Prostaglandin E2 activates the mTORC1 pathway through an EP$_4$/cAMP/PKA- and EP$_1$/Ca$^{2+}$-mediated mechanism in the human pancreatic carcinoma cell line PANC-1

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Obesity is an established risk factor for pancreatic cancer (4), a remarkably aggressive and fatal disease with an overall 5-yr survival rate of only ~5% (46). Although the link between obesity and cancer is compelling, the mechanisms driving this association remain poorly understood. A number of factors, such as the proinflammatory state associated with excess adiposity, as well as the elevated levels of growth hormones, i.e., insulin and insulin-like growth factor type 1 (IGF-1), are implicated (21). Compensatory high levels of insulin during obesity-associated insulin resistance are known to upregulate 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 activating insulin and IGF-1 receptors and the downstream phosphoinositide 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) 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 anticancer 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 eukaryotic translation initiation factor 4E-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 proinflammatory 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 nonsteroidal anti-inflammatory drug, 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 (PGE$_2$), a key inflammatory lipid mediator generated by cyclooxygenase enzymes (COX-1 and -2), the best-characterized targets of nonsteroidal
anti-inflammatory drugs (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 activation of 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, EP2, EP3, and EP4 (50). Previously, we showed 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 αi (Gqi), which is 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 interconnected. Links between critical signaling pathways deserve more attention. In particular, a potential cross talk 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 activation of mTORC1 (3, 22). Therefore, by activating EP receptors, PGE2 may stimulate mTORC1 and potentiate its growth-promoting action. We have uncovered a novel cross talk between PGE2 signaling and the mTORC1 pathway in pancreatic cancer cells. We further investigated the mechanisms through which PGE2 can stimulate mTORC1 and, potentially, augment the protumorigenic effects of insulin/IGF-1. Since PGE2 signaling and the IGF-1/Akt/mTORC1 pathway are overactivated in obesity-associated cancers, this novel cross talk 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 purchased from Cell Signaling Technology (Danvers, MA): phosphorylated (Thr389) p70S6K Ab (catalog no. 9205), phosphorylated (Ser235/236) S6 ribosomal protein (S6rp) Ab (catalog no. 2211), phosphorylated (Ser240/244) S6rp MAb (catalog no. 5364), S6rp MAb (catalog no. 2317), phosphorylated (Ser137) cAMP response element-binding protein (CREB) MAb (catalog no. 9198), phosphorylated (Ser72) Akt Ab (catalog no. 9271), phosphorylated (Thr308) Akt Ab (catalog no. 9275), Akt Ab (catalog no. 9272), phosphorylated (Thr202/Tyr204) MAPK (Erk1/2) MAb (catalog no. 4370), phosphorylated (Ser180) p90S6K (also known as RSK) MAb (catalog no. 9335), phosphorylated (Thr37/46) 4E-BP1 (236B4) MAb (catalog no. 2118). PGE2, forskolin, H-89, butaprost, the EP2 agonist CAY10850, the EP3 antagonist PF04418984, the EP4 antagonist ONO-AE3-208, ionicomycin, and BAPTA-AM were purchased from Cayman Chemical (Ann Arbor, MI); rapamycin from Santa Cruz Biotechnology (Santa Cruz, CA); EP1 small interfering RNA (siRNA; catalog no. L-005711, ON-TARGETplus human PTGER1 siRNA-SMARTpool) from GE Dharmacon (Lafayette, CO); and recombinant human IGF-1 from Sigma-Aldrich (St. Louis, MO).

**Cell culture and treatments.** The human cancer cell lines AsPC-1, BxPC-3, Capan-2, HPAF-II, MIA PaCa-2, and PAN-1 (pancreatic cancer), PC-3 (prostate cancer), MCF-7 (breast cancer), and DLD-1 and SW480 (colon cancer) were obtained from the American Tissue Type Culture Collection. MIA PaCa-2 and PAN-1 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS and 1× penicillin-streptomycin-glutamine (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 1× penicillin-streptomycin-glutamine (Life Technologies) and cultured at 37°C and 5% CO2. For Western blot analysis, cells were seeded in six-well plates. After serum starvation for 18 h, cells were treated as indicated.

**Western blotting.** After treatments, cells were harvested and lysed in lysis buffer (1× Tris buffer, pH 7.4, 1% Triton X-100, and 0.25% sodium deoxycholate) supplemented with protease and phosphatase inhibitor cocktails (Thermo Scientific, Rockford, IL). Forty micrograms of proteins [quantified using a 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. After incubation with secondary antibodies, proteins were detected by ECL reagents (Thermo Scientific) and exposed to CL-XPosure X-ray films (Thermo Scientific).

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

**Intracellular Ca2+ concentration measurement.** Cells were seeded on coverslips. After serum starvation, the coverslips were incubated with 5 μM fura 2-AM diluted in prewarmed Hanks’ 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 (0.5 ml volume) 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 (340-nm excitation to 380-nm excitation) images were obtained every second by a digital camera (AxioCam MRm) attached to the microscope, which was operated with associated software (AxioVision, all components from Carl Zeiss, Thornwood, NY). Intracellular Ca2+ concentration is proportional to 35-Γ0 ratio excitation at 340 nm to excitation at 380 nm. In some experiments, cells were plated onto coverslips that fit inside cuvettes, which in turn were loaded into a temperature-controlled fluorometer (Hitachi). At selected times, agonist was introduced into the cuvette. Ratio values for the entire cuvette were determined as described above.

**siRNA transfection.** Cells were seeded in six-well plates and incubated overnight in Dulbecco’s modified Eagle’s medium with 10% FBS. Transfection of siRNA was carried out on the following day with Lipofectamine RNAiMAX reagent (Life Technologies) following the manufacturer’s recommendations. Cells were incubated for an additional 3 days and then serum-starved and stimulated with PGE2.

**Quantitative reverse-transcription PCR.** Relative transcript expression levels of EP1, EP2, EP4, and IL-23 were determined by quantitative RT-PCR (RT-qPCR) using a SYBR Green-based method. Briefly, the PureLink RNA Mini Kit (Life Technologies) was used to extract total RNA from cells. Reverse transcription was performed with the iScript reverse-transcription supermix (Bio-Rad Laboratories) 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 iQ5 system, and the amplifications were done using the iTag Universal SYBR Green Supermix (Bio-Rad). Gene-specific oligonucleotide primers for EP1, EP2, EP4, and IL-23 (IL-23, α-subunit p19) and internal reference ACTB (β-actin) or RNAS18S (RNA, 18S ribosomal) are as follows: 5’-ACCTTCTTGGGCGGTCTC-3’ (forward) and 5’-CCACCAACCACTGTGGCGG-3’ (reverse) for EP1 (exons 2 and 3), 5’-GTCTCTTTGGCTCCAGATT-3’ (forward) and 5’-AG-
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GATGGCAGAACCCACG-3' (reverse) for EP2 (exons 1 and 2), 5'-CGCTCTGTTGCGAGGATATT-3' (forward) and 5'-AGGGCTTACAGTGGGCTT-3' (reverse) for EP4 (exons 2 and 3), 5'-CAGTATGGACGACCGATTT-3' (forward) and 5'-TATCATCTCATGTTGAGCTTGCCTT-3' (reverse) for ACTB (exons 1 and 2), and 5'-AGCTCTGCTGGGTATGTCAACA-3' (forward) and 5'-CGATCCGAGGGTCTCATA-3' (reverse) for RNA18S5. 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.

Cytokine array. PANC-1 cells were seeded in six-well plates and then serum-starved. Cells were incubated in culture medium (2 ml) with or without PGE2 and IFG-1, alone or in combination. Cell culture supernatants collected after 24 h of 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, interleukin-1 (IL-1), IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-16, IL-17, IL-23, IL-27, IL-32a, CXCL10/inducible protein 10, CXCL11/interferon-inducible T cell α chemotactant (I-TAC), CCL2/monocyte chemotactant protein (MCP)-1, macrophage migration inhibitory factor (MIF), CCL3/macrophage inflammatory protein (MIP)-1α, CCL4/ MIP-1β, CCL5/regulated on activation, normal T cell expressed and secreted (RANTES), CCL12/stromal cell-derived factor (SDF)-1, serotonin (5-HT) receptor expressed on myeloid cells (TREM)-1. After exposure to horseradish peroxidase 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 version 3.0 software (Fujifilm Life Sciences).

Statistical analyses. Values are means ± SD. To determine statistical significance, one-way ANOVA and two-tailed Student’s t-tests were performed assuming unequal variances. P < 0.05 was considered significant.

RESULTS

PGE2 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 PGE2 stimulates mTOR signaling in pancreatic cancer cells, PANC-1 cells were treated with PGE2, and then mTORC1 activation was assessed by Western blot analysis of phosphorylation levels of downstream molecules. As shown in Fig. 1, A and B, PGE2 dose- and time-dependently induced the phosphorylation of S6rp at Ser235/236, which correlated with the increased Thr389 phosphorylation of p70S6K, a major mTORC1 target upstream of S6rp. These effects were robust after 15 min of stimulation and observed at 0.1 μM PGE2.

Although Ser235/236 phosphorylation of S6rp can also be accomplished by p90RSK independently of mTORC1, p90RSK activation assessed by Ser380 phosphorylation was unchanged in response to PGE2 treatment (Fig. 1B). Also, phosphorylation of ERK, a key regulator of p90S6K, was not increased by PGE2 in these cells (Fig. 1A). Furthermore, phosphorylation of S6rp at Ser240/244, known to be a p70S6K-specific site (1, 44), was markedly induced by treatment with PGE2 (Fig. 1, B and C). Moreover, treatment of PANC-1 cells with 1 nM rapamycin, a potent allosteric inhibitor of mTORC1, suppressed PGE2-induced S6rp phosphorylation (Fig. 1C), corroborating the finding that phosphorylation of S6rp at Ser240/244 in response to PGE2 is mediated by mTORC1. Taken together, these results indicate that PGE2 stimulates mTORC1 activity in pancreatic cancer cells. Interestingly, PGE2 did not increase Akt phosphorylation (Thr308 and Ser473) (Fig. 1B), implying that the cross talk to the mTORC1 cascade is downstream of Akt and does not affect mTORC2 [as judged by phosphorylated (Ser473) Akt]. These findings prompted us to explore the mechanism(s) underlying PGE2-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 was seen at 0.09 μM, and maximal effects were obtained at ~1 μM PGE2. Notably, PGE2 was effective at submicromolar 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 CREB (Fig. 1, A and B), which is known to be positively regulated by PKA, the activation of which 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 adenylyl 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 the idea that cAMP is involved in this cross talk. Also, the effect of forskolin was blocked in cells preincubated with H-89 (Fig. 2C), a preferential inhibitor of PKA.

Among the four subtypes of GPCRs for PGE2, EP2 and EP4 are known to couple to Goα and, thus, are responsible for stimulating cAMP production via activation of adenylyl 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 EP2 agonist CAY10580 promoted mTORC1 activation, as assessed by S6rp phosphorylation (Fig. 4A). Importantly, PGE2-activated S6rp phosphorylation (at Ser235/236 or Ser240/244) was prevented by the specific EP2 antagonist ONO-AE3-208 (Fig. 4B). These results reinforce the notion that PGE2-induced cAMP accumulation and subsequent responses are mainly mediated by the EP4 receptor in PANC-1 cells.

The cAMP-dependent PKA is a central target of cAMP. Therefore, we used the PKA inhibitor H-89 to test whether PGE2-stimulated mTORC1 activation is PKA-dependent. Pretreatment of cells with 5 μM H-89 attenuated baseline and...
PGE2-activated S6rp phosphorylation (Fig. 4C), indicating a role for PKA in the cross talk between PGE2 signaling and the mTORC1 pathway. This finding is in agreement with previous results showing that PGE2-induced activation of p70S6K and S6rp paralleled an increase in the phosphorylation of CREB, a substrate of PKA (Fig. 1).

Ca2+ signaling contributes to PGE2-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 PGE2-induced S6rp phosphorylation (at Ser235/236 or Ser240/244) (Fig. 4C). These results suggest that, besides cAMP, PGE2 may activate an additional pathway leading to mTORC1 activation. Ca2+ signaling has been implicated in the regulation of mTORC1 activation independently of PI3K/Akt (15, 31, 34). Consequently, PGE2 might exert some of its cAMP-independent effects through activation of EP1 receptors, which couple to Gs, and, thereby, promote phospholipase C-mediated formation of diacylglycerol and inositol trisphosphate, second messengers leading to PKC activation and Ca2+ mobilization, respectively (19). Initially, we examined whether PGE2 induces Ca2+ mobilization in PANC-1 cells. In agreement with this hypothesis, we found that addition of PGE2 to fura2-loaded PANC-1 cells induced a rapid and transient increase in intracellular Ca2+ levels in PANC-1 cells (Fig. 5A). This microscopy-based measurement allowed us to monitor the Ca2+ response in individual cells and revealed that ~62% of the cells (8 of 13) were responsive to PGE2 to varying extents (see representative traces in Fig. 5A).

Additionally, ionomycin, a Ca2+ ionophore that raises intracellular Ca2+ level, induced phosphorylation of p70S6K and downstream S6rp (Ser235/236 and Ser240/244) in these cells. This
effect was markedly suppressed by BAPTA-AM, an intracellular Ca\textsuperscript{2+}/H\textsuperscript{11001} chelator (Fig. 5B). Importantly, ionomycin was used in this experiment at a concentration (50 nM) that elicited an increase in intracellular Ca\textsuperscript{2+}/H\textsuperscript{11001} comparable to that induced by PGE\textsubscript{2} (data not shown).

To study the possible role of Ca\textsuperscript{2+} signaling in PGE\textsubscript{2}-induced mTORC1 activation, cells were pretreated with BAPTA-AM prior to PGE\textsubscript{2} stimulation. As shown in Fig. 5C, phosphorylation (Ser\textsuperscript{235/236} and Ser\textsuperscript{240/244}) of S6rp was significantly decreased by depletion of intracellular Ca\textsuperscript{2+}. To further confirm that the G\textsubscript{q}/H\textsuperscript{9251} coupled EP\textsubscript{1} receptor is involved in PGE\textsubscript{2}-stimulated mTORC1 activation, we utilized a genetic approach to specifically knock down the EP\textsubscript{1} receptor subtype in these cells. Successful gene knockdown was validated by RT-qPCR assessing the levels of EP\textsubscript{1} mRNA transcripts in EP\textsubscript{1} siRNA-transfected vs. control siRNA-transfected PANC-1 cells (Fig. 5D). As a result, siRNA-mediated knockdown of EP\textsubscript{1} blunted the PGE\textsubscript{2}-induced phosphorylation of p70S6K and S6rp (Ser\textsuperscript{235/236} or Ser\textsuperscript{240/244}) (Fig. 5E). These results suggest that, besides cAMP/PKA signaling, a Ca\textsuperscript{2+}-dependent pathway contributes to PGE\textsubscript{2}-induced mTORC1 activation via the EP\textsubscript{1} receptor. This finding is reinforced by the data showing that PGE\textsubscript{2}-stimulated S6rp phosphorylation (Ser\textsuperscript{235/236} and Ser\textsuperscript{240/244}) was abolished when both cAMP/PKA and Ca\textsuperscript{2+} signaling were blocked by the combination of H-89 and BAPTA-AM but were only partially inhibited by either treatment alone (Fig. 5F).

PGE\textsubscript{2}-induced mTORC1 activation requires both cAMP/PKA and Ca\textsuperscript{2+} pathways. Our data suggest that both cAMP/PKA and Ca\textsuperscript{2+} pathways are important in mediating PGE\textsubscript{2}-induced activation of mTORC1. To test whether the effects of PGE\textsubscript{2} on mTORC1 activation represent a cell type-specific or a more general phenomenon, we conducted an RT-qPCR screening of EP\textsubscript{1}, EP\textsubscript{2}, and EP\textsubscript{4} expression in a variety of human cancer cell lines (Fig. 6A) derived from pancreatic
EP4 and PKA are involved in PGE2-induced mTORC1 activation. A: PANC-1 cells were treated with the EP4 agonist CAY10580 (10 μM) for 15 min. B: PANC-1 cells were pretreated with the EP4 antagonist ONO-AE3-208 (1 μM) for 1 h and then stimulated with or without PGE2 for 15 min. C: PANC-1 cells were pretreated with the PKA inhibitor H-89 (5 μM) for 1 h and stimulated with or without PGE2 (1 μM) for 15 min. After treatments, cell lysates were collected and subjected to immunoblotting with indicated antibodies. GAPDH was used as a loading control.

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 proinflammatory signaling and hormone/growth factor pathways that are upregulated in obese and insulin-resistant subjects. Specifically, the results presented here identified a signaling cross talk between PGE2 and mTORC1 pathways in pancreatic cancer cells. Our data show that stimulation of PANC-1 cells with PGE2 increased the phosphorylation of S6rp (Fig. 6C), indicating that 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 upregulated by the combination of PGE2 and IGF-1, PAI-1 (also known as serpin E1), and IL-23, both of which are known to be proinflammatory (Fig. 7B). Further validation by RT-qPCR showed that IL-23 transcription in PANC-1 cells was promoted by treatment for 24 h with PGE2 and IGF-1 in combination, but not PGE2 or IGF-1 alone (Fig. 7C). Importantly, the upregulation of IL-23 expression was inhibited by rapamycin, confirming that IL-23 production is a consequence of the cross talk between PGE2 and IGF-1 signaling converging at mTORC1. Together, our studies reveal a novel cross talk between PGE2 signaling and the IGF-1/mTORC1 pathway (Fig. 7D). By regulating cytokine production, this cross talk may be of importance in amplifying the inflammatory response in the tumor microenvironment.
Fig. 5. Ca\(^{2+}\) 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 \(\mu\)M PGE\(_2\) at the times marked by the arrows. Each trace represents results from a single cell. Top, middle, and bottom: different levels of responses in individual cells. B: PANC-1 cells were preincubated with the cell-permeable Ca\(^{2+}\) chelator BAPTA-AM (10 \(\mu\)M) 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 preincubated with 10 \(\mu\)M BAPTA-AM for 1 h and then stimulated with or without PGE\(_2\) (1 \(\mu\)M) for 15 min. After treatments, cell lysates were collected and subjected to immunoblotting with indicated antibodies. D: PANC-1 cells were transfected with negative control siRNA or EP1 siRNA. After 4 days of incubation, total RNA was extracted from the cells and subjected to quantitative RT-PCR (RT-qPCR) using gene-specific primers for EP1 and ACTB (\(\beta\)-actin), which was used as an internal reference gene to normalize results. *\(P < 0.05\) (by Student’s t-test). E: PANC-1 cells were transfected with negative control siRNA or EP1 siRNA and then stimulated with or without PGE\(_2\) (1 \(\mu\)M) for 15 min. After treatment, cell lysates were collected and subjected to immunoblotting with indicated antibodies. GAPDH was used as a loading control. F: PANC-1 cells were pretreated with H-89 (5 \(\mu\)M) and BAPTA-AM (10 \(\mu\)M), alone or in combination, and then stimulated with or without PGE\(_2\) (1 \(\mu\)M) for 15 min. After treatments, cell lysates were collected and subjected to immunoblotting with indicated antibodies.
Epac (exchange protein directly activated by cAMP), thereby augmenting the effect of insulin on PI3K/Akt signaling (2). In PANC-1 cells, however, PGE2/cAMP-induced activation of mTORC1 seems to be independent of PI3/Akt, as phosphorylation of Akt remained unchanged with PGE2 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 phosphorylated ERK levels upon PGE2 treatment in PANC-1 cells. Subsequently, we showed that the effect of PGE2 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).

On the basis of the studies using agonist/antagonist for EP2 and EP4, our data suggest that, in PANC-1 cells, the PGE2-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 compared with adjacent benign pancreatic tissues (13). Also, EP4-mediated mTORC1 activation by PGE2 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 found that PGE2-induced mTORC1 activation is also mediated by a Ca2+ dependent mechanism, which is elicited through the Goq-coupled EP1 receptor, as supported by our results from the EP1 knockdown experiments. Although the role of EP1 in cancer development is less documented than the role of EP2 or EP4 (16), several studies have identified EP1 as a critical receptor (20, 52, 55). Furthermore, on the basis of

Fig. 6. A: expression levels of EP1, EP2, and EP4 in a panel of cancer cell lines were determined by RT-qPCR. ACTB was used as an internal reference gene to normalize results. mRNA expression of each gene is presented relative to that of PANC-1 cells (set as 1-fold). B: PC-3 cells were incubated with DMSO or PGE2 (1 μM) for 1 min, and levels of intracellular cAMP in the cell lysates were determined. C: intracellular Ca2+ response to PGE2 in PC-3 cells. Trace represents averaged response of all cells. Arrow marks addition of PGE2. D: PC-3 cells were pretreated with H-89 (5 μM) or BAPTA-AM (10 μM), alone or in combination, and then stimulated with or without PGE2 (1 μM) for 15 min. After treatments, cell lysates were collected and subjected to immunoblotting with indicated antibodies. E: PC-3 cells were pretreated with rapamycin (1 nM) for 30 min and then stimulated with or without PGE2 (1 μM) for 15 min. After treatments, cell lysates were collected and subjected to immunoblotting with indicated antibodies. F: BxPC-3 cells were treated with PGE2 (1 μM), alone or in combination with ionomycin (500 nM), for 15 min. Top: cell lysates were collected and subjected to immunoblotting with indicated antibodies. Bottom: quantitative densitometry of p-S6rp Western blots. Values are expressed relative to untreated control (set as 1-fold). NS, not significant. *P < 0.05 (by paired Student’s t-test).
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/EP4 and EP1 in the cell lines (PANC-1 and PC-3) that prominently displayed PGE2-activated mTORC1 signaling, we discovered the cooperative roles of cAMP/PKA and Ca2+/H11001 pathways in PGE2-induced mTORC1 activation. The expression patterns of EP receptors in PANC-1 (high in EP1 and EP4), PC-3 (high in EP1 and EP2), and BxPC-3 (high in EP2 only) cells suggest that, to induce PGE2-mediated mTORC1 activation, the functional integrity of both the EP2/Ca2+ pathway and the 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 PGE2-induced mTORC1 activation is mediated by the coordinated operation of the EP2/cAMP/PKA and EP1/Gαq/Ca2+ pathways in PANC-1 cells (Fig. 7D).

Signaling pathways usually interact and cross talk 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 shown in pancreatic cancer cells that the Akt/mTORC1 pathway mediates a cross talk between insulin receptor and GPCR signaling systems, where insulin can potentiate Ca2+/H11001 signaling triggered by Gαq-coupled receptor agonists (24). Remarkably, further studies revealed that such cross talk is disrupted by metformin, a widely used antidiabetes drug that negatively regulates mTORC1 through AMP kinase activation, leading to inhibition of pancreatic cancer growth (23). In the present study we have identified a similar, but reverse, cross talk between PGE2-mediated pathways and IGF-1 signaling, converging at mTORC1 and creating a positive reinforcement (Fig. 6D). Interestingly, PGE2 and IGF-1 in combination, but not alone, induced IL-23 mRNA transcription in PANC-1 cells, and this effect was abrogated by the mTORC1 inhibitor rapamycin. This finding highlights the significance of the reinforcing cross talk between PGE2 and IGF-1 signaling through subsequent mTORC1 activation. IL-23, a proinflammatory cytokine, has been shown to promote tumor growth by driving protumorigenic inflammation, as well as impairing T cell function.
cell-mediated immune surveillance (25, 38). Also, a recent study suggests 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 protumorigenic properties through regulation of pro-inflammatory cytokine production by augmentation of mTORC1 function, which is already supported by enhanced IGF-1/Akt signaling under certain conditions (e.g., the obese state and neoplasm). Overall, the signaling cross talk identified above would lead to an exacerbated inflammatory tumor microenvironment and, thereby, contribute 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 cross talk 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 cross talk is highly relevant to obesity-associated cancer, where proinflammatory 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 cross talk 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.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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