Do altering in ornithine decarboxylase activity and gene expression contribute to antiproliferative properties of COX inhibitors?

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Two isoforms of cyclooxygenase (COX) participate in growth control; COX-1 is constitutively expressed in most cells, and COX-2 is an inducible enzyme in response to cellular stimuli. An induction of COX-2 found in neoplastic tissues results in increased cell growth, inhibition of apoptosis, activation of angiogenesis, and decreased immune responsiveness. Although both COX-1 and COX-2 inhibitors are suppressors of cell proliferation and appear to be chemopreventive agents for tumorigenesis, the molecular mechanisms mediating antiproliferative effect of COX inhibitors are still not well defined. This study contrasts and compares the effects of aspirin and celecoxib, inhibitors of COX-1 and COX-2, in rat hepatoma HTC-IR cells. The following were assessed: cell proliferation and apoptosis, ornithine decarboxylase (ODC) activity, and pattern expression of three immediate-early genes, c-myc, Egr-1, and c-fos. We have shown that the treatment of hepatocytes in vitro with the selective COX-2 inhibitor, celecoxib, was associated with induction of apoptosis and complete inhibition of cellular proliferation. Aspirin exhibited a small antiproliferative effect that was not associated with apoptosis. Treatment with celecoxib produced dose- and time-dependent decrease in ODC activity. In addition, at higher drug concentration the decrease in ODC activity was greater in proliferating than in resting cells. Much lesser inhibitory effect on ODC activity was observed in aspirin-treated cells. The two COX inhibitors did not change c-myc expression, significantly decreased the expression of Egr-1, and differentially altered expression of c-fos; aspirin did not change, but celecoxib dramatically decreased the levels of c-fos-mRNA. Our study revealed that celecoxib and aspirin share the ability to inhibit ODC activity and alter the pattern of immediate-early gene expression. It seems that some of the observed effects are likely to be related to COX-independent pathways. The precise mechanisms of action of COX inhibitors should be defined before using these drugs for cancer chemopreventive therapy.

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Mammalian cells express two related but unique isoforms of cyclooxygenase (COX), COX-1 and COX-2, the rate-limiting enzymes in prostaglandin (PG) biosynthesis; they participate in both normal and neoplastic growth responses (Bennett et al, 1977; Maxwell et al, 1990; Kokoglu et al, 1998; Denkert et al, 2001). While COX-1 is a constitutively expressed enzyme that is generally involved in cell function of most tissues, COX-2 is an inducible enzyme in response to cellular stimuli, including mitogens, tumour promoters, cytokines, and other inflammatory mediators (Eberhart and DuBois, 1995; Smith et al, 1996; Beejay and Wolfe, 1999; Whittle et al, 2000). Although the molecular mechanisms causing the overexpression of COX-2 in various cancer cells are not understood, it is known that increased level of COX-2 results in enhanced PG production.

Both COX-1 and COX-2 inhibitors are suppressors of cell proliferation and appear to be chemopreventive agents for tumorigenesis (Thun et al, 1991; Scheinemachers and Everson, 1994; Kawamori et al, 1998; Taketo, 1998a, b; Elder et al, 2000; Joki et al, 2000; Lui et al, 2000). It is clear that inhibitors of COX-2, but not COX-1, strongly suppress cell growth by inducing apoptosis (Erickson et al, 1999; Ding et al, 2000; Elder et al, 2000; Lui et al, 2000; Uefuji et al, 2000; Williams et al, 2000; Grosch et al, 2001), which may result from blocking the cell cycle, enhancing c-myc expression, and diminishing bcl-2 expression (Elder et al, 2000). In addition, among genes differentially expressed in cells treated with the specific inhibitor of COX-2, several other genes involved in the regulation of cell adhesion, cell cycle progression, apoptosis, and differentiation were found (Zhang and DuBois, 2001). While these results provided evidence that antineoplastic effects of nonsteroidal anti-inflammatory drugs (NSAIDs) may result from altered expression of genes that regulate various biological processes, the molecular mechanisms mediating antiproliferative effect of COX inhibitors are still not well defined.
Induction of cell proliferation is associated with transcriptional stimulation of growth-related genes that are required for G1/S transition (Kavin et al., 1997). One of them is ornithine decarboxylase (odc) gene encoding a key regulatory enzyme in the biosynthesis of polyamines that are essential for cell proliferation (Pegg, 1986). ODC and polyamines can also act as facilitating factors in triggering apoptosis (Tiberio et al., 2001). Thus, inhibition of ODC might be an important event among antiproliferative and proapoptotic effects of COX inhibitors.

In this study, we have examined cell proliferation, apoptosis, and ODC activity in rat hepatoma HTC-IR cells that were treated with NSAIDs, aspirin and celecoxib. In addition, an expression of immediate-early genes, c-myc, Egr-1, and c-fos, was assessed. We have shown that NSAIDs inhibit activity of ODC and, for the first time, differentially alter expression of c-fos.

**MATERIALS AND METHODS**

**Cells**

Rat hepatoma cells, HTC-IR, were grown in plastic cell culture flasks in DME media supplemented with 10% FBS, 2 mM glutamine, penicillin (100 U ml⁻¹), streptomycin (0.01%), and humidified with 694% CO₂/air gas mixture. Cells were routinely subcultured using trypsin solution. Aspirin and celecoxib were dissolved in DMSO as 1000 x 1000 stock solutions and then diluted in DMEM for the experiments. The final DMSO concentration was maintained at 0.1%.

Celecoxib was kindly provided by Pharmacia Corporation (St Louis, MO, USA).

**Cell proliferation assays**

The effect of aspirin and celecoxib on HTC-IR cell growth was determined by MTT cell proliferation assay, incorporation of [³H] thymidine into DNA, and cell counting.

Exponentially growing cells were harvested, seeded at a density 5 x 10⁵ in 96-well plates and then grown in DMEM containing 10% FBS. After 24 h, fresh medium without or with aspirin or celecoxib was added. Cell growth was monitored at 24, 48, and 72 h by the cell proliferation assay, MTT CellTiter 96 (Promega). Three independent experiments were performed and all assays were repeated in octuplicate. Results were expressed as the percentage of control cells (means ± s.d.).

5 x 10⁴ cells per well were seeded in 96-well plates and grown for 24 h in DMEM containing 10% FBS. Then, cells were supplemented with fresh medium containing aspirin or celecoxib or DMSO and 72 h later 0.1 μCi of [³H] thymidine was added to each well for an additional 4 h. Finally, cells were harvested, DNA was collected on GPC filters, and the radioactivity was determined by scintillation counting. Three independent experiments were performed and all assays were repeated in octuplicate.

Cells were seeded at a density 10⁴ cells per T25 flask. After 24 h, medium was removed, DMEM without or with aspirin or celecoxib was added and cells were incubated for 72 h. Then, floating and adherent cells were harvested and combined cell populations were counted using a haemocytometer. In addition, cell viability was evaluated by trypan blue exclusion cell staining. Results were expressed as the percentage of control cells (means ± s.d.) of three independent experiments, each performed in duplicate.

**Measurement of apoptosis**

Cells were grown in eight-chamber culture slides until 50% of confluency was obtained, and then were supplemented with fresh medium containing aspirin or celecoxib or DMSO. At different time points, the adherent cells were stained on chamberslides with fluorescein-conjugated annexin V and propidium iodide using Annexin-V-FLUOS Staining Kit (Roche) as per the manufacturer’s instructions and the fluorescence of individual cells was assayed by fluorescence microscopy. The percentage of annexin V- and propidium iodide-stained cells within a minimum of 400 cells was determined.

**Assay of ornithine decarboxylase activity**

ODC activity was assayed by determination of [¹⁴C]CO₂ formation as previously described (Ostrowski et al., 2000). Briefly, HTC-IR cell pellets were homogenised in buffer containing 50 mM HEPES–NaOH (pH = 7.5), 2.5 mM DTT, 0.25 mM pyridoxal 5-phosphate, and 0.1 mM EDTA. The reaction mixture consisted of 20 μl of homogenate’s supernatant, 0.25 mM pyridoxal 5-phosphate, 2.5 mM DTT, 50 mM HEPES–NaOH (pH = 7.5), 0.1 mM EDTA with 0.2 μCi of [¹⁴C]ornithine hydrochloride (Amersham International) in a total volume of 40 μl. The reaction tube was sealed with plastic pipette tip containing a 0.5 x 4.0 cm² of Whatman No. 1 filter paper soaked with 40 μl of β-phenylethylamine. Tubes were incubated at 37°C for 60 min and the reaction was then stopped by adding 200 μl of 2 M citric acid. After further 60 min of incubation, the filter paper was removed, placed in 10 ml of scintillation liquid, and counted in a scintillation counter. Results were expressed as nmol of [¹⁴C]CO₂ released per hour per mg of protein. All assays were performed in triplicate.

**RT – PCR**

Cells were grown in T25 flasks until 60% of confluency was obtained. Then cells were made quiescent by 24 h serum deprivation, and aspirin (5 μg ml⁻¹) or celecoxib (5 μg ml⁻¹), or DMSO were added for an additional 24 h. Finally, cells were treated with 15% FBS for 0, 15, 30, 60, 180, and 360 min. Total RNA was prepared from cell pellets by acid-guanidium thiocyanate/phenol–chloroform extraction using TRIzol reagent. Five micrograms of total RNA was reverse transcribed using SuperScript II RT (GIBCO-BRL) and oligo-dT in 20 μl volume as per the manufacturer’s protocol. RT reactions were diluted 1:10 with water, and PCR was carried out using 2 μl of cDNAs and primers for c-fos, Egr-1, c-myc, and odc. [α-³²P]dCTP (NEN) was used to label the PCR products. PCR products were resolved on native 5% polyacrylamide gels, then the gels were dried and the PCR products were quantified using a phosphorimager. Densitometric analysis was performed using OptiQuantSTM Image Analysis Software (Packard). Levels of band intensities after background subtraction were expressed in Digital Light Units (DLU).

**Statistical analysis**

Results are presented as means ± s.d. Significant difference between mean values was assessed by means of analysis of variance (ANOVA). P-values for differences from control results were calculated using Bonferroni method. Means were considered to be different if P < 0.05.

**RESULTS**

**Inhibition of cell proliferation by celecoxib and aspirin in rat hepatocytes in vitro**

MTT test reflects the combined effects of cell proliferation and survival, and the colour development results from the reduction of tetrazolium salts to formazans by living cells. HTC-IR cells were treated with increasing concentration of aspirin or celecoxib and MTT metabolisation was determined after 1, 2, and 3 days of the treatment. Celecoxib inhibited MTT metabolisation in a dose-dependent manner. The suppressive effect was observed even with the lowest COX-2 inhibitor concentration (2.5 μg ml⁻¹) at 24 h of
the treatment, and almost complete inhibition of MTT metabolisation was seen in 48 h-treated cells with the higher concentration of celecoxib (50 \(\mu\)g/ml\(^{-1}\)) (Figure 1A). In contrast, no inhibition in MTT test was observed with low doses of aspirin (2.5 and 5 \(\mu\)g/ml\(^{-1}\)), and only some inhibitory effect was observed after 48 and 72 h of the aspirin treatment with the higher drug concentrations (25 and 50 \(\mu\)g/ml\(^{-1}\)) (Figure 1B).

The results of MTT test were further confirmed by two other proliferation tests. Incorporation of \(^{3}H\)thymidine into DNA of cultured HTR-IR cells was strongly inhibited by the treatment with celecoxib and only moderately diminished by higher concentrations of aspirin (Figure 2). Also, direct cell counting revealed that aspirin did not affect cell proliferation at low (2.5 – 5 \(\mu\)g/ml\(^{-1}\)) concentrations (Figure 3). However, minor but significant decline in the cell yield was evident at higher (25 – 50 \(\mu\)g/ml\(^{-1}\)) concentrations. Over a 3-day incubated period, none of the aspirin concentration used in the study affected cell viability (as detected by trypan blue exclusion) and there was no increase in the number of nonadherent cells relative to control cells.

[Cancer Research UK image]

![MTT test (%) of control](image)

**Figure 1** The effects of celecoxib and aspirin on proliferation of rat hepatoma HTC-IR cells determined by cell viability. Cells which were grown in 96-well plates in DMEM containing 10% FBS were treated with increasing concentrations (2.5, 5, 25, and 50 \(\mu\)g/ml\(^{-1}\)) of celecoxib (A) or aspirin (B), and cell viability was monitored by MTT test 24, 48, and 72 h later. Three independent experiments were performed and all assays were repeated in octuplicate. Results are expressed as the percentage of control cell viability and represent means \(\pm\) s.d. a, b, c indicate significant decrease (a, \(P<0.05\); b, \(P<0.01\); c, \(P<0.001\)) in cell viability in aspirin- and celecoxib-treated cells compared to cells treated with DMSO.

In contrast, celecoxib showed dose-dependent inhibitory effect on the cell yield (Figure 3). Three-day treatment with celecoxib at low (2.5 – 5 \(\mu\)g/ml\(^{-1}\)) concentrations resulted in less than 40% of cell viability and more than 50% of floating cells; after treatment with higher celecoxib concentrations (25 – 50 \(\mu\)g/ml\(^{-1}\)), most of the cells were floating and dead (not shown). Since the proportion of floating to adherent cells was found to be a measure of apoptosis (Eberhart and DuBois, 1995), the HTC-IR cell growth inhibition by celecoxib was likely to take place through the induction of apoptosis.

**Induction of apoptosis by celecoxib**

To confirm that dominant antiproliferative effect of celecoxib is the induction of apoptosis, the quantitative analysis of apoptotic HTC-IR cells that were treated with aspirin or celecoxib was performed using fluorescence microscopy. In the early stages of apoptosis, phosphatidylserine (PS) translocates from the inner side of the plasma membrane to the external surface of the cell. Annexin V that binds to PS with a high affinity is suited to detect early apoptotic cells. Apoptotic cells in cultures undergo secondary necrosis (Vermes et al, 1997) and the nuclei of late apoptotic and necrotic cells can be labelled with propidium iodide. Thus, the discrimination between early apoptotic and necrotic cells was possible with the use of combined cell staining with annexin V and propidium iodide.

The adherent HTC-IR cells grown under standard conditions showed about 5% of total cells that were stained with annexin V, and less than 4% that were stained with propidium iodide. In repeated and quantified experiments, treatment with aspirin did not change the level of either early apoptosis or necrosis (not shown), while treatment with celecoxib caused a statistically significant, concentration- and time-dependent increase of apoptosis and necrosis (Figure 4).

**COX inhibitors suppress ODC activity**

ODC activity was determined by monitoring formation of \(^{14}C\)CO\(_2\) from \(^{14}C\)ornithine. Fetal calf serum is an essential agent for cell growth in culture, supplying the cells with required proliferation signals and growth factors. Since ODC is thought to play an important role in the mitogenic responses, we determined activity of this enzyme in serum-deprived growth-arrested cells and in proliferating cells in vitro. As shown in Figure 5, ODC activity was, unexpectedly, significantly higher in cells fasting for 24 h than in proliferating cells.

Treatment with celecoxib produced dose- and time-dependent decrease of ODC activity (Figure 6). After treatment with low celecoxib concentration (5 \(\mu\)g/ml\(^{-1}\)), the kinetics of enzyme inhibition was the same regardless of resting or proliferating cells were used for experiments (Figure 6A). However, at higher celecoxib concentration (50 \(\mu\)g/ml\(^{-1}\)) the decrease in ODC activity was significantly higher in proliferating than in resting cells (Figure 6B). Compared to celecoxib, a much smaller degree of ODC inhibition was observed in aspirin-treated cells; until sixth hour of incubation aspirin did not inhibit ODC activity, and the significant decrease in the enzyme activity was found only at the last time point measured, 24 h (Figure 7A, B).

**COX inhibitors do not affect expression of mRNA ODC**

Under serum-deprived conditions, cultured cells become growth-arrested, but following serum supplementation they re-enter the cell cycle. HTC-IR cells were growth-arrested by 48-h serum starvation, and then were treated with 15% fetal calf serum. At given time points after treatment, cells were harvested, total RNA was isolated, and odc expression was assayed by semiquantitative RT-PCR. As shown in Figure 8, serum treatment of starved
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Figure 2 The effects of celecoxib and aspirin on proliferation of rat hepatoma HTC-IR cells determined by \(^{[3]H}\) thymidine incorporation. Cells were grown in 96-well plates in DMEM containing 10% FBS. After 24 h, cells were exposed to varying concentrations (2.5, 5, 25, and 50 \(\mu\)g ml\(^{-1}\)) of celecoxib or aspirin, and 72 h later 0.1 \(\mu\)Ci of \(^{[3]H}\)thymidine was added to each well for additional 4 h. The radioactivity of collected cellular DNA was determined by scintillation counting. Three independent experiments were performed and the results represent means \(\pm\) s.d. of radioactivity counts expressed in d.p.m. a, b, c indicate significant decrease (a, \(P<0.05\); b, \(P<0.01\); c, \(P<0.001\)) in \(^{[3]H}\)thymidine incorporation in aspirin- and celecoxib-treated cells compared to cells treated with DMSO.

Figure 3 Growth inhibition of HTC-IR cells by celecoxib or aspirin treatment. Cells were grown for 72 h with or without COX inhibitors, and combined populations of floating and adherent cells were counted using a haemocytometer. Results are expressed as the percentage of control cell yield (means \(\pm\) s.d.) of three independent experiments, each performed in duplicate. b, c indicate significant decrease (b, \(P<0.01\); c, \(P<0.001\)) in cell yield in aspirin- and celecoxib-treated cells compared to cells treated with DMSO.

HTC-IR cells progressively induced the expression of \(odc\) that achieved maximum at 6 h, the last time point measured. At this time point, \(odc\) was induced 3.5-fold. Pretreatment for 24 h with aspirin or celecoxib at a concentration of 5 \(\mu\)g ml\(^{-1}\) affected neither basal nor serum-stimulated levels of mRNA ODC. Therefore, the alterations in ODC activity, described above, caused by COX inhibitors occurred through post-transcriptional events.

COX inhibitors differentially alter gene expression

The mitogenic stimulation of various cells is accompanied by rapid induction of immediate-early genes (Herschman, 1991). We examined the effects of celecoxib and aspirin on the serum-induced gene transcription of three early genes: \(Egr-1\), \(c-fos\), and \(c-myc\).

Semiquantitative RT–PCR analysis showed that serum treatment of starved HTC-IR cells induced the expression \(c-myc\), \(Egr-1\), and \(c-fos\) (Figure 9). Aspirin and celecoxib did not significantly change basal and serum-stimulated \(c-myc\) expression (Figure 9A). Both aspirin and celecoxib changed the \(Egr-1\) (early growth regulated gene) expression pattern (Figure 9B). In fasting cells, and in those serum-stimulated for 15 min, the \(Egr-1\) mRNA levels of control and drug-treated cells were similar. However, in serum-stimulated cells for 30 min, the \(Egr-1\) expression was significantly lower in drug-treated cells as compared to control cells.

In contrast to the gene expression pattern of \(Egr-1\), expression of \(c-fos\) was drastically reduced by celecoxib at each time point measured, while the treatment with aspirin did not alter the levels of \(c-fos\) mRNA in unstimulated cells as well as in those stimulated by serum for 15 and 30 min (Figure 9C). To test whether the effect of celecoxib on \(c-fos\) expression might be COX-2-dependent or -independent, we examined the expression of this gene in HTC-IR cells that were treated with nimesulide, another COX-2 inhibitor. As shown in Figure 10, nimesulide did not significantly alter both basal and serum-stimulated \(c-fos\) expression levels.
DISCUSSION

This study contrasts and compares the effects of COX-1 and COX-2 inhibitors on mitogenesis, apoptosis, ODC activity, and pattern expression of selected growth-related genes in rat hepatocyte cell line, HTC-IR (expressing COX-2 mRNA as detected by RT–PCR – not shown).

COX-2 plays the dominant role among mechanisms regulating cell survival, cell proliferation, and oncogenesis. Its induction that was observed in human carcinomas of various organs including colon, breast, prostate, lung, oesophagus, pancreas, head, neck, and brain resulted in increased cell growth, inhibition of apoptosis, activation of angiogenesis, and decreased immune responsiveness (Subbaramaiah et al, 1996; Zimmermann et al, 1999).

The final product of COX-2 is PGH2 that acts as the immediate precursor for other PGs and thromboxanes involved in controlling cell proliferation (Herschman, 1994). The major product of COX-2 is PGE2, which induces expression of Bcl2, inhibitor of apoptosis (Sheng et al, 1998). Antiproliferative effect of NSAIDs was thought to be caused predominantly by COX-2 inhibition. However, the decreased production of PGE2 induced by COX-2 inhibitors did not correlate with the inhibition of cell proliferation (Erickson et al, 1999), and exogenous PGE2 did not prevent the antiproliferative or proapoptotic effects of COX inhibitors (Hanif et al, 1996; Elder 2003, Experimental Therapeutics).
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Figure 8 The effects of celecoxib and aspirin on the relative mRNA levels for ODC during serum stimulation. Untreated serum-starved HTC-IR cells and those treated for 24 h with 5 μg ml⁻¹ celecoxib or 5 μg ml⁻¹ aspirin were exposed to 15% FBS for 0, 15, 30, 60, 180, and 360 min. After harvesting the cells, total RNA was isolated and used in RT. PCR was carried out using [α³²P]dCTP (0.5 μCi reaction⁻¹) and odc primers (sense: 5’-GGGCCCCATCTGGCAATCGTCTC-3’; antisense: 5’-GGGCCCCATCTGGCAATCGTCTC-3’). PCR products were resolved by native PAGE and were quantified using a phosphorimager. Results are expressed as percentage mRNA level from serum-starved, untreated cells and represent means ± s.d. of two separate experiments.

Figure 9 The effects of celecoxib and aspirin on the relative mRNA levels for c-myc (A), Egr-1 (B), and c-fos (C) during serum stimulation. Untreated serum-starved HTC-IR cells and those treated for 24 h with 5 μg ml⁻¹ celecoxib or 5 μg ml⁻¹ aspirin were exposed to 15% FBS for 0, 15, and 30 min. After harvesting the cells, total RNA was isolated and used in RT. PCR was carried out using [α³²P]dCTP (0.5 μCi reaction⁻¹) and primers for c-myc (sense: 5’-ACGAAAAGCCCCAAGGTAGTT-3’; antisense: 5’-AAGGAAAAAAGAAAAAGATGGG-3’), Egr-1 (sense: 5’-GGGGGCCCACCTACACTCC-3’; antisense: 5’-GGGGGCCCACCTACACTCC-3’), and c-fos (sense: 5’-TTTGCCCTTCTGCGGATGCCTCT-3’; antisense: 5’-TTTGCCCTTCTGCGGATGCCTCT-3’). PCR products were resolved by native PAGE and were quantified using a phosphorimager. Results are expressed as percentage mRNA level from serum-starved, untreated cells and represent means ± s.d. of six separate experiments. a, b, c indicate significant decrease (a, P < 0.05; b, P < 0.01; c, P < 0.001) in mRNA levels in celecoxib- or aspirin-treated cells compared to control cells.

et al, 2000) suggesting that these processes may be unrelated. COX-2 inhibitors can reduce cell proliferation and survival also in cells that do not express COX-2 (Smith et al, 2000; Williams et al, 2000; Grosch et al, 2001) and cells transfected with an expression vector carrying cDNA of either COX-1 or COX-2 possessed a similar stimulation of growth rates (Williams et al, 2000). Therefore, both COX-2-dependent and -independent mechanisms may contribute to antiproliferative and proapoptotic effects of NSAIDs.

Celecoxib is a specific inhibitor of COX-2, inhibiting this enzyme at concentration of 10 μM. In contrast, aspirin acts as nonspecific COX inhibitor; at 10 μM it inhibits COX-1 while at a concentration higher than 100 μM inhibits also COX-2 (Dermott et al, 1999). In our experiments, aspirin at the inhibitory concentrations for COX-1 (2.5 and 5 μg ml⁻¹) did not exhibit any effect on HTR-IR cell proliferation. In contrast, celecoxib even at low concentrations (2.5 and 5 μg ml⁻¹) drastically inhibited proliferation of these cells. The extent of growth inhibition by celecoxib is related, at least in part, by proapoptotic effect induced by COX-2 inhibitors. As reported recently by others (Kusunoki et al, 2002; Yamazaki et al, 2002), the proapoptotic effect of celecoxib seems to be unrelated to the inhibition of COX-2.

ODC is the first and rate-limiting enzyme in the polyamine pathway and, therefore, it is a key regulatory enzyme in growth processes (Pegg, 1986). The enzyme function is to convert ornithine to putrescine, and changes in ODC activity reflect the rate of macromolecular synthesis. Cancer cells display upregulation of ODC, and sustained high level of ODC activity is implicated as an essential component of tumour development. Indeed, increased expression of ODC mRNA and high activity of ODC were observed in rat (Huber et al, 1989; Ostrowski et al, 2000) and human hepatocellular carcinoma (Tamori et al, 1994). ODC activity correlated also with growth rate in a panel of hepatoma
phases in G1 of the cell cycle and represent diverse functional
diminish the transcription of
observed in aspirin-treated cells. Since COX inhibitors did not
activity. Much lesser inhibitory effect on ODC activity was
1990).
S phase along with RNA and DNA synthesis (Heby and Persson,
of the cell cycle, whereas spermine and spermidine accumulate in
findings, inhibition of ODC activity by COX inhibitors may be a
consequence of at least dual events: COX-2 inhibitory-dependent
cells stronger than in growth-arrested cells suggesting that this
inhibitory effect of celecoxib may depend on the phase of
cell cycle. In contrast, the inhibitory effect of aspirin did not
considerate to proliferative state of the cell. In regard to these
findings, inhibition of ODC activity by COX inhibitors may be a
consequence of at least dual events: COX-2 inhibitory-dependent
cell death and some unknown COX-independent mechanism(s). In
fact, as reported recently (Turcchonowa et al, 2001), treatment of
colon cancer cells with indomethacin (a nonselective COX-1 and
COX-2 inhibitor) induced an irreversible cascade of events leading
to oxidative stress, activation of spermidine/spermine-acetyltrans-
erase (SSAT), polyamine depletion, and cell death. Thus, decreased
ODC protein level and its enzymatic activity caused by
indomethacin were rather a consequence of impaired proliferation
than a direct effect of indomethacin on ODC protein.

Growth signals are linked to gene expression. Immediate-early genes are
activated in a protein synthesis-independent manner and are
involved in cell proliferation, differentiation, apoptosis, and
oncogenic transformation (Jochum et al, 2001). They regulate later
phases in G1 of the cell cycle and represent diverse functional
classes including transcription factors. In this study, we
determined the expression of three immediate-early genes encoding
transcription factors, c-myc, Egr-1, and c-fos. c-myc target genes
include several genes involved in growth control and therefore
c-myc is implicated as a direct regulator of cell cycle machinery
(Schmidt, 1999). One of c-myc-dependent genes is odc (Bello-
Fernandez et al, 1993). During normal mitogenesis c-myc
expression transiently increases in G1. Egr-1 is a zinc-finger
transcription factor that is rapidly activated by a variety of
extracellular signals or tissue injury (Szabo et al, 2001).
Members of Fos family of transcription factors are thought to
have a primary function in activating transcription of delayed-
early genes expressed subsequently in the growth response (Taub,
1996).
c-fos, Egr-1, and several other immediate-early genes contain in
their promoter region serum response element (SRE) that is
rapidly activated in response to serum treatment. Serum activates
SRE-driven pathways via Ras-MAP kinase pathway (Tice et al,
2002). In our study, as expected, following serum readmission to
the serum-deprived HTC-IR cells, the expression of the immediate-
early genes significantly increased at 15–30 min.

Treatment of cells with aspirin or celecoxib did not alter basal
and serum-stimulated c-myc expression. Also, the treatment of rats
with the selective COX-2 inhibitor, NS-398 did not change c-myc
mRNA levels in colon mucosa as compared to untreated animals,
although in rat treated with a colon-specific carcinogen, azoxy-
methane, NS-398 significantly decreased the expression of c-myc
elevated by carcinogen (Kishimoto et al, 2002). Thus, neither the
short-term treatment in vitro nor the long-term treatment in vivo
with COX-2 inhibitors altered c-myc expression. In contrast,
indomethacin can reduce c-myc protein level in colon cancer cells
(Turchanowa et al, 2001). Since COX inhibitors did not alter the
transcription of odc, it is unlikely that the inhibition of ODC
activity might be a consequence of a direct relation between c-myc
and ODC.

Both aspirin and celecoxib inhibited serum-induced Egr-1
mRNA levels and these results are consistent with the inhibitory
effect of indomethacin and NS-398 on vascular endothelial growth
factor-stimulated expression of Egr-1 in human microvascular
endothelial cells (Szabo et al, 2001).

While c-fos expression was drastically reduced by celecoxib
at each time point measured, aspirin treatment did not alter the levels
of c-fos mRNA in fasting and serum-stimulated cells. This
inhibitory effect of celecoxib on c-fos expression seems to be a
celecoxib-specific and COX-2-independent since the treatment
with nimesulide, another highly selective COX-2 inhibitor, did not
significantly alter both basal and serum-stimulated c-fos expres-
sion levels.

A distinct subset of genes is regulated by the COX-2 inhibitor
(Zhang and Dubois, 2001), and alteration in gene expression
programming could be a target in the anticancer activity of COX
inhibitors. The diminishing of Egr-1 and c-fos by celecoxib may
reduce their target gene activation involved in controlling cellular
proliferation. However, changes in the array of gene expression
might not be a cause of antiproliferative and/or proapoptotic
effects of NSAIDs if other genes could compensate the down-
regulation of the immediate-early genes.

In summary, we have shown that the treatment of HTC-IR
hepatoma cells with the selective COX-2 inhibitor, celecoxib, was
associated with induction of apoptosis and complete inhibition of
cell proliferation, suppression of ODC activity, and diminished
c-fos expression. Aspirin exhibited a small antiproliferative effect
with moderate inhibition of ODC activity that was not associated
with the proapoptotic effect or alteration in c-fos expression. Both
inhibitors significantly decreased the expression of Egr-1.
Although celecoxib and aspirin share the ability to inhibit one or
both COX isozymes, the molecular mechanisms of antiproliferative
and proapoptotic effects of NSAIDs are still not well understood.
Some of the observed effects are likely to be related to COX-
independent pathways and may be drug-specific. Thus, the precise
mechanisms of NSAIDs action should be defined before using
these drugs for cancer chemopreventive therapy.
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REFERENCES

Beejer U, Wolfe MM (1999) Cyclooxygenase 2 selective inhibitors: panacea or flash in the pan. Gastroenterology 117: 1002–1014

Bello-Fernandez C, Packham G, Cleveland JL (1993) The ornithine decarboxylase gene is a transcriptional target of c-Myc. Proc Natl Acad Sci USA 90: 7804–7808

Bennett A, Taccia MD, Stamford IF, Zebro T (1977) Prostaglandins from tumours of human large bowel. Br J Cancer 35: 881–884

Denkert C, Kobel M, Berger S, Siegert A, Leclere A, Trefzer U, Hauptmann S (2001) Expression of cyclooxygenase 2 in human malignant melanoma. Cancer Res 61: 303–308

Dermott JM, Reddy RMV, Onesime D, Reddy EP, Dhanasekaran N (1999) Oncogenic mutant of Gs2 stimulates cell proliferation through cyclooxygenase-2 signaling pathway. Oncogene 18: 7185–7189

Ding XZ, Tong WG, Adrian TE (2000) Blockade of cyclooxygenase-2 inhibitors proliferation and induces apoptosis in human pancreatic cancer cells. Anticancer Res 20: 2625–2631

Eberhart CE, DuBois RN (1998) Prostaglandin E2 levels in human brain tumor tissues and expression in rat colon carcinogenesis induced by azoxymethane. (2002) Effects of cyclooxygenase-2 inhibitor NS-398 on APC and c-myc carcinogenesis. Cancer Res 62: 2742–2744

Hanif R, Pittas A, Feng Y, Koutsos MI, Qiao L, Stainano-Coico L, Shiff SI, Rigas B (1996) Effects of nonsteroidal anti-inflammatory drugs on proliferation and on induction of apoptosis in colon cancer cells by a prostaglandin-independent pathway. Biochem Pharmacol 26: 237–245

Heby O, Persson L (1990) Molecular genetics of polypamine synthesis in eukaryotic cells. Trends Biochem Sci 4: 153–158

Herschman HR (1991) Primary response genes induced by growth factors. J Biol Chem 266: 281–319

Herschman HR (1994) Regulation of prostaglandin synthase-1 and cyclooxygenase-2. Cancer Metastasis Rev 13: 241–256

Huber BE, Heilman CA, Thorgerisson SS (1989) Poly(A)+ RNA levels of growth-, differentiation- and transformation-associated genes in the progressive development of hepatocellular carcinoma in the rat. Hepatology 9: 756–762

Jochum W, Passegue E, Wagner EF (2001) AP-1 in mouse development and cancer. J Cell Biol 9: 240–246

Kawamura T, Rao CV, Seibert K, Reddy BS (1998) Chemopreventive activity of celecoxib, a specific cyclooxygenase-2 inhibitor, against colon carcinogenesis. Cancer Res 58: 409–412

Kishimoto Y, Yashima K, Morisawa T, Shiota G, Kawasaki H, Hasegawa J (2002) Effects of cyclooxygenase-2 inhibitor NS-398 on APC and c-myc expression in rat colon carcinogenesis induced by azoxymethane. J Gastroenterol 37: 186–193

Kokolou E, Tuter Y, Sandicki KS, Yazici Z, Ulakoglu EZ, Sonmez H, Ozurt E (1998) Prostaglandin E2 levels in human brain tumor tissues and arachidonic acid levels in plasma membrane of human brain tumors. Cancer Lett 132: 17–21

Kuwonoski N, Yamanouchi R, Kawai S (2002) Induction of apoptosis in rheumatoid synovial fibroblasts by celecoxib, but not by other selective cyclooxygenase 2 inhibitors. Arthritis Rheum 46: 3159–3167

Lui KH, Kirschbaum A, Yao S, Lee R, Holland JF, Levine AC (2000) Inhibition of cyclooxygenase-2 suppresses angiogenesis and the growth of prostate cancer in vivo. J Urol 164: 820–825

Maxwell WJ, Kelleher D, Keating JJ, Hogan FP, Bloomfield FJ, MacDonald GS, Keeling PW (1990) Enhanced secretion of prostaglandin E2 by tissue-fixed macrophages in colonar carcinoma. Digestion 47: 160–166

Ostrowski J, Wosszychnski M, Kowalczyk P, Wocial T, Henning E, Trzeciak L, Janik P, Bomatzky K (2000) Increased activity of MAP, p70s6, and p90rs kinases is associated with AP-1 activation in spontaneous liver tumors, but not in adjacent tissue in mice. Br J Cancer 82: 1041–1050

Pegg AE (1986) Recent advances in the biochemistry of polyamines in eukaryotes. Biochem J 234: 249–262

Scheinemachers DM, Everson RB (1994) Aspirin use and lung, colon and breast cancer incidence in a prospective study. Epidemiology 5: 138–146

Schmid EV (1999) The role of c-myc in cellular growth control. Oncogene 18: 2988–2996

Sheng H, Shao J, Morrow JD, Beauchamp RD, DuBois RN (1998) Modulation of apoptosis and Bcl-2 expression by prostaglandin E2 in human colon cancer cells. Cancer Res 58: 362–366

Smith ML, Hawcroft G, Hull MA (2000) The effects of non-steroidal anti-inflammatory drugs on human colorectal cancer cells: evidence of different mechanisms of action. Eur J Cancer 36: 664–674

Smith WL, Garavito RM, DeWitt DL (1996) Prostaglandin endoperoxide H synthase (cyclooxygenase)-1 and 2. J Biol Chem 271: 33157–33160

Subbaramiah K, Telang N, Ramonetti JT, Araki R, DeVito B, Wessels BK, Danenberg AJ (1996) Transcription of cyclooxygenase-2 is enhanced in transformed mammary epithelial cells. Cancer Res 56: 4424–4429

Szabo IL, Pai R, Soreghan B, Jones MK, Baatar D, Kawanaka H, Tarnowski AS (2001) NSAIDs inhibit the activation of egr-1 gene in microvascular endothelial cells. A key to inhibition of angiogenesis? J Physiol 95: 379–383

Takyo MM (1998A) Cyclooxygenase inhibitors in tumorigenesis (Part I). J Natl Cancer Inst 90: 1529–1536

Taketo MM (1998B) Cyclooxygenase inhibitors in tumorigenesis (Part II). J Natl Cancer Inst 90: 1609–1615

Tamori A, Nishiguchi S, Kuroki T, Seki S, Kobayashi K, Kinoshita H, Otani S (1994) Relationship of ornithine decarboxylase activity and histological findings in human hepatocellular carcinoma. Hepatology 20: 1179–1186

Taub R (1996) Transcriptional control of liver regeneration. FASEB J 10: 413–427

Thun MJ, Namboodiri MM, Heath CW (1991) Aspirin use and reduced risk of fatal colon cancer. N Engl J Med 325: 1593–1596

Tiberio L, Maier JA, Schiaffonati L (2001) Down-regulation of c-myc expression by phorbol ester protects CEM T leukaima cells from starvation-induced apoptotic role of ornithine decarboxylase and polyamins. Cell Death Differ 9: 967–976

Tice DV, Soloviev I, Polakis P (2002) Activation of the Wnt pathway interferes with SRE-driven transcription of immediate early genes. J Biol Chem 277: 6118–6123

Turchanowa L, Dauletbayev N, Milovic V, Stein J (2001) Nonsteroidal anti-inflammatory drugs stimulate spermidine/spermine acetyltransferase and deplete polypamine content in colon cancer cells. Eur J Clin Invest 31: 887–893

Uefuji K, Ichikura T, Shinomiya N, Mochizuki H (2000) Down-regulation of c-myc expression by phorbol ester protects CEM T leukemia cells from starvation-induced apoptotic role of ornithine decarboxylase and polyamins. Cell Death Differ 9: 967–976

Vermes I, Haanen C, Bichel DJ, Schaafsm MR, Kalebek-Batenburg E, Reutelingsperger CP (1997) Apoptosis and secondary necrosis of lymphocytes in culture. Acta Haematol 98: 8–13

Whittle BJ (2000) COX-1 and COX-2 products in the gut: therapeutic impact of COX-2 inhibitors. Gut 47: 320–325

Williams CS, Watson AJ, Sheng H, Heo J, Shao J, DuBois RN (2000) Celecoxib prevents tumor growth in vivo without toxicity to normal gut;
lack of correlation between in vitro and in vivo models. *Cancer Res* 60: 6045–6051
Williams-Ashman HG, Coppoc LL, Weber G (1972) Imbalance ornithine metabolism in hepatomas of different growth rates expressed in formation of putrescine, spermidine, and spermine. *Cancer Res* 32: 1924–1932
Yamazaki R, Kusunoki N, Matsuzaki T, Hashimoto S, Kawai S (2002) Selective cyclooxygenase-2 inhibitors show a differential ability to inhibit proliferation and induce apoptosis of colon adenocarcinoma cells. *FEBS Lett* 531: 278–284
Zhang Z, DuBois RN (2001) Detection of differentially expressed genes in human colon carcinoma cells treated with a selective COX-2 inhibitor. *Oncogene* 20: 4450–4456
Zimmermann KC, Sarbia M, Weber AA, Borchard F, Gabbert HE, Schror K (1999) Cyclooxygenase-2 expression in humam esophageal carcinoma. *Cancer Res* 59: 198–204

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