Cyclooxygenase-independent down-regulation of multidrug resistance–associated protein-1 expression by celecoxib in human lung cancer cells

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Abstract

The recent finding of a link between cyclooxygenase-2 (COX-2) and p-glycoprotein expression suggests that COX-2 is involved in the development of the multidrug resistance (MDR) phenotype. MDR-associated protein 1 (MRP1) is another major MDR-related protein that is frequently overexpressed in cancer patients, including those with lung cancer. Based on our observation that among four human epithelial lung cell lines both MRP1 and COX-2 protein were highly expressed only in A549 cells, we have investigated whether COX-2 regulates the expression of MRP1. The COX-2 inhibitor celecoxib down-regulated the expression of MRP1 protein in A549 cells, which was accompanied by increased accumulation and enhanced cytotoxicity of doxorubicin, an MRP1 substrate. However, enforced expression of COX-2 in human H460 lung carcinoma cell lines, which express minimal level of COX-2, did not cause enhancement in MRP1 expression. Celecoxib down-regulation of MRP1 was observed independent of COX-2 expression. Moreover, in COX-2-overexpressing cell lines, celecoxib down-regulation of MRP1 was observed only at a concentration far exceeding that required for inhibiting COX activity, and exogenous addition of prostaglandin E_2 did not restore MRP1 expression. These results suggest that celecoxib down-regulates MRP1 expression in human lung cancer cells in a COX-independent manner. The use of celecoxib for adjuvant therapy in lung cancer patients may contribute to their decreased resistance to chemotherapeutic drugs transported by MRP1. [Mol Cancer Ther 2005;4(9):1358–63]

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Introduction

Cyclooxygenases (COX) are the key enzymes that catalyze the conversion of arachidonic acid to prostaglandins and other eicosanoids. In most tissues, COX-1 is expressed constitutively, whereas COX-2 is induced by growth factors, cytokines, and carcinogens. Epidemiologic and experimental studies have shown that COX-2 inhibitors are effective chemopreventive agents, reducing the risks of many types of tumors, including colon, lung, prostate, and gastric cancers. Recently, COX-2 inhibitors have also gained attention, either alone or in combination with other chemotherapeutic agents and/or radiation therapy, in the treatment of cancer (1). For example, a COX-2-selective inhibitor celecoxib exerted synergistic antitumor effects when combined with gemcitaine or 5-fluorouracil in patients with advanced pancreatic cancer (2), and it enhanced the response to paclitaxel and carboplatin in early-stage non–small cell lung cancer (3). The mechanism underlying the antitumor activity of COX-2 inhibitors is thought to involve inhibition of COX-2 enzyme activity and induction of apoptosis, but it is unclear whether COX-2 inhibition is required to induce apoptosis (4).

Intrinsic or acquired resistance to chemotherapeutic drugs is one of the major obstacles to effective cancer treatment. The most frequent form of resistance observed in cancer patients is multidrug resistance (MDR), which is characterized by cross-resistance to a wide variety of structurally unrelated drugs, including the anthracyclines, some *Vinca* alkaloids, and the epipodophyllotoxins (5). Several mechanisms of MDR have been identified, including the overexpression of the ATP-binding cassette superfamily of transporters, which function as pumps to extrude anticancer drugs from cancer cells. Among the ATP-binding cassette transporters frequently overexpressed in drug-resistant cancer cells are multidrug resistance protein 1 (MRP1) and p-glycoprotein; the latter is encoded by the human *MDR1* gene.

A close association between MDR and COX-2 has been reported in human hepatocellular carcinoma (6) and rat renal mesangial cells (7). Transfection into these cells of an expression vector encoding COX-2 increased p-glycoprotein expression, and this was blocked by COX-2 inhibitors. A strong correlation between expression of COX-2 and MDR-1 was also found in tumor specimens derived from breast cancer patients (8). Furthermore, it has been suggested that COX-2 inhibitors sensitize cells to chemotherapeutic drugs by a functional blockade of p-glycoprotein (9). All of these studies strongly suggest that COX-2 modulates p-glycoprotein expression and is involved in the development of the MDR phenotype (10).

COX-2 is frequently overexpressed in lung cancer (11). In addition, overexpression of MRP1 but not p-glycoprotein is

frequently observed in lung cancers and has been associated with poor prognosis (12). These findings prompted us to investigate whether COX-2 plays a role in regulating MRP1 expression in lung cancer. Our results suggest that COX-2 does not regulate MRP1 expression, but that the COX-2 inhibitor celecoxib dose dependently decreases MRP1 expression, which is likely to involve COX-independent mechanisms.

Materials and Methods

Cell Lines and Cultures

The human H460, A549, and H358 non–small cell lung cancer cell lines; HCT116 colon cancer cell lines; and BEAS-2B immortalized bronchial epithelial cells were purchased from the American Type Culture Collection (Manassas, VA). MCF-7/ADR cells were kindly provided by K.H. Cowan. (National Cancer Institute, Bethesda, MD). BEAS-2B cells were grown in a Bullet kit (Clonetics, Walkersville, MD) containing serum-free bronchial epithelial cell growth medium. Cancer cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT) and 100 units/mL each of penicillin and streptomycin. Cells were grown in incubators in a humid atmosphere of 95% air/5% CO_2.

Reagents and Antibodies

Antibodies to human COX-2 and prostaglandin E_2 (PGE_2) were obtained from Cayman Chemical (Ann Arbor, MI). Doxorubicin and celecoxib was obtained from LKT Laboratories (Minneapolis, MN). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was purchased from Sigma (St. Louis, MO). Antibodies to human MRP1 and p-glycoprotein were obtained from Calbiochem (La Jolla, CA). Antibodies to human COX-1 and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals were of reagent grade and used without further purification.

Reverse Transcription-PCR analysis for MRP1 Expression

Total cellular RNA was extracted from cells using a Qiagen RNeasy mini kit (Qiagen, Santa Clarita, CA). Total RNA (2 μg) was reverse transcribed for 1 hour at 37°C in a 25-μL reaction mixture containing RNase inhibitor (Invitrogen, Carlsbad, CA), 0.5 mmol/L deoxynucleotide triphosphate, oligo dT primer, 1× reverse transcriptase buffer, and Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI). The mixture was placed at room temperature for 10 minutes, 42°C for 45 minutes, and 90°C for 3 minutes and then rapidly cooled on ice. The amplification reaction was carried out with 2 μL cDNA product for 27 cycles, and each cycle consisted of 94°C for 45 seconds, 57°C for 50 seconds, and 72°C for 50 seconds followed by a final 1-minute elongation at 72°C. The final PCR products were electrophoresed in 1% agarose gel. Analysis of the resulting PCR products on agarose gels showed single-band amplification products with expected sizes. Primers used for analysis of human MRP1 were 5′-CGTGTACTCCAACGCTGAC-3′ (forward)

and 5′-CTGGACCGCTGACGCCCGTGAC-3′ (reverse). Primers for glyceraldehyde-3-phosphate dehydrogenase was used as an internal standard (forward 5′-GTCAACG-GATTTGGTCGTATT-3′ and reverse antisense primer 5′-AGTCTTCTGGGTGGCAG-TGAT-3′).

Transfection of COX-2 cDNA

Cells were plated in six-well plates. Twenty-four hours later, cells were transfected either with empty pSG5 vector or 2.0 μg of pSG5-COX-2 plasmid, which contains a full-length COX-2 cDNA in the pSG expression vector (a gift of Dr. Robert Kulmacz, University of Texas Medical School, Houston, TX) and 0.4 μg of pcDNA3.1, which contains a hygromycin B-resistant marker (Invitrogen). Transfection was done with Effectine reagent (Qiagen) according to the manufacturer's protocol. To obtain stable transfectants, the cells were cloned by limiting dilution in 96-well plates in RPMI 1640 containing culture medium containing 500 μg/mL hygromycin B. After culture for 3 to 4 weeks, wells containing a single colony were chosen, and the expression of each protein was monitored by Western blotting. The established clones were expanded and used for the experiments described below.

Immunoblotting

Treated cells were scraped from the culture, washed twice with PBS, and incubated for 15 to 30 minutes on ice in lysis buffer containing 150 mmol/L NaCl, 10 mmol/L Tris, 0.2%Triton X-100, 0.3% NP40, 0.2 mmol/L Na_3VO_4, and protease inhibitors (pH 7.4; Roche, Nutley, NJ). After centrifugation at 16,000 × g for 15 minutes at 4°C, supernatants were collected and the protein concentration in each was measured by the Bradford method. Aliquots of supernatants containing equal amounts of protein were boiled in SDS-reducing buffer for 5 minutes, electrophoresed on SDS-polyacrylamide gels, and transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat dry milk and probed with specific primary antibodies followed by incubation with appropriate peroxidase-conjugated secondary antibodies. Blots were developed with Enhanced Chemiluminescence Plus reagent (Amersham, Arlington Heights, IL) according to the manufacturer's protocol.

PGE_2 Release and COX Activity Assay

Cells (5×10^4 cells/mL) plated in 24-well tissue culture plates in RPMI 1640 with 5% fetal bovine serum were treated with celecoxib (stock in DMSO at 50 mmol/L). After overnight incubation, the supernatant conditioned medium was then harvested and assayed for PGE_2 levels using a specific EIA kit according to the manufacturer's instructions (Cayman Chemical). Medium alone without cells was incubated under the same conditions and used as blank control for the EIA. Levels of PGE_2 were normalized to the number of cells.

For analyzing COX activity, the supernatant was removed and cells were resuspended with fresh medium containing 10 μmol/L arachidonic acid. After 30 minutes, the medium was collected and subjected for analysis of PGE_2.
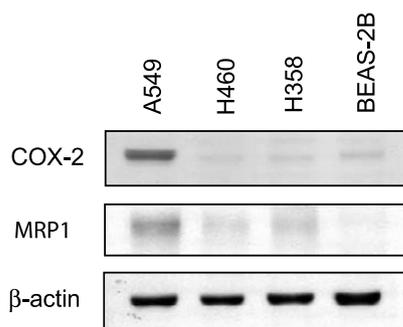
Figure 1. COX-2 and MRP1 protein expression in lung cancer cell lines and immortalized lung epithelial cell lines. Cell lysates were obtained from exponentially growing cells and subjected to immunoblotting with appropriate antibodies. Immunoblotting with an antibody to β-actin was used to ensure equal loading of proteins in each lane (*bottom*).

Intracellular Accumulation of Doxorubicin

Cells seeded on 24-well plates were treated with increasing doses of celecoxib. After pretreatment, cells were washed and equilibrated with assay buffer (122 mmol/L sodium chloride, 25 mmol/L sodium bicarbonate, 10 mmol/L glucose, 10 mmol/L HEPES, 1.2 mmol/L magnesium sulfate, 1.4 mmol/L calcium chloride, and 0.4 mmol/L potassium phosphate biphasic) containing 20 μmol/L doxorubicin. After incubation at 37°C, cells were washed thrice with ice-cold PBS and then lyzed with 1% Triton 100-X in PBS. Following centrifugation, the supernatant was collected. Aliquots were subjected to spectrofluorometric analysis for determining doxorubicin concentration at excitation 488 nm/emission 570 nm in a fluorescence microplate reader (Gemini EM, Molecular Devices, Basel, Switzerland). Extracts from untreated cells were used as blank. Doxorubicin concentration was expressed as ng doxorubicin equivalents per 100 μg of protein.

Cytotoxicity Assay

Cells (4–8 × 10³ per well in 0.1 mL medium) were seeded into 96-well plates. On the following day, the cells were treated with increasing doses of doxorubicin together with celecoxib or vehicle (DMSO) alone. Cell growth and viability were measured after 48 hours using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, as described previously (13). The formation of formazan crystals by active mitochondrial respiration in cells was determined using a microplate spectrophotometer (BioTek, Winooski, VT) after dissolving the crystals in DMSO.

Statistical Analysis

Statistically significant differences between values obtained under different experimental conditions were determined using two-tailed unpaired Student's *t* tests. Synergism was assessed by the method of Chou and Talalay (14) using a nonfixed ratio experimental design and the Calcusyn software (Biosoft, Ferguson, MO). Combination index values of <1.0 indicate synergism.

Results

Celecoxib Effect on MRP1 Expression

When we assayed four lung cancer cell lines for expression of COX-2 and MRP1 protein by immunoblotting, we found that expression of COX-2 protein was much higher in human A549 lung cancer cells than in the other cell lines (Fig. 1). Furthermore, MRP1 protein was highly expressed only in A549 cells, suggesting the possibility that MRP1 expression is associated with that of COX-2 in lung cancer.

To further investigate the potential link between COX-2 and MRP1, we tested whether COX-2 inhibition affects MRP1 expression. To address this issue, we treated A549 cells with the COX-2 inhibitor celecoxib overnight. We found that celecoxib reduced the expression of MRP1 mRNA and protein in a dose-dependent manner (Fig. 2A and B). Celecoxib also slightly reduced p-glycoprotein expression but only at the highest concentration used (Fig. 2A).

We next questioned whether MRP1 down-regulation by celecoxib led to augmented accumulation and cytotoxicity of MRP1 substrate drugs in A549 cells. When we assayed the intracellular accumulation of doxorubicin, an MRP1 substrate drug, with or without celecoxib pretreatment, we observed that 50 and 100 μmol/L celecoxib increased the intracellular accumulation of doxorubicin by 1.8- and 2.0-fold ($P < 0.05$), respectively (Fig. 3A). Moreover, after 48 hours of incubation, cotreatment with 50 μmol/L celecoxib, increased the cytotoxicity of 100 nmol/L doxorubicin by 4.0-fold ($P < 0.01$; Fig. 3B). At the indicated concentrations, celecoxib alone inhibited cell
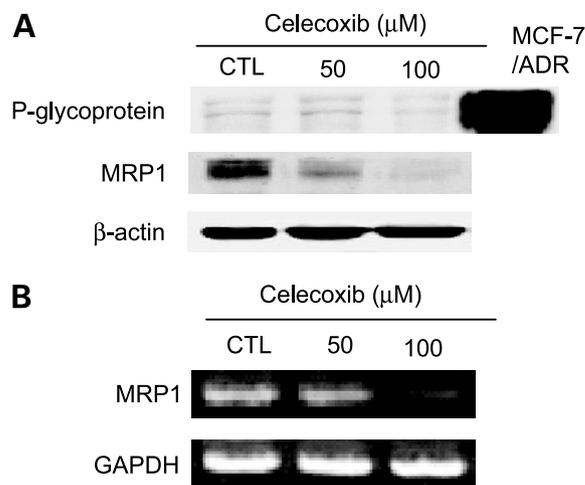

Figure 2. Celecoxib down-regulated the MRP1 protein (**A**) and mRNA (**B**) expression in A549 cells. **A**, cell lysates obtained after overnight incubation with vehicle alone or 50 or 100 μmol/L celecoxib were subjected to immunoblotting with appropriate antibodies. **B**, total RNA was isolated from A549 cells treated with the indicated doses of celecoxib and reverse transcribed and amplified for expression of MRP1 mRNA, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), an internal control, as described in the text, with expression of GAPDH mRNA serving as a loading control.
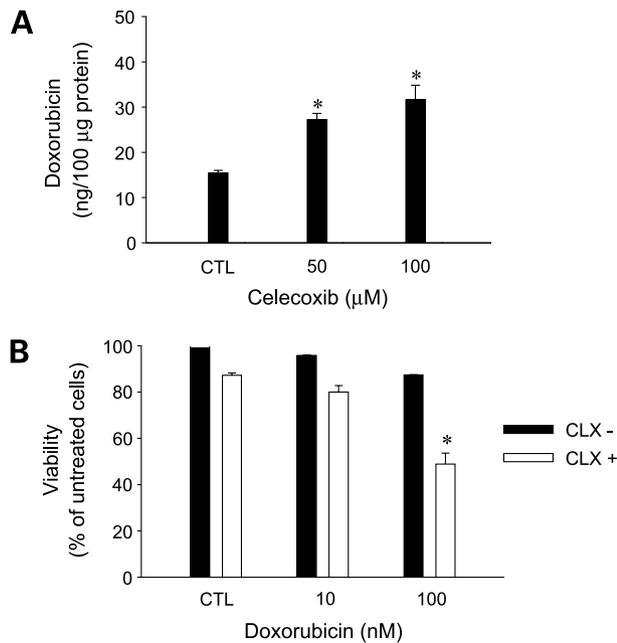
Figure 3. Augmented accumulation (**A**) and cytotoxicity (**B**) of doxorubicin induced by celecoxib in A549 cells. **A,** cells plated at 100-cm^2 dishes were treated with indicated doses of celecoxib for 16 h, cells were washed with PBS, and incubated with 10 μmol/L doxorubicin for 3 h. Cells were harvested, washed thrice with PBS, lyzed with 1% Triton X-100, and processed for quantitative determination of intracellular concentration of doxorubicin as described in the text. *, $P < 0.05$, controls versus celecoxib treatment by unpaired t test ($n = 3$). **B,** cells were seeded at a density of 5×10^3 per well in 96-well plates and starting 24 h later were incubated with varying doses of doxorubicin, in the presence or absence of 50 μmol/L celecoxib. At 48 h after incubation, cell growth and viability of cells were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. *Columns,* mean percentage growth of triplicate wells relative to untreated cells; *bars,* SD. *, $P < 0.05$ by unpaired t test ($n = 3$).

growth of <15%. The combination index was 0.898, suggesting that celecoxib and doxorubicin are synergistic. Collectively, these data suggested the possibility that COX-2 may regulate MRP1, similar to its regulation of p-glycoprotein.

Effect of Forced COX-2 Expression on MRP1 Expression

We next sought to examine whether forced COX-2 expression up-regulates MRP1 expression. To address this issue, we stably transfected a vector expressing human COX-2 cDNA into H460 lung carcinoma cells, which express minimal endogenous COX-2. In addition to HCT116 colon carcinoma cells, which are COX-2 negative, were also transfected with COX-2 c-DNA because every lung cell line used in this study were COX-2 positive (Fig. 1). From the hygromycin-resistant clones, we chose those expressing the highest level of COX-2 (Fig. 4A). We found that forced expression of COX-2 significantly increased COX activity (Fig. 4B) and the resultant PGE$_2$ release (Fig. 4C) in both cell lines (18.1- and 5.5-fold increases in PGE$_2$ release in HCT116 and H460 cells, respectively). Nonetheless, COX-2 overexpression did not up-regulate the expression of MRP1 or

p-glycoprotein in these cell lines (Fig. 5), and the cellular accumulation and resultant cytotoxicity of doxorubicin was not affected by forced COX-2 expression (data not shown). These data suggest that COX-2 does not play an important role in regulating MRP1 expression.

COX Dependency in Mediating the Effect of Celecoxib

Our transfection results suggested that COX-2 is not involved in mediating the celecoxib-induced down-regulation of MRP1. Recent evidence has indicated that COX-2 inhibitors exert their antiproliferative and chemopreventive effects through targets other than COX, depending on the cellular context (15). We therefore questioned whether the MRP1-reducing effect of celecoxib is independent of its ability to inhibit COX and thereby decrease PGE$_2$ production.

When we assayed COX activity in A549 cells after celecoxib treatment, we found that celecoxib concentrations as low as 0.5 μmol/L significantly suppressed COX activity, reaching a maximum at 5 μmol/L (Fig. 6A). In contrast, 50 μmol/L but not 5 μmol/L celecoxib reduced MRP1 expression (Fig. 6B), indicating that celecoxib inhibits MRP1 expression only at concentrations far exceeding those required to inhibit COX. Furthermore, addition of exogenous PGE$_2$ to the medium did not reverse the MRP1-reducing effect of celecoxib (Fig. 6C). In line with these data, celecoxib down-regulated MRP1
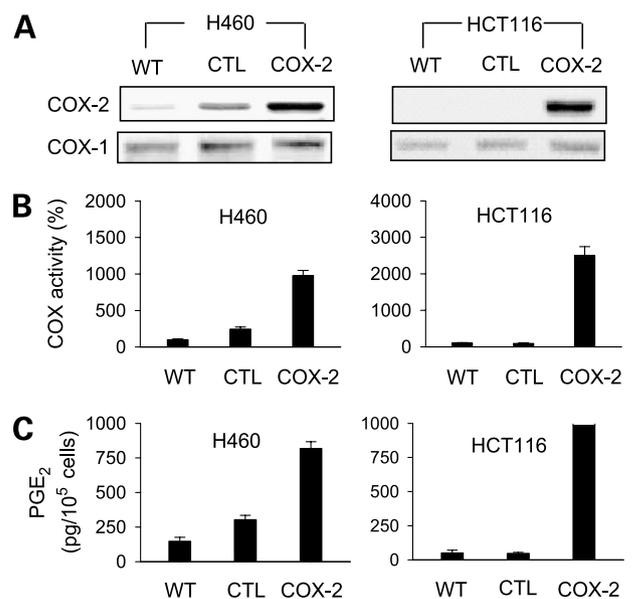

Figure 4. Characterization of COX-2 stably transfected cell lines. **A,** immunoblot analysis of COX expression in H460 and HCT116 cancer cell line stably transfected with the COX-2 DNA. Cells were transfected with empty pSG5 plasmid or pSG-5-COX-2 plasmid as described in the text. Analysis of COX activity (**B**) and PGE$_2$-producing activity (**C**) in H460 and HCT116 cell lines stably transfected with the COX-2 DNA. PGE$_2$ was measured as described in Materials and Methods. COX activity (%) is expressed as the ratio of PGE$_2$ produced in mock or COX-2-transfected cells to that in wild-type (*WT*) cells.

mRNA to a similar extent in H460 cells, regardless of COX-2 transfection (data not shown). Considered together, our findings imply that the MRP1-down-regulation by celecoxib in lung cancer cells is independent of its COX-inhibitory activity.

Discussion

MRP1 is an important member of the ATP-binding cassette transporters that function as pumps to extrude anticancer drugs from cancer cells, thereby causing MDR in cancer patients. This study tested the hypothesis that COX-2 plays a role in regulating MRP1 expression in human lung cancer cells, because overexpression of COX-2 and MRP1 has been frequently observed in established lung cancer cell lines (11) and tumor specimens obtained from lung cancer patients (12). However, our result, that forced COX-2 expression did not up-regulate MRP1 expression, was not in agreement with our initial hypothesis. We found, however, that forced COX-2 expression also did not cause up-regulation of p-glycoprotein expression, which is inconsistent with results from earlier studies using human hepatocellular and renal rat mesangial cells (6, 7). This discrepancy may be due to the difference among species, tissue origins, or cell types.

In addition to inhibiting COX activity, celecoxib modulates the expression or function of apoptosis-related proteins, such as death receptor-5 (16) and protein kinase Akt (17) with or without COX dependency. In addition, our present results indicated that celecoxib down-regulates MRP1 expression. This latter effect is likely to be mediated through a mechanism independent of the celecoxib inhibition of COX and the resulting production of PGE$_2$. This is supported by our data showing that celecoxib reduced MRP1 expression at concentrations much higher than those required to inhibit COX, that MRP1 down-regulation by celecoxib was similarly observed regardless of COX-2 expression status, and that the addition of exogenous PGE$_2$ did not reverse the celecoxib-induced down-regulation of MRP1.

Recently, phosphatidylinositol 3'-kinase was shown to modulate *MRP1* gene expression in prostate cancer cells, leading to chemoresistance (18). Celecoxib, at concentrations similar to those used in our study, has been found to induce the inactivation of Akt, a major
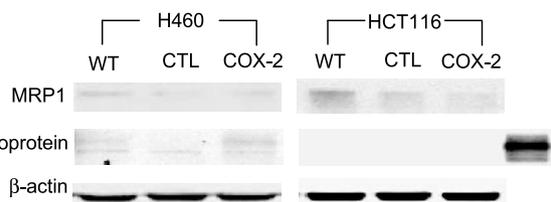
Figure 5. Enforced COX-2 expression did not cause upregulation of MRP1. MRP1 and p-glycoprotein expressions were compared among wild-type (*WT*), mock-transfected, and COX-2-transfected H460 and HCT116 cells. Cell lysates obtained from exponentially growing cells were subjected to immunoblotting with appropriate antibodies.
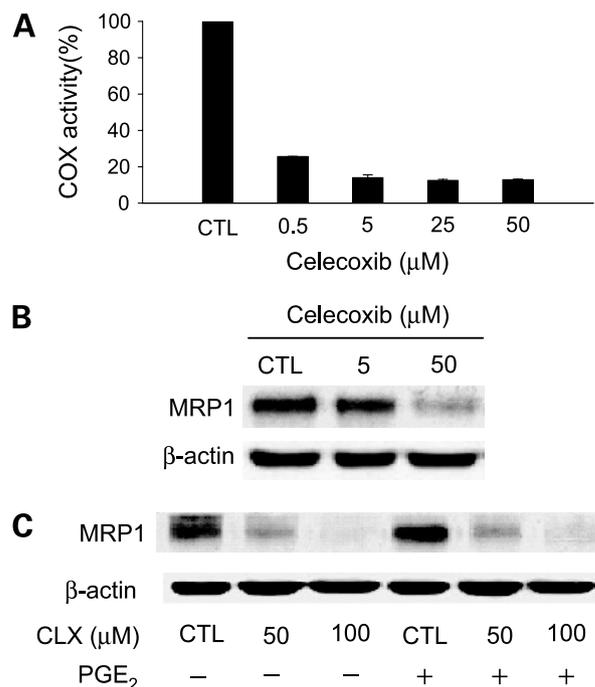
Figure 6. Celecoxib-induced MRP1 down-regulation in A549 cells was independent from its activity to inhibit PGE$_2$ production. **A,** reduced COX activity by treatment with celecoxib in A549 cells. After 2 h treatment with indicated doses of celecoxib, the supernatant was removed and cells were resuspended with fresh medium containing 10 μmol/L arachidonic acid for 30 min at 37°C. The supernatant was collected and PGE$_2$ was measured as described in the text. COX activity (%) is expressed as the ratio of PGE$_2$ produced in the presence of celecoxib to that with vehicle alone. **B,** immunoblot analysis of MRP1 expression in A549 cells after overnight treatment with 5 and 50 μmol/L celecoxib. **C,** effect of exogenous addition of PGE$_2$ on the MRP1-down-regulation effect of celecoxib in A549 cells. Cells were plated for 24 h and incubated with indicating doses of celecoxib (*CLX*) in the presence or absence of PGE$_2$. After overnight incubation, cell lysates were obtained and subjected to immunoblot analysis. Immunoblotting with an antibody to β-actin was used to ensure equal loading of proteins in each lane.

downstream molecule of the phosphatidylinositol 3'-kinase pathway, in a wide range of cancer cells (19). We also observed the dose-dependent inactivation of Akt by 50 and 100 μmol/L celecoxib.[1] It is therefore of interest to determine whether phosphatidylinositol 3'-kinase/Akt down-regulation by celecoxib is linked to MRP1 down-regulation.

COX inhibitors exert diverse effects on the expression and function of drug efflux pumps such as MRP1 and p-glycoprotein. For example, indomethacin directly inhibited the MRP1 efflux pumps without altering their level of expression (20). Similarly, celecoxib blocked the function of p-glycoprotein in experimentally induced mammary tumor (9). In contrast, sulindac dose dependently induced MRP1 expression in colon cancer cells (21). In this study, we have presented evidence that celecoxib down-regulates MRP1 expression in human lung cancer

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[1] Unpublished data.

cells. It is therefore likely that the diverse effects of COX inhibitors depend on the specific drug used, despite their common ability to inhibit COX, and thus that their MDR-regulating effects are COX independent.

COX-2 inhibitors are currently being tested as potential adjuvants in chemotherapy with cytotoxic drugs. For example, the combination of celecoxib and standard cancer chemotherapeutic agents has entered randomized trials, based on the preliminary results of phase II studies (22). Although the molecular mechanism underlying their chemoadjuvant effect is not fully understood, this effect has been also observed in established cancer cell lines (9). In this study, we have shown that treatment with celecoxib augmented the intracellular accumulation and cytotoxicity of doxorubicin in human A549 lung cancer cells. The results presented here suggest that celecoxib enhanced the cytotoxicity of doxorubicin in A549 cells by inhibiting the expression of MRP1, thereby inhibiting the MRP1 efflux pump. However, we cannot exclude the possibility that celecoxib also regulated other signaling molecules related to the cytotoxic effects of doxorubicin.

The celecoxib concentrations used here are much higher than the concentrations found in plasma samples from patients or animals after treatment (3–8 μmol/L after a single dose of 400–800 mg; refs. 23, 24). With this regard, it should be considered that celecoxib has a high protein binding capacity (25) thus limiting the free and therefore effective celecoxib concentrations in our cell culture systems, which include 10% serum. We observed that the reduction of MRP1 protein by celecoxib at a dose as low as 10 μmol/L when experiments were done under culture conditions containing 0.5% serum.[1] Furthermore, several celecoxib derivatives are currently under development (26). It will therefore be of interest to compare the MRP1-down-regulating effects of these derivatives with that of celecoxib. Further studies are needed to determine whether celecoxib or its derivatives at therapeutic doses will inhibit MRP1 expression and chemoresistance *in vivo*.

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