Overexpression of Cyclooxygenase-2 Induces Cell Cycle Arrest

EVIDENCE FOR A PROSTAGLANDIN-INDEPENDENT MECHANISM*

Ovidiu C. Trifan, Robert M. Smith, Brian D. Thompson, and Timothy Hla‡

From the Center for Vascular Biology, Department of Physiology, University of Connecticut Health Center, Farmington, Connecticut 06030-3505

(Received for publication, July 14, 1999, and in revised form, August 9, 1999)

The immediate-early gene cyclooxygenase 2 (Cox-2) is induced in a variety of hyperplastic pathological conditions, including rheumatoid arthritis and colorectal cancer. Although a causal role for Cox-2 has been proposed, mechanisms by which Cox-2 function contributes to the pathogenesis of hyperplastic disease are not well defined. We constructed a green fluorescent protein-tagged Cox-2 (Cox-2-GFP) to examine its effects on a variety of cell types upon overexpression. Subcellular localization and enzymatic and pharmacological properties of Cox-2-GFP polypeptide were indistinguishable from those of the wild-type Cox-2 polypeptide. Overexpression of the Cox-2-GFP or the Cox-2 polypeptide by transient transfection suppressed the population of cells in the S phase of the cell cycle, with a concomitant increase in G1/G0 population. In contrast, transient overexpression of GFP had no effect on cell cycle distribution, whereas endoplasmic reticulum-retained GFP (GFP-KDEL) overexpression was associated with only a minor decrease of cells in S phase. Interestingly, neither NS-398 (a Cox-2-specific inhibitor) nor indomethacin could reverse the effect of Cox-2-GFP overexpression on cell cycle progression. Furthermore, two mutants of Cox-2, S516Q and S516M, which lack the cyclooxygenase activity, exhibited the same effect as Cox-2-GFP. The cell cycle effect of Cox-2-GFP was observed in ECV-304, NIH 3T3, COS-7, bovine microvascular endothelial cells, and human embryonic kidney 293 cells. These findings suggest that Cox-2 inhibits cell cycle progression in a variety of cell types by a novel mechanism that does not require the synthesis of prostaglandins.

Cyclooxygenase is the rate-limiting enzyme in the production of prostaglandins and thromboxanes, which are involved in numerous physiologic and pathologic processes such as inflammation, pain, angiogenesis, and the regulation of vascular tone. The Cox1 isoenzymes (the “constitutive” Cox-1 and the “inducible” Cox-2) have both overlapping as well as distinct physiologic and pathologic functions (1, 2). They have similar subcellular localization in the ER and on the inner and outer membranes of the nuclear envelope (3). The Cox enzymes catalyze two distinct reactions: 1) the conversion of AA to PGH2 via the cyclooxygenase activity and 2) the reduction of PGG2 to PGGH2 via the peroxidase activity. PGH2 is converted by distinct isomerases into biologically active prostaglandins, including PGD2 and PGE2. Nonsteroidal anti-inflammatory drugs such as aspirin and indomethacin inhibit the cyclooxygenase activity but not the peroxidase activity (4, 5). Recently developed Cox-2-specific inhibitors such as NS-398, celecoxib (SC-58635), and rofecoxib likewise inhibit the cyclooxygenase activity of the Cox-2 isoenzyme and thus inhibit prostanooid synthesis (6). Most biologically active prostanooids act on plasma membrane-localized G-protein-coupled receptors (7). Some prostanooids, such as 15-deoxy-12,14-PGJ2, act via the nuclear peroxisomal proliferator activator receptor (8, 9).

Overexpression of Cox-2 is associated with a variety of proliferative diseases, such as rheumatoid arthritis, colorectal cancer, and gastric cancer (1, 2). Cox-2-selective inhibitors may be chemopreventive in colorectal cancer development (10, 11).

Vascular endothelial cells are critical for the initiation and maintenance of the angiogenic response, a major process by which new blood vessels are formed in the adult (19). We originally cloned the Cox-2 eDNA from human umbilical vein endothelial cells and showed that Cox-2 expression is induced by various angiogenic factors, such as fibroblast growth factor-1, the tumor promoter PMA, and the inflammatory cytokine.
interleukin-1 (2, 20, 21). PMA, which inhibits growth of human umbilical vein endothelial cells and promotes angiogenesis in vitro as well as in vivo, induces an increase in Cox-2 mRNA expression, with minimal change in Cox-1 mRNA levels. Activation of endothelial cell Cox-2 expression results in the formation of various prostanoids, such as prostacyclin and PGE₂, as well as HETEs (22). Thus, Cox-2-derived prostanoids were thought to mediate endothelial cell functions including inhibition of thrombosis and regulation of vascular tone and permeability (23). Indeed, PGE₂ induces vascular endothelial cell growth factor expression and promotes angiogenesis (24). Overexpression of Cox-2 is associated with chronic inflammatory diseases such as rheumatoid arthritis and solid tumor development (2, 10, 24), both of which are characterized by enhanced angiogenesis.

To study the effects of Cox-2 function, we attempted to derive endothelial and nonendothelial cells overexpressing Cox-2. Our efforts were routinely unsuccessful or resulted in clones that transiently expressed the transgene at low levels (17). Such cells grew at reduced rates, and loss of transgene expression resulted in growth enhancement. These anecdotal observations suggested that Cox-2 overexpression may confer a growth disadvantage. In this report, we studied this phenomenon in detail using a GFP-tagged Cox-2 chimeric protein.

MATERIALS AND METHODS

Cell culture and Transfection—ECV-304 cells were obtained from Dr. Thomas Maciag (Maine Medical Center, Portland, ME). They were cultured in M199 medium (Cellgro, Mediatech) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone) and antimiycotic-antibiotic mixture (Life Technologies, Inc). Bovine microvascular endothelial cells (Cell Systems) were grown in fibronectin-coated dishes in M199 supplemented with 20% fetal bovine serum. NIH 3T3 (ATCC CRL-6361), HEK 293 (ATCC CRL-1570), and COS-7 cells were grown in Dulbecco’s modified Eagle’s medium (Cellgro, Mediatech) plus 10% fetal bovine serum and antimiycotic/antibiotic mixture. All plasmid-mediated transfections were performed on attached cells at 30–40% confluence using LipofectAMINE Plus (Life Technologies, Inc.) or NovaFECTOR (VennNova, Inc.). Indomethacin was purchased from Sigma, and NS-398 was purchased from Calbiochem. Cox-2-GFP and other Constructs—pCDNA/Neo and pCDNA/Zeo 3.1 were obtained from Invitrogen. Cox-2, Cox-2-S516Q, and S516M mutants (25) in pOSML were kindly gifts of Dr. David L. DeWitt and William Smith (Michigan State University). GFP-KDEL cDNA was generously provided by Dr. Mark Terasaki (UConn Health Center) in pSP64 (26) and subsequently cloned in the pCDNA Zeo 3.1 expression vector. 9-β-D-Desatursase cDNA (27) cloned in pEGFP-N1 was kindly provided by Dr. Juris Ozols (UConn Health Center). Cox-2-GFP, S516Q-Cox-2-GFP (S516Q), and S516M-Cox-2-GFP (S516M) constructs were made by cloning the polymerase chain reaction-amplified GFP open reading frame (excluding the initiator methionine and the termination codon) into the BspEI site of the human Cox-2 cDNA (position 1854 (20)). The insertion did not interrupt the open reading frame and was in the C-terminal 18-amino acid Cox-2-specific region (Fig. 1A).

Prostanoid Analysis—PGE₂ release in the culture medium by cells stimulated for 15 min with 30 μM AA was used as an indicator of Cox-2 activity. PGE₂ was determined by radioimmunoassay utilizing the kit, with the following modifications. BrdUrd concentration was 50 μM, and BrdUrd was counted. ECV cells transfected with GFP or GFP-KDEL were nontreated with an anti-GFP antibody. ECL detection reagents (Amersham Pharmacia Biotech) were used to visualize immunoblot signals.

Flow Cytometry Cell Cycle Analysis, BrdUrd Incorporation—48 h after transfection, cells were washed with PBS, trypsinized, fixed with 70% ethanol, and stored at −20 °C. Later cells were stained for 30 min at room temperature with a 20 μg/ml PI (Sigma) solution in PBS containing 0.1% Triton X-100 (Sigma) and 0.2 mg/ml DNase-free RNase A (Sigma). Cells were analyzed on a FACSCalibur flow cytometer (Becton and Dickenson). For each sample, at least 10,000 fluorescent cells were counted. ECV cells transfected with GFP or GFP-KDEL were sorted first in a FACS StarPlus cell sorter into green fluorescent and non-green fluorescent populations. After ethanol fixation, nuclear DNA was stained with PI, and cells were analyzed by FACS. After gating out cellular aggregates and debris, cell cycle distribution of fluorescent or nonfluorescent cells was analyzed using ModFitLT V2.0 software. For each condition, at least three different experiments were performed.

Pulse labeling with BrdUrd was conducted in 3-cm glass-bottom dishes using the Roche Molecular Biochemicals labeling and detection kit, with the following modifications: BrdUrd concentration was 50 μM, cells were fixed in 70% ethanol in H₂O₂, the incubation with the primary antibody was increased to 45 min, and the secondary anti-mouse antibody was conjugated with tetramethylrhodamine isothiocyanate. To visualize the nuclei, all samples were stained for 5 min with 1 μg/ml Hoechst 33342 (Sigma). For both green fluorescent and nonfluorescent populations, BrdUrd-positive cells (red nuclei) were counted and expressed as percentage of the total corresponding population (blue nuclei).

**FIG. 1.** Cox-2-GFP chimeric protein. A, Cox-2-GFP was obtained by inserting 720 base pairs (bp) of the GFP open reading frame (ORF) (from pEGFP-N1 vector; CLONTECH) into the BspEI site of Cox-2, near to the 3’ end of the open reading frame. This corresponds to the insertion of 240 amino acids (aa) from GFP to the C-terminal end of Cox-2 polypeptide (between amino acids Ser-586 and Gly-587). The salient features of the Cox-2-GFP polypeptide are indicated (T = N-linked glycosylation sites). B, ECV-304 cells were transiently transfected with pOSML vector (Ctrl), Cox-2-GFP (C2g), and Cox-2 in POSML expression vector or pEGFP-N1 plasmid for 14–16 h. 48 h later cell extracts were prepared and analyzed by immunoblotting with an anti-GFP antibody as described. The predicted molecular mass of the Cox-2-GFP chimeric protein is 99–101 kDa.

nontreated with an anti-GFP antibody. ECL detection reagents (Amersham Pharmacia Biotech) were used to visualize immunoblot signals.
Cells growing on glass coverslips or in glass-bottom 3-cm dishes were subjected to transient transfection with different variants of Cox-2 using lipofection reagents. 24–48 h later, cells were washed with PBS and fixed for 15 min with a 4% paraformaldehyde solution in PBS. For visualization of the nucleus, cells were stained for 5 min with 1 μg/ml Hoechst 33342 in PBS. Immunohistochemistry was carried out as follows. Cells were permeabilized for 5 min with 0.2% Triton X-100, washed with PBS, and incubated for 90 min with various primary antisera. After several washes, the secondary antibody, fluorescein isothiocyanate- or tetramethylrhodamine isothiocyanate-conjugated antibody was incubated with the sample for 30 min. If necessary, additional Hoechst 33342 staining was performed. All specimens were mounted in 80% glycerol and examined/photographed with a Zeiss-Axiovert 100 fluorescence microscope. All antibodies used for immunohistochemistry or Western blotting were purchased from Santa Cruz and Cayman.

RESULTS AND DISCUSSION

Characterization of the Cox-2-GFP Chimeric Polypeptide—
The Cox-2-GFP chimeric protein was constructed by insertion of the 27-kDa GFP polypeptide into the C-terminal 18-amino acid insert region of the Cox-2 polypeptide. This region, known to reside in the lumen of the ER, is dispensable for Cox-2 enzymatic activity (4, 25). The GFP open reading frame, excluding the initiator methionine and the termination codon, was polymerase chain reaction-amplified and cloned into the BspEI site of the human Cox-2 cDNA (Fig. 1A). We next delineated the biochemical characteristics and the enzymatic function of the Cox-2-GFP polypeptide. As shown in Fig. 1B, transfection of Cox-2-GFP into ECV-304 cells resulted in the production of a band at approximately 100 kDa, which was detected by the anti-GFP antibody. COS-7 cells transiently transfected with Cox-2 or Cox-2-GFP yielded comparable amounts of PGE$_2$; this synthesis was completely inhibited by NS-398 (Fig. 2A). These data suggest that the Cox-2-GFP polypeptide is enzymatically active. Incubation of Cox-1, Cox-2, or Cox-2-GFP-transfected ECV-304 cells with [14C]AA led to the release of prostanoids, which were further analyzed by TLC and autoradiography. As shown in Fig. 2B, a similar pattern of prostanoids and HETEs was produced by all three transfections, suggesting that the Cox-2-GFP polypeptide is capable of coupling to isomerases in a manner similar to the wild-type protein. As shown previously, the human Cox-2 mRNA is highly unstable and does not accumulate to high levels in transfected cells (20). Cox-2-GFP expression level, as determined by enzyme activity measurements, was slightly higher than the native Cox-2.

Fluorescence microscopic imaging of Cox-2-GFP-transfected cells was conducted to determine the subcellular localization of the Cox-2-GFP. As shown in Fig. 1B, the Cox-2-GFP chimeric protein was constructed by insertion of the 27-kDa GFP polypeptide into the C-terminal 18-amino acid insert region of the Cox-2 polypeptide. This region, known to reside in the lumen of the ER, is dispensable for Cox-2 enzymatic activity (4, 25). The GFP open reading frame, excluding the initiator methionine and the termination codon, was polymerase chain reaction-amplified and cloned into the BspEI site of the human Cox-2 cDNA (Fig. 1A). We next delineated the biochemical characteristics and the enzymatic function of the Cox-2-GFP polypeptide. As shown in Fig. 1B, transfection of Cox-2-GFP into ECV-304 cells resulted in the production of a band at approximately 100 kDa, which was detected by the anti-GFP antibody. COS-7 cells transiently transfected with Cox-2 or Cox-2-GFP yielded comparable amounts of PGE$_2$; this synthesis was completely inhibited by NS-398 (Fig. 2A). These data suggest that the Cox-2-GFP polypeptide is enzymatically active. Incubation of Cox-1, Cox-2, or Cox-2-GFP-transfected ECV-304 cells with [14C]AA led to the release of prostanoids, which were further analyzed by TLC and autoradiography. As shown in Fig. 2B, a similar pattern of prostanoids and HETEs was produced by all three transfections, suggesting that the Cox-2-GFP polypeptide is capable of coupling to isomerases in a manner similar to the wild-type protein. As shown previously, the human Cox-2 mRNA is highly unstable and does not accumulate to high levels in transfected cells (20). Cox-2-GFP expression level, as determined by enzyme activity measurements, was slightly higher than the native Cox-2.

Fluorescence microscopic imaging of Cox-2-GFP-transfected cells was conducted to determine the subcellular localization of the Cox-2-GFP. As shown in Fig. 2C, the Cox-2-GFP exhibited green fluorescence at the ER and the nuclear membrane, similar to the localization of the wild-type Cox-2 protein. Immunostaining of the Cox-2-GFP-transfected ECV-304 cells with anti-Cox-2 antibody, followed by detection with rhodamine-conjugated secondary antibodies, showed extensive colocalization of the green fluorescence and the Cox-2 epitopes, suggesting that...
Cox-2 and Growth Arrest

Overexpression of Cox-2-GFP Causes G₀/G₁ Arrest—Next we determined the effect of Cox-2-GFP overexpression on cell cycle progression. ECV-304 cells were transiently transfected with the Cox-2-GFP, and cell cycle distribution was analyzed by DNA staining with PI followed by FACS analysis. We analyzed the cell cycle characteristics of GFP-positive (Cox-2-GFP expressing; Fig. 3A) as well as GFP-negative (Cox-2-GFP nonexpressing; Fig. 3B) cells from the same plate by gating the high and low intensity green fluorescence signal in the analysis software. Cox-2-GFP expression into ECV-304 cells was associated with a significant decrease in S phase population (from 50% to 17%) and a 2-fold increase in G₀/G₁ population (from 23% to 50%) compared with the nonfluorescent cells (Fig. 3C).

Cell cycle characteristics of GFP-negative cells are similar to those of untransfected or vector-transfected counterparts.

To assess the specificity of G₀/G₁ arrest subsequent to the overexpression of Cox-2-GFP, we conducted several control experiments. First, we overexpressed the cytosolic GFP and ER-localized GFP-KDEL proteins in ECV-304 cells (27). As shown in Fig. 3C, GFP expression did not alter cell cycle distribution, whereas GFP-KDEL caused a minor decrease in S phase, from 55 to 41%. To determine the fate of cells transfected with Cox-2-GFP, ECV-304 green fluorescent cells were sorted with a FACS StarPlus cell sorter and maintained in culture for several days. Two distinct phenomena were observed. Some cells lost their fluorescence (implying a loss of Cox-2-GFP expression), and many fluorescent cells rounded and died. After 1 week in culture, less than 1% of the cells maintained their fluorescence. In no case was proliferation of Cox-2-GFP-positive cells seen, whereas GFP-positive colonies were routinely observed. These data strongly suggest that Cox-2-GFP overexpression in ECV-304 cells results in G₀/G₁ arrest, which leads to cell growth disadvantage and, ultimately, to cell death.

We also overexpressed Cox-2-GFP in other cell lines such as HEK 293, COS-7, and NIH 3T3 cells. Cox-2-GFP expression caused a variable extent of growth arrest as indicated by a decrease in the population of cells in S phase (Fig. 3D). For COS-7 cells, there was a 3-fold decrease in the percentage of cells in S phase, from 30 to 11%; for NIH 3T3, from 28 to 16%; and for HEK 293 cells, from 21% to 13%. Interestingly, HEK 293 and COS-7 cells exhibited a concomitant increase in the G₀/M population. Fluorescence microscopic visualization of Cox-2-GFP-positive cells indicates an increase in binucleated cells, which appear as a part of the G₀/M population in the FACS analysis. This is further documented in Fig. 4.

To validate the cell cycle data obtained by FACS analysis, we utilized the BrdUrd incorporation assay to determine the population of cells in the S phase. Quantitation of BrdUrd incorporation by Cox-2-GFP-positive and -negative cells is shown in Fig. 3E. Both Cox-2 and Cox-2-GFP induced a similar degree of S-phase suppression in ECV-304 cells. Bovine microvascular endothelial cells exhibited a similar behavior with Cox-2-GFP overexpression but not in response to the overexpression of 9-N-desaturase (an ER resident enzyme involved in fatty acid metabolism (27)). These data strongly suggest that overexpression of transfected Cox-2 enzyme results in growth arrest of a variety of cell types.

We also observed that Cox-2-GFP overexpression is associated with changes in nuclear morphology. Untransfected ECV-304 cells exhibit primarily round nuclei. Occasionally, mitotic, kidney-shaped, or apoptotic nuclei are observed. ECV cells transfected with GFP-KDEL have a similar nuclear morphology (Fig. 4A). In contrast, a large number of Cox-2-GFP positive

**Fig. 3.** FACS analysis of the cell cycle. Transfected cells were fixed with 70% ethanol at -20 °C, stained for 30 min at room temperature with a PI-Triton-RNase PBS solution, and analyzed on a FACS-Calibur flow cytometer. A, typical histogram of PI staining intensity of ECV-304 cells transfected with Cox-2-GFP after gating only the high intensity green fluorescent cells. B, the corresponding nonfluorescent population. C, comparison of cell cycle distribution of ECV-untransfected (CTRL) and -transfected cells (expressing (GFP⁺)) or not expressing the transfrectant protein (GFP⁻) when transfected with Cox-2-GFP, GFP, and GFP-KDEL. D, comparison of cell cycle distribution of COS-7, NIH 3T3, and HEK 293 cells transfected with Cox-2-GFP (C2g). Statistical significance of the S phase reduction in green fluorescent population comparative with nonfluorescent population was calculated using the paired Student t test. p values are as follow: COS-7 cells, p < 0.01; NIH 3T3 cells, p < 0.01; HEK 293 cells, p < 0.01. E, S-phase evaluation by pulse-labeling with BrdUrd. ECV-304 cells were transfected with Cox-2 and Cox-2-GFP (C2g), and bovine microvascular endothelial cells (BMEC) were transfected with Cox-2-GFP (C2g) and GFP-tagged 9-N-desaturase. For each experimental condition, transfected (striped columns) and untransfected cells (solid columns) were compared for their ability to incorporate BrdUrd as described. In all experiments except bovine microvascular endothelial cells transfected with GFP tagged 9-N-desaturase, BrdUrd incorporation was significantly decreased in green fluorescent population; p < 0.05. The results are presented as the mean ± S.D. of 2–6 independent triplicate transfections.
cells exhibit prominent kidney-shaped, budding, or multilobular nuclei (Table I) or are binucleated (Fig. 4B). Similar morphological changes were induced by Cox-2 transfection visualized with anti-Cox-2 antibody (Fig. 4C). As shown in Table I, 44% of the Cox-2-GFP-positive cells undergo various degrees and types of nuclear morphological changes (including specific apoptotic nuclear morphology) compared with 11% in nonfluorescent cells. Out of the green fluorescent ECV cells, 12% are binucleated, compared with only 1.5% in the nonfluorescent cell population. These data suggest that overexpression of Cox-2-GFP or Cox-2 results in growth arrest as well as structural changes in the nuclear architecture, most likely due to defective nuclear envelope breakdown and/or cytokinesis. This may eventually result in increased cell death, which accompanies Cox-2-GFP overexpression. Similar to ECV Cox-2-GFP-transfected cells, COS-7 (Fig. 4D) and HEK 293 cells (Fig. 4E) displayed various changes of nuclear morphology, in particular a very high frequency of binucleated cells. Such a phenotype is most likely generated by a defect in cytokinesis. This may explain the increase in G2/M population (as opposed to G1/G0) for COS-7 and HEK 293 cells transfected with Cox-2-GFP (Fig. 3D).

A Prostanoid-independent Mechanism Is Involved in G0-G1 Arrest Caused by Cox-2—We next determined whether prostanoid secretion by the Cox-2-GFP-transfected cells is neces-

![Fig. 4. Abnormal nuclear morphology of cells transiently transfected with Cox-2 or Cox-2-GFP.](image)

**Table I**

*Nuclear morphology changes induced in ECV-304 cells by transient transfection*

| Experimental condition | Population | Apoptotic nuclei | Advanced kidney shape | Incomplete lobulation (budding, shape changes) | Binuclear or multiple lobes | Total abnormal nuclei |
|------------------------|------------|------------------|-----------------------|-----------------------------------------------|-----------------------------|----------------------|
| **Cox-2-GFP**          | GFP+       | 6.5 ± 1.0        | 10.6 ± 2.8            | 14.7 ± 1.6                                    | 11.9 ± 1.8                  | 43.7 ± 1.9           |
|                        | GFP−       | 3.6 ± 0.8        | 3.5 ± 1.2             | 2.5 ± 0.5                                     | 1.5 ± 0.9                   | 11.1 ± 1.2           |
| **GFP-KDEL**           | GFP+       | 3.2 ± 0.3        | 5.0 ± 1.9             | 3.9 ± 2.9                                     | 2.8 ± 1.7                   | 14.9 ± 0.8           |
|                        | GFP−       | 2.1 ± 0.3        | 4.1 ± 0.5             | 5.4 ± 1.6                                     | 0.4 ± 0.4                   | 11.9 ± 2.2           |
| **Cox-2-GFP + indomethacin** | GFP+    | 6.5 ± 0.5        | 8.5 ± 2.4             | 16.9 ± 5.5                                    | 9.8 ± 2.6                   | 42.0 ± 7.3           |
|                        | GFP−       | 4.2 ± 1.1        | 2.5 ± 0.6             | 3.5 ± 2.6                                     | 0.9 ± 0.4                   | 11.1 ± 3.3           |
| **Cox-2-GFP + NS-398** | GFP+       | 4.4 ± 2.2        | 9.8 ± 2.1             | 15.9 ± 0.7                                    | 13.3 ± 1.0                  | 43.4 ± 2.8           |
|                        | GFP−       | 4.1 ± 0.7        | 2.0 ± 0.6             | 2.4 ± 0.9                                     | 0.8 ± 0.3                   | 9.2 ± 1.1            |
Cox-2 and Growth Arrest

Fig. 5. Cell cycle arrest induced by Cox-2-GFP is PG-independent. A, protein extracts from ECV-304 cells transfected with GFP, Cox-2, Cox-2-GFP (C2g), S516Q-Cox-2-GFP (SQ), and S516M-Cox-2-GFP (SM) were immunoblotted with anti-GFP antibody. The predicted molecular mass of the Cox-2-GFP chimeric protein is 99–101 kDa. B, after 48 h, ECV-304 cells untransfected or transfected overnight with Cox-2, Cox-2-GFP (C2g), and S516Q and S516M mutants were incubated for 15 min with 12.5 μM [3H]AA at 37 °C. Lipids were then extracted from cellular supernatant medium and analyzed further by TLC and autoradiography. C, comparison of cell cycle distribution of ECV cells expressing (GFP+) or not expressing (GFP−) the transfectant protein, when transfected with Cox-2-GFP, S516Q, and S516M. In two experimental groups, cells transfected with Cox-2-GFP were treated for 48 h with 2 μM indomethacin and 10 μM NS-398 before harvesting for FACS. Statistical significance of the S-phase reduction and G0-G1 increase in green fluorescent population compared with the nonfluorescent population was calculated using the paired student t test. P values are as follows. ECV cells transfected with Cox-2-GFP, for S phase, p < 0.001, and for G0-G1 phase, p < 0.001. In indomethacin-treated cells, for S phase, p < 0.01, and for G0-G1 phase, p < 0.01. In NS-398-treated cells, for S phase, p < 0.01, and for G0-G1 phase, p < 0.01. In S516Q-transfected cells, for S phase, p < 0.01, and for G0-G1 phase, p < 0.01. In S516M-transfected cells, for S phase, p < 0.001, and for G0-G1 phase, p < 0.001. In S516Q-transfected cells, for G0-G1 phase, p < 0.01. In S516M-transfected cells, for S phase, p < 0.001, and for G0-G1 phase, p < 0.001. D, S-phase evaluation by pulse labeling with BrdUrd. ECV-304 cells were transfected overnight in glass-bottom dishes with Cox-2 and two Cox-2-GFP mutants, S516Q and S516M. 24 h after transfection, cells were fixed in 70% ethanol and stained with anti-BrdUrd antibody, followed by tetramethylrhodamine isothiocyanate-conjugated secondary antibody and Hoechst 33342. For each cellular population, expressing (stippled columns) or not expressing (solid columns) the green fluorescent transfectant protein, BrdUrd incorporating red-stained nuclei are expressed as percentage from total number of nuclei (blue-stained). The reduction in S phase is statistically significant, with p < 0.05 for all three experimental condition. All results are presented as mean ± S.D. of 2–6 independent replicate transfections.

sary for the cell cycle arrest caused by Cox-2-GFP. As shown in Fig. 5A, pretreatment of Cox-2-GFP-transfected cells with 2 μM indomethacin and 10 μM NS-398 for 48 h did not inhibit cell cycle arrest. Such treatment resulted in near complete (>95%) inhibition of prostanoid secretion of the Cox-2-GFP-transfected cells (Fig. 2A and data not shown). To further demonstrate the independence of prostanoid synthesis, we utilized two active-site mutants of Cox-2, initially characterized by Smith and coworkers (4, 25). These mutants, S516Q and S516M, mimic the aspirin-treated Cox-2 and do not produce any PGs (25). However, the S516M mutant possesses a partial oxidative activity, resulting in the production of cell-associated 15(R)-HETE (4, 25). Western blot analysis with anti-GFP antibody on protein extracts from ECV-304 cells transfected with S516Q and S516M yielded an immunoreactive band at the same molecular weight as Cox-2-GFP (Fig. 5A). TLC analysis of the lipids released into the culture medium confirmed the lack of cyclooxygenase activity in cells transfected with these two mutants (Fig. 5B). Since 15(R)-HETE is poorly secreted, we could not detect significant increases of the 15(R)-HETE band in the medium. However, in some experiments, a radioactive band consistent with an esterified 15-HETE was observed near the solvent front (data not shown). As shown in Fig. 5, C and D, overexpression of S516M and S516Q mutants induced cell cycle arrest as determined by both FACS analysis and BrdUrd incorporation. These data unequivocally demonstrate that a nonprostanoid-dependent function of Cox-2 results in G0/G1 arrest of transfected cells.

It is well established that the Cox-2 gene is growth factor-, cytokine-, and tumor promoter-inducible (1, 2). Prostaglandin release by the Cox isoenzymes is limited by the self-inactivation or suicide mechanism of irreversible inactivation (1, 2, 4). Therefore, de novo synthesis of Cox isoenzymes is required to restore the PG biosynthetic capacity. It is not known whether the sustained presence of the Cox-2 isoenzyme, which is observed during cytokine stimulation, is involved in regulation of cell growth. Indeed, treatment of vascular endothelial cells with the cytokine interleukin-1 or tumor promoter PMA results in concomitant increase in Cox-2 expression and inhibition of cell growth (21). Data in this report suggest that sustained overexpression of Cox-2 induces growth arrest of a variety of cell types. Interestingly, the ability of Cox-2 to induce growth arrest is independent of prostanoid secretion. This is consistent with the fact that interleukin-1 and PMA-induced endothelial cell growth arrest is not reversed by nonsteroidal anti-inflammatory drugs that block prostanoid synthesis (Ref. 21, and data not shown). These data suggest a novel mechanism of Cox-2 function.

It is generally accepted that Cox-2 overexpression and activation of the relevant phospholipases result in the extracellular secretion of prostanoids such as PGE2 (1, 2). However, the peroxidase activity of the Cox-2 isoenzyme may also play a role in signal transduction; for example, it was shown recently that the redox-sensitive transcription factor NFκB is regulated by the peroxidase activity of the Cox enzyme (16). Thus, such a pathway may be involved in the growth arrest mechanism. Alternatively, interaction of Cox-2 polypeptide with cell cycle pathway may be involved in the growth arrest mechanism. Nevertheless, in this report show unequivocally that Cox-2 overexpression induces G0/G1 growth arrest by an uncharacterized nonprostanoid-dependent signaling pathway. Further studies are required to define at a molecular level this novel mechanism of Cox-2 function and to assess its physiological relevance.

Acknowledgments—We thank Drs. David DeWitt and William Smith.
of Michigan State University and Mark Terasaki and Juris Ozols of University of Connecticut Health Center for their kind gift of plasmids. We also acknowledge the help of Dr. Juris Ozols for critical reading of the manuscript and Dr. R. D. Berlin for helpful comments.

REFERENCES

1. Dubois, R. N., Abramson, S. B., Crofford, L., Gupta, R. A., Simon, L. S., Van De Putte, L. B., and Lipsky. P. E. (1998) FASEB J. 12, 1063–1073
2. Hla, T., Ristimaki, A., Appleby, S., and Barricciusal, J. G. (1993) Ann. N. Y. Acad. Sci. 696, 197–204
3. Spencer, A. G., Woods, J. W., Arakawa, T., Singer, I. I., and Smith, W. L. (1998) J. Biol. Chem. 273, 9886–9893
4. Smith, W. L., and DeWitt, D. L. (1996) Adv. in Immunology (Dixon, F. J., ed) Vol. 62, pp. 167–215, Academic Press, Orlando, FL
5. Laneuville, O., Breuer, D. K., Dewitt, D. L., Hla, T., Funk, C. D., and Smith, W. L. (1994) J. Pharmacol. Exp. Ther. 271, 927–934
6. Warner, T. D., Giuliano, F., Vojnovic, I., Bukasa, A., Mitchell, J. A., and Vane, J. R. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 7563–7568
7. Coleman, R. A., Smith, W. L., and Naramiya, S. (1994) Pharmacol. Rev. 46, 205–229
8. Forman, B. M., Tontonoz, P., Chen, J., Brun, R. P., Spiegelman, B. M., and Evans, R. M. (1995) Cell 83, 803–812
9. Kliwer, S. A., Lenhard, J. M., Wilson, T. M., Patel, I., Morris, D. C., and Lehmann, J. M. (1995) Cell 83, 813–819
10. Kawamori, T, Rao, C. V., Seibert, K., and Reddy, B. S. (1998) Cancer Res. 58, 409–412
11. Sheng, H., Shao, J., Kirkland, S., I. C., Isakson, P., Coffey, R. J., Morrow, J., Beauchamp, R. D., and DuBois, R. N. (1997) J. Clin. Invest. 99, 2254–2259
12. Oshima, M., Dinechuk, J. E., Karpman, S. L., Oshima, H., Hancock, B., Kwong, E., Trzaskos, J. M., Evans, J. P., and Taketo, M. M. (1996) Cell 87, 803–809
13. Tsuji, M., and DuBois, R. N. (1995) Cell 83, 493–501
14. Tsuji, M., Kawano, S., Tsuji, S., Sawaska, H., Hori, M., and DuBois, R. N. (1997) Cell 83, 795–716
15. Hla, T., Bishop-Bailey, D., Liu, C. H., Schafer, H., and Trifan, O. C. (1999) Int. J. Biochem. Cell Biol. 31, 551–557
16. Munroe, D. G., Wang, E. Y., Macintyre, J. P., Tam, S. S., Lea, D. H., Taylor, G. R., Zhou, L., Plante, R. K., Kazmi, S. M., Bauerle, P. A., and Lau, C. (1995) J. Inflamm. 45, 260–268
17. Narko, K., Ristimaki, A., MacPhee, M., Smith, E., Haudenschild, C. C., and Hla, T. (1997) J. Biol. Chem. 272, 21455–21460
18. Ballif, B. A., Mincek, N. V., Barratt, J. T., Wilson, M. L., and Simmons, D. L. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 5544–5549
19. Folman, J., and Klagesbn, M. (1987) Science 235, 442–447
20. Hla, T., and Neilson, K. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 7384–7388
21. Ristimaki, A., Garfinkel, S., Wessendorf, J., Macing, T., and Hla, T. (1994) J. Biol. Chem. 269, 11769–11775
22. Lopez, S., Vila, L., Breviario, F., and de Castellarnau, C. (1993) Biochim. Biophys. Acta 1170, 17–24
23. Murohara, T., Horowitz, J. R., Silver, M., Tsurumi, Y., Chen, D., Sullivan, A., and Imer, J. M. (1998) Circulation 97, 99–107
24. Ben-Av, P., Crofford, L. J., Wilder, R. L., and Hla, T. (1995) FEBS Lett. 372, 83–87
25. Lecomte, M., Laneuville, O., Ji, C., DeWitt, D. L., and Smith, W. L. (1994) J. Biol. Chem. 269, 13207–13215
26. Terasaki, M., Jaffe, L. A., Hanzicutt, G. R., and Hammer, J. A., 3rd (1996) Dev. Biol. 179, 320–328
27. Heinemann, F. S., and Ozols, J (1998) Mol. Biol. Cell 9, 3445–3453
28. Mitchell, J. A., Akarasereenont, P., Thiemermann, C., Flower, R. J., and Vane, J. R. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 11693–11697
29. Bailey, J. M., Muza, B., Hla, T., and Salata, K. (1985) J. Lipid Res. 26, 54–61