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Regulation of cyclooxygenase-2 (COX-2) expression in human pancreatic carcinoma cells by the insulin-like growth factor-I receptor (IGF-IR) system

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Abstract

Both the insulin-like growth factor-I receptor (IGF-IR) and cyclooxygenase-2 (COX-2) are frequently overexpressed in pancreatic cancer. We hypothesized that IGF-IR is directly involved in induction of COX-2 and sought to investigate signaling pathways mediating this effect. Pancreatic cancer cells (L3.6pl) were stably transfected with a dominant-negative receptor (IGF-IR DN) construct or empty vector (pcDNA). Cells were stimulated with IGF-I to determine activated signaling intermediates and induction of COX-2. Signaling pathways mediating COX-2 induction were identified using signaling inhibitors. IGF-I up-regulated COX-2 selectively via the MAPK/ (Erk1/2) pathway. In addition, IGF-IR DN cells showed a marked decrease in constitutive COX-2 and a blunted response to IGF-I. Similarly, treatment with an anti-IGF-IR antibody effectively inhibited IGF-IR and MAPK/Erk activation and decreased COX-2 in parental cells. In conclusion, activation of IGF-IR mediates COX-2 expression in human pancreatic cancer cells.

Keywords

cyclooxygenase-2; pancreatic cancer; insulin-like growth factor-I receptor; IRS-1; signaling

1. Introduction

The insulin-like growth factor-I receptor (IGF-IR) has been proven an important promoter of tumour growth in various cancer entities [1,2], and therefore has been considered a target for therapy [3,4]. Overexpression of IGF-IR and its ligands (IGF-I, -II), have been demonstrated in a variety of gastrointestinal cancers, including human pancreatic cancer [5-7]. In pancreatic cancer, IGF-IR mediates several processes involved in tumor progression and metastases [8,

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9], which, in part, involves mechanisms that lead to an autocrine activation of the IGF-I receptor [7,8,10]. Furthermore, the IGF-I/IGF-IR system may contribute to increased angiogenesis in tumours by inducing the expression of vascular endothelial growth factor (VEGF) [7,11,12]. In our previous study, we were able to demonstrate that IGF-IR mediates VEGF expression and angiogenesis in pancreatic cancer [7], suggesting that targeting IGF-IR could be valuable for therapy of this aggressive cancer entity.

Furthermore, expression of certain proinflammatory mediators, such as prostaglandins, has been reported to be up-regulated in cancer tissues which could be linked to invasive cancer growth [13-15]. One important step of prostaglandin synthesis is the conversion of arachidonic acid to distinct prostaglandins and other eicosanoids, a process facilitated by cyclooxygenases (COXs) [16]. Overexpression of cyclooxygenase-2 (COX-2) has been described for several different malignancies, including lung, colon, prostate, breast and pancreatic cancers [17,18]. Importantly, evidence exists that COX-2 represents an attractive target in pancreatic cancer since it is highly up-regulated and involved in anti-apoptotic mechanisms [19]. Moreover, experimental studies suggest that COX-2 plays an important role in tumour progression [19-21]. The importance of decreasing COX-2 in tumours is also supported by several experimental models using conditional COX-2 knockout mice and COX-2-selective inhibitors, in which a reduction in COX-2 resulted in decreased tumour growth in colorectal tumour models [22-25].

Although COX-2 regulation has been studied in several tumour types, its regulation in pancreatic cancer has not been fully elucidated. The correlation of IGF-IR activation and COX-2 expression has been noted in a study in colon cancer cells in which exposure to recombinant IGF-II increased COX-2 [26]. Due to the fact that IGF-IR may activate specific pathways that have previously been shown to lead to an induction of COX-2 in other models [7,27], we hypothesized that IGF-I/IGF-IR could mediate COX-2 expression in human pancreatic cancer cells.

2. Materials and Methods

2.1 Cells and stable transfection

The human pancreatic cancer cell line L3.6pl was kindly provided by I. J. Fidler, DVM, PhD, (The University of Texas M. D. Anderson Cancer Center, Houston, TX) [28]. The human pancreatic cancer cell lines HAPF-II, BxPC-3, and the human colon cancer cell line HT29 were obtained from the ATCC, Manassas, Virginia, USA. Cells were cultured and maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), and supplements and incubated in 5% CO₂-95% air at 37°C, as described elsewhere [7]. For experiments investigating effects of IGF-IR inhibition *in vitro*, L3.6pl cells were stably transfected with a mutated dominant-negative (DN) construct of IGF-IR (truncated at position 952 in the β -subunit transmembrane region) (the generous gift of Dr. Diane Prager M.D., Cedars-Sinai Medical Center-UCLA School of Medicine, Los Angeles, CA) [11,12,29], or an empty vector (pcDNA3), as previously described [7].

2.2 Materials and antibodies

Recombinant human (rh) IGF-I was purchased from R&D Systems Inc. (Minneapolis, MN). The chimeric anti-IGF-IR blocking antibody A12 was provided by ImClone Systems Inc. (New York, NY) [30,31]. Activated signaling pathways were identified by using the following antibodies: anti-extracellular signal-regulated kinase (Erk)-1/2 (p42/44) (Oncogene Research Products); and anti-phosphospecific Erk-1/2 (p42/44), anti-Akt, anti-phosphospecific Akt, anti-phosphospecific c-jun amino-terminal kinase (JNK), anti-P38, anti-phosphospecific P38, and anti-phosphospecific IGF-IR antibody from Cell Signaling Technologies (Beverly, MA);

anti-phospho tyrosine antibody 4G10 and anti-IRS-1 antibody from Upstate Biotechnology (Lake Placid, NY). For inhibition of signaling pathways, the following reagents were used: SB203580 (P38 MAPK inhibitor; 25 μ M), LY 294002 (PI-3K/Akt inhibitor; 25 μ M) and SP600125 (JNK inhibitor; 25 μ M) from Calbiochem Co. (La Jolla, CA); U0126 and PD98059 (MEK inhibitors; 10 μ M) from New England Biolabs, Inc. (Beverly, MA), and wortmannin (PI-3K/Akt inhibitor; 200 nM) from Sigma Biochemicals (St. Louis, MO). COX-2 antibody was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The β -actin antibody was purchased from Sigma Biochemicals.

2.3 Western blot analysis

Signaling pathways activated by rhIGF-I in parental L3.6pl cells were investigated by Western blotting, as described elsewhere [7]. Briefly, cells were grown to 60-70% cell confluence, incubated overnight in 1% FBS-MEM (to reduce effects of secreted growth factors) and treated thereafter for indicated time points with rhIGF-I (100 ng/ml) in 1% FBS-MEM. For experiments using the IGF-IR antibody (A12) or selective signaling inhibitors (see above), cells were pretreated for one hour in 1% FBS-MEM containing either A12 (1 μ g/ml), signaling inhibitor or PBS (control) prior to the addition of rhIGF-I (100 ng/ml). Protein was extracted from cell lysates with RIPA buffer, and 50- μ g protein samples were subjected to Western blot analysis by SDS-PAGE on a denaturing 10% gel, as described [27]. Bands were visualized using the appropriate HRP-conjugated secondary antibody and a chemiluminescence kit (Amersham Biosciences, Piscataway, NJ). For the detection of COX-2 expression in IGF-IR DN- and pcDNA-transfected L3.6pl cells, cells were incubated with or without rhIGF-I (100 ng/ml) for 4 h in 1% FBS-MEM prior to protein extraction.

2.4 Northern blot analysis

For Northern blot analyses, total RNA was extracted from cells using TRIzol Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. Northern blot analysis was performed as described previously [27]. Briefly, probes for COX-2 (Cayman Chemical Co.) or glyceraldehyde-3-phosphate dehydrogenase (American Type Culture Collection, Manassas, VA) were radiolabeled by the random primer technique with a commercially available kit (Rediprime II, Amersham Biosciences). Nylon membranes (Hybond-N+, Amersham Biosciences) were subsequently hybridized overnight (Rapid-hyb Solution, Amersham Biosciences) at 65°C. Autoradiography was performed thereafter in the linear range of the film (Hyperfilm MP, Amersham Biosciences). To investigate COX-2 induction by rhIGFI, parental or transfected L3.6pl cells were grown to 50-60% confluence in standard medium as described above and then incubated in 1% FBS-containing medium overnight. Cells were then incubated in the presence or absence of rhIGF-I (100 ng/ml) for one hour, as induction of COX-2 mRNA expression in this cell line was maximal by this time. To determine the relative importance of specific signaling pathways on COX-2 induction, signaling inhibitors were added to parental cells in 1%FBS-MEM one hour prior to stimulation with rhIGF-I (100 ng/ml), as described [27].

2.5 Transient transfection for RNAi of HIF-1 α

In order to inhibit HIF-1 α (Gene U22431), we utilized HIF-1 α Stealth™ RNAi (Invitrogen, Karlsruhe, Germany) (RNA#1: CCAUGAGGAAAUGAGAGAAAUGCUU) that was designed using an online RNAi-designer application (www.invitrogen.com). Pancreatic cancer cells were plated at 40 to 50% density and transiently transfected (100/200 nmol RNAi, 24 hours) using Lipofectamine (Invitrogen).

2.6 Immunoprecipitation of IRS-1

Phosphorylation of IRS-1 after stimulation with rhIGF-I (100 ng/ml) in the presence or absence of A12 antibody (one hour pre-incubation), was detected by immunoprecipitation of 300 µg protein with an antibody to IRS-1 in the presence of agarose beads (A/G Plus Agarose, Santa Cruz Biotechnology Inc.). Protein samples were heat denatured (95°C, 5 min) and separated on a denaturing 6% SDS-PAGE, as described [11]. Membranes were subsequently probed with anti-phospho-tyrosine and anti-IRS-1 antibodies, as described [7].

2.7 Densitometric quantification of western blot analyses

Densitometric analysis of autoradiographs was performed with NIH Image Analysis software (V1.62) from the National Institutes of Health (Bethesda, MD) to quantify the results of Northern and Western blot analyses.

3. Results

3.1 Effect of IGF-IR activation on COX-2 expression in human pancreatic cancer cells

We first investigated whether IL-1β could up-regulate COX-2 in pancreatic cancer cells, as demonstrated in our previous work using human colon cancer cells[27]. Indeed, IL-1β markedly increased COX-2 protein in a time dependent manner in colon (HT29) and pancreatic cancer cells (L3.6pl) (Fig. 1A). Using this finding as a positive control, we next investigated whether activation of IGF-IR by recombinant IGF-I would increase COX-2 expression in L3.6pl human pancreatic cancer cells. For this purpose, cells were stimulated for various time points with rhIGF-I. Stimulation of L3.6pl cells with rhIGF-I led to time-dependent induction of COX-2 mRNA and COX-2 protein (Fig. 1B, C). Hence, time points of maximal induction were used for subsequent studies using increasing doses of IGF-I. Western blot analyses showed that induction of COX-2 also occurs in a dose dependent manner (Fig. 1D). This finding of IGF-I-mediated up-regulation of COX-2 was confirmed in two other pancreatic cancer cell lines (Fig. 1E); results from signaling experiments are displayed for L3.6pl throughout the manuscript.

In order to determine the impact of IGF-IR on COX-2 expression in pancreatic cancer cells, IGF-IR was selectively inhibited by stably transfecting pancreatic cancer cells with a dominant-negative IGF-IR construct (IGF-IR DN), as previously reported [7]. By Northern blotting, untreated IGF-IR DN cells exhibited a ~80% reduction in constitutive COX-2 mRNA expression, as compared to controls (pcDNA). Moreover, COX-2 mRNA expression in IGF-IR DN cells could not be induced by the addition of rhIGF-I (Fig. 1A). Similar findings were observed in Western blot analyses of COX-2 protein, where IGF-IR DN-transfected L3.6pl cells showed a ~60% reduction in constitutive COX-2 expression, and a blunted response to rhIGF-I (Fig. 1B). Experiments were repeated and confirmed by using additional IGF-IR DN-transfected cell clones (data not shown). We, therefore, concluded that IGF-IR is a direct mediator of COX-2 expression in human pancreatic cancer cells.

3.2 Identification of signaling pathways mediating IGF-I-induced COX-2 expression in pancreatic cancer cells

In order to determine IGF-I activated signaling pathways involved in the regulation of COX-2 expression in pancreatic cancer, cells were stimulated with rhIGF-I for various time points and activated signaling intermediates were determined. Western blotting showed time-dependent phosphorylation of MAPK/(Erk1/2), PI-3K/Akt, P38 and SAPK signaling pathways with a peak roughly at 15-30 min (data not shown). These results were consistent to findings observed in our previous studies [7].

To identify regulatory pathways of COX-2, specific inhibitors to either MAPK/Erk (UO126), PI-3K/Akt (Wortmannin, LY294002), P38 MAPK (SB203580), or SAPK (SP600125) were used. Results showed that inhibition of MAPK/Erk1/2 diminished IGF-I-mediated induction of both COX-2 protein and mRNA expression (~80% by densitometry) in pancreatic cancer cells (Fig. 3). In contrast, inhibition of PI-3K/Akt by either Wortmannin or LY294002 did not inhibit the effect of IGF-I induction of COX-2 protein or mRNA expression, but surprisingly led to a modest increase in constitutive and inducible COX-2 expression (Fig. 3 B, C). This effect was even more pronounced when using the SAPK inhibitor (Fig. 3B). However, inhibition of the P38 pathway did not abrogate IGF-I mediated induction of COX-2 (Fig. 3). Hence, an IGF-IR/MAPK/Erk signaling cascade appears to be a critical mediator of COX-2 induction in pancreatic cancer, whereas the SAPK/JNK pathway might play a role as a negative mediator role on the COX-2 regulation process.

Since our previous work demonstrated that HIF-1 α is involved in the IGF-IR signaling cascade, we next addressed whether a specific molecular blockade of HIF-1 α could lower COX-2 expression in pancreatic cancer cells. Indeed, RNAi of HIF-1 α led to a decrease in COX-2 expression in L3.6pl pancreatic cancer cells (Fig. 3D). However, an IGF-I-mediated induction of COX-2 protein could not be inhibited by solely blocking HIF-1 α , suggesting that additional transcription factors are involved in this process. A proposed regulatory pathway for COX-2 expression in pancreatic cancer is illustrated in Figure 4.

3.3 Effects of a novel anti-IGF-IR antibody on cell signaling and COX-2 expression

To confirm the findings from experiments with IGF-IR DN cells and to rule out non-specific molecular alterations caused by transfection and clonal selection, we next examined the effects of a function blocking anti-IGF-IR-specific antibody (A12) in parental L3.6pl cells on signaling and COX-2. This characteristics of this antibody have been published [30,32]. The efficacy of A12 in regards to blocking IGF-IR function was first investigated by Western blot analyses. A12 treatment inhibited IGF-IR activation upon stimulation with rhIGF-I (Fig. 5A). A12 did not alter expression of IGF-IR itself (data not shown). As a consequence of IGF-IR inhibition, A12 inhibited IGF-I-mediated phosphorylation IRS-1 of in pancreatic cancer cells (Fig. 5B). In addition, A12 led to a marked reduction in IGF-I-mediated MAPK/Erk activation in L3.6pl cells. Further, when cancer cells were incubated for 24 hours in complete-media (10% FBS-MEM), treatment with A12 also decreased constitutive MAPK/Erk phosphorylation in comparison to untreated cells (data not shown). This constitutive activation of Erk is thought to be the result of an autocrine loop activation of IGF-IR, as we have reported previously [7].

In contrast to experiments with IGF-IR DN cells, A12 antibody treatment only delayed the activation of PI-3K/Akt upon stimulation with rhIGF-I, resulting in final phosphorylation levels of Akt that were comparable to controls (Fig. 5A). Importantly, A12 antibody treatment of pancreatic cancer cells substantially reduced constitutive and inducible COX-2 mRNA expression compared to controls (Fig. 5C). Similar findings were observed by Western blot analyses of COX-2 protein (data not shown). Interestingly, activation of SAPK and P38 pathways were also diminished by the addition of A12 antibody (data not shown). We conclude from these experiments, that the IGF-IR system represents an important modulator of COX-2 in human pancreatic cancer.

DISCUSSION

In the current study, activation of IGF-IR in human pancreatic cancer cells increased COX-2 expression which was mediated via the MAPK/Erk1/2 signaling pathway. Selective inhibition of IGF-IR function, which was achieved by either transfection with a dominant-negative IGF-IR construct or by using an anti-IGF-IR antibody (A12), reduced constitutive and inducible COX-2 expression in cancer cells.

The proliferative effects of the IGF-I/IGF-IR system and its implication in malignant tumor progression have been described by various groups [33-36]. Importantly, recent studies demonstrated that activation of the IGF-IR system promotes VEGF expression, and thereby angiogenesis, in various solid malignancies, including pancreatic cancer [7,11,36]. Hence, the IGF-IR system has become an attractive target for cancer therapy [3]. Recent evidence suggested that IGF-IR may additionally modulate expression of certain intermediates in the arachidonic acid pathway, such as COX-2, in various cancer cells [26,37]. However, the exact role of IGF-IR in the regulation of COX-2 in pancreatic cancer has not been well defined.

In our previous study, we were able to identify the IGF-IR system as an important regulator of angiogenesis in human pancreatic cancer [7]. This effect was mediated through an autocrine activation of IGF-IR in pancreatic cancer cells which in turn lead to induction of HIF-1 α and VEGF in these cells. *In vivo*, specific blockade of IGF-IR function resulted in a dramatic inhibition of tumor growth and angiogenesis in an orthotopic model in mice, which in part could relate to decreases in COX-2 [7]. Our results now indeed show that activation of IGF-I receptor by IGF-I up-regulates COX-2 in pancreatic cancer cells, which is mediated through MAPK/Erk1/2. Interestingly, we also identified HIF-1 α as an additional modulator of COX-2, as its selective inhibition of HIF-1 α by RNAi decreased COX-2 expression levels. However, sole inhibition of HIF-1 α was not sufficient to suppress IGF-I-mediated activation of COX-2, suggesting that other transcription factors are involved in mediating this down-stream effect of MAPK/Erk.

In gastrointestinal cancer, IGF-IR mediated regulation of COX-2 and PGE₂ synthesis has been best characterized in human colon cancer cells [26]. Di Popolo and colleagues used recombinant IGF-II to stimulate IGF-IR in Caco-2 colon carcinoma cells. IGF-II led to induction of Cox-2 via phosphorylation of Akt. In addition, transfection of Caco-2 cells with a dominant-negative IGF-IR construct abrogated this effect and increased cell apoptosis rates *in vitro* [26]. However, as IGF-I (and not IGF-II) is highly overexpressed in human pancreatic cancer, we used recombinant IGF-I for investigating IGF-IR activation in pancreatic cancer cells, which may explain the difference in signaling pathways mediating COX-2 induction in colon and pancreatic cancers. Interestingly, in our study, inhibitors of PI-3K/Akt, or SAPK (JNK) signaling pathways did not block IGF-I mediated induction of COX-2, but rather increased its constitutive expression. We speculate that this finding could mediate a potential negative regulatory feedback mechanism, which might be mediated through PI-3K/Akt and SAPK; this finding has not been reported to date in other studies.

In pancreatic cancer, COX-2 overexpression is frequently observed [18,19,38] which has been associated with increased angiogenesis in this malignancy [38,39]. Importantly, autocrine loops for IGF-IR activation have additionally been described, suggesting that this receptor system might be a valuable molecular target for therapy [7,8,10]. The potential implication of IGF-IR in cancers in regards to COX-2, has been addressed in two recent reports that postulated an interplay of COX-2 and the IGF-I/IGF-IR axis in some malignancies. Levitt et al. elegantly demonstrated that anti-apoptotic effects of IGF-I, which were mediated through PI-3K/Akt activation, reduced or abrogated apoptosis induced by administration of different COX-2 inhibitors in human pancreatic cancer cells (BxPC-3) *in vitro* [37]. The authors concluded that alterations of IGF-I levels or IGF-I receptor signal transduction in pancreatic cancer may significantly modulate the antineoplastic actions of COX-2 inhibitors [37]. In another study, Pold et al. described that overexpression of COX-2 in A549 NSCLC cells (stably transfected with a COX-2 construct) led to enhanced anti-apoptotic and proliferative effects of IGF-I. Furthermore, COX-2 facilitated the autophosphorylation of IGF-IR [40]. Our study demonstrates for the first time that there is a direct link between IGF-IR activation and COX-2 expression in pancreatic cancer cells. In addition, effective inhibition of both IGF-IR and COX-2 in cancer cells can be achieved with an anti-IGF-IR antibody that does not lead to an

initial IGF-IR activation, as described for other antibodies [41]. Thus targeting IGF-IR may be a valuable approach for reducing angiogenic activity and COX-2 in human pancreatic cancer. In conclusion, the IGF-IR system is an important mediator of tumor angiogenesis and growth of pancreatic cancer, and it also functions as positive regulator of COX-2 expression in this cancer entity. Hence, blockade of IGF-IR function may be a promising approach for the treatment of patients with advanced and/or metastatic pancreatic cancer.

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3The abbreviations used are

PC, pancreatic cancer
 COX-2, cyclooxygenase-2
 PG, prostaglandin
 VEGF, vascular endothelial growth factor
 EC, endothelial cell
 EGF, epidermal growth factor
 MEM, minimum essential medium
 FBS, fetal bovine serum
 HIF-1 α , hypoxia-inducible factor-1 α
 IGF-I, insulin-like growth factor-I
 IGF-IR, IGF-I receptor
 IRS-1, insulin receptor substrate-1
 rhIGF-I, recombinant human IGF-I
 MAPK, mitogen-activated protein kinase
 PI-3K, phosphatidylinositol-3' kinase
 SAPK, stress-activated protein kinase
 JNK, c-Jun NH₂-terminal kinase
 MEK, MAP/ERK kinase
 MTT, 3-[4, 5-Dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide
 PBS, phosphate-buffered saline

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Figure 1. Impact of IGF-I stimulation on COX-2 expression in pancreatic cancer cells

Effects of IGF-IR activation on COX-2 expression were investigated by stimulating human pancreatic cancer cells with rhIGF-I. *A*) IL-1 β , a known inducer of COX-2 served as a positive control. Stimulation with IL-1 β led to a time dependent up-regulation of COX-2 in human colon (HT29) and pancreatic cancer cells (L3.6pl), as determined by Western blotting. *B*) Pancreatic cancer cells were stimulated with rhIGF-I for the indicated times and RNA was isolated. Treatment of L3.6pl cells with rhIGF-I led to a time dependent increase in COX-2 mRNA, as determined by Northern blot analysis. *C*) Similarly, Western blot analysis confirmed this up-regulation of COX-2 upon exposure to rhIGF-I. *D*) Pancreatic cancer cells were incubated with increasing doses of rhIGF-I (4 h) and protein was harvested for Western blot

analysis. IGF-I dose-dependently up-regulated COX-2 protein in L3.6pl cells. *E*) IGF-IR mediated induction of COX-2 was studied in two additional human pancreatic cancer cell lines. HPAF-II and BxPC-3 cells were incubated with rhIGF-I for the indicated times. IGF-I stimulation led to an increase in COX-2 protein. β -actin served as a loading control.

Figure 2. Effects of molecular IGF-IR inhibition on COX-2 expression

Effects of a specific molecular inhibition of IGF-IR on COX-2 expression were investigated by stably transfecting L3.6pl cells with a dominant-negative IGF-IR construct (IGF-IR DN) or an empty vector (pcDNA). *A*) Northern blot analysis of rhIGF-I induced COX-2 mRNA expression in transfected cells. Cells were stimulated for one hour with rhIGF-I or PBS (control) before RNA was extracted. Changes in COX-2 mRNA expression were quantified by densitometry. *B*) Western blot analysis for COX-2 protein. Transfected cells were treated for four hours with rhIGF-I or PBS and protein was subsequently extracted. Inhibition of IGF-IR function significantly reduced constitutive COX-2 expression in cells and blunted the response to rhIGF-I.

Figure 3. Identification of signaling pathways of IGF-I mediated COX-2 expression in pancreatic cancer cells

A) Western blot analysis for COX-2. Cells were pretreated with signaling inhibitors to MAPK/Erk-1/2 (UO126), or PI-3K/Akt (wortmannin, WT), as described in Material and Methods. Cells were subsequently stimulated for four hours by adding rhIGF-I. IGF-I treatment resulted in a 5-fold increase in COX-2 protein expression, which was selectively mediated by the MAPK (Erk-1/2) signaling pathway. Inhibition of PI-3K/Akt signaling did not reduce COX-2 expression. B) COX-2 expression was investigated following treatment with inhibitors to PI-3K (LY), P38 (SB) and SAPK (SP). IGF-I mediated COX-2 induction was not blunted by pretreatment with these inhibitors prior to IGF-I exposure. C) Northern blot analysis for COX-2

mRNA expression. Cells were pretreated with inhibitors as described above, except that cells were treated for one hour with rhIGF-I. Stimulation with rhIGF-I increased COX-2 mRNA in control cells, whereas this response was blunted after inhibition of MAPK/Erk1/2 by UO126. In addition, constitutive COX-2 mRNA expression was lowered by 70% upon treatment with UO126. In contrast, inhibition of PI-3K/Akt by LY did not reduce constitutive or inducible COX-2 mRNA expression. *D)* Effect of HIF-1 α inhibition on COX-2 expression. L3.6pl cells were transiently transfected with RNAi for HIF-1 α . After 24 hours, cells were exposed to rhIGF-I. RNAi to HIF-1 α markedly reduced constitutive COX-2 expression without altering the inducible response to IGF-I. β -actin served as a loading control.

Figure 4. Summary of IGF-I activated signaling pathways in pancreatic cancer cells and their impact on COX-2 expression

IGF-I leads to induction of multiple signaling intermediates down-stream of IGF-IR and IRS-1 (□). For investigating signaling pathways involved in IGF-I mediated COX-2 expression, inhibitors to MAPK/Erk1/2, PI-3K/Akt, P38, or SAPK were studied. The results show that MAPK/Erk is the predominant pathway for mediating IGF-I-induced COX-2 expression in pancreatic cancer cells (bold arrow). HIF-1α is downstream of the IGF-I/IGF-IR signaling cascade, as previously reported (dotted arrow) [7]. However, selective inhibition of HIF-1α using RNAi, decreased constitutive COX-2 expression, but had no effect on inducible COX-2 up-regulation suggesting that other transcription factors are involved in IGF-IR mediated Cox-2 induction. In contrast, PI-3K and SAPK appear to function as negative regulatory pathways for COX-2 expression since selective inhibition leads to increases in constitutive COX-2. The P38 pathway is activated upon stimulation with IGF-I, but inhibition of this protein had no effect of COX-2 expression in pancreatic cancer cells.

Figure 5. Effect of an anti-IGF-1 receptor antibody on IGF-I induced signaling pathway activation and COX-2 expression

A) Western blot analysis of activated IGF-IR and signaling intermediates upon rhIGF-I treatment under the presence or absence of a specific antibody to IGF-IR (A12). Cells were treated for indicated time points with rhIGF-I under presence or absence of A12. A12 inhibited phosphorylation of IGF-IR and lead to a subsequent decrease in Erk1/2 activation. Phosphorylation of Akt was only delayed upon A12 treatment. Loading was verified by probing for β -actin. B) Immunoprecipitation for IRS-1 as a downstream effector of IGF-1R. Cells were treated with rhIGF-I under the presence or absence of A12. IRS-1 phosphorylation was determined by immunoprecipitating IRS-1 and blotting for phospho-Tyrosine (p-Tyr). A12 effectively inhibited IRS-1 activation upon rhIGF-I treatment. C) Northern blot analysis for COX-2 mRNA expression. L3.6pl cells were treated for one hour with rhIGF-I or PBS and RNA was extracted. A12 lead to a 50% reduction in constitutive COX-2 mRNA expression and abrogated the response to rhIGF-I.