Cyclooxygenase-2 (COX-2) is constitutively expressed in restricted subpopulations of kidney cells, where it presumably acts as an antiapoptotic factor. In conditions that are characterized by inflammation, COX-2 expression also has been described in glomerular mesangial cells (GMC), where COX-2 is not expressed constitutively. It was shown previously that adenovirus-mediated gene transfer of COX-2 into rat GMC led to increased expression and activity of multidrug resistance protein 1 (MDR-1), a membrane transporter that functions as an efflux pump for chemotherapeutic drugs, including Adriamycin (ADR). In ADR nephrotoxicity, a pathologic change in glomeruli could be partially explained by ADR-mediated changes in GMC. Here it is demonstrated that ADR (also known as doxorubicin; 1 µg/ml) induced apoptosis in 15.3 ± 2.2% of GMC, whereas after adenovirus-mediated COX-2 expression, only 6.6 ± 0.4% of ADR-treated cells underwent apoptosis. This protective effect was nullified by treatment with NS398, specific COX-2 inhibitor. ADR efflux is greater in COX-2-overexpressing cells, when compared with control, which is attributed to the increased MDR-1 expression. Addition of PSC833, the specific MDR-1 inhibitor, completely abolished the protective effect of COX-2 overexpression and increased the level of apoptosis in GMC that were exposed to ADR. These data suggest that COX-2 protects GMC from ADR-mediated apoptosis via transcriptional upregulation of MDR-1 and that induced COX-2 expression would lessen ADR nephrotoxicity.

Cyclooxygenase 1 (COX-1) and cyclooxygenase 2 (COX-2), also known as prostaglandin endoperoxide synthases 1 and 2, are enzymes that are required for the first step in the conversion of arachidonic acid to prostaglandins (1). COX-1 is expressed constitutively in most tissues, whereas COX-2 is the inducible form of the enzyme that is produced upon stimulation with growth factors and cytokines (e.g., at sites of inflammation) (1,2). Notably, constitutive expression of COX-2 is observed in restricted subpopulations of cells, which in kidney include macula densa, associated cortical thick ascending limb of Henle cells, and medullary interstitial cells (3). Although glomerular mesangial cells (GMC) as a rule do not display constitutive expression of COX-2, in conditions that are characterized by inflammation, COX-2 expression is induced (4). Traditional nonsteroidal anti-inflammatory drugs inhibit both isozymes of the enzyme but have their anti-inflammatory, analgesic, and antipyretic effects as a result of inhibition of COX-2. It seems that these drugs have undesirable side effects, such as gastrointestinal ulceration and bleeding and platelet dysfunctions caused by inhibiting COX-1. Because a new class of COX-2 selective inhibitors preferentially inhibit the COX-2 enzyme, thereby reducing side effects, these inhibitors have emerged as important therapeutic tools for treatment of pain and arthritis (5). The initial enthusiasm about COX-2 selective inhibitors has diminished recently because of reports suggesting an increased cardiovascular risk associated with their use (6,7).

Remarkably, tumor progression often is accompanied by increased COX-2 expression, and selective COX-2 inhibitors protect against the formation of multiple tumor types in experimental animals (8). COX-2–mediated resistance to apoptosis of cancer cells is among several mechanisms of COX-2–related tumor promotion (9,10). Because anticancer drugs typically possess proapoptotic properties and their efficiency is linked to the ability to induce apoptotic cell death in cancer cells (11,12), COX-2 expression antagonizes anticancer treatment, making cells resistant to apoptosis, and therefore prevents the success of therapy. Given that the ability of selective COX-2 inhibitors to facilitate action of anticancer drugs and combat inflammation is accompanied by undesirable cardiotoxicity, it is important to uncover the molecular mechanisms of antiapoptotic action of COX-2 both in transformed and normal mammalian cells. Our previous studies have shown that the overexpression of COX-2 leads to increased expression of multidrug resistance protein 1 (MDR-1) in rat GMC, and this increase depends on...
COX-2 activity (13). MDR-1 (also termed P-glycoprotein, P-gp, and ABCB1) belongs to the ATP-binding cassette family of transporter molecules that require hydrolysis of ATP to run the transport mechanism. Elevated expression of MDR-1 is one of the important mechanisms of multidrug resistance of cancer cells. Consequently, the ability of COX-2 expression to cause the upregulation of MDR-1 suggests the role of COX-2 in regulation of drug efflux and multidrug resistance phenotype of tumors (14).

In normal kidney, MDR-1 is found in proximal tubules, mesangium, thick limb of Henle’s loops, and collecting ducts (15). It is likely that the MDR-1 function in kidney is to excrete toxic xenobiotics and metabolites into urine, preventing their accumulation in the body. One of the MDR-1 substrates is Adriamycin (ADR; also called doxorubicin), an anthracycline chemotherapeutic agent with severe cardio- and nephrotoxicity linked to its proapoptotic properties (16). It is of note that in cultured human GMC that were treated with ADR, the inhibition of MDR-1 resulted in increased cell damage (17). ADR-induced nephropathy is a widely used model for idiopathic nephrotic syndrome (18–20). It seems that in ADR nephrotoxicity, a pathologic change in glomeruli could be explained partially by ADR-mediated changes in GMC. In fact, ADR decreased contractile function of GMC, presumably as a result of effects on endoplasmic reticulum (21).

It seems that COX-2 inhibitors aggravate ADR-mediated cardiac injury (22). The purpose of this study was to determine the ability of COX-2 to rescue rat GMC from apoptosis that is induced by cardio- and nephrotoxic chemotherapeutic agent ADR, a substrate of MDR-1. We used adenovirus-mediated transfer of COX-2 and studied the ability of ADR to induce apoptosis in GMC. Using flow cytometry, Western blotting with antibodies against active caspase-3, and analysis of nuclear fragmentation by means of Hoechst staining, we demonstrate that COX-2 rescues rat GMC from apoptosis that is induced by ADR, at least partially as a result of upregulation of MDR-1, causing enhanced efflux of ADR out of the cells.

Materials and Methods

Unless otherwise stated, all chemicals were obtained from Sigma Chemicals Co. (St. Louis, MO) and Invitrogen (Carlsbad, CA). MDR-1 antibody was obtained from RDI (Flanders, NJ). COX-2 antibody was obtained from Santa Cruz (La Jolla, CA). Active caspase-3 antibody was obtained from Cell Signaling (Beverly, MA). PSC-833 was a gift of Novartis (Basel, Switzerland). NS398 was obtained from Cayman Chemical (Ann Arbor, MI).

Cell Culture, Recombinant Adenoviral Vectors, Adenoviral Infection, and Western Blot Analysis

Rat GMC were cultured as described previously (13,23). Construction of the recombinant adenovirus vector AdCOX-2 and adenovirus-mediated gene transfer into rat GMC was described previously (13). Experiments were carried out in serum-free media or in media supplemented with 0.5% FBS. Western blot analysis for MDR-1 and COX-2 was carried out as described previously (13). Samples that were standardized for protein (BCA; Pierce, Rockford, IL) were separated by SDS-PAGE using 7 or 15% acrylamide (Criterion Gels; Bio-Rad, Hercules, CA).

Rhodamine 123 Efflux Assay

Adherent rat mesangial cells were washed once with prewarmed HEPES-buffered solution (37°C) that contained 125 mM NaCl, 5 mM KCl, 1 mM MgSO4, 1.36 mM Na3PO4, 10 mM sodium acetate, 5 mM HEPES, 1.8 mM CaCl2, and 8 mM glucose, titrated to pH 7.4. The cells were incubated for 1 h at 37°C in HEPES buffer that contained 1 μM rhodamine 123 (R123). The R123 solution was removed, and cells were detached from the cell culture flask by incubation in a trypsin-containing, Ca2+-free, phosphate-buffered solution with 1 μM R123 and 100 μM vinblastine to avoid leak of R123 to the extracellular solution. Trypsin digestion was stopped by addition of cold HEPES buffer supplemented with newborn bovine serum, 1 μM R123, and 100 μM vinblastine. Cell aliquots were centrifuged at 1000 x g for 5 min at 4°C. The supernatant was removed, and 2 ml of cold HEPES buffer was added to resuspend cells, which were kept on ice. R123 uptake (load control) was measured at 0°C. Efflux of R123 was initiated by incubating the cells at 25°C on an orbital shaker for 2 h. In some experiments, the cells were resuspended in HEPES buffer that contained 1 μg/ml PSC833. Efflux was stopped by putting samples on ice until assayed. Fluorescence of R123 was collected through a 530/30-nm bandpass filter on a FACS Calibur flow cytometer (Becton-Dickinson). After gating for live cells, 10,000 cells were recorded for each sample and processed by Cell Quest software (Becton-Dickinson). Samples were analyzed after data collection by Western blotting to verify MDR-1 expression.

Adriamycin Efflux Assays

Adherent rat mesangial cells were washed once with prewarmed RPMI culture medium and incubated for 1 h in RPMI medium that contained Dox (10 μg/ml) at 37°C. For efflux measurement, the ADR-containing medium was removed and replaced with RPMI medium alone or medium that contained PSC833 for 2 h of efflux at 37°C. The cells were washed and collected by trypsinization in RPMI medium that contained newborn bovine serum. Samples were centrifuged, resuspended in RPMI medium, and kept on ice until measurement. Fluorescence of ADR was collected through a 585/42-nm bandpass on a FACS Calibur flow cytometer (Becton-Dickinson). After gating for live cells, 10,000 cells were recorded for each sample and processed by Cell Quest software (Becton-Dickinson).

Luciferase Reporter Assay

MDR1b promoter construct that represented nucleotides –250 to 23 of the rat pgl2/mdr1b gene within the pGL2 basic vector was a gift from John D. Schuetz (St. Jude Children’s Research Hospital, Memphis, TN). The plasmids were transfected into rat GMC using the Lipofectamine2000 reagent (Invitrogen) as per the manufacturer’s instructions. For luciferase activity measurements, cells were washed twice in PBS, incubated for 15 min at room temperature with agitation in 400 μl of Reporter Lysis buffer (Promega, Madison, WI), and scraped from the culture dishes. Luciferase activity was assayed on 20 to 40 μl of lysate using a luciferase kit (Promega) following the manufacturer’s instructions and using a Opticomp Luminometer (MGM Instruments, Hamden, CT) with a 10-s counting window and was normalized to protein.

Hoechst Staining

Cells were seeded in six-well dishes to 90% confluence and infected with 200 multiplicity of infection (MOI) in serum-free medium the following day. Medium was changed 24 h later, and the cells were left for additional 24 h of incubation. The next day, cells were left untreated or treated with 1 μg/ml ADR for 48 h or treated with 1 μg/ml PSC-833 alone or together with ADR for 24 h. Cells were washed with PBS and
fixed in a 1:1 methanol:acetone solution at −20°C for 20 min. Cells were washed with PBS twice and stained with a 5-μg/ml Hoechst 33342/PBS solution for 10 min. Cells were washed in PBS, and apoptotic cells were scored as condensed and/or fragmented nuclei as a percentage of normal intact nuclei.

Results

We first studied whether physiologic induction of endogenous COX-2 can control upregulation of endogenous MDR-1 in GMC. Endogenous COX-2 expression is increased after stimulation with proinflammatory cytokines, such as IL-1 and TNF-α, in many cell types, including synoviocytes, endothelial cells, mesangial cells, chondrocytes, osteoblasts, and monocytes/macrophages (23,24). Lysates from GMC that were incubated with a combination of TNF-α and IL-1β in the presence and absence of specific COX-2 inhibitor NS398 were subjected to SDS-PAGE and Western blotted with antibodies against MDR-1 and COX-2 (Figure 1). Increase in COX-2 expression was evident at 8 and 24 h after stimulation, regardless the presence of NS398. Cytokine-dependent stimulation of COX-2 expression was accompanied by an increase of MDR-1 expression in GMC, and this upregulation was at least partially caused by COX-2 enzymatic activity, because NS398 treatment significantly attenuated induced MDR-1 expression (Figure 1).

Our previous studies showed that COX-2 overexpression leads to an increase in MDR-1 (P-gp) expression and activity (13). Here we provide evidence that this upregulation is at least partially due to increased transcription activity of Mdr1b promoter. We carried out luciferase reporter assays in cells that were infected either with wild-type adenovirus or with adenovirus encoding COX-2 and transiently transfected with either plasmid carrying the MDR1b promoter linked to the luciferase gene or control plasmid. Cells that were infected with adenovirus encoding COX-2 displayed a seven-fold greater enhancement of MDR1b promoter activity, when compared with cells that were infected with wild-type adenovirus (Figure 2). As expected, Western blot analysis of GMC cells that were infected with adenovirus encoding COX-2 revealed enhanced expression of MDR-1 protein, when compared with cells that were infected with control adenovirus (data not shown).

COX-2–mediated increase of expression of MDR-1 resulted in a dose-dependent enhancement of R123 efflux from cells that were infected with AdCox-2 (Figure 3). Cells that were infected with control adenovirus AdNull (QBiogene, Morgan Irvine, CA) failed to demonstrate a comparable increase of efflux of R123 (Figure 3). As shown, the loading with R123 was similar in AdNull and AdCox-2–infected cells. At 200 MOI, AdCox-2 increased the ability of cells to pump out R123 approximately 3 times.

R123 efflux data suggest that COX-2 upregulation enhances the ability of cells to pump out drugs, substrates of MDR-1. The anticancer drug ADR, with severe cardio- and nephrotoxicity, represents one of the MDR-1 substrates by which intracellular accumulation could be measured with flow cytometry on the basis of detection of intracellular ADR fluorescence. We investigated the ability of MDR-1 to pump out ADR using an efflux assay similar to the previously used R123 assay. Figure 4 shows that the portion of cells with decreased intracellular ADR concentration (resulting from enhanced efflux of fluorescence ADR) is several times higher in a population that is infected with AdCox-2 than in a population that is infected with AdWT.
Because this increase in ADR efflux was blocked when cells were treated with COX-2 inhibitor NS398 for a 48-h infection period, we conclude that COX-2 enzymatic activity is required.

To confirm that the observed decrease in intracellular concentrations of R123 and ADR in rat mesangial cells is indeed mediated by MDR-1 activity, we used specific inhibitor of MDR-1 function PSC833 (Novartis). As shown in Figure 5, treatment of GMC with PSC833 efficiently blocked the efflux of both R123 and ADR from GMC, providing evidence that observed R123 and ADR efflux in GMC depends on MDR-1 function.

Having established that COX-2 expression causes the decrease of intracellular concentration of ADR, our next set of experiments was designed to determine whether this COX-2 effect would be sufficient to reduce the ADR-mediated apoptosis of GMC. The ability of ADR to induce apoptosis in several cell systems is well documented (16,25). ADR induced apoptosis in rat GMC, as was revealed by Western blotting with antibodies that were generated against the active fragments of caspase-3 (Figure 6A), visual analysis of morphologic changes (data not shown), and Hoechst staining to score cells for condensed and/or fragmented nuclei (Figure 6B). Adenovirus-mediated transfer of COX-2 cDNA into GMC efficiently decreased the ability of ADR to induce an appearance of active caspase-3 fragments, a recognized hallmark of apoptotic cell death (Figure 6A). The diminished ability of ADR to induce apoptosis in these cells was confirmed further by quantification of appearance of GMC with condensed and/or fragmented nuclei (Figure 6B), resulting from activation of apoptotic pathways downstream of caspase-3. Cells that were infected with AdCox-2 were compared with cells that were infected with the same amount of control AdNull or AdGFP virus particles. The
protective effect of COX-2 expression was significantly attenuated by incubation with specific COX-2 inhibitor NS398. Slightly higher protection was observed when the amount of AdCox-2 that was used for infection was increased (up to 400 MOI) with corresponding increase of the level of COX-2 expression (data not shown). At this concentration, however, control adenovirus encoding reporter gene green fluorescence protein (GFP) had some effect on caspase-3 cleavage in the absence of ADR.

To provide additional evidence that the antiapoptotic effect of COX-2 expression in cells that were treated with ADR is coupled to the functional activity of MDR-1, we studied the ability of ADR to induce apoptosis in cells that were infected with AdCox-2 in the presence and absence of specific MDR-1 inhibitor PSC833. Whereas PSC833 did not have any proapoptotic effect when added to GMC by itself, it completely abolished the protective effect of COX-2 expression in cells that were treated with ADR (Figure 6B). Treatment of GMC with ADR in the presence of PSC833 caused an even higher percentile of cells with fragmented/condensed nuclei when compared with cells that were treated with ADR alone, and enforced COX-2 expression had no protective effect (Figure 6B). These data provide additional evidence that MDR-1 activity plays crucial role in resistance of GMC to ADR-induced apoptosis.

Figure 4. NS398 blocks Adriamycin (ADR) efflux that is induced by COX-2 overexpression. GMC were infected with AdWT or AdCox-2 and treated with 25μM NS398 for 48 h before being subjected to ADR efflux assay. (A) Histograms of the flow cytometric analysis; the x axis shows ADR fluorescence, and the y axis represents cell counts. Data show that NS398 is able to block the COX-2–mediated increase in ADR efflux. Data are representative of three independent experiments. (B) ADR efflux was quantified in terms of the fraction of cells in the M1 region, represented as a percentage of the total cells that were analyzed by flow cytometry. Data represent mean ± SEM of three independent experiments. Statistical analysis revealed a significance difference (*P < 0.008) between AdWT- and AdCox-2–infected cells. Statistical difference also was revealed between AdCox-2–infected cells and AdCox-2–infected cells that were treated with NS398 (**P < 0.02).
Discussion

A previous study that was conducted in our laboratory provided evidence that adenoviral mediated transfer of COX-2 cDNA results in increased MDR-1 mRNA and protein expression (13). In this study, we demonstrate that COX-2–mediated increase of MDR-1 mRNA is due to transcriptional upregulation. Furthermore, we show that increased functional expression of an energy-dependent drug pump MDR-1 results in enhanced efflux of an anthracycline chemotherapeutic agent ADR, concomitantly increasing cellular resistance to ADR-induced apoptosis.

The ability of enforced expression of COX-2 to stimulate transcriptional upregulation of mdr1b promoter in GMC by COX-2 is in excellent agreement with reported capacity of prostaglandins PGE$_2$ and PGF$_{2\alpha}$ to upregulate mdr1b gene expression in primary rat hepatocyte cultures (26). Induction of R123 efflux by PGE$_2$ was reported recently in human acute myeloid leukemic HL-60 cells (27), proving that both rat and human MDR-1 promoters could be regulated by products of COX activity. We reported previously that PGE$_2$ and PGI$_2$ seem to be the principal PG metabolites that are induced by adenovirus-mediated transfer of COX-2 cDNA to rat GMC (23). Therefore, increased transcriptional activation of mdr1b promoter argues that the elevated level of MDR-1 mRNA is not a consequence of suppressed degradation of MDR-1 mRNA but results, at least partially, from enhanced transcriptional activity of promoter. These findings raise the possibility that administration of prostaglandins can protect from ADR-induced damage. Correspondingly, inhibition of COX-2 would aggravate ADR-induced damage, as was shown for ADR-induced cardiac cell injury (22).

Our data suggest that induction of endogenous COX-2 by cytokines results in stimulation of expression of endogenous MDR-1 in GMC. Because of the ability of COX-2 inhibitor to attenuate this stimulation, it seems that stimulation depends on COX-2 enzymatic activity. These data are in accordance with the previously reported ability of COX-2 inhibitors to mediate significant inhibition of EGF-induced mdr1b mRNA overexpression in primary rat hepatocyte cultures (26). Because multiple pathways could be involved in regulation of MDR-1 expression in cytokine-treated GMC, the complete inhibition of MDR-1 upregulation by COX-2 inhibitors is not expected.

Figure 5. PSC833 blocks R123 and ADR efflux. GMC were infected with AdCox-2 for 48 h to upregulate MDR1 and subjected to R123 efflux assay (A) and ADR efflux assay (B) in the presence or absence of 1 µg/ml PSC833. Figures show histograms of the flow cytometric analysis; x axis shows drug fluorescence, and the y axis represents cell counts. Data show that PSC833 is a powerful inhibitor of MDR1 activity and can block R123 and ADR efflux. Data are representative of three independent experiments.
Expression of COX-2 is controlled by a variety of signaling pathways, including major mitogen-activated protein kinase cascades and NF-κB (28,29). In cardiomyocytes, cell injury by H₂O₂ and ADR is limited by an increase in prostacyclin formation that reflects induction of COX-2 that is mediated by extracellular signal–regulated kinase 1/2 activation (30).

Understanding of interconnection among ADR, MDR-1, and COX-2 is complicated by the fact that in a number of studies, ADR significantly upregulated MDR-1 expression and function (27,31,32) and also increased COX-2 expression and prostaglandin release (27). In our experiments, ADR treatment did not result in expression of endogenous COX-2 protein (Figure 6A), probably because of shorter time of ADR treatment (24 to 48 h in our experiments versus 72 h in studies of Puhlmann et al. [27]). Because incubation of cells with COX-2 inhibitor NS398 did not enhance ADR-mediated caspase-3 cleavage in the absence of AdCox-2, it seems that endogenous COX-2 did not play a significant role in ADR-treated GMC in our experimental design.

The existence of a causal link between COX-2 activity and MDR-1 expression suggests the role for COX-2 in multidrug resistance phenotype (14). Indeed, the strong correlation has been established between COX-2 expression and resistance to chemotherapeutic agents (and corresponding expression of...
MDR-1) in a number of human tumors (14). It is likely that COX-2-mediated protection of GMC from ADR-induced apoptosis via upregulation of MDR-1 expression is an illustration of a general mechanism by which COX-2 regulates drug efflux and multidrug resistance phenotype and exerts its antiapoptotic action in tumors. It is of note that MDR-1 is an essential regulator of cell survival (33–36), and cells that were induced to express MDR-1 were reported to maintain resistance to cell death that is induced by such death stimuli as FasL, glucocorticoid hormones, TNF, and ultraviolet irradiation (35,37). Accordingly, COX-2 expression and activity could be protective against apoptosis that is induced by these agents via upregulation of MDR-1. We previously reported COX-2-mediated resistance from TNF-α–induced apoptosis (23). Whether a protective effect of COX-2 with regard to TNF-α is related to induced overexpression of MDR-1 remains to be determined. It is intriguing that PGE2, being one of the major products of COX-2, protects GMC from TNF-α–induced apoptosis (23).

This study suggests that the co-administration of any MDR-1 inhibitor along with chemotherapeutic drugs, substrates of MDR-1, may have toxic effects on the mesangial cell population as a result of inhibition of endogenous multidrug resistance. Even though MDR-1 is known to mediate secretion of endogenously produced platelet-activating factor in human mesangial cells (38), additional experiments are necessary to define the entire role of MDR-1 in GMC.

The mechanism by which COX-2 protects cells from ADR-induced apoptosis remains to be fully elucidated and is likely to consist of several antiapoptotic components. In human normal mammary epithelial cells, COX-2 protection from ADR-induced apoptosis was linked to COX-2 interaction with p53 in vitro and in vivo, resulting in regulation of its transcriptional activity as evidenced by suppression of p53 target gene induction by COX-2 co-transfection (39). The modulation of expression of the antiapoptotic protein Bcl-2 often is recognized as a mechanism of COX-2 antiapoptotic effect (40,41).

Our data represent the first indication of the ability of ADR to induce apoptosis in GMC. Our data also are the first demonstration of an antiapoptotic effect of COX-2 that is maintained via upregulation of functional expression of an energy-dependent drug pump. Presented studies have several implications among which two seem to be particularly important. First, administration of COX-2 selective inhibitors should enhance the intracellular concentration and, accordingly, chemotherapeutic activity of drugs, which are usually pumped out of target cells by MDR-1. Therefore, the pretreatment with selective COX-2 inhibitors may be useful in the prevention of multidrug resistance in response to cancer chemotherapy (42) but might have undesirable consequences for normal cells. Second, the observed cardiovascular risk that is associated with usage of COX-2 inhibitors could be due to increased sensitivity of normal cells to drugs that usually are pumped out by MDR-1. It seems that sensitivity of GMC to ADR is dramatically increased in the presence of COX-2 inhibitors, presumably as a result of attenuating of MDR-1 function. Because it is likely to be true for other types of cells as well, an increased cardiovascular risk that is associated with use of COX-2 inhibitors could be partially explained by inhibition of MDR-1 expression.

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References
15. Ernest S, Rajaraman S, Megyesi J, Bello-Reuss EN: Expres-