Insulin-like Growth Factor-I Antagonizes the Antiproliferative Effects of Cyclooxygenase-2 Inhibitors on BxPC-3 Pancreatic Cancer Cells

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ABSTRACT

Cyclooxygenase (COX)-2 inhibitors demonstrate modest antineoplastic activity in experimental models of human malignancies, but little is known about factors that may confer resistance to their antiproliferative actions. We observed that fetal bovine serum antagonizes growth inhibition and G_1 arrest induced by two COX-2 inhibitors (NS-398 and celecoxib) on BxPC-3 pancreatic cancer cells. We investigated the hypothesis that insulin-like growth factor I (IGF-I), a major survival factor present in serum, mediates these effects. Treatment of BxPC-3 cells with 25 μM celecoxib in 1% fetal bovine serum-containing medium for 48 h resulted in a ~40% decrease in cell viability. Coincubation of BxPC-3 cells with 25 μM celecoxib and 50 ng/ml IGF-I resulted in complete attenuation of the celecoxib-associated decrease in cell viability. Cell cycle analysis revealed that this IGF-I-induced increase in cell viability was correlated with an IGF-I-induced inhibition of celecoxib-mediated G_1 arrest. Similar results were observed when another COX-2 inhibitor (50 μM NS-398) was used. When IGF-binding protein-3 (an inhibitor of IGF-I bioactivity) was added in combination with 25 μM celecoxib, enhanced growth inhibition was observed (~60% decrease in cell viability). Treatment of BxPC-3 cells with a higher dose (50 μM) of celecoxib for 24 h resulted in the induction of apoptosis, as assayed by flow cytometry and poly(ADP-ribose) polymerase cleavage. Addition of 50 ng/ml IGF-I resulted in a complete attenuation of celecoxib-induced apoptosis. The protection from celecoxib-induced apoptosis by IGF-I correlated with an increase in the levels of the activated antiapoptotic protein Akt. These results suggest that alterations of IGF-I levels or IGF-I receptor signal transduction modulate the antineoplastic actions of COX-2 inhibitors.

INTRODUCTION

A large body of data has suggested a role for NSAIDs[2] in the treatment or prevention of various cancers. The classic actions of NSAIDs involve inhibition of the rate-limiting enzyme in the prostaglandin synthesis cascade, COX. This enzyme exists in two isoforms: COX-1 and COX-2. The expression of COX-1 is constitutive in most tissues, whereas the expression of COX-2 is inducible by cytokines, growth factors, and tumor promoters (1).

Several reports show that many types of malignant tissues overexpress the COX-2 enzyme (2, 3), and that NSAIDs inhibit proliferation and/or induce apoptosis in various cancer cell lines both *in vitro* (4, 5) and *in vivo* (6, 7). It is believed that these antineoplastic effects are because of inhibition of prostaglandin synthesis and modification of other non-COX targets (reviewed in Ref. 8). Administration of COX-2 selective NSAIDs (COX-2 inhibitors) to patients minimizes the gastrointestinal tract toxicity associated with COX-1 inhibition (9), and it is hopeful that COX-2 inhibitors can be used in the clinic in combination with other agents for the treatment of various cancers.

Although the antineoplastic actions of COX-2 inhibitors have been well documented in the literature, little is known about factors that may confer resistance to their effect. Chang and Weng (10) have proposed that the cytotoxic effect of COX-2 inhibitors on cancer cells may be influenced by the extracellular environment. This hypothesis was based on their results showing that NS-398 induced apoptosis in A549 lung cancer cells under serum-free conditions, whereas this drug induced G_1 arrest rather than apoptosis in cells treated in 10% serum medium.

We wished to additionally clarify whether serum has an effect on the antineoplastic activity of COX-2 inhibitors. We chose to investigate this hypothesis in a pancreatic cancer cell line, because treatment of pancreatic cancer represents an unmet medical need. Furthermore, COX-2 inhibitors have been shown to inhibit the growth of several pancreatic cancer cell lines *in vitro* (11, 12), and COX-2 levels are elevated in human pancreatic adenocarcinomas when compared with the surrounding stroma (13). As well, COX-2 inhibitors have been shown to enhance the cytotoxic effect of gemcitabine on pancreatic cancer cells *in vitro* (14).

We demonstrate that IGF-I, a component of serum, inhibits the antiproliferative effects of two COX-2 inhibitors (NS-398 and celecoxib) on BxPC-3 pancreatic cancer cells. Conversely, we show that IGFBP-3, a protein that inhibits IGF-I receptor activation by sequestering IGF-I (15), enhances celecoxib-induced growth inhibition. Furthermore, we show that IGF-I protects BxPC-3 cells from celecoxib-induced apoptosis. IGF-I is known to activate the antiapoptotic protein Akt (16), and because down-regulation of Akt activity has been hypothesized as one of the mechanisms involved in celecoxib-induced apoptosis (4), we also show that IGF-I and celecoxib have opposing effects on Akt activation in our experimental system.

MATERIALS AND METHODS

Cell Line and Treatment. BxPC-3 pancreatic cancer cells (American Type Culture Collection, Manassas, VA) were cultured in RPMI 1640 supplemented with 10% FBS at 37°C and 5% CO_2. These cells have been shown previously to express high levels of COX-2 protein and are growth inhibited by NSAIDs (11, 12).

Celecoxib was obtained from Searle Pharmacia (St. Louis, MO). A stock solution of 50 mM in DMSO was used. NS-398 was purchased from Cayman Chemical (Ann Arbor, MI). A stock solution of 100 mM in DMSO was used. Stock solutions were added to the test medium at dilutions of at least 1:1000, and control samples were treated with vehicle (DMSO). The concentration of DMSO in the test medium never exceeded 0.1% (v/v). IGF-I and recombinant human IGFBP-3 were obtained from Protigen Incorporation (Mountain View, CA) and were added to test medium to a final concentration of 50 ng/ml and 1 μg/ml, respectively.

Cell Proliferation Assay. We used a MTT assay to determine cell proliferation (cell viability). Cells (3×10^5) were plated in six-well plates in medium containing 10% FBS. After 24 h, the cells were ~40% confluent, and the medium was changed to test medium specific for each experiment. After 48 h, MTT (Sigma Chemical Co., St. Louis, MO) was added to a final concentration of 1 mg/ml, and the reaction mixture was incubated for 3 h at 37°C. The resulting crystals were dissolved in 0.04% HCl in isopropanol, and the absorbance was read at 562 nm.

Flow Cytometry to Assay Cell Cycle Distribution and Apoptosis. Cells were plated in medium containing 10% FBS, and after 24 h, the cells were

~40% confluent and the medium was changed to test medium specific for each experiment. After 24 h, adherent cells were collected using trypsin-EDTA, and floating cells were collected by centrifugation. The cells were combined and washed twice with ice-cold PBS, and fixed in 70% ethanol at $-20°C$ overnight. For cell cycle analysis, the cells were washed twice with ice-cold PBS and resuspended in propidium iodide buffer (PBS, 0.1% Triton X-100, 0.1 mM EDTA, 0.05 mg/ml RNase A, and 50 μM propidium iodide). After 30 min at room temperature, the cell cycle distribution was determined by flow cytometry with a FACScan (Beckman Coulter, Fullerton, CA). The proportion of cells in the hypodiploid (sub-G_1) area were considered to be apoptotic.

Western Blotting. After each treatment, both floating and adherent cells were lysed in radioimmunoprecipitation assay buffer (0.1 mM dibasic sodium phosphate, 1.7 mM monobasic phosphate, 150 mM NaCl, 1% NP40, 0.5% SDS, 0.2 mM sodium vanadate, 0.2 mM phenylmethylsulfonyl fluoride, and aprotinin at 0.2 units/ml). Protein from clarified lysates (40 μg) was resolved electrophoretically on denaturing 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. Membranes were probed with antibodies specific for cleaved PARP (Biosource, Camarillo, CA), COX-2 (Cayman Chemical, Ann Arbor, MI), phospho-[473]Ser-Akt (New England Biolabs, Beverly, MA), or total Akt (New England Biolabs). The position of protein was visualized with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). To confirm equal loading, membrane was stripped and reprobed using an antibody specific for α-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA).

Statistical Analysis. All of the data are shown as means ± SE. To assess the statistical significance of observed differences, we used Student's t test. All of the tests were two-sided, and $Ps < 0.05$ were considered to be statistically significant.

RESULTS AND DISCUSSION

To determine whether modulation of FBS concentration in culture medium influences the antiproliferative actions of COX-2 inhibitors on pancreatic cancer cells, we treated BxPC-3 cells for 48 h with 50 μM NS-398, 25 μM celecoxib, or 50 μM celecoxib in medium containing either a low (1%) or high (10%) FBS concentration. As seen in Fig. 1A, increasing the FBS concentration in the culture medium significantly diminished the efficacy of the COX-2 inhibitors to inhibit BxPC-3 proliferation. This effect was most dramatic for the 50 μM celecoxib treatment condition, where treatment under 1% FBS conditions resulted in virtually no viable cells after 48 h, whereas treatment under 10% FBS conditions resulted in ~50% of the cells remaining viable. Fig. 1B illustrates that the protective effect of FBS against 50 μM celecoxib-induced growth inhibition is consistent over many FBS concentrations.

To additionally investigate this observation, cell cycle analysis was performed after 24-h treatment. As seen in Fig. 2A, treatment with 50 μM NS-398 or 25 μM celecoxib in 1% FBS-containing medium results in the induction of G_1 arrest (18% increase in the proportion of cells in G_1 for 50 μM NS-398 and 14% increase for 25 μM celecoxib). Repeating these treatment conditions in 10% FBS-containing medium (Fig. 2B) results in a decrease in the induction of G_1 arrest (9% increase in the proportion of cells in G_1 for 50 μM NS-398, and 5% increase for 25 μM celecoxib). These results are consistent with those of Yip-Schneider *et al.* (14), who have shown that NSAIDs induce G_1 arrest in BxPC-3 cells by decreasing the expression of various cyclins and increasing the expression of the cyclin-dependent kinase inhibitors p21 and p27.

Under 1% FBS conditions, treatment with 50 μM celecoxib resulted in a 40% increase in the proportion of cells undergoing apoptosis, with no induction of G_1 arrest (Fig. 2A). In the presence of 10% FBS, the apoptosis induction by 50 μM celecoxib was completely blocked (Fig. 2B), although G_1 arrest was observed (25% increase in the proportion of cells in G_1). The switch from apoptosis to G_1 arrest as a function of serum concentration was correlated with an attenuation of the
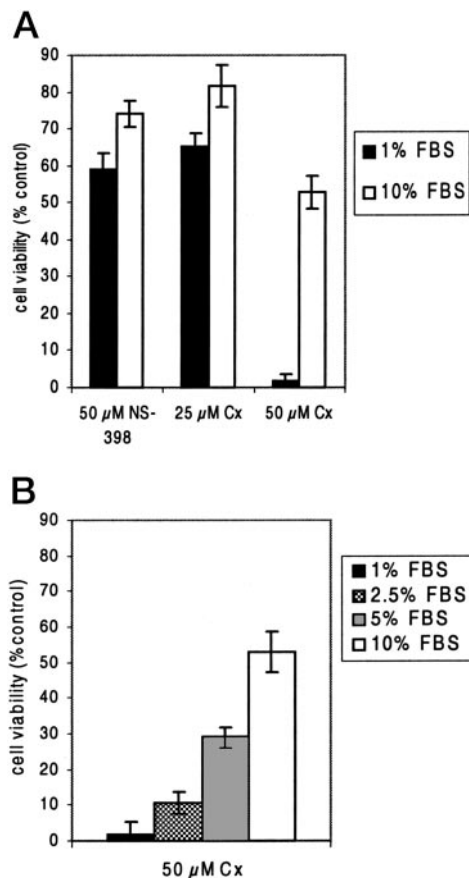

Fig. 1. FBS antagonizes NSAID-induced growth inhibition. BxPC-3 cells were treated for 48 h in the presence of either 50 μM NS-398, 25 μM celecoxib (Cx), or 50 μM Cx using various FBS concentrations. Cell viability was determined by the MTT assay, and the results are expressed as a percentage of the control sample with identical FBS concentration. All changes in cell viability because of increasing the FBS concentration were statistically significant with $P < 0.05$; *bars*, ±SD.

growth inhibitory actions of 50 μM celecoxib, as demonstrated by the cell viability data in Fig. 1.

There are two possible explanations for the observed effects of FBS. One hypothesis is that growth factors present in FBS could increase COX-2 expression, overcoming the NSAID-induced inhibition of COX-2 activity. This hypothesis was disproved by Western blotting showing that COX-2 protein levels remained constant when the FBS concentration in the culture medium was varied between 1% and 10% (data not shown). An alternate hypothesis is that components of FBS are inducing survival signals in BxPC-3 cells, protecting them from the effects of the COX-2 inhibitors. One candidate molecule present in FBS is IGF-I. Activation of the IGF-I receptor by IGF-I results in the induction of the phosphatidylinositol 3'-kinase/Akt and mitogen-activated protein kinase pathways, which are known to promote mitogenesis and survival in a variety of cell types (reviewed in Ref. 16).

To determine whether IGF-I could be mediating the observed effects of FBS in this system, BxPC-3 cells were treated with COX-2 inhibitors in 1% FBS, with or without exogenous IGF-I (50 ng/ml). It should be noted that no significant changes in COX-2 protein expression were detected on IGF-I addition in this system (data not shown). As seen in Fig. 3A, treatment with 50 μM NS-398 or 25 μM celecoxib for 48 h resulted in a ~40% decrease in cell viability. Coincubation with the NSAID and IGF-I resulted in a complete attenuation of the NSAID-associated decrease in cell viability. This effect was accompanied by a complete blocking of the NSAID-induced G_1 arrest (Fig. 3B).
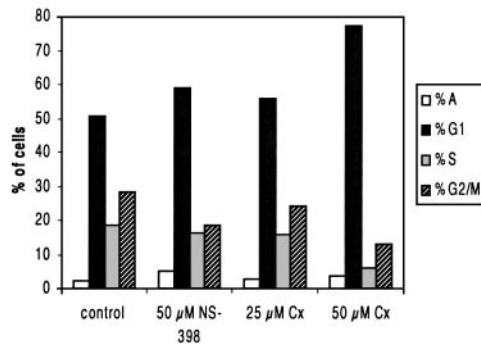
A) 1% FBS
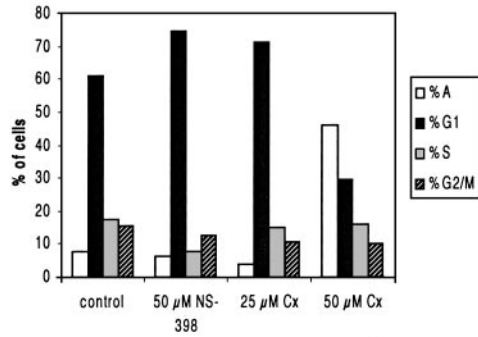
B) 10% FBS

Fig. 2. FBS protects BxPC-3 cells from undergoing NSAID-induced G_1 arrest and apoptosis. BxPC-3 cells were treated with the indicated concentrations of either NS-398 or celecoxib (Cx) for 24 h in either 1% FBS (A) or 10% FBS (B). Cell cycle analysis was determined using propidium iodide staining and flow cytometry. Data are presented as percentage of cells in a particular stage of the cell cycle with the cells in the hypodiploid (sub-G_1) area representing apoptotic cells.

After having established that IGF-I antagonizes COX-2 inhibitor-induced growth inhibition, we wished to determine whether an inhibitor of IGF-I would be able to enhance the antiproliferative actions of COX-2 inhibitors. To study this hypothesis, we chose to use IGFBP-3, a known inhibitor of IGF-I bioactivity, which acts by sequestering IGF-I and preventing its activation of the IGF-I receptor (15). Fig. 3C (*Lane 4*) shows that 1 μg/ml IGFBP-3 alone has a modest growth-inhibitory effect on BxPC-3 cells growing in 1% FBS-containing medium (~20% decrease in cell viability). When 1 μg/ml IGFBP-3 was added in combination with 25 μM celecoxib, additive growth inhibition was observed with the combination treatment resulting in a ~60% decrease in cell viability (Fig. 3C, *Lane 5*). As expected, when 1 μg/ml IGFBP-3 was added to cells treated with both IGF-I and celecoxib, the protective effect of IGF-I was completely abolished (Fig. 3C, *Lane 6*), as the growth inhibition was similar to that observed for celecoxib alone (~40% decrease in cell viability).

As seen in Fig. 3A, IGF-I has no effect on cells treated with 50 μM celecoxib in 1% FBS-containing medium. However, when the background FBS concentration was increased to 5% FBS (Fig. 4A), addition of 50 ng/ml IGF-I resulted in a 16% increase in cell viability. This observation raises the possibility that IGF-I is working in synergy with another growth factor in FBS to confer resistance to the antiproliferative actions of 50 μM celecoxib. Treatment with 50 μM celecoxib in 5% FBS-containing medium results in a 16% increase in the proportion of apoptotic cells compared with control (Fig. 4B, panel 2). Coincubation of 50 ng/ml IGF-I with 50 μM celecoxib resulted in a complete block of apoptosis induction (Fig. 4B, panel 3). However, in the presence of IGF-I, 50 μM celecoxib did induce a substantial G_1

arrest (17% increase in the proportion of cells in G_1 compared with control). This result parallels our prior observation that increasing the FBS concentration from 1% to 10% attenuates 50 μM celecoxib-induced growth inhibition by promoting a switch from apoptosis to G_1 arrest (Fig. 2).

To verify the antiapoptotic effect of IGF-I, a second apoptosis end point was used, namely PARP cleavage. As seen in Fig. 4C, treatment with 50 μM celecoxib in 5% FBS-containing medium for 24 h resulted in an increase in the amount of cleaved PARP (Fig. 4C, *Lane 2*) indicating the induction of apoptosis. PARP cleavage was completely blocked by the addition of IGF-I (Fig. 4C, *Lane 4*). The lack of apoptosis induction by 50 μM celecoxib in 10% FBS containing
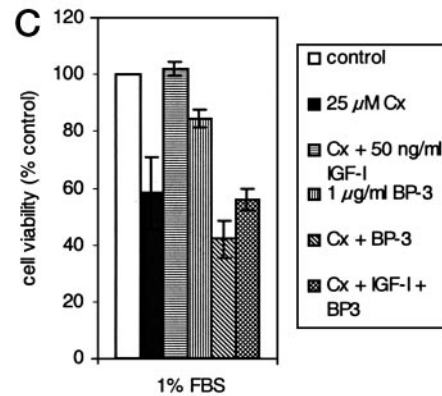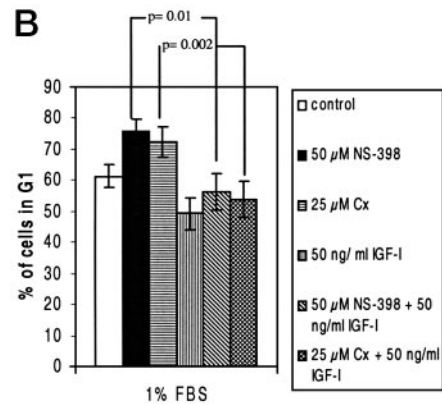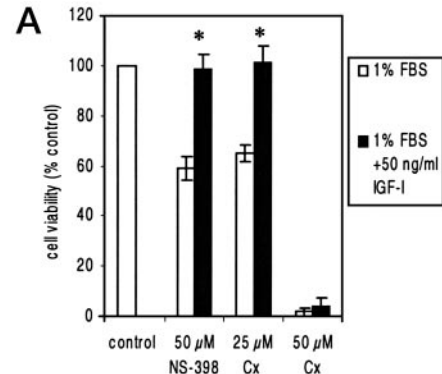

Fig. 3. IGF-I antagonizes NSAID-induced growth inhibition. *A,* BxPC-3 cells were treated for 48 h with the indicated NSAIDs with or without the addition of 50 ng/ml IGF-I. Cell viability was determined by the MTT assay, and the results are expressed as a percentage of the control sample. Statistically significant changes in cell viability ($P < 0.05$) are marked with *. *B,* BxPC-3 cells treated for 24 h were subjected to cell cycle analysis by propidium iodide staining and flow cytometry. Data shown are the percentage of cells in the G_1 phase under each condition. *C,* BxPC-3 cells were treated for 48 h with 25 μM celecoxib, with or without the addition of 50 ng/ml IGF-I and/or 1 μg/ml IGFBP-3. Cell viability was determined by the MTT assay, and the results are expressed as a percentage of the control sample; *bars,* ±SD.
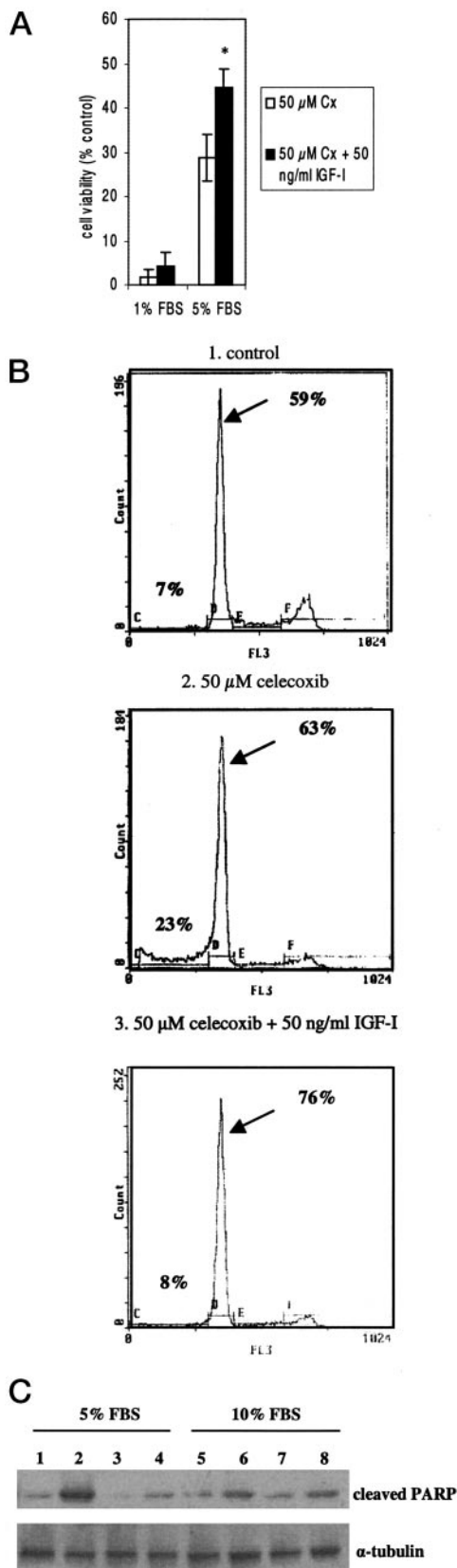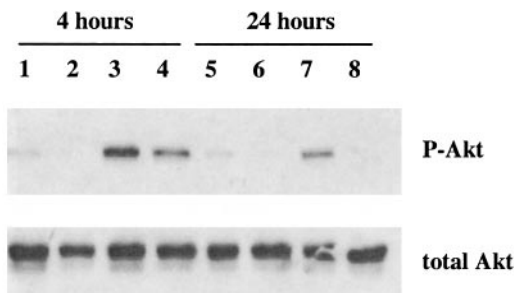
Fig. 5. Antagonistic effects of IGF-I and celecoxib on Akt phosphorylation. BxPC-3 cells were treated with DMSO vehicle (control: *Lanes 1* and *5*), 50 μM celecoxib *(Lanes 2* and *6*), 50 ng/ml IGF-I *(Lanes 3* and *7*) or 50 μM celecoxib and 50 ng/ml IGF-I in combination *(Lanes 4* and *8*) in 5% FBS-containing medium for either 4 or 24 h. Whole cells lysates were electrophoresed and probed by Western blot with anti-P-^{473}Ser-Akt and anti-Akt antibodies. The minor variations observed in total Akt levels *(lower panel)* are negligible when compared with the variations in P-Akt levels.

Fig. 4. IGF-I antagonizes 50 μM celecoxib-induced apoptosis. *A,* MTT assay of BxPC-3 cells treated for 48 h with 50 μM celecoxib in 5% FBS containing medium $\pm$ 50 ng/ml IGF-I. *B,* cell cycle analysis of BxPC-3 cells: *histogram 1,* 5% FBS control; *histogram 2,* 50 μM celecoxib; *histogram 3,* 50 μM celecoxib +50 ng/ml IGF-I. Percentage of apoptotic cells (sub-G$_1$) and cells in G$_1$ are indicated. *C,* Western blot for PARP cleavage from cell lysates of BxPC-3 cells treated for 24 h in either 5% or 10% FBS-containing medium: *Lanes 1* and *5,* control; *Lanes 2* and *6,* 50 μM celecoxib; *Lanes 3* and *7,* 50 ng/ml IGF-I; *Lanes 4* and *8,* 50 μM celecoxib +50 ng/ml IGF-I; *bars,* $\pm$SD.

medium observed in Fig. 2 was confirmed using the PARP cleavage end point. As expected, no induction of apoptosis was observed (Fig. 4*C, Lane 6*).

The fact that IGF-I protected BxPC-3 cells from 50 μM celecoxib-induced apoptosis is consistent with other publications that demonstrate the protective actions of IGF-I against apoptosis induced by other means (17, 18). We next wished to investigate the downstream effectors of IGF-I signaling that lead to protection from apoptosis in our system. As down-regulation of Akt activity has been hypothesized as one of the mechanisms involved in celecoxib-induced apoptosis (4), one strong candidate was the IGF-I receptor/phosphatidylinositol 3'-kinase/Akt pathway, which leads to phosphorylation and activation of the serine/threonine kinase Akt (16). P-Akt subsequently phosphorylates and inactives several proapoptotic proteins such as pro-caspase-9 and Bad (19).

As seen in Fig. 5, treatment of BxPC-3 cells with 50 μM celecoxib for 4 h reduced the low level of P-Akt present under control conditions (Fig. 5, *Lane 2 versus Lane 1*). As expected, IGF-I (50 ng/ml) significantly up-regulated P-Akt levels (Fig. 5, *Lane 3*). Importantly, coincubation with celecoxib and IGF-I revealed titratable effects of their opposing effects on P-Akt levels (Fig. 5, *Lane 4*). The IGF-I-induced increase in P-Akt could at least partially explain the anti-apoptotic effect of IGF-I in this system. These results are consistent with those of Hsu *et al.* (4), who have shown that overexpression of constitutively active Akt in PC-3 prostate cancer cells protected against celecoxib-induced apoptosis.

At the 24-h time point, the observed decrease in P-Akt by celecoxib (Fig. 5, *Lane 6*) and increase in P-Akt by IGF-I (Fig. 5, *Lane 7*) are similar to those observed at 4 h. However, when IGF-I and celecoxib were added in combination for 24 h (Fig. 5, *Lane 8*), we observed virtually no P-Akt present. Therefore, the titration of P-Akt levels by IGF-I and celecoxib is time dependent, with IGF-I having an earlier effect than celecoxib. These results are consistent with the possibility of the activation of a celecoxib-induced phosphatase that acts on Akt.

In this report, we show that the peptide hormone IGF-I interferes with the ability of COX-2 inhibitors to promote G$_1$ arrest and induce apoptosis in pancreatic cancer cells. These results suggest that growth factors present in serum, such as IGF-I, can influence the antiproliferative actions of COX-2 inhibitors and attenuate their activity. Such studies may lead to therapeutic strategies to optimize the use of COX-2 inhibitors in the treatment of pancreatic cancer.

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