Regulation of MDR-1 (P-glycoprotein) by cyclooxygenase-2.

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Running Title: Regulation of P-gp by Cox-2

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Summary

Cyclooxygenase-2 (Cox-2), an inducible form of the enzyme that catalyses the first step in the synthesis of prostanoids, has been shown to be over expressed in a wide range of tumors and possesses pro-angiogenic and anti-apoptotic properties. To understand the molecular mechanism of Cox-2 action we used adenovirus mediated transfer of rat Cox-2 cDNA into rat renal mesangial cells (RMC) and determined the differential gene expression using cDNA microarrays. One of the several genes that were highly up regulated by over expressed Cox-2 was \textit{MDR1}. MDR1 or P-glycoprotein (P-gp), the product of the \textit{MDR1} gene is implicated as the primary cause of multi-drug resistance (MDR) in tumors where it acts as an efflux pump for chemotherapeutic agents. It is also expressed in normal tissues of the liver and kidney where it functions to actively transport lipophilic xenobiotics. RT-PCR analysis confirmed the results of the microarray, showing increased mRNA levels for MDR1 in Cox-2 over expressing cells. This increase in mRNA translated to an increase in MDR1 protein expression which was dose dependent on Cox-2 expression. Furthermore, using rhodamine 123 efflux assay we observed a significant increase in P-gp activity in Cox-2 over expressing RMC. The specific Cox-2 inhibitor NS398 was able to block the Cox-2 mediated increase in MDR1 expression and activity, suggesting that Cox-2 products may be implicated in this response. These results prove the existence of a causal link between Cox-2 and P-gp activity which would have implications for kidney function and multi-drug resistance in tumors where Cox-2 is over expressed.
Introduction

Cyclooxygenase (Cox), also known as prostaglandin endoperoxide synthase, is the key enzyme required for the conversion of arachidonic acid to prostaglandins (1). There are two isoforms of the enzyme that have been identified, Cox-1 and Cox-2. In most tissues, the Cox-1 enzyme is produced constitutively, whereas Cox-2 is highly inducible by growth factors and cytokines (e.g., at sites of inflammation) (1,2). Traditional nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit both enzymes leading to their anti-inflammatory, analgesic and antipyretic effects. However, a new class of Cox-2 selective inhibitors (COXIBs) preferentially inhibit the Cox-2 enzyme thereby reducing side effects such as gastrointestinal ulceration and bleeding and platelet dysfunctions caused by inhibiting Cox-1. These specific Cox-2 inhibitors have emerged as important therapeutic tools for treatment of pain and arthritis (3).

There is compelling evidence linking Cox-2 and carcinogenesis, where selective Cox-2 inhibitors have been shown to have strong chemopreventative actions against colon carcinogenesis in rats, inhibiting tumors to greater degree than conventional NSAIDs (4). Cox-2 derived prostaglandins were found to modulate the production of angiogenic factors by colon cancer cells thereby inducing new blood vessel formation to sustain tumor growth (5). Also, over expression of Cox-2 either in epithelial cells or in PC12 cells has been shown to lead to resistance to apoptosis which would lead to a dysregulation in cell death and cell growth (6,7). Apart from the colon, elevated Cox-2 levels have been observed in cancer of the lung, breast, gastric and prostate.

Multidrug resistance (MDR) has been a major obstacle to successful cancer chemotherapy. One important mechanism of MDR involves the multidrug transporter, P-glycoprotein (P-gp), which confers upon cancer cells the ability to resist lethal doses of certain cytotoxic drugs by
actively pumping the drugs out of the cells and thus reducing their cytotoxicity (8,9). P-gp belongs to the ATP-binding cassette (ABC) family of transporter molecules which require hydrolysis of ATP to run the transport mechanism. The substrates of P-gp may be endogenous (steroid hormones, cytokines) or xenobiotics (cytostatic drugs). A number of studies have demonstrated a negative correlation between P-gp expression levels and chemosensitivity or survival in a range of human malignancies. P-gp is encoded by a group of related genes termed MDR; only MDR1 is known to confer the drug resistance, and its over expression in cancer cells has been a therapeutic target to circumvent the resistance. Only recently has the role of P-gp expressed in normal tissues as a determinant of drug pharmacokinetics and pharmacodynamics been examined. P-gp is present in organ systems that influence drug absorption (intestine), distribution to site of action (central nervous system and leukocytes), and elimination (liver and kidney), as well as several other tissues. In the kidney, P-gp is present in the brush border membrane of the proximal tubule (9,11), a site compatible with a role in xenobiotic secretion. It is also expressed in the mesangium, the thick ascending limb of Henle's loop, and the collecting duct (11), locations that are not traditionally associated with drug excretion.

The purpose of this study was to determine the mechanism of the biological actions of Cox-2 within RMC. We employed cDNA expression microarrays for this purpose and observed an increase in P-gp gene expression within cells with enforced expression of Cox-2. This translated to increased protein expression and functional activity of P-gp. This is the first report that has found a direct link between Cox-2 expression and P-gp and further analysis into the mechanism of this relationship would help us to understand the nature of multi-drug resistance and possible strategies to block this effect.
Experimental Procedures

Materials

Unless otherwise stated all chemicals were obtained from Sigma Chemicals Co. (St. Louis, MO) or Gibco-BRL (Carlsbad, CA). MDR1 antibody was obtained from RDI (Flanders, NJ). Cox-2 antibody was obtained from Santa Cruz (La Jolla, CA).

Cell Culture

Rat glomerular mesangial cells were cultured as described previously reported (12,13). Briefly both kidneys from male Sprague Dawley rats (120 to 170 g) were removed and decapsulated under sterile conditions. Cortical tissue was cut away from the medulla and minced in isolation buffer solution (IBS) containing 5 mM KCl, 2 mM CaCl₂, 130 mM NaCl, 10 mM glucose, 20 mM sucrose, and 10 mM Tris (pH 7.4). Glomeruli were isolated by sequential sieving and collected on a 50-µm sieve. After incubation with 0.1% collagenase in IBS for 30 min at 37 °C to remove epithelial cells and obtain glomerular cores consisting mostly of mesangium and capillary loops. The glomeruli suspension was centrifuged at 2200 rmp for 7 min at room temperature. Isolated glomeruli were plated in RPMI 1640 medium supplemented with 17% fetal bovine serum, 100 units/ml penicillin, 100 units/ml streptomycin, 5 µg/ml each insulin and transferin, and 5 ng/ml selenite. The flasks were incubated at 37 °C and 5% CO₂ in a humidified atmosphere in a CO₂-controlled incubator for 3 to 6 wk, the medium was changed every third day. RMC were used between passages 5 and 24.
Recombinant Adenoviral Vectors and Adenoviral Infection

The recombinant adenovirus vector AdCox-2, expressing COX-2, was constructed from replication-deficient adenovirus type 5 (Ad5) with deletions in the E1 and E3 genes, Addl327, and a plasmid containing Ad5 sequences from 22 to 5790 with a deletion of the E1 region from bp 342 to 3523, a polycloning site under control of the cytomegalovirus promoter, the COX-2 cDNA, and the simian virus 40 polyadenylation signal. Rat mesangial cells were incubated with serum free media for 24 hours prior to infection with AdCox-2, Ad5-based green fluorescent protein expression vector (AdGFP) or a wild type Ad5 based virus containing no transgene at a varying multiplicity of infection (MOI) for 1 h. The cells remain in serum free conditions for 48 hours after infection before experiments were performed.

Gene Array Analysis and RT-PCR

AdCox2 or AdGFP infected Rat mesangial cells were grown for 48 hours in serum free media. mRNA was isolated from two 100-mm-diameter plates of each condition, using TRIzol reagent. $^{32}$P-labeled cDNA probes were prepared and hybridised to the nylon Atlas Rat Stress cDNA expression array (Clontech) as instructed by the manufacturer. Membranes were exposed to Kodak BioMax MS X-ray film with a BioMax MS intensifying screen at $-70^\circ$C for 24 h.

MDR1 gene expression was determined by reverse transcriptase polymerase chain reaction (RT-PCR) analysis using the Advantage RT-for-PCR kit (Clontech). Total RNA was prepared and RT-PCR was performed as instructed by the manufacturer using rat MDR1 and $\beta$-actin primers (Clontech).
Western Blot Analysis

Western blot analysis was carried out as described previously (14). Cells were lysed in 50 mM HEPES, pH 7.5, containing 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 200 µM orthovanadate. Samples standardized for protein (BCA; Pierce, Rockford, IL) were separated by SDS-polyacrylamide gel electrophoresis using 7% acrylamide (Criterion Gels, Bio-Rad, Hercules, CA). Protein was transferred to nitrocellulose and probed with the appropriate polyclonal antibody.

Rhodamine 123 Efflux Assay

Rat mesangial cells were washed once with prewarmed Hepes-buffered solution (37°C) consisting of 125 mM NaCl, 5 mM KCl, 1 mM MgSO₄, 1.36 mM Na₂HPO₄, 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 the same solution containing 1 µM rhodamine 123 (R123). The R123 solution was removed from the extracellular medium, and cells detached from the cell culture flask by incubation in a trypsin-containing, Ca²⁺-free, phosphate-buffered solution with 1 µM R123. Trypsin digestion was stopped by addition of cold HEPES-buffered solution supplemented with newborn bovine serum, 1 µM R123. Cell aliquots were placed in 12 x 75-mm polystyrene Falcon tubes (Becton Dickinson, Lincoln Park, NJ) and centrifuged at 1000 x g for 5 min at 4°C. The supernatant was removed by aspiration, and 2 ml of the cold incubation solution was added to the tube and aliquots were kept at 4°C. Loading was measured at 0°C. The efflux of R123 was initiated by and incubating the cells at 25°C. In some experiments, the cells were resuspended in incubation medium containing 100 µM of the P-gp blockers (Verapamil and Cyclosporin).
Samples were kept at 4°C until assayed. Fluorescence of R123 was collected through a 530/30nm bandpass on a FACscalibur 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).
Results

Our first objective was to confirm our ability to introduce the transgene of interest into rat mesangial cells with the use of adenovirus infection. Figure 1A shows GFP infected cells at 100 MOI. Under the fluorescence microscope 90% of the cells show fluorescence however, more sensitive flow cytometry analysis revealed 100% transgene expression by the cells. Figure 1B (middle panel) shows a western blot of Cox-2 expression in rat mesangial cells infected with GFP and Cox-2 adenovirus. The figure shows that we are able to increase the expression of Cox-2 within the cells using this system and that basal levels of Cox-2 expression (AdGFP treated) were negligible 48 hours after infection. To identify the differential gene expression as a result of Cox-2 over expression, Atlas™ rat stress cDNA expression arrays (Clontech) were screened with 32P-labelled cDNA generated from mRNA from RMC infected with AdCox-2 or AdGFP. A difference of more that 3 fold was considered significant. Signals were normalized to signals from housekeeping genes (data not shown). Of genes which were up regulated in RMC MDR1 or P-gp showed the most significant increase and because of the roles of Cox-2 and P-gp in cancer we investigated this correlation further. In order to confirm the results of the cDNA microarray we analyzed the samples using RT-PCR with primers specific for rat P-gp and actin for control purposes. Figure 1C shows the result of this experiment and confirmed the data that we obtained with the cDNA arrays by showing increased expression of the MDR1 gene.

It was necessary to investigate whether this increased gene expression resulted in an increased translation to protein. RMC were infected with AdGFP or AdCox-2 and P-gp and Cox-2 expression analyzed by western immunoblot. Figure 1B shows the results of this initial experiment where we observed a basal level of P-gp expression which increased in RMC over expressing Cox-2. The P-gp protein is 170kDa but is hyperglycosylated and as such appears as a
diffuse band on the western immunoblot. Using a new preparation of RMC and AdCox-2 we were able to reproduce these results and we subsequently found that the P-gp protein expression was depended on Cox-2 levels as shown in Figure 2.

This relationship was studied further using an assay to measure specific P-gp activity. The assay measures P-gp mediated transport by the decrease in the intracellular fluorescence of rhodamine 123 (R123, a P-gp substrate) using flow cytometry. Intracellular rhodamine 123 was measured at time 0 (load) and after 2 hours of efflux in RMC infected with AdCox-2 and wild type adenovirus. AdGFP was not used due to fluorescence from GFP interfering with R123 measurements. Figure 3A shows the results of this experiment with control RMC (top panel) effluxing R123 consistent with the basal protein expression observed in Fig. 2B. Efflux was measured by the number of cells in the M1 region of the plot. In the RMC infected with AdCox-2 (Fig 3A, bottom panels) there was an increase in R123 efflux from the cells indicating greater P-gp activity. This experiment was carried out several times and each time the increase in R123 efflux in Cox-2 expressing RMC over control levels was measured. The average percentage increase in R123 efflux from the Cox-2 over expressing RMC was 75% ± 10 (p < 0.0001). To confirm the specificity of the R123 efflux from the RMC we employed two P-gp blockers, verapamil (VRP) and cyclosporin A (CsA). The R123 efflux assays were performed in the presence of these inhibitors and the results are shown in figure 3B. The inhibitors at concentrations of 100µM were able to significantly reduce the efflux of R123 from the RMC thus confirming that the increase in efflux form Cox-2 over expressing RMC is due to increase in P-gp expression and activity.

The effect of the specific Cox-2 inhibitor NS398 on the Cox-2 mediated MDR1 activity and expression was assessed. Figure 4A and 4B show the results of R123 efflux assays measuring
MDR1 activity in RMC infected with the Cox-2 adenovirus and treated with increasing concentrations of NS398. The histograms in figure 4A show a dose dependent decrease in R123 efflux as measured by the number of cells in the M1 region of the plot. Figure 4B gives the percentage values for the efflux in all treatments. They confirm that we see a greater efflux in Cox-2 over-expressing cells (39% vs 14%) and that with the treatment of NS398 the efflux in in Cox-2 over expressing cells is reduced to control levels (14%). Our next objective was to see if the reduced MDR1 activity with NS398 treatments was due to reduced MDR1 expression. Figure 4C shows a representative western blot of RMC infected with control and Cox-2 adenovirus plus or minus NS398 (25µM). We again confirm that Cox-2 over expressing RMC have increased MDR1 expression. We also observed that the addition of NS398 blocked the up regulation of the MDR1 protein, consistent with the R123 efflux assay showing reduced MDR1 activity. These additional results show that the Cox-2 mediated MDR1 response is dependent on Cox-2 activity.
Discussion

We have shown that the over expression of Cox-2 leads to increased P-gp expression and activity in RMC and this effect is dependent on Cox-2 activity. The products of Cox-2 activity that may be implicated in this response are varied. They include prostaglandin endoperoxide H2 (PGH$_2$), PGD$_2$, PDE$_2$, PGF$_{2\alpha}$, PGI$_2$ (prostacyclin) or TxA$_2$ (thromboxane A$_2$). P-glycoprotein is the product of the multidrug drug resistance (MDR) gene has been of great interest due to its role in multidrug resistance in a variety of cancers (8,9,15). It has been shown to be over expressed in cancer cells that actively extrude chemotherapeutic agents (15). Aberrant up regulation of Cox-2 expression is also increasingly implicated in the pathogenesis of epithelial cell carcinomas such as human colorectal adenocarcinomas and colorectal tumors (16). Previous studies in rat medullary interstitial cells have shown that Cox-2 over expression leads to a number of effects that could be associated with tumorogenesis: increased adhesion to extracellular matrix proteins, inhibition of butyrate-induced apoptosis, decreased expression of both E-cadherin and transforming growth factor β2 receptor, and stimulation of Bcl-2 protein expression (6). Cox-2 expressing cells also produce high levels of angiogenic factors that stimulate angiogenesis (5). The results we have shown suggest another possible role of Cox-2 in carcinogenesis by promoting multi-drug resistance.

Immunohistochemical analyses of human breast tumor specimens revealed a strong correlation between expression of Cox-2 and P-gp. In drug resistant cell lines that over-express P-gp there was also significant expression of Cox-2 expression (17). Studies indicate that the use of non-steroidal anti-inflammatory drugs (NSAIDs) may enhance the anti-tumor activity of cancer chemotherapeutic agents and reduce the risk of many cancers. The best known function of NSAIDs is to block Cox and stop the conversion of arachidonic acid to prostaglandins. We have
shown the first report of direct causal relationship between Cox-2 expression and P-gp regulation. These results may give us an indication as to the nature of multi-drug resistance in tumors where Cox-2 is over expressed.

MDR1 is also expressed in normal human tissues including the kidney, liver, pancreas and colon (10). MDR1 expression has been shown to be up regulated by many extracellular stimuli including UV irradiation, heat shock, and growth factors, and by a number of drugs. In the kidney it is localized at sites compatible with its role in xenobiotic secretion. Human mesangial cells express P-pg in culture where it is localized to the plasma membrane (18) and functionally transports xenobiotics. Our results would have implications as to how up regulated Cox-2 activity may mediate these effects in the kidney, notably the excretion of drugs by the kidney via P-gp.

P-gp has also been implicated in a role in augmenting cell survival of a number of cell systems (19,20). Up regulation of P-gp via nuclear factor-kappaB (NF-kB) activation protects kidney proximal tubule cells from cadmium- and reactive oxygen species-induced apoptosis which was blocked by the MDR1 inhibitors such as PSC833, cyclosporine A, or verapamil (19). It is also possible that MDR1 is capable of preventing transformation by pumping out xenobiotic carcinogens. Cox-2 and its products are also implicated in cell survival. Treatment of human colon cancer cells (HCA7) with SC-58125, a highly selective Cox-2 inhibitor results in inhibition of growth and increased apoptosis that is reversed by PGE2 (21). Cox-2 promotes resistance to trophic withdrawal apoptosis in PC12 cells by stimulation of dynein light chain expression and inhibition of neuronal nitric oxide synthase activity (22). It has been established that MDR1 can translocate a wide variety of short-chain lipid molecules, including ceramides, across the plasma membrane to the cell surface (23). It was also shown that TNFα-induced ceramide generation and apoptosis was restored by MDR1 inhibitors in TNFα-resistant leukemia cells (24). Apoptosis
can be triggered by ceramide generation and Cox-2 anti-apoptotic activity is sometimes assigned
to prevention of ceramide formation due to arachidonic acid depletion. (25). Our results suggest
that one of the mechanisms of Cox-2 anti-apoptotic action is mediated via P-gp.

To date there has been no evidence of Cox-2 mediated P-glycoprotein expression. We hope
that uncovering of this link and further studies will help to devise strategies to combat multidrug
resistance in tumors over expressing the Cox-2 protein.
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Figure Legends

Figure 1. **Transgene expression in adenovirus infected RMC and MDR1 gene and protein expression in Cox-2 over expressing RMC.** A) Micrographs of rat mesangial cells infected with adenovirus encoding the GFP transgene for 48 hours. Infection was carried out at 100 MOI. Left micrograph shows the phase contrast view of GFP-infected RMC and the micrograph to the right is the view showing the fluorescence generated by the introduced GFP transgene (×100). B) Lysates from RMC infected with 200 MOI of AdGFP or AdCox-2 for 48 hours were separated on SDS 7% PAGE and analysed by western immunoblot for MDR1 and Cox-2 expression. Samples were normalised for protein loading as shown by Ponceau S staining of the membrane bound protein. A) RT-PCR analysis of RNA from RMC infected with AdGFP and Cox-2. *MDR1* analysis was over 30 cycles and *actin* analysis over 18 cycles using rat specific primers.

Figure 2. **P-gp protein expression in RMC is Cox-2 dependent.** Lysates from RMC infected with increasing concentrations of AdGFP and AdCox-2 for 48 hours were separated on SDS 7% PAGE and analysed by western immunoblot for MDR1 and Cox-2 expression. Samples were normalised for protein loading as shown by Ponceau S staining of the membrane bound protein. Experiments were repeated three times with similar results.

Figure 3. **Rhodamine 123 efflux assay measuring P-gp activity.** RMC infected with wild type adenovirus or Cox-2 for 48 hours were loaded with rhodamine 123 for 1 hour followed by efflux for 2 hours. After which the immunofluorescence of the cell
population was measured by flow cytometry by counting cells in the M1 region of the plot. Plots shows the level of fluorescence vs cell count. A) The plots show the loading and efflux of rhodamine 123 from RMC infected with control virus (top panels) and AdCox-2 (bottom panels), with greater efflux in Cox-2 over expressing cells. B) The effect of inhibitors of P-gp activity on rhodamine 123 efflux from AdCox-2 infected RMC was tested using 100µM verapamil (VRP) and cyclosporin A (CSA) during the efflux stage.

Figure 4. **Effect of the Cox-2 inhibitor, NS398 on MDR1 activity and expression.** A) R123 efflux assay measuring MDR1 activity in RMC infected with AdCox-2 and incubated with increasing concentrations of NS398. Cells were loaded with R123 for 1 hour followed by effluxing for 2 hours. The histograms show reduced efflux (cells in M1 region) by cells incubated with increasing concentrations of NS398. B) Table showing the percentage R123 efflux values from the experiment in (A). C) Western blot of lysates from RMC infected with AdGFP and AdCox-2 for 48 hours amd treated with or without NS398 were separated on SDS 7% PAGE and analysed by western immunoblot for MDR1 expression. Samples were normalised for protein loading as shown by Ponceau S staining of the membrane bound protein.
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Figure 1

A

Phase Contrast  Fluorescence

B

AdGFP  AdCox-2

MDR1 (170kDa)

Cox-2 (72kDa)

Ponceau Stain

C

AdGFP  AdCox-2

MDR1 (1253bp)

Actin (794bp)
### Figure 2

| MOI | 0  | 90 | 180 | 270 | 360 | 450 | 90 | 180 | 270 | 360 | 450 |
|-----|----|----|-----|-----|-----|-----|----|----|-----|-----|-----|
| MDR1| 170kDa |   |     |     |     |     |   |   |     |     |     |
| Cox-2| 72kDa |   |     |     |     |     |   |   |     |     |     |
| Ponceau S |   |   |     |     |     |     |   |   |     |     |     |

The figure shows the expression levels of MDR1 (170kDa), Cox-2 (72kDa), and Ponceau S at different MOIs (multiples of infection) for AdGFP and AdCox-2.
Figure 3

A

Load (Control)

Efflux (Control)

Load (Cox-2)

Efflux (Cox-2)

B

Load

Efflux

Efflux + VRP

Efflux + CsA
Figure 4

A

![Graphs showing rhodamine efflux](image)

B

| Sample          | % Rhodamine 123 Efflux |
|-----------------|------------------------|
|                 | Control    | Cox-2          |
| Load            | 1.33       | 2.20           |
| Efflux          | 14.41      | 39.87          |
| NS398 1µM       | 15.52      | 30.23          |
| NS398 10µM      | 9.49       | 19.66          |
| NS398 25µM      | 10.83      | 14.50          |

C

|       | AdGFP | AdCox-2   |
|-------|-------|-----------|
| -     | +     | -         | +         |
|       |       | NS398 (25µM) |
|       |       | MDR1 (170kDa) |
|       |       | Ponceau S   |
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