection with plasmids or infection by adenovirus vector or treatment with different reagents, the cells were scraped down from plates and homogenized in Hepes-EDTA buffer. The homogenates were then centrifuged to remove cell debris at 14,000 rpm for 20 min. To minimize contamination of secretary PLA$_2$ and calcium-independent PLA$_2$, supernatant was concentrated by an Amicon Y30 filter, followed by incubation for 20 min with bromoelanol lactone, a calcium-independent PLA$_2$ inhibitor. 10 µl of cell lysate was finally subjected to the assay in the 96-well mode, and the OD value was measured at 414 nm.

[3H]Arachidonic Acid Release—The cells seeded on 6-well plates were treated with various reagents or infected with adenovirus in the presence or absence of cytokine mixture for 24 h. [5,6,8,9,11,12,14,15-3H]Arachidonic acid (0.5 µCi/ml) was simultaneously added into culture medium containing 1% serum and left for incorporation into cell membranes overnight. On the next day, the culture media were removed, and the cells were rinsed five times with medium, followed by incubation with medium containing 0.5% serum for 1–2 h. The
culture medium was then collected and centrifuged at 14,000 rpm for 20 min to remove suspension cells. \(^3\)H activity was measured using a liquid scintillation counter.

**PGE\(_2\) Production Assay**—The cells were treated with different reagents or infected with adenovirus for 24 h. The culture medium was then collected and centrifuged at 14,000 rpm for 20 min to exclude suspension cells. 50 \(\mu\)l of supernatant, with triplicate wells for each sample, was utilized for enzyme immunoassay. Triplicate blank wells and nonspecific binding wells were included to minimize the interference of background color. The binding of PGE\(_2\) with antibody lasted for 2 h at room temperature, which was followed by five rinses with wash buffer to maximally eliminate unbound or nonspecifically bound antibody. 150 \(\mu\)l of enzyme substrate was added into each well for 30 min, and the OD value was measured at 630 nm using an enzyme-linked immunosorbent assay reader. The actual PGE\(_2\) concentration was calculated according to the standard curve ranging from 0 to 320 pg/50 \(\mu\)l. The samples with too high concentration of PGE\(_2\) were diluted appropriately for further assay.

**Detection of S-Nitrosylation of cPLA\(_2\)α**—A biotin switch assay was utilized to measure the S-nitrosylation of cPLA\(_2\)α according to the previously described methods (34, 35) with modifications. For this assay, the unmodified thiols, which are first blocked by S-methyl methanethiosulfonate; then reduced and labeled with biotinylated protein. This affinity chromatography method utilizes N-(6-(biotinamido)hexyl)-3-(2'-pyridyldithio)propionamide (1.5 mM) at room temperature for 150 min to exclude suspension cells. 50 \(\mu\)l of neutralization buffer (20 mm Hepes-NaOH, 100 mm NaCl, 1 mm EDTA, 0.5% Triton X-100, pH 7.7) and 30 \(\mu\)l of immobilized streptavidin beads were added into the solution, and the mixture was rotated for 1 h at room temperature. The formed streptavidin bead-protein complex was then washed five times with neutralization buffer plus NaCl (neutralization buffer with 500 mm NaCl) and two times with neutralization buffer, 30 \(\mu\)l of elution buffer (20 mm Hepes-NaOH, 100 mm NaCl, 1 mm EDTA, 100 mm 2-mercaptoethanol, pH 7.7) was applied to the complex to elute proteins from streptavidin beads for 20 min at room temperature. The obtained proteins were then subjected to Western blotting analysis. All of the procedures, except Western blotting, were performed in the absence of direct light. The absence of ascorbate was applied as the assay negative control.

**Purification of Human Recombinant cPLA\(_2\)α Protein**—Affi-Gel 10-activated affinity support (catalog number 153-6099; Bio-Rad) was applied to purify recombinant human cPLA\(_2\)α protein. This affinity chromatography method utilizes monoclonal anti-cPLA\(_2\) α antibody, which is covalently coupled to the Affi-Gel 10 gel. In the presence of the expression plasmid was transfected into HEK293 cells, the endogenous cPLA\(_2\)α protein was co-precipitated. During this period, Affi-10 gel (500 \(\mu\)g, SC-454; Santa Cruz Biotechnology, CA) for 2–4 h at 4 °C. The crude lysate was applied to the crude lysate was washed three times with Hepes buffer containing 100 \(\mu\)m Hepes, pH 8.0. The obtained protein was immediately desalted by passing through a Micro Bio-spin P-6 column (pre-equilibrated with 100 \(\mu\)m Hepes, pH 8.0) and then subjected to SDS-PAGE for characterization of its purity. 50 \(\mu\)g of purified cPLA\(_2\)α protein was applied to a biotin switch assay or in vitro cPLA\(_2\) activity assay.

**Site-directed Mutagenesis of the Cysteine Residues in cPLA\(_2\)α Protein**—The cPLA\(_2\)α protein contains nine cysteine residues (Cys-139, -141, -152, -220, -324, -331, -620, -634, and -726). These cysteine residues were mutated into alanine residues by using the QuikChange II site-directed mutagenesis kit (catalog number 200523; Stratagene, La Jolla, CA). The procedures were carried out according to the manufacturer’s instructions. The primer sets for the nine mutations were as follows: Cys-139 (5'-GTC TCT TGA AGT TGC CTC ATG CCC AGA-3'; 5'-GTC TGG GCA TGG AAC TTC AAG AGA C-3'); Cys-141 (5'-GAA GTT TGC TCA GCC CCA GAC CTA CG-3'; 5'-CGT AGG TCT GGG GCT GAA ACT AC-3'); Cys-152 (5'-GTA TGG CTC TGG CTT ACG ATC AGG AGA AG-3'; 5'-CTT CTC CTC ATC AGC ACG AGC CAT AC-3'); Cys-220 (5'-GGA ATT CTG GAT CTA CTC ATC AGC AGC-3'); Cys-324 (5'-GTT AAT ACT GCA CAA GCC CCT TTA CCT CTG TTT GAC AGG TAA AGG GCC TTG TGC AGT AT-3'); Cys-331 (5'-CCT CTT TTC
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ACC GCT TTT CAT GTC AAA CCA-3'; 5'-GGT TTG ACA TGA AGA GCG GTG AAA AGA GG-3'); Cys-620 (5'-GCT GAA GGA GCC CTA TGT CTT TAA ACC-3'; 5'-GTT TTA AAG ACA TAG GCC TCC TTC AGC-3'); Cys-634 (5'-GGA GAA AGA TGC CCC AAC CAT CC-3'; 5'-GGG TGA TGG TTG GGG CAT CTT TCT CC-3'); Cys-726 (5'-GGA TCT GTT GCC TCT GCC CTT C-3'; 5'-GGG AAA CAG AGG CAC GAG ATG G-3'). 500 ng of wild-type cPLA$_2$α plasmid template and 125 ng of each primer were used in the PCR system for 16 cycles. Prior to transformation into XL1-Blue competent cells, the PCR product was digested for 1 h by DpnI enzyme at 37 °C to damage the wild-type template plasmid. All mutated plasmids were confirmed by DNA sequencing.

Immunoprecipitation and Western Blotting—Cells were lysed in Nonidet P-40 buffer (50 mM Tris-HCl, 2 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, pH 7.2) and homogenized on ice. Cell debris was removed by centrifugation at 14,000 rpm for 20 min at 4 °C. Whole cell lysates (~500 μg in 100 μl) were recovered and incubated with mouse anti-human cPLA$_2$α antibody (5 μl, 200 μg/ml) or mouse IgG for 1 h at 4 °C, followed by the addition of protein A/G-agarose beads (30 μl) for an extra 1 h. The protein-bead complex was washed four times with Nonidet P-40 buffer and subjected to regular Western blotting. 20 μl of sample buffer was added into protein-bead complex, and the mixture was boiled at 90 °C for 5 min. All of the supernatant was loaded onto polyacrylamide gel for electrophoresis. Transferred proteins in polyacrylamide gels were transferred onto nitrocellulose membrane (Bio-Rad), and the blot was blocked with 5% milk PBS-T (0.5% Tween 20) at room temperature. The membrane was incubated with primary antibodies (1:1000) for 1 h, followed by repeated washing with PBS-T. The membranes were incubated with the secondary antibody (1:1000) for 1 h at room temperature. After washing, the blots were developed using the ECL Western blotting detection system and exposed to Eastman Kodak Co. MR radiographic film.

Statistical Analysis—Statistical analysis was performed using Microsoft Excel 2003 software. Comparisons were performed using two-tailed unpaired Student's t test. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Overexpression of iNOS Induces cPLA$_2$α S-Nitrosylation and Activation—Given the documented role of cPLA$_2$α in AA metabolism and the known cross-interactions between iNOS and prostaglandin signaling pathways in human cells (25, 26), we sought to determine whether iNOS could regulate AA metabolism through S-nitrosylation of cPLA$_2$α protein. For this purpose, two human epithelial cell lines with high constitutive cPLA$_2$α expression (SG231 and A549) were transduced with the iNOS adenovirus expression vector or the control Y5 vector to determine the occurrence of cPLA$_2$α S-nitrosylation. As shown in Fig. 1A, forced expression of iNOS induced S-nitrosylation of cPLA$_2$α in both SG231 cells and A549 cells. Overexpression of iNOS had no effect on cPLA$_2$α protein level (Fig. 1A) or phosphorylation (see below). The level of cPLA$_2$α S-nitrosylation is directly proportional to the amount of iNOS protein expressed in those cells. Furthermore, iNOS overexpression significantly increased the release of AA from both SG231 and A549 cells (Fig. 1B). These data suggest that modulation of cPLA$_2$α by iNOS-mediated S-nitrosylation probably increases cPLA$_2$α enzymatic activity. Indeed, in vitro cPLA$_2$α activity assays confirmed that iNOS overexpression enhanced cPLA$_2$α activity by 3-fold in SG231 cells and 2-fold in A549 cells (Fig. 1C). These results provide the first evidence that iNOS is able to S-nitrosylate and activate cPLA$_2$α in human cells. The observation that the iNOS-induced increase of cPLA$_2$α activity is blocked by the specific iNOS inhibitor, 1400W, suggests that iNOS-mediated production of NO is required for cPLA$_2$α S-nitrosylation (Fig. 1C).

NO Donor Causes S-Nitrosylation and Activation of cPLA$_2$α—Next we utilized NO donor to evaluate the direct effect of NO on cPLA$_2$α S-nitrosylation and enzymatic activity. The addition of the NO donor GSN0 to the cell lysates isolated from SG231 cells resulted in S-nitrosylation of cPLA$_2$α, in vitro (Fig. 2A). The assay specificity was confirmed by the observation that omission of ascorbic acid in the assay system completely abolished the detection of S-nitrosylated cPLA$_2$α. Accordingly, we transduced SG231 cells with GSN0 significantly increased the activity in vitro (Fig. 2B). These observations document that NO donors are capable of directly S-nitrosylating and activating cPLA$_2$α as well as from A549 cells (Fig. 2C). Furthermore, l-Arg, a NO donor) also increased the enzyme activity of purified cPLA$_2$α (Fig. 2B). The iNOS-induced cPLA$_2$α S-nitrosylation and activity, as well as the availability of NO.

To further characterize the direct effect of NO on cPLA$_2$α protein S-nitrosylation and to exclude the potential influence of other molecules, cPLA$_2$α expression plasmid was transfected into HEK293 cells, and the recombinant cPLA$_2$α protein was purified by affinity chromatography. The purified recombinant cPLA$_2$α protein was then incubated with the NO donor to determine its S-nitrosylation and enzyme activity, in vitro. As shown in Fig. 3A, GSN0 induces the S-nitrosylation of purified cPLA$_2$α protein. Accordingly, the NO donors (GSNO and NOC-18) also increased the enzyme activity of purified cPLA$_2$α (Fig. 3B). These results demonstrate that S-nitrosylation and activation of cPLA$_2$α in cells is in direct proportion to the level cPLA$_2$α protein as well as the availability of NO.

The iNOS-induced cPLA$_2$α S-Nitrosylation and AA Release Is Enhanced by COX-2—In light of the documented functional coupling between cPLA$_2$α and COX-2 in human cells (5, 21–24), we further examined whether COX-2 might influence the iNOS-induced cPLA$_2$α S-nitrosylation. For this purpose, HEK293 cells were transfected with the expression vector for cPLA$_2$α, COX-2 and iNOS, either alone or in combination, to determine the S-nitrosylation of cPLA$_2$α. HEK293 cells were...
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**FIGURE 1.** Effect of iNOS overexpression on cPLA$_2\alpha$ S-nitrosylation and activity. A, iNOS overexpression induces S-nitrosylation of cPLA$_2\alpha$. SG231 and A549 cells were infected with different titers of iNOS or control Y5 adenovirus for 24 h, and cell lysates were collected to determine cPLA$_2\alpha$ S-nitrosylation by biotin switch as described under “Experimental Procedures.” The same samples were also subjected to regular Western blotting for detection of total cPLA$_2\alpha$ and iNOS. β-Actin was utilized to normalize protein loading. B, iNOS overexpression increases AA release. SG231 and A549 cells infected with different titers of Y5 or iNOS adenovirus were incubated with [5,6,8,9,11,12,14,15-$^3$H]arachidonic acid (0.5 µCi/ml culture medium) for 24 h. The cells were then washed five times to measure AA release as described under “Experimental Procedures.” The same samples were also subjected to regular Western blotting for detection of total cPLA$_2\alpha$ and iNOS. β-Actin was utilized to normalize protein loading. C, iNOS overexpression enhances cPLA$_2\alpha$ activity in vitro. SG231 and A549 cells were infected with Y5 (100 MOI for SG231 cells, 500 MOI for A549 cells) or iNOS adenovirus for 24 h with or without 1400W (100 µM) and then subjected to a cPLA$_2\alpha$ activity assay in vitro (a, p < 0.05 compared with Y5; b, p < 0.01 compared with lower titer iNOS or Y5; c, p < 0.01 compared with higher titer iNOS alone; n = 3).
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**FIGURE 2.** The effect of exogenous NO on cPLA$_2$$\alpha$ S-nitrosylation, activity, and AA release. A, the NO donor, GSNO, induces cPLA$_2$$\alpha$ S-nitrosylation, in vitro. The cell lysates isolated from SG231 cells were incubated with GSNO (20 $\mu$M) for 30 min. The samples were then subjected to the biotin switch assay in the presence or absence of ascorbate to determine cPLA$_2$$\alpha$ S-nitrosylation. B, the effect of NO donor and iNOS substrate on cPLA$_2$ activity. SG231 cells were incubated with GSNO (20 $\mu$M), l-arginine (1 mM) with or without 1400W (100 $\mu$M) for 30 min. The cell lysate was then collected and subjected to a cPLA$_2$ activity assay ($a$, $p < 0.05$ compared with vehicle; $b$, $p < 0.05$ compared with l-arginine treatment; $n = 3$). C, the effect of NO donor and iNOS substrate on AA release. SG231 and A549 cells were incubated with [5,6,8,9,11,12,14,15-$^3$H]arachidonic acid (0.5 $\mu$Ci/ml culture medium) for 24 h. The cells were then washed five times with culture medium, followed by incubation with GSNO (20 $\mu$M), NOC-18 (100 $\mu$M), l-Arg (1 mM), or vehicle Me$_2$SO for 1 h. The supernatant was collected to measure $^3$H radioactivity ($a$, $p < 0.05$ compared with vehicle; $n = 3$). D, the effect of NO donor on cPLA$_2$ activity in CCLP1 cells. CCLP1 cells were transfected with the cPLA$_2$$\alpha$ expression plasmid or the control MT2 plasmid for overnight. The cell lysate was then collected and incubated with GSNO (20 $\mu$M) or N-$\alpha$-monomethyl-l-arginine (250 $\mu$M) for 30 min at 4 °C. The samples were subsequently analyzed for cPLA$_2$ activity as described under “Experimental Procedures” ($a$, $p < 0.05$ compared with MT2; $b$, $p < 0.05$ compared with cPLA$_2$$\alpha$ transfection plus vehicle Me$_2$SO treatment; $n = 3$).

**FIGURE 3.** Purified cPLA$_2$$\alpha$ activity and S-nitro cPLA$_2$$\alpha$. A, the NO donor, GSNO, induces cPLA$_2$$\alpha$ activity in vitro. HEK293 cells were transfected with the cPLA$_2$$\alpha$ expression plasmid, and the cPLA$_2$$\alpha$ protein was then subjected to a cPLA$_2$ activity assay. The supernatant was collected to measure $^3$H radioactivity ($a$, $p < 0.05$ compared with vehicle; $n = 3$). B, the NO donors, GSNO and NOC-18, enhance the activity of purified cPLA$_2$$\alpha$. The purified cPLA$_2$$\alpha$ protein was then subjected to a cPLA$_2$ activity assay. The supernatant was collected to measure $^3$H radioactivity ($a$, $p < 0.05$ compared with vehicle; $n = 3$).
The effect of iNOS on the activation of cPLA2α in HEK293 cells. A, overexpression of COX-2 enhances iNOS-induced cPLA2α S-nitrosylation. HEK293 cells were transfected with the cPLA2α expression plasmid, COX-2 expression plasmid, or the control MT2 plasmid, with or without iNOS adenovirus. The cell lysates were subjected to a biotin switch assay to determine cPLA2α S-nitrosylation and conventional Western blotting to detect cPLA2α and COX-2, and iNOS. B, induction of COX-2 by cytokine mixture. HEK293 cells with or without overexpression of cPLA2α or iNOS were treated with cytokine mixture (50 ng/ml IL-1β, 50 ng/ml TNF-α, 100 ng/ml IFN-γ) for 24 h. The cell lysates were collected for SDS-PAGE and Western blotting. C, induction of COX-2 enhances iNOS-induced cPLA2α S-nitrosylation. HEK293 cells transfected with cPLA2α expression plasmid or the control MT2 plasmid, infected with iNOS or Y5 adenovirus were treated with cytokine mixture for 24 h. Some cells were treated with the iNOS inhibitor, 1400W (100 μM). The cells were processed for biotin switch assay and conventional Western blotting. D, effect of iNOS, cPLA2α, and COX-2 on AA release. HEK293 cells labeled with [3H]AA were transfected with cPLA2α expression plasmid or the control MT2 plasmid, infected with iNOS or Y5 adenovirus, or treated with the cytokine mixture for 24 h. At the end of treatment, the cells were washed five times, followed by culture with regular medium containing 0.5% FBS for 1 h. The culture medium was then collected for [3H]AA measurement using a liquid scintillation counter (a, p < 0.05 compared with MT2 transfection; b, p < 0.01 compared with cPLA2α + YS transfection; c, p < 0.01 compared with cPLA2α + YS + cytokines; d, p < 0.01 compared with cPLA2α + iNOS + cytokines; n = 3).

iNOS Induces Maximal PGE2 Production in the Presence of cPLA2α and COX-2—Since cPLA2α activation leads to the release of AA, providing substrate for COX-2-mediated PGE2 synthesis, we further examine the effect of iNOS, cPLA2α, and COX-2 in different combinations on PGE2 production. In HEK293 cells, combined iNOS expression and COX-2 induction in the absence of cPLA2α induced only a 2-fold increase of PGE2. (Fig. 7). When the cells were transfected with the cPLA2α expression vector, either iNOS expression or COX-2 induction induced a 2–3-fold increase of PGE2. Interestingly, the combination of cPLA2α, COX-2, and iNOS dramatically enhanced PGE2 production by more than 80-fold (Fig. 7). It is noteworthy that overexpression of cPLA2α and iNOS in combination with COX-2 induction achieved a much higher increase of PGE2 production than either iNOS plus cPLA2α or cPLA2α plus COX-2. A similar synergistic effect was also observed in the cells with endogenous expression of cPLA2α (SG231 and A549) (Fig. 8). iNOS overexpression plus COX-2 induction increased the production of PGE2 by nearly 40-fold in SG231 cells (Fig. 8A) and ~100-fold in A549 cells (Fig. 8B). These results demonstrate that maximal PG synthesis is achieved by the synergistic interactions among iNOS, cPLA2α, and COX-2.

Cys-152 of cPLA2α Is Essential for S-Nitrosylation—Upon completion of the above experiments, we further characterized the contribution of individual cysteine residues of cPLA2α to iNOS-induced S-nitrosylation. To this end, site-directed
mutagenesis was performed to replace each of the 9 cysteine residues with alanine (Cys-139, -141, -152, -220, -324, -331, -620, -634, and -726). The generated Cys-mutated cPLA₂α expression plasmid or wild type vector was transfected into HEK293 cells to determine cPLA₂α S-nitrosylation, enzyme activity, and AA release. As shown in Fig. 9A, mutation of Cys-152 markedly reduced the iNOS-induced cPLA₂α S-nitrosylation, whereas mutation of other cysteine residues exhibited no apparent effect. Accordingly, mutation of Cys-152, but not other cysteine residues, prevented iNOS-induced cPLA₂α activation (Fig. 9B) and arachidonic acid release (Fig. 9C). These results demonstrate that Cys-152 is the primary site for iNOS-induced cPLA₂α S-nitrosylation and enzymatic activation.

**DISCUSSION**

NO is a bioactive molecule with a single unpaired electron. The two major mechanisms whereby NO influences its intra-cellular targets are stimulation of guanylyl cyclase by direct binding of NO to iron in heme at the active site of guanylyl cyclase (37) and S-nitrosylation of protein targets on appropriate cysteines (34, 36, 38). S-Nitrosylation refers to the NO adduct with cysteine residue; it is increasingly recognized as an important posttranslational modification mechanism that mediates the diverse actions of NO. Our data in this study demonstrate that iNOS/NO S-nitrosylates and activates cPLA₂α, which represents a novel mechanism for the regulation of cPLA₂α in human cells. This assertion is based on the following observations: 1) iNOS overexpression induces cPLA₂α S-nitrosylation, enhances its catalytic activity, and increases the

**FIGURE 5. Influence of iNOS and COX-2 on the activation of endogenous cPLA2**

A, co-immunoprecipitation of cPLA₂α with iNOS in HEK293 cells. HEK293 cells were transfected with cPLA₂α or MT2 plasmid, infected with iNOS (50 MOI) or Y5 adenovirus, or treated with cytokine mixture (50 ng/ml IL-1β, 50 ng/ml TNF-α, 100 ng/ml IFN-γ) for 24 h. The cell lysates were collected and incubated with anti-cPLA₂α antibody or mouse IgG (negative control) for 1 h at 4°C, followed by incubation with protein A/G-agarose beads for 40 min. After five washes with lysis buffer, the protein-bound beads were subjected to Western blotting (WB) for detection of iNOS. B, co-immunoprecipitation of cPLA₂α with iNOS in SG231 cells. SG231 cells were transfected with iNOS (100 MOI) or control Y5 adenovirus and/or treated with cytokine mixture (50 ng/ml IL-1β, 50 ng/ml TNF-α, 100 ng/ml IFN-γ) or vehicle Me₂SO for 24 h. The cell lysates were then processed for immunoprecipitation and Western blot analysis. C, effect of the COX-2 inhibitor, NS-398, on cPLA₂α and iNOS association in SG231 cells. SG231 cells were infected with iNOS adenovirus (100 MOI), with or without cytokines (50 ng/ml IL-1β, 50 ng/ml TNF-α, 100 ng/ml IFN-γ) or NS-398 (25 μM) treatment for 24 h. The cell lysates were collected and subjected to immunoprecipitation and Western blotting. D, effect of iNOS on cPLA₂α and COX-2 association. SG231 cells were exposed to cytokines (50 ng/ml IL-1β, 50 ng/ml TNF-α, 100 ng/ml IFN-γ) with or without infection of iNOS adenovirus (100 MOI) for 24 h. The cell lysates were then collected and subjected to cPLA₂α immunoprecipitation and COX-2 Western blotting. Each figure is representative of three separated experiments.
release of AA from cells; 2) mutation of Cys-152 of cPLA₂α abolishes iNOS-induced cPLA₂α S-nitrosylation and enzymatic activation; 3) the level of cPLA₂α activity is blocked by the presence of denitrosylation, thus far beyond control and prediction under random conditions (46).

This observation is consistent with other studies showing that S-nitrosylated proteins display alterations in enzymatic activity, trafficking through intracellular compartments, stability in intracellular environment, binding with proteins, and other functions (34, 36, 39–45).

The occurrence of S-nitrosylation and concomitant modulation of protein function are regulated by factors including NO concentration fluctuation, other redox signaling, proximity of NO with targeted protein, intracellular location of protein, iron concentration, and the presence of denitrosylation, thus far beyond control and prediction under random conditions (46). In this study, the association between iNOS and cPLA₂α provides a mechanistic explanation for the noticeable effect of iNOS on cPLA₂α S-nitrosylation, which is consistent with the documented proximity between NOS and other targeted proteins for efficient S-nitrosylation (27, 36).
Activation of cPLA$_2$$\alpha$ through S-Nitrosylation

FIGURE 9. Cys-152 of cPLA$_2$$\alpha$ is critical for COX-2-mediated cPLA$_2$$\alpha$ S-nitrosylation

A. Site-directed mutagenesis was performed to replace each of the nine cysteine residues of cPLA$_2$$\alpha$ (Cys-139, -141, -152, -220, -234, -331, -620, -634, and -726). The generated mutants and wild-type cPLA$_2$$\alpha$ expression plasmid were then transfected into HEK293 cells to determine cPLA$_2$$\alpha$ S-nitrosylation, enzyme activity, and the release of arachidonic acid. A, Cys-152 mutation prevents iNOS-induced cPLA$_2$$\alpha$ S-nitrosylation. HEK293 cells were transfected with the mutated or wild-type cPLA$_2$$\alpha$ expression plasmid and infected with iNOS adenovirus (100 MOI) for 24 h. The cell lysates were collected and subjected to a biotin switch assay to detect cPLA$_2$$\alpha$ S-nitrosylation. Regular Western blotting was performed to determine the levels of cPLA$_2$$\alpha$ and iNOS proteins. B, mutation of Cys-152 prevents iNOS-induced cPLA$_2$$\alpha$ activity. HEK293 cells transfected with the mutated or wild-type cPLA$_2$$\alpha$ expression plasmid were infected with iNOS adenovirus (100 MOI) and exposed to cytokine mixture (50 ng/ml IL-1\(\beta\), 50 ng/ml TNF-\(\alpha\), 100 ng/ml IFN-\(\gamma\)) for 24 h. The cell lysates were subjected to a cPLA$_2$$\alpha$ activity assay, in vitro, as described under "Experimental Procedures" (*, p < 0.01 versus wild-type cPLA$_2$$\alpha$; n = 3). C, mutation of Cys-152 prevents iNOS-induced AA release. HEK293 cells transfected with the mutated or wild-type cPLA$_2$$\alpha$ expression plasmid were infected with iNOS adenovirus (100 MOI) and exposed to cytokine mixture (50 ng/ml IL-1\(\beta\), 50 ng/ml TNF-\(\alpha\), 100 ng/ml IFN-\(\gamma\)) for 24 h. The cells were analyzed for the release of [3H]arachidonic acid as described under "Experimental Procedures" (*, p < 0.01 versus wild-type cPLA$_2$$\alpha$; n = 3).

observed that transduction of iNOS adenovirus vector led to more S-nitrosylation of cPLA$_2$$\alpha$ than NO donors; this effect is most likely due to the higher concentration of NO accumulated at specific organelle or intracellular membrane in iNOS-overexpressed cells or due to the physical interaction between iNOS and cPLA$_2$$\alpha$, which allows efficient transfer of produced NO to cPLA$_2$$\alpha$.

Another important aspect of this study is the dramatic synergistic effect of COX-2 on iNOS-induced cPLA$_2$$\alpha$ S-nitrosylation and activation. Our results show that although cPLA$_2$$\alpha$ between COX-2 and iNOS (27) and our observation that iNOS is essential for communoprecipitation of cPLA$_2$$\alpha$ and COX-2, it is likely that iNOS may be the central molecule that recruits cPLA$_2$$\alpha$ and COX-2 to the binding complex.

The synergistic effect among iNOS, cPLA$_2$$\alpha$, and COX-2 for PGE$_2$ synthesis is highly significant, given that cPLA$_2$$\alpha$ and COX-2 are rate-limiting key enzymes for PG synthesis and that iNOS is the key enzyme for production of NO. Under inflammatory processes, activation of these key enzymes results in simultaneous production of NO and PGs. Therefore, it is pos-

S-nitrosylation is present in cells expressing iNOS and cPLA$_2$$\alpha$, the level of S-nitrosylated cPLA$_2$$\alpha$ is much higher in the presence of COX-2. For example, in HEK283 cells, a combination of iNOS, cPLA$_2$$\alpha$, and COX-2 induced a 20-fold increase of AA release, in contrast to the 4-fold increase induced by iNOS plus cPLA$_2$$\alpha$. Accordingly, the combination of cPLA$_2$$\alpha$, iNOS, and COX-2 dramatically enhanced PGE$_2$ production by more than 80-fold, in contrast to the 3-fold increase by iNOS plus cPLA$_2$$\alpha$ and less than 2-fold increase by COX-2 plus iNOS. A similar synergistic effect was also seen in two other epithelial cells (SG231 and A549). These findings indicate that COX-2 not only the ability to enhance iNOS-induced cPLA$_2$$\alpha$ S-nitrosylation and maximal PG synthesis is improved by the synergistic interaction among iNOS, cPLA$_2$$\alpha$, and COX-2. It is worth mentioning that COX-2-mediated cPLA$_2$$\alpha$ activation requires the presence of iNOS, further highlighting the synergistic effect of COX-2 on iNOS-induced cPLA$_2$$\alpha$ S-nitrosylation.

Since COX-2 increases the formation of cPLA$_2$$\alpha$-iNOS binding complex, it appears that COX-2-induced augmentation of cPLA$_2$$\alpha$ S-nitrosylation may be mediated at least in part through increased association between iNOS and cPLA$_2$$\alpha$. However, it is not clear at this time whether cPLA$_2$$\alpha$ directly binds to COX-2. We were able to coimmunoprecipitate COX-2 with cPLA$_2$$\alpha$ antibody in cells expressing cPLA$_2$$\alpha$, COX-2, and iNOS but not in cells without iNOS. Thus, it is possible that a multiprotein complex is formed, containing these three proteins with or without other molecules. Given the documented direct binding
References

1. Ghosh, M., Tucker, D. E., Burchett, S. A., and Leslie, C. C. (2006) Lipid Res. 48, 159–163.
2. Schaloske, R. H., and Dennis, E. A. (2006) J. Biol. Chem. 281, 19289–19297.
3. Murakami, M., and Kudo, I. (2002) J. Biol. Chem. 277, 145–182.
4. Capper, E. A., and Marshall, L. A. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 13977–13983.
5. Fitzpatrick, F. A., and Soberman, R. (2001) Trends Immunol. 22, 665–674.
6. Funk, C. D. (2001) Annu. Rev. Biochem. 70, 570–571.
7. Sambrano, G. R., and Angi, J. (2001) Biochem. J. 350, 1–11.
8. Li, G., Anderson, W. C., and Huang, Y. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 13977–13983.
9. Fujishima, H., Sanchez-Mejia, R. O., Ouchi, Y., Miyazaki, J., and Shimizu, T. (2000) J. Biol. Chem. 275, 618–622.
10. Nakatani, N., Uozumi, N., Kume, K., Murakami, M., Maki, K., Kudo, I., and Shimizu, T. (2002) Nat. Med. 8, 480–484.
11. Nagase, T., Uozumi, N., Ishii, S., Kume, K., Izumi, T., Ouchi, Y., and Shimizu, T. (2002) Nat. Med. 8, 480–484.
12. Tabuchi, S., Uozumi, N., Ishii, S., Shimizu, Y., Watanabe, T., and Shimizu, T. (2002) Biochim. Biophys. Acta 150–166.
13. Marusic, S., Leach, M. W., Pelker, J. W., Azoitei, M. L., Uozumi, N., Cui, J., Shen, M. W., DeClercq, C. M., Miyashiro, J. S., Carito, B. A., Thakker, P., Hogen, M., Sun, L., Uozumi, N., Kume, K., Goad, M. E., Nickerson-Nutter, R., and Stamler, J. S. (2007) Proc. Natl. Acad. Sci. U. S. A. 104, 20450–20463.
14. Hegen, M., Sun, L., Uozumi, N., Kume, K., Goad, M. E., Nickerson-Nutter, R., and Stamler, J. S. (2007) Biochemistry 46, 7503–7511.
15. Han, C., Demetris, A. J., Liu, Y., Shelhamer, J. H., and Wu, T. (2006) FEBS Lett. 579, 387–391.
16. Bonventre, J. (2004) Neuron 45, 1297–1302.
17. Miyaura, C., Inada, M., Matsumoto, C., Ohshiba, T., Uozumi, N., Shimizu, T., and Kume, K. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 96, 1297–1302.
18. Komagata, Y., Maki, K., Ikuta, K., Ouchi, Y., Miyazaki, J., and Shimizu, T. (2002) J. Biol. Chem. 277, 579, 618–622.
19. Oikawa, Y., Yamato, E., Tashiro, F., Yamamoto, M., Uozumi, N., Shimada, T., and COX-2, it is possible that this mechanism may be implicated in the synthesis of active prostanoids and leukotrienes.
20. Nakatani, N., Uozumi, N., Kume, K., Murakami, M., Maki, K., Kudo, I., and Shimizu, T. (2002) Nat. Med. 8, 480–484.
21. Komagata, Y., Maki, K., Ikuta, K., Ouchi, Y., Miyazaki, J., and Shimizu, T. (2002) J. Biol. Chem. 277, 579, 618–622.
22. Marushima, K., Kambe, T., Shimbara, S., and Kudo, I. (1999) J. Biol. Chem. 274, 169–172.
23. Schaloske, R. H., and Dennis, E. A. (2006) J. Biol. Chem. 281, 19289–19297.
24. Schaloske, R. H., and Dennis, E. A. (2006) J. Biol. Chem. 281, 19289–19297.
25. Schaloske, R. H., and Dennis, E. A. (2006) J. Biol. Chem. 281, 19289–19297.
26. Schaloske, R. H., and Dennis, E. A. (2006) J. Biol. Chem. 281, 19289–19297.
27. Schaloske, R. H., and Dennis, E. A. (2006) J. Biol. Chem. 281, 19289–19297.
28. Schaloske, R. H., and Dennis, E. A. (2006) J. Biol. Chem. 281, 19289–19297.
29. Schaloske, R. H., and Dennis, E. A. (2006) J. Biol. Chem. 281, 19289–19297.
30. Schaloske, R. H., and Dennis, E. A. (2006) J. Biol. Chem. 281, 19289–19297.
31. Schaloske, R. H., and Dennis, E. A. (2006) J. Biol. Chem. 281, 19289–19297.
32. Schaloske, R. H., and Dennis, E. A. (2006) J. Biol. Chem. 281, 19289–19297.
33. Schaloske, R. H., and Dennis, E. A. (2006) J. Biol. Chem. 281, 19289–19297.
34. Schaloske, R. H., and Dennis, E. A. (2006) J. Biol. Chem. 281, 19289–19297.
35. Schaloske, R. H., and Dennis, E. A. (2006) J. Biol. Chem. 281, 19289–19297.
36. Schaloske, R. H., and Dennis, E. A. (2006) J. Biol. Chem. 281, 19289–19297.
37. Schaloske, R. H., and Dennis, E. A. (2006) J. Biol. Chem. 281, 19289–19297.
38. Schaloske, R. H., and Dennis, E. A. (2006) J. Biol. Chem. 281, 19289–19297.
39. Schaloske, R. H., and Dennis, E. A. (2006) J. Biol. Chem. 281, 19289–19297.
40. Schaloske, R. H., and Dennis, E. A. (2006) J. Biol. Chem. 281, 19289–19297.
41. Schaloske, R. H., and Dennis, E. A. (2006) J. Biol. Chem. 281, 19289–19297.
42. Schaloske, R. H., and Dennis, E. A. (2006) J. Biol. Chem. 281, 19289–19297.
43. Schaloske, R. H., and Dennis, E. A. (2006) J. Biol. Chem. 281, 19289–19297.
44. Schaloske, R. H., and Dennis, E. A. (2006) J. Biol. Chem. 281, 19289–19297.
45. Schaloske, R. H., and Dennis, E. A. (2006) J. Biol. Chem. 281, 19289–19297.
46. Schaloske, R. H., and Dennis, E. A. (2006) J. Biol. Chem. 281, 19289–19297.
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