Prostaglandin E2 activates the mTORC1 pathway through an EP4/cAMP/PKA and EP1/Ca\textsuperscript{2+}-mediated mechanism in the human pancreatic carcinoma cell line PANC-1

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H-H.C. designed and performed experiments, analyzed data, and prepared the manuscript; G.E., E.R., and O.J.H. supervised the project; G.E. and E.R. edited the manuscript; S.H.Y., J.SS., and K.M.H. provided technical support and conceptual advice; S.H.Y., C.E.C., and A.M. assisted with some experiments.

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PGE2 activates mTORC1 in pancreatic cancer cells

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Abstract

Obesity, a known risk factor for pancreatic cancer, is associated with inflammation and insulin resistance. Pro-inflammatory PGE$_2$, and elevated IGF-1 related to insulin resistance, are both shown to play critical roles in pancreatic cancer progression. We aimed at exploring a potential crosstalk between the PGE$_2$ signaling and IGF-1/Akt/mammalian target of rapamycin complex 1 (mTORC1) pathway in pancreatic cancer, which may be a key to unraveling the obesity-cancer link. In PANC-1 human pancreatic cancer cells, we showed that PGE$_2$ stimulated mTORC1 activity independently of Akt, as evaluated by downstream signaling events. Subsequently using pharmacological and genetic approaches, we demonstrated that PGE$_2$-induced mTORC1 activation is mediated by EP4/cAMP/PKA, as well as an EP1/Ca$^{2+}$-dependent pathway. The cooperative roles of the two pathways were supported by the maximal inhibition achieved with the combined pharmacological blockade, and the co-existence of highly expressed EP1 (mediating Ca$^{2+}$ response) and EP2 or 4 (mediating cAMP/PKA pathway) in PANC-1 and a prostate cancer line PC-3, which also robustly exhibited PGE$_2$-induced mTORC1 activation, as identified from a screen in various cancer cell lines. Importantly, we showed a reinforcing interaction between PGE$_2$ and IGF-1 on mTORC1 signaling, with an increased IL-23 production as a cellular outcome. Together, our data reveal a previously unrecognized mechanism of PGE$_2$-stimulated mTORC1 activation mediated by EP4/cAMP/PKA and EP1/Ca$^{2+}$ signaling, which may be of great importance in elucidating the promoting effects of obesity in pancreatic cancer. Ultimately, a precise understanding of these molecular links may provide novel targets for efficacious interventions devoid of adverse effects.

(Word count: 248)

Keywords

PGE$_2$, mTORC1, pancreatic cancer, obesity
Introduction

Obesity is an established risk factor for pancreatic cancer (4), a remarkably aggressive and fatal disease with an overall 5-year survival rate of only about 5% (46). Although the link between obesity and cancer is compelling, the mechanisms driving this association remain poorly understood. A number of factors are implicated, such as the pro-inflammatory state associated with excess adiposity, as well as the elevated levels of growth hormones, i.e. insulin and insulin-like growth factor-1 (IGF-1) (21). Compensatory high levels of insulin during obesity-associated insulin resistance, is known to up-regulate hepatic IGF-1 synthesis and suppress IGF-binding protein, leading to increased bioavailable IGF-1 (10). The elevated levels of insulin/IGF-1 may contribute to cancer development by activation of insulin and IGF-1 receptors and the downstream phosphoinositide-3-kinase (PI3K)/Akt/mammalian target of rapamycin complex 1 (mTORC1) cascade, a key signaling module in the regulation of cell growth and survival (26, 41). In accordance with this notion, type 2 diabetes, hyperinsulinemia, and increased circulating IGF-1 are established risk factors for pancreatic and other types of cancers (17, 48). Additionally, the IGF-1/Akt/mTORC1 pathway has been shown to be important in promoting proliferation of pancreatic cancer cells (45), and recently has been implicated in the anti-cancer effects of caloric restriction and the pro-cancer effects of an obesity-inducing diet on mouse models of pancreatic cancer (28). Triggered by growth factors (e.g. IGF-1), mTORC1 is activated through the canonical PI3K/Akt module, and can phosphorylate a number of substrates involved in protein synthesis and cell growth, such as ribosomal protein S6 kinase (p70S6K) and eIF4E binding protein (4E-BP1) (26). Therefore, the insulin/IGF-mTOR axis may, at least in part, account for the link between obesity and pancreatic cancer.

In addition to systemic hormonal changes, increasing attention has focused on the local and systemic effects of inflammation. Obesity is recognized as a chronic inflammatory state with leukocyte infiltration into adipose and other tissues, accompanied by increased local and systemic pro-inflammatory mediators such as cytokines and prostaglandins (21, 39). Chronic
inflammation has long been associated with cancer development and progression (30). Our
previous study also showed that oral administration of nimesulide, a non-steroidal
anti-inflammatory drug (NSAID), significantly delayed the progression of Kras-driven early
pancreatic neoplasia in a conditional mouse model (11). A critical player in the
obesity-inflammation-cancer axis is prostaglandin E2 (PGE2), which is a key inflammatory lipid
mediator generated by cyclooxygenase enzymes (COX-1 and -2), the best characterized targets
of NSAIDs (32). Overexpression of COX-2 and elevated levels of PGE2 are often observed in
human cancers, including pancreatic cancer, as well as in obese individuals (9, 35, 47, 49).
Secreted by tumor cells or infiltrating inflammatory cells, PGE2 through activating its receptors
and downstream signaling may promote cell proliferation, survival, migration, invasion,
angiogenesis, inflammation, and immune evasion (14, 18, 37). PGE2 exerts its biological
functions through binding to one of the four subtypes of G protein-coupled receptors (GPCRs),
EP1 to EP4 (50). Previously, we have shown that the growth-promoting effects of PGE2 on
pancreatic cancer cells are mediated by EP2 and/or EP4 (12), both of which are coupled with G
protein αs (Gαs) known to stimulate cAMP formation and subsequently activate protein kinase A
(PKA) (7).
Overall, the mechanisms underlying obesity-promoted pancreatic cancer are most likely to be
multifaceted and interrelated. Links between critical signaling pathways deserve more attention.
In particular, a potential crosstalk between the PGE2/EP/cAMP and IGF-1/Akt/mTOR pathways
in pancreatic cancer has not been explored. It has been demonstrated in other cell systems that
cAMP or PKA can lead to the activation of mTORC1 (3, 22). Therefore, by activating EP
receptors, PGE2 may stimulate mTORC1 and potentiate its growth-promoting action. Herein, we
uncovered a novel crosstalk between PGE2 signaling and mTORC1 pathway in pancreatic
cancer cells. We further investigated the mechanisms through which PGE2 can stimulate
mTORC1 and potentially augment the pro-tumorigenic effects of insulin/IGF-1. Since both, PGE2
signaling and IGF-1/Akt/mTORC1 pathway, are over-activated in obesity-associated cancers,
this novel crosstalk may be of great importance in elucidating the tumor-promoting effects of obesity and inflammation in pancreatic and other types of cancers.

**Materials and Methods**

**Antibodies and chemical reagents:** The following primary antibodies were all purchased from Cell Signaling Technology (Danvers, MA). Phospho-p70 S6 Kinase (Thr389) antibody (#9205), phospho-S6 ribosomal protein (Ser235/236) antibody (#2211), phospho-S6 ribosomal protein (Ser240/244) mAb (#5364), S6 ribosomal protein mAb (#2317), phospho-CREB (Ser133) mAb (#9198), phospho-Akt (Ser473) antibody (#9271), phospho-Akt (Thr308) antibody (#9275), Akt antibody (#9272), phospho-p44/p42 MAPK (Erk1/2) (Thr202/Tyr204) mAb (#4370), phospho-p90RSK (Ser380) mAb (#9335), phospho-4E-BP1 (Thr37/46) (236B4) mAb (#2855), and GAPDH mAb (#2118). PGE2, forskolin, H-89, butaprost, EP4 agonist CAY10580, EP2 antagonist PF-04418948, EP4 antagonist ONO-AE3-208, ionomycin, and BAPTA-AM were all purchased from Cayman Chemical (Ann Arbor, MI). Rapamycin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). EP1 siRNA (L-005711, ON-TARGETplus human PTGER1 siRNA - SMARTpool) was purchased from GE Dharmacon (Lafayette, CO). Recombinant human IGF-1 was purchased from Sigma-Aldrich (St. Louis, MO).

**Cell culture and treatments:** Human cancer cell lines AsPC-1, BxPC-3, Capan-2, HPAF-II, MIA PaCa-2, and PANC-1 (pancreatic cancer), PC-3 (prostate cancer), MCF-7 (breast cancer), DLD-1 and SW480 (colon cancer) were obtained from the American Tissue Type Culture Collection (ATCC). MIA PaCa-2 and PANC-1 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS and 1x Penicillin-Streptomycin-Glutamine from Life Technologies (Grand Island, NY), and cultured at 37 °C and 10% CO2. AsPC-1, BxPC-3, Capan-2, HPAF-II, PC-3, MCF-7, DLD-1 and SW480 cells were maintained in RPMI 1640 medium supplemented with 10% FBS and 1x Penicillin-Streptomycin-Glutamine (Life
Technologies), and cultured at 37 °C and 5% CO₂. For Western blotting analysis, cells were seeded in 6-well plates. After serum-starvation for 18 h, cells were treated as indicated.

**Western blotting:** Following treatments, cells were harvested and lysed in lysis buffer (1x Tris buffer pH 7.4, 1% Triton-X 100, 0.25% sodium deoxycholate) supplemented with protease and phosphatase inhibitor cocktails (Thermo Scientific, Rockford, IL). Forty μg of proteins (quantified using BCA Protein Assay Kit (Thermo Scientific)) were loaded onto 10% Mini-PROTEAN® TGX™ Precast Gels (Bio-Rad Laboratories, Hercules, CA). The proteins were electrophoretically transferred onto nitrocellulose membranes. The membranes were blocked and incubated with primary antibodies according to the manufacturer’s recommendations. Following incubation with secondary antibodies, proteins were detected by ECL reagents (Thermo Scientific), and exposed to CL-XPosure X-ray Films from Thermo Scientific.

**Intracellular cAMP measurement:** Cells were seeded in 6-well plates or 35 mm dishes and then serum-starved. Following treatments, cells were lysed in 0.1 M HCl (150 μl per well). The lysates were centrifuged at 1,000 x g, 4°C for 10 min. Cyclic AMP levels in the cell lysates were then measured by a cAMP EIA kit from Cayman Chemical (Ann Arbor, MI) following the manufacturer’s instructions. The values of cAMP levels were normalized to the protein concentrations of the samples.

**Intracellular [Ca²⁺] measurement:** Cells were seeded on coverslips. Following serum-starvation, the coverslips were incubated with 5 μM Fura 2-AM diluted in pre-warmed Hank’s Balanced Salt Solution (HBSS) containing 20 mM HEPES (pH 7.4) for 1 h at 37°C. Coverslips were then mounted in an experimental chamber (volume: 0.5 ml) placed on the stage of an inverted microscope (Axio Observer.A1). The chamber was perfused (1 ml/min) at 37°C with buffered HBSS. At selected times the perfusion fluid was changed to HBSS containing agonists (PGE₂). Ratio images (340 nm excitation divided by 380 nm excitation) were obtained every second by a digital camera (AxioCam MRm) attached to the microscope, which was operated with associated software (AxioVision, all components Carl Zeiss, Thornwood, NY).
Intracellular $[\text{Ca}^{2+}]$ is proportional to the 340 nm/380 nm ratio. In some experiments, cells were plated onto coverslips that fit inside cuvettes, which in turn were loaded into a temperature controlled fluorimeter (Hitachi). At selected times, agonist was introduced into the cuvette. Ratio values for the entire cuvette were determined as above.

**Small interfering RNA (siRNA) transfection:** Cells were seeded in 6-well plates and incubated overnight in DMEM with 10% FBS. Transfection of siRNA was carried out the next day with Lipofectamine® RNAiMAX Reagent (Life Technologies, Grand Island, NY) following the manufacturer’s recommendations. Cells were then incubated for an additional 3 days before serum-starved and stimulated with PGE$_2$.

**Quantitative reverse transcription PCR (RT-qPCR):** Relative transcript expression levels of $EP1$, $EP2$, $EP4$ and $IL-23$ were determined by RT-qPCR using a SYBR Green-based method. Briefly, total RNA was extracted from cells by using the PureLink® RNA Mini Kit (Life Technologies, Grand Island, NY). Reverse transcription was performed with the iScript reverse transcription supermix (Bio-Rad Laboratories, Hercules, CA), using 1 μg of total input RNA. The synthesized cDNA samples were used as templates for the following real-time PCR analysis. All reactions were performed using the Bio-Rad iQ™5 system and the amplifications were done using the iTaq™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA). Gene-specific oligonucleotide primers for $EP1$, $EP2$, $EP4$ and $IL-23$A (interleukin 23, alpha subunit p19) and internal reference $ACTB$ (actin, beta) or RNA18S5 (RNA, 18S ribosomal 5) are as follows: $EP1$ forward, 5'-ACCTTCTTTGGCGGCTCTC-3', and reverse, 5'-CCAACACCAGCATGGGCT-3' (exons 2&3); $EP2$ forward, 5'-GCTCCTTGCGCCTTC-3', and reverse, 5'-CCAACACCAGCATGGGCT-3' (exons 2&3); $EP4$ forward, 5'-AGGGGTCTAGGATGGGGTTC-3' (exons 1&2); $IL-23$A forward, 5'-CACTAGTGGGACACATGGATCT-3', and reverse, 5'-GGTGGATCCTTTGCAAGCAG-3' (exons 1&2); $ACTB$ forward, 5'-GCACAGAGCCTCGCCTTT-3', and reverse, 5'-TATCATCATCCATGGTAGCT-3'.
1&2); RNA18S5 forward, 5'-AGTCCCTGCCCTTTGTACACA-3', and reverse, 5'-CGATCCGAGGGCCTCACTA-3'. The relative mRNA transcript levels are calculated using the 2^ΔΔCt method (29) to determine the fold change in indicated genes normalized to ACTB or RNA18S5 gene.

**Cytokine array:** PANC-1 cells were seeded in 6-well plates and then serum-starved. Cells were incubated in culture medium (2 ml) with or without PGE₂ and IGF-1, alone or in combination. Cell culture supernatants collected after 24 h incubation were profiled using the Human Cytokine Array Kit, Panel A (R&D systems, Minneapolis, MN) following the manufacturer’s instructions. The membrane-based proteomic array detects relative levels of 36 different cytokines, chemokines, and acute phase proteins including complement 5/5a, CD40 ligand, G-CSF, GM-CSF, CXCL1/GROα, CCL1/I-309, ICAM-1, IFN-γ, IL-1α, IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-16, IL-17, IL-17E, IL-23, IL-27, IL-32α, CXCL10/IP-10, CXCL11/I-TAC, CCL2/MCP-1, MIF, CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES, CCL12/SDF-1, Serpin E1/PAI-1, TNFα, and TREM-1. Following exposure to HRP substrate as the final step, the array membranes were imaged using a chemiluminescence image analyzer LAS-4000 mini (Fujifilm Life Sciences, Tokyo, Japan), and the intensity of signals normalized to the references was quantified with Multi Gauge V3.0 software (Fujifilm Life Sciences, Tokyo, Japan).

**Statistical analyses:** Data are presented as means ± SD. To determine statistical significance, one way ANOVA and two-tailed Student’s t-tests were performed assuming unequal variances. A P value less than 0.05 was considered significant and was indicated with an asterisk (*).
Results

PGE\textsubscript{2} activates mTORC1 in human pancreatic cancer cells.

PANC-1 cells have been used extensively as a model system to study the effects of growth factors on the biological behavior of human pancreatic cancer cells. To determine whether PGE\textsubscript{2} stimulates mTOR signaling in pancreatic cancer cells, PANC-1 cells were treated with PGE\textsubscript{2}, and then mTORC1 activation was assessed by Western blot analysis of phosphorylation levels of downstream molecules. As shown in Fig. 1A and 1B, PGE\textsubscript{2} dose- and time-dependently induced the phosphorylation of S6 ribosomal protein (S6rp) at Ser\textsuperscript{235/236}, which correlated with the increased Thr\textsuperscript{389} phosphorylation of p70S6K, a major mTORC1 target upstream of S6rp. These effects were robust after 15 min of stimulation and observed at a concentration of PGE\textsubscript{2} of 0.1 μM.

Although S6rp Ser\textsuperscript{235/236} can also be phosphorylated by p90S6 kinase (p90S6K, also known as RSK) independently of mTORC1, p90S6K activation assessed by Ser\textsuperscript{380} phosphorylation, was unchanged in response to PGE\textsubscript{2} treatment (Fig. 1B). Also, phosphorylation of ERK, a key regulator of p90S6K, was not increased by PGE\textsubscript{2} in these cells (Fig. 1A). Furthermore, phosphorylation of S6rp at Ser\textsuperscript{240/244}, known to be a p70S6K-specific site (1, 44), was markedly induced by treatment with PGE\textsubscript{2} (Fig. 1B and 1C). Moreover, treatment of PANC-1 with 1 nM of rapamycin, a potent allosteric inhibitor of mTORC1, suppressed PGE\textsubscript{2}-induced S6rp phosphorylation (Fig. 1C), corroborating that the phosphorylation of S6rp at Ser\textsuperscript{240/244} in response to PGE\textsubscript{2} is mediated by mTORC1. Taken together, these results indicate that PGE\textsubscript{2} stimulates mTORC1 activity in pancreatic cancer cells. Interestingly, PGE\textsubscript{2} did not increase Akt phosphorylation (Thr\textsuperscript{308} and Ser\textsuperscript{473}) (Fig. 1B), implying that the crosstalk to the mTORC1 cascade is downstream of Akt and does not affect mTORC2 (as judged by p-Akt Ser\textsuperscript{473}). These findings prompted us to explore the mechanism(s) underlying PGE\textsubscript{2}-induced mTORC1 activation in pancreatic cancer cells.
PGE₂ activates mTORC1 via the EP4/cAMP/PKA pathway.

As a first step to examine the mechanism(s) by which PGE₂ induces mTORC1 activation in PANC-1 cells, we determined the effects of PGE₂ on intracellular cAMP levels. A representative dose-response curve is shown in Fig. 2A. A half-maximal increase (EC₅₀) was seen at a concentration of 0.09 μM and maximal effects were obtained at ~1 μM of PGE₂. Notably, PGE₂ was effective at sub-micromolar concentrations, suggesting that the responses were mediated by specific binding to the EP receptors. We verified that treatment of these cells with PGE₂ increased the phosphorylation of cAMP response element-binding protein (CREB) (Fig. 1A and 1B), known to be positively regulated by PKA, whose activation is dependent on cAMP.

Next, we determined whether the elevation of the intracellular levels of cAMP is sufficient to induce mTORC1 activation. Exposure to the adenylate cyclase activator forskolin induced a marked accumulation of cAMP in MIA PaCa-2 and PANC-1 cells (Fig. 2B). Importantly, forskolin mimicked the effect of PGE₂ on S6rp phosphorylation in both cell lines (Fig. 2C), supporting that cAMP is involved in this crosstalk. Also, the effect of forskolin was blocked in cells pre-incubated with H-89 (Fig. 2C), a preferential inhibitor of PKA.

Among the four subtypes of G protein-coupled receptors (GPCRs) for PGE₂, EP2 and EP4 are known to couple to Gαₛ and thus, are responsible for stimulating cAMP production via activation of adenylate cyclase (50). To determine the contribution of EP2 and EP4, receptor agonists and antagonists were utilized. As shown in Fig. 3A, PGE₂-induced cAMP accumulation was blocked by the EP4 antagonist ONO-AE3-208, but not by the EP2 antagonist PF-04418948 in PANC-1 cells. Besides, an EP4 agonist (CAY10580), rather than an EP2 agonist (butaprost), markedly increased cAMP production in these cells (Fig. 3B), indicating the importance of EP4 in mediating cAMP responses in PANC-1 cells.

We next used pharmacological approaches to further substantiate a link between cAMP and mTORC1. The EP4 agonist CAY10580 promoted mTORC1 activation, as assessed by S6rp phosphorylation (Fig. 4A). Importantly, PGE₂-activated S6rp phosphorylation (either at Ser²³⁵/²³⁶


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or Ser\(^{240/244}\) was prevented by the specific EP4 antagonist ONO-AE3-208 (Fig. 4B). These results reinforce the notion that PGE\(_2\)-induced cAMP accumulation and subsequent responses are mainly mediated by the EP4 receptor in PANC-1 cells.

The cAMP-dependent protein kinase, PKA, is a central target of cAMP. Therefore, we used the PKA inhibitor H-89 to test whether PGE\(_2\)-stimulated mTORC1 activation is PKA-dependent. Pre-treatment of cells with H-89 at 5 μM attenuated baseline and PGE\(_2\)-activated S6rp phosphorylation (Fig. 4C), indicating a role of PKA in the crosstalk between PGE\(_2\) signaling and the mTORC1 pathway. This finding is in agreement with previous results showing that PGE\(_2\)-induced activation of p70S6K and S6rp paralleled an increase in the phosphorylation of CREB, a substrate of PKA (Fig. 1).

Calcium signaling contributes to PGE\(_2\)-induced mTORC1 activation.

We noticed that treatment with H-89, at a concentration that completely blunted the forskolin-induced S6rp phosphorylation, did not fully inhibit PGE\(_2\)-induced S6rp phosphorylation (either at Ser\(^{235/236}\) or Ser\(^{240/244}\)) (Fig. 4C). These results suggest that, besides cAMP, PGE\(_2\) may activate an additional pathway leading to mTORC1 activation. Calcium signaling has been implicated in the regulation of mTORC1 activation independently of PI3K/Akt (15, 31, 34). Consequently, PGE\(_2\) might exert some of its cAMP-independent effects through activation of EP1 receptors, which couple to G\(_{\alpha_q}\) and thereby promote phospholipase C (PLC)-mediated formation of diacylglycerol (DAG) and IP3, second messengers leading to PKC activation and Ca\(^{2+}\) mobilization, respectively (19). Initially, we examined whether PGE\(_2\) induces Ca\(^{2+}\) mobilization in PANC-1 cells. In agreement with this hypothesis, we found that addition of PGE\(_2\) to Fura-2-loaded PANC-1 cells induced a rapid and transient increase in intracellular Ca\(^{2+}\) levels in PANC-1 cells (Fig. 5A). This microscopy-based measurement allowed us to monitor Ca\(^{2+}\) response in individual cells, and revealed that ~62% (8/13) cells were responsive to PGE\(_2\) to varying extents. The representative traces are shown in Fig. 5A.
Additionally, ionomycin, a Ca\(^{2+}\) ionophore that raises intracellular Ca\(^{2+}\) level, induced phosphorylation of p70S6K and downstream S6rp (Ser\(^{235/236}\) and Ser\(^{240/244}\)) in these cells. This effect was markedly suppressed by BAPTA-AM, an intracellular Ca\(^{2+}\) chelator (Fig. 5B). Importantly, ionomycin was used in this experiment at a concentration (50 nM) that elicited a comparable increase of intracellular Ca\(^{2+}\) as induced by PGE\(_2\) (data not shown).

To study the possible role of Ca\(^{2+}\) signaling in PGE\(_2\)-induced mTORC1 activation, cells were pre-treated with BAPTA-AM prior to PGE\(_2\) stimulation. As shown in Fig. 5C, phosphorylation of S6rp (Ser\(^{235/236}\) and Ser\(^{240/244}\)) was significantly decreased by depletion of intracellular Ca\(^{2+}\). To further confirm that G\(_\alpha_q\)-coupled EP1 receptor is involved in PGE\(_2\)-stimulated mTORC1 activation, we utilized a genetic approach to specifically knockdown the EP1 receptor subtype in these cells. Successful gene knockdown was validated by RT-qPCR assessing the levels of EP1 mRNA transcripts in EP1 siRNA-transfected versus control siRNA-transfected PANC-1 cells (Fig. 5D). As a result, siRNA-mediated knockdown of EP1 blunted the PGE\(_2\)-induced phosphorylation of p70S6K and S6rp (either at Ser\(^{235/236}\) or Ser\(^{240/244}\)) (Fig. 5E). These results suggest that, besides cAMP/PKA signaling, a Ca\(^{2+}\)-dependent pathway contributes to PGE\(_2\)-induced mTORC1 activation, via the EP1 receptor. This finding is reinforced by the data that PGE\(_2\)-stimulated S6rp phosphorylation (Ser\(^{235/236}\) and Ser\(^{240/244}\)) was abolished when both cAMP/PKA and Ca\(^{2+}\) signaling were blocked by the combination of H-89 and BAPTA-AM, but only partially inhibited by either treatment alone (Fig. 5F).

**PGE\(_2\)-induced mTORC1 activation requires both cAMP/PKA and Ca\(^{2+}\) pathways.**

Our data suggest that both cAMP/PKA and Ca\(^{2+}\) pathways are important in mediating PGE\(_2\)-induced activation of mTORC1. To test whether the effects of PGE\(_2\) on mTORC1 activation represent a cell type-specific or more general phenomenon, we conducted an RT-qPCR screening of EP1, 2, and 4 expression in a variety of human cancer cell lines (Fig. 6A) derived from pancreatic (AsPC-1, BxPC-3, Capan-2, HPAF-II, MIA PaCa-2, and PANC-1),
prostate (PC-3), breast (MCF-7), and colon (DLD-1 and SW480) cancers, all of which are notably associated with obesity. We found that the responsiveness of mTORC1 activation to PGE₂ correlated well with the co-existence of highly expressed EP1 (mediating Ca²⁺ response) and EP2 or 4 (mediating cAMP response), as exemplified by PANC1 and PC-3 cells (Fig. 6A). In PC-3 cells, PGE₂ robustly stimulated intracellular cAMP (Fig. 6B) and Ca²⁺ (Fig. 6C) levels, and the phosphorylation of S6rp (Fig. 6D). Also, similar to the results seen in PANC-1, the phosphorylation of S6rp was attenuated by H-89, BAPTA-AM, and rapamycin (Fig. 6D&E). Further, in BxPC-3 cells expressing abundant EP4 while substantially lacking EP1 transcripts, PGE₂-induced phosphorylation of S6rp (Ser²³⁵²³⁶) became only evident when intracellular Ca²⁺ level was increased with ionomycin to mimic EP1 receptor-mediated Ca²⁺ signaling (Fig. 6F). These findings suggest that PGE₂-mediated mTORC1 activation depends on two separate signals, namely cAMP (mediated by EP2 or EP4) and intracellular Ca²⁺ (mediated by EP1).

**PGE₂ potentiates the effect of IGF-1 through mTORC1 activation.**

Our data suggest that, in addition to the canonical insulin/IGF-1-mediated signaling, mTORC1 can be co-activated by the inflammatory PGE₂ pathway, which might potentiate the effects of insulin (and/or IGF-1). To investigate the interaction between these pathways, cells were treated with PGE₂ in combination with IGF-1. As shown in Fig. 7A, PGE₂ enhanced the effect of IGF-1 on the phosphorylation of p70S6K and 4E-BP1 downstream of mTORC1, suggesting reinforcement by the crosstalk between the two pathways.

We next investigated phenotypical responses of these signaling events. There is considerable evidence suggesting the contribution of PGE₂ to tumor development in many aspects of the immune and inflammation responses (18, 37). Since PGE₂ mediates inflammation via cytokine production, we used a human cytokine protein array to measure a panel of cytokines released from PANC-1 cells exposed to PGE₂, IGF-1, or the combination of the two stimuli. Among the 36 cytokines, chemokines, and acute phase proteins, we identified two proteins that were especially
up-regulated by the combination of PGE$_2$ and IGF-1. As shown in **Fig. 7B**, they were plasminogen activator inhibitor-1 (PAI-1) also known as serpin E1, and interleukin 23 (IL-23), both of which are known to be pro-inflammatory. Further validation by RT-qPCR showed that *IL-23* transcription in PANC-1 was promoted by the treatment with combined PGE$_2$ and IGF-1 for 24 h, but not PGE$_2$ or IGF-1 alone (**Fig. 7C**). Importantly, the up-regulation of *IL-23* expression was inhibited by rapamycin, confirming IL-23 production as a consequence of the crosstalk between PGE$_2$ and IGF-1 signaling converging at mTORC1. Together, our studies reveal a novel crosstalk between PGE$_2$ signaling and IGF-1/mTORC1 pathway as illustrated in **Fig. 7D**. By regulating cytokine production, this crosstalk may be of importance in amplifying inflammatory response in the tumor microenvironment.

**Discussion**

Obesity is a contributing factor in the development of a number of cancers, but the mechanisms driving this association remain unclear. Recent *in vivo* studies from our group and others strongly support the importance of the inflammatory environment in obesity-promoted pancreatic cancer (5, 40). To further elucidate the molecular mechanisms, we explored a potential link between pro-inflammatory signaling and hormone/growth factor pathways that are up-regulated in obese and insulin-resistant subjects. Specifically, the results presented here identified a signaling crosstalk between PGE$_2$ and mTORC1 pathways in pancreatic cancer cells. Our data showed that stimulating PANC-1 cells with PGE$_2$ increased the phosphorylation of molecules downstream of mTORC1, including p70S6K, S6rp, and 4E-BP1, indicating that PGE$_2$ induces activation of mTORC1.

Associated with G$\alpha_s$, EP2 and EP4 are known to mediate PGE$_2$-induced intracellular cAMP production. Several previous studies linked a cAMP-dependent pathway to the PI3K/Akt/mTORC1 signaling module, but the underlying mechanisms vary among diverse
cellular systems, implying cell context-specific processes (2, 3, 22). In epinephrine-stimulated rat skeletal muscle cells, cAMP can activate PI3K/Akt via Epac (exchange protein directly activated by cAMP), thereby augmenting the effect of insulin on PI3K/Akt signaling (2). In PANC-1 pancreatic cancer cells, however, PGE$_2$/cAMP-induced activation of mTORC1 seems to be independent of PI3/Akt, as phosphorylation of Akt remained unchanged with PGE$_2$ or forskolin stimulation. Also, cAMP-activated Epac is known to activate the Ras-associated protein-1 (Rap1)/B-Raf/ERK cascade (6, 27), which may be an alternative route to mTORC1 activation (33). However, there was no increase in p-ERK levels upon PGE$_2$ treatment in PANC-1 cells. Subsequently, we showed that the effect of PGE$_2$ or forskolin on mTORC1 activity was attenuated by H-89, suggesting the involvement of PKA, a major target of cAMP. This finding is in agreement with a previous study investigating cAMP-mediated mTORC1 signaling in thyroid cells induced with thyroid-stimulating hormone (51).

Based on the studies using agonist/antagonist for EP2 and EP4, our data suggest that in PANC-1 pancreatic cancer cells the PGE$_2$-stimulated cAMP responses are predominantly mediated by EP4. Although the significance of EP4 in vivo remains to be determined, this finding is consistent with the notion that EP4 plays a major role in colorectal carcinogenesis (36, 42), and with a recent study showing that EP4 expression is markedly elevated in human pancreatic tumors as compared with adjacent benign pancreatic tissues (13). Also, EP4-mediated mTORC1 activation by PGE$_2$ has been recently demonstrated in colon (8) and prostate (53) cancer cells, reinforcing our findings in pancreatic cancer cells as a common concept, especially in obesity-associated cancers. In those previous reports, however, the significance of cAMP/PKA downstream of EP4 was not delineated. Importantly, in addition to the EP4/cAMP/PKA pathway, we uncovered that PGE$_2$-induced mTORC1 activation is also mediated by a Ca$^{2+}$-dependent mechanism, which is elicited through the G$_{q1}$-coupled EP1 receptor, as supported by our results from the EP1 knock-down experiments. Although the role of EP1 in cancer development is less
documented as compared to EP2/4 (16), several studies have identified EP1 as a critical receptor (20, 52, 55). Further, we discovered the cooperative roles of cAMP/PKA and Ca\(^{2+}\) pathways in PGE\(_2\)-induced mTORC1 activation, based on the maximal inhibition achieved by the combined blockade of both arms of signaling with H-89 and BAPTA-AM, as well as the concurrence of high levels of EP2/4 and EP1 in the cell lines (PANC-1 and PC-3) that prominently displayed PGE\(_2\)-activated mTORC1 signaling. The expression patterns of EP receptors in PANC-1 (high in EP1 & 4), PC-3 (high in EP1 & 2), and BxPC-3 (high in EP2 only) suggest that, to induce PGE\(_2\)-mediated mTORC1 activation, the functional integrity of both EP1/Ca\(^{2+}\) pathway and cAMP response is critical, and the latter may be evoked through either EP2 or EP4. Collectively, our studies using pharmacological and genetic approaches demonstrate that PGE\(_2\)-induced mTORC1 activation is mediated by the coordinated operation of the EP4/cAMP/PKA and EP1/G\(_{\alpha_q}\)/Ca\(^{2+}\) pathways in PANC-1 cells (Fig. 7D).

Signaling pathways usually interact and crosstalk with each other. A precise understanding of these complex networks may reveal critical mechanistic targets for therapeutic intervention devoid of unwanted side effects or resistance. For example, it has been previously shown in pancreatic cancer cells that the Akt/mTORC1 pathway mediates a crosstalk between insulin receptor and GPCR signaling systems, where insulin can potentiate Ca\(^{2+}\) signaling triggered by G\(_{\alpha_q}\)-coupled receptor agonists (24). Remarkably, further studies revealed that such crosstalk is disrupted by metformin, a widely-used anti-diabetic drug that negatively regulates mTORC1 through AMP kinase activation, leading to inhibition of pancreatic cancer growth (23). Herein, we identified a similar but reverse crosstalk between PGE\(_2\)-mediated pathways and IGF-1 signaling, converging at mTORC1 and creating a positive reinforcement (Fig. 6D). Interestingly, PGE\(_2\) and IGF-1 in combination, but not alone, induced \textit{IL}-23 mRNA transcription in PANC-1, and this effect was abrogated by the mTORC1 inhibitor rapamycin. This finding highlights the significance of the reinforcing crosstalk between PGE\(_2\) and IGF-1 signaling through subsequent mTORC1
activation. IL-23, a pro-inflammatory cytokine, has been shown to promote tumor growth by driving pro-tumorigenic inflammation, as well as impairing T cell-mediated immune surveillance (25, 38). Also, a recent study has suggested that PGE2 secreted from breast tumor cells, through cAMP/PKA signaling, induces IL-23 expression in the tumor microenvironment, leading to Th17 cell expansion (43). These novel findings raise the attractive possibility that PGE2 exerts its pro-tumorigenic properties through regulating pro-inflammatory cytokine production by means of augmenting mTORC1 function, which is already supported by enhanced IGF-1/Akt signaling under certain conditions (e.g. obese state and neoplasm). Overall, the signaling crosstalk identified above would lead to an exacerbated inflammatory tumor microenvironment thereby contributing to cancer development.

It is noteworthy that we also detected PGE2-induced phosphorylation of S6rp in mouse primary PanIN (pancreatic intraepithelial neoplasia) cells, which are precancerous cells isolated from the conditional KrasG12D mouse model of pancreatic cancer (data not shown). These findings suggest that the crosstalk between PGE2 and mTORC1 is not restricted to cancer cells but is also operational in early stages of pancreatic tumor development. Since both inflammation and insulin/growth hormones are key factors shared by obesity and cancer, this signaling crosstalk is highly relevant to obesity-associated cancer, where pro-inflammatory PGE2 and systemic high levels of IGF-1 are conducive to tumor development. Given the well-known, potentially harmful cardiovascular side effects of COX-2 inhibitors (32) and feedback mechanisms of resistance associated with mTOR inhibitors (54), a deeper understanding of the molecular pathways involved in this link will facilitate the development of novel efficacious interventions while circumventing adverse effects.

In summary, our data link the PGE2 signaling to the IGF-1/Akt/mTORC1 module. The operation of this crosstalk strongly supports the hypothesis that inflammation-associated PGE2 can
reinforce the tumor-promoting effects of insulin/IGF-1 signaling. Since both pathways are implicated in obesity as well as in cancer development, this notion may be of importance in elucidating the connection between obesity and enhanced cancer risk. Ultimately, a detailed understanding of these molecular links may provide novel targets for improved interventions for pancreatic cancer in an increasingly obese population.

Grants

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Disclosures

None.
References


9. El-Bayoumy K, Iatropoulos M, Amin S, Hoffmann D, and Wynder EL. Increased expression of cyclooxygenase-2 in rat lung tumors induced by the tobacco-specific nitrosamine


Figure Captions

**Figure 1.** PGE2 activates mTORC1. PANC-1 cells were (A) incubated with PGE2 at the indicated concentrations for 15 min, (B) incubated with 1 µM of PGE2 for the indicated times, (C) pre-treated with the mTORC1 inhibitor rapamycin at 1 nM for 30 min, and then stimulated with or without PGE2 (1 µM) for 15 min. Following treatments, cell lysates were collected and subject to immunoblotting with indicated antibodies. GAPDH was used as a loading control.

**Figure 2.** Forskolin, a cAMP stimulator, mimics the effects of PGE2. (A) Dose-response curve of PGE2-mediated cAMP induction. PANC-1 cells were incubated with PGE2 at increasing concentrations (0, 0.03, 0.1, 0.3, 1, 3, 10, 30, 100 µM) for 1 min at 37°C and then lysed in 0.1 N HCl. Intracellular levels of cAMP in the cell lysates were determined by an enzyme immunoassay (EIA). All values were normalized to protein concentrations of the cell lysates. (B) Forskolin increases intracellular levels of cAMP in MIA PaCa-2 and PANC-1 cells. The cells were incubated with forskolin (10 µM) for 5 min. Levels of cAMP in the cell lysates were determined as...
described. (C) Forskolin stimulates phosphorylation of S6rp in MIA PaCa-2 and PANC-1 cells. Cells were treated with forskolin (10 µM) for 2 h, with or without H-89 (a PKA inhibitor). Following treatments, cell lysates were collected and subject to immunoblotting with indicated antibodies.

Figure 3. EP4 receptor mediates PGE$_2$-induced cAMP response. (A) PANC-1 cells were pre-treated with antagonists for EP4 or EP2 (ONO-AE3-208 and PF-04418948, respectively) at the indicated concentrations for 1 h, and then stimulated with PGE$_2$ (1 µM) for 1 min. Levels of cAMP in the cell lysates were determined as described. Values are relative to the untreated control, and are means ± SD. *P<0.05 (Student’s t-tests). (B) PANC-1 cells were incubated with agonists for EP4 or EP2 (CAY10580 and Butaprost, respectively) for 1 min. Levels of cAMP in the cell lysates were determined. Values are means ± SD. *P<0.05 (Student’s t-tests).

Figure 4. EP4 and PKA are involved in PGE$_2$-induced mTORC1 activation. (A) PANC-1 cells were treated with an EP4 agonist CAY10580 at 10 µM for 15 min. (B) PANC-1 cells were pre-treated with the EP4 antagonist ONO-AE3-208 at 1 µM for 1 h, and then stimulated with or without PGE$_2$ for 15 min. (C) PANC-1 cells were pre-treated with the PKA inhibitor H-89 at 5 µM for 1 h, and then stimulated with or without PGE$_2$ (1 µM) for 15 min. Following treatments, cell lysates were collected and subject to immunoblotting with indicated antibodies. GAPDH was used as a loading control.

Figure 5. Calcium signaling is involved in PGE$_2$-induced mTORC1 activation. (A) PANC-1 cells were loaded with the fluorescent Ca$^{2+}$ indicator Fura 2-AM, and stimulated with 1 µM PGE$_2$ at the times marked by the arrows. Each line represents an individual tracing from a single cell. Different levels of responses in individual cells were observed and shown (top, middle, and bottom panels). (B) PANC-1 cells were pre-incubated with a cell permeable Ca$^{2+}$ chelator
BAPTA-AM at 10 μM for 1 h, and then treated with 50 nM ionomycin (an ionophore that raises intracellular Ca\(^{2+}\) levels) for 15 min. (C) PANC-1 cells were pre-incubated with 10 μM BAPTA-AM for 1 h, and then stimulated with or without PGE\(_2\) (1 μM) for 15 min. Following treatments, cell lysates were collected and subject to immunoblotting with indicated antibodies. (D) PANC-1 cells were transfected with negative control siRNA or EP1 siRNA. Following 4 days incubation, total RNA was extracted from the cells and subject to RT-qPCR using gene-specific primers for EP1 and ACTB; the latter was used as an internal reference gene to normalize the results. Values are means ± SD. *P<0.05 (Student’s t-tests). (E) PANC-1 cells were transfected with negative control siRNA or EP1 siRNA, and then stimulated with or without PGE\(_2\) (1 μM) for 15 min. Following treatments, cell lysates were collected and subject to immunoblotting with indicated antibodies. GAPDH was used as a loading control. (F) PANC-1 cells were pre-treated with rapamycin (1 nM) and BAPTA-AM (10 μM) as described, alone or in combination, and then stimulated with or without PGE\(_2\) (1 μM) for 15 min. Following treatments, cell lysates were collected and subject to immunoblotting with indicated antibodies.

Figure 6. (A) Expression levels of EP1, 2, and 4 in a panel of cancer cell lines (as indicated) were determined by RT-qPCR. ACTB was used as an internal reference gene to normalize the results. The mRNA expression of each gene is presented relative to that of PANC-1 (set as 1-fold). Values are means ± SD. (B) PC-3 cells were incubated with DMSO or PGE\(_2\) (1 μM) for 1 min, and levels of intracellular cAMP in the cell lysates were determined as described. (C) Intracellular Ca\(^{2+}\) response to PGE\(_2\) in PC-3 cells was measured as described. The trace represents the averaged response of all cells. The arrow marks the time PGE\(_2\) was added. (D) PC-3 cells were pre-treated with rapamycin (1 nM) or BAPTA-AM (10 μM) as described, alone or in combination, and then stimulated with or without PGE\(_2\) (1 μM) for 15 min. Following treatments, cell lysates were collected and subject to immunoblotting with indicated antibodies. (E) PC-3 cells were pre-treated with rapamycin (1 nM) for 30 min, and then stimulated with or without...
PGE$_2$ (1 µM) for 15 min. Following treatments, cell lysates were collected and subject to
immunoblotting with indicated antibodies. (F) BxPC-3 cells were treated with PGE$_2$ (10 µM),
alone or in combination with ionomycin (500 nM) for 15 min. Following treatments, cell lysates
were collected and subject to immunoblotting with indicated antibodies. Quantitative
densitometry of p-S6rp Western blots was shown as a bar graph. Values are relative to the
untreated control (set as 1-fold), and are means ± SD. *$P<0.05$ (paired Student’s t-tests).

Figure 7. Potentiating crosstalk between PGE$_2$ and IGF-1 signaling pathways through mTORC1.
(A) PANC-1 cells were stimulated with IGF-1 (10 ng/ml) in the absence or presence of PGE$_2$ (1
µM) for 15 min. Following treatments, cell lysates were collected and subject to immunoblotting
with indicated antibodies. (B) Relative levels of PAI-1 and IL-23 in PANC-1 cell supernatants
collected after 24 h of indicated treatments, determined using a cytokine array. Values are signal
intensity (arbitrary units), and are averages ± SD quantified from duplicated spots on the array
membrane. (C) Following indicated treatments for 24 h, relative transcript expression levels of
IL-23 in PANC-1 were determined by RT-qPCR as described. Values are means ± SD. *$P<0.05$
(Student’s t-tests). (D) A schematic model showing crosstalks between the PGE$_2$ and IGF-1
pathways converging at mTORC1 implicated in obesity-associated tumor promotion.
Figure 1

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<td>Total S6rp</td>
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35 kDa
Figure 7

A

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<th></th>
<th>PGE₂</th>
<th>-</th>
<th>+</th>
<th>IGF-1</th>
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<tr>
<td>p-p70S6K ⁵³⁸⁹</td>
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<td>Total p70S6K</td>
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<td>p-4E-BP1 ⁵³⁷/⁴⁶</td>
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<td>p-Akt ³⁰⁸</td>
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<tr>
<td>p-CREB ¹¹³³</td>
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B

Serpine E1 (PAI-1) vs IL-23

Signal intensity (Arbitrary units)

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<th>PGE₂</th>
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<th>+</th>
<th>IGF-1</th>
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<td>Serpine E1</td>
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<td>IL-23</td>
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Graphs showing the signal intensity of Serpine E1 and IL-23 under different conditions of PGE₂ and IGF-1.
**Figure 7 (Continued)**

**C**

**IL-23A**

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<td>IGF-1</td>
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<td>Rapamycin</td>
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Relative transcript expression (fold change)

**D**

**Inflammation** $\leftrightarrow$ **PGE$_2$** $\leftrightarrow$ **IGF-1** $\leftrightarrow$ **Hyperinsulinemia**

- EP1
- EP4
- [Ca$^{2+}$]$_i$$\uparrow$
- cAMP$\uparrow$
- PKA
- mTORC1
- 4E-BP1
- p70S6K
- elf4E
- S6rp

Protein synthesis and cytokine production