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Activation of PPARγ increases PTEN expression in pancreatic cancer cells

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Abstract

The PI3K pathway contributes to the invasive properties and apoptosis resistance that epitomize pancreatic cancers. PPARγ is a ligand-activated transcription factor with anti-inflammatory and anti-tumor effects; the mechanisms of tumor suppression are unknown. The purpose of this study was to examine whether activation of PPARγ can increase the expression of the tumor suppressor PTEN and inhibit PI3K activity. AsPC-1 human pancreatic cancer cells, transfected with a PPRE-luciferase construct, demonstrated increased luminescence following treatment with PPARγ ligands, indicating the presence of functional PPARγ protein. The selective PPARγ ligand rosiglitazone increased PTEN expression in AsPC-1 cells; concurrent treatment with GW9662, which inhibits PPARγ activation, prevented the increase in PTEN protein levels. Levels of phosphorylated Akt decreased as PTEN levels increased, indicating inhibition of PI3K activity. Taken together, our results suggest that activation of PPARγ may represent a novel approach for the treatment of pancreatic cancer by increasing PTEN levels and inhibiting PI3K activity.

Keywords: Pancreatic cancer; PPARγ; Rosiglitazone; PTEN; Phosphatidylinositol-3 kinase; Metastasis

Pancreatic cancer is the fourth leading cause of cancer death in the US [1]. Five-year survival is less than 5% because pancreatic cancer has a propensity to invade adjacent organs, which prevents surgical resection in greater than 80% of patients [2]. Most pancreatic cancers are resistant to conventional chemotherapy due to constitutively active factors that stimulate growth and inhibit apoptosis [3]. The phosphatidylinositol-3 kinase (PI3K) pathway mediates both the invasive and anti-apoptotic properties of pancreatic cancer through a family of downstream Akt kinases, known as Akt1 and Akt2 [4]. Akt2 is overexpressed in pancreatic cancer [5], and activated Akt can facilitate cell survival by phosphorylating and inactivating pro-apoptotic proteins such as Bad and caspase-9 [6,7]. Akt kinases also promote pancreatic cancer cell invasion in vitro through upregulation of the insulin-like growth factor (IGF)-1 receptor [8]. Akt activation by PI3K plays an important role in the biologic behavior of pancreatic cancer and its inhibition may provide effective treatment against pancreatic cancer.

Phosphatase and tensin homologue deleted on chromosome ten (PTEN) is a tumor suppressor protein that dephosphorylates inositol phospholipid intermediates of the PI3K pathway, inhibiting activation of downstream targets including Akt [9]. Loss of PTEN activity has been suggested to cause enhanced cell proliferation, decreased apoptosis, and increased tumor angiogenesis [10,11]. Mutations in the PTEN gene produce Cowden syndrome, characterized by a high incidence of breast and thyroid cancers. PTEN mutations have also been described in sporadic malignancies including brain and colorectal cancers [12,13]. Despite studies describing the importance of PTEN in suppressing tumor growth [14], little is known about its regulation. Induction of PTEN and increasing the activity of the functional PTEN protein may provide an effective adjuvant treatment for a variety of malignancies.

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone receptor superfamily of ligand-activated transcription factors, which

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are present in three isoforms (α, δ, and γ) in humans [15]. Ligand-activated PPARγ binds to a specific DNA binding site, termed PPRE (peroxisome proliferator response element), to regulate the transcription of numerous target genes [16]. Ligands for PPARγ include the arachidonic acid metabolite 15-deoxy-prostaglandin J_2 (15d-PGJ_2), and the thiazolidenediones (e.g., troglitazone, pioglitazone, and rosiglitazone) [17]. Both rosiglitazone and pioglitazone are currently approved for the treatment of type II diabetes [18]. In pancreatic cancer, PPARγ ligands inhibit cell growth, cause cell cycle arrest at G0/G1 via induction of $p21^{waf1}$, and increase expression of differentiation markers [19,20], however, the mechanisms for these effects are poorly understood. Therefore, the purpose of this study was to examine whether activation of PPARγ can increase the expression of PTEN protein in pancreatic cancer cells, which may mediate some of the reported anti-tumor effects of PPARγ and provide a novel therapeutic approach against pancreatic cancer.

Methods

Cell lines and reagents. Human pancreatic cancer cells AsPC-1 (ATCC, Manassas, VA) and SUIT-2 (provided by Dr. Takeshi Iwamura, Miyazaki Medical College, Miyazaki, Japan) were grown in RPMI 1640 with 10–20% fetal bovine serum (FBS) and penicillin/streptomycin. Unless otherwise noted, experiments were conducted in 1% FBS media without antibiotics. Rosiglitazone and the PPARγ inhibitor, GW9662, were purchased from Cayman chemical (Ann Arbor, MI). Troglitazone was purchased from BioMol (Plymouth Meeting, PA). 15d-PGJ_2 was purchased from Calbiochem (La Jolla, CA). Mouse monoclonal antibodies for PTEN (clone 2) were from BD Signal Transduction Laboratories (San Diego, CA) and phosphorylated Akt (Ser473) rabbit polyclonal antibody was obtained from Cell Signaling Technology (Beverly, MA). Peroxidase conjugated goat anti-mouse IgG was purchased from Pierce (Rockford, IL) and goat anti-rabbit IgG was purchased from Upstate Biotech (Lake Placid, NY).

Cell transfection and luciferase assay. Both AsPC-1 and SUIT-2 cells were plated at 100,000 cells/60 mm plate in 5% FBS/DMEM/PSA and transfected with a 3× PPRE-luciferase vector (gift from Dr. Ronald Evans, The Salk Institute, La Jolla, CA; 2 mg/60 mm plate) and *Renilla* luciferase (0.01 ng/plate; Promega, Madison, WI) using FuGene (Roche Molecular Biochemicals, Indianapolis, IN) as the transfectant in a 1:3 (mg DNA:ml FuGene) ratio. On the following day, cells were treated with either 1:1000 dilution of DMSO (control) or PPARγ ligands at a final concentration of 1 μM. Twenty-four hours later, cells were dissolved in lysis buffer and firefly luciferase and *Renilla* luciferase activities were determined using a standard luminometer. Data are reported as normalized luciferase activity. A PPARγ construct was co-transfected with the PPRE-luciferase vector in one group of cells to serve as a positive control in the presence of rosiglitazone.

Western blotting. AsPC-1 cells were treated with PPARγ ligands and lysed, and whole cell protein was extracted using Cell Lysis Buffer (Cell Signaling, Beverly, MA) as we have previously described [21]. Briefly, protein was resolved on a 10% Tris–glycine gel and transferred to PVDF membrane. Membranes were blocked using 2% milk and blotting performed using 1–2% milk in TBST. Signals were detected by enhanced chemiluminescence (ECL Plus) (Amersham Biosciences, Buckinghamshire, UK).

Results and discussion

Thiazolidenediones and 15d-PGJ_2 activate PPARγ in AsPC-1 cells

Previously, we have shown that the PPARγ ligand 15d-PGJ_2 can inhibit pancreatic cancer cell growth and induce apoptosis [22]. Others have shown that the thiazolidenediones (e.g., troglitazone, rosiglitazone, and pioglitazone), which selectively bind PPARγ and stimulate its transcriptional activity, inhibit the growth of other cancer cell lines (e.g., breast, prostate, and lung cancer cells) [23–25]; the mechanisms responsible for these effects are not known. In this study, we determined whether functional PPARγ protein was present in pancreatic cancer cell lines. Using ligands known to activate PPARγ, we transfected a PPRE-luciferase construct into AsPC-1 and SUIT-2 cells; addition of PPARγ ligands, 15d-PGJ_2, troglitazone or rosiglitazone, increased luciferase activity in AsPC-1 cells (Fig. 1). The greatest activation of PPARγ was noted with rosiglitazone, which is consistent with a higher affinity for this ligand compared with troglitazone. Co-transfection with a construct which contains the PPARγ gene, in conjunction with the PPRE construct, resulted in an approximate 2.5-fold higher luciferase activity following the addition of rosiglitazone. Another pancreatic cancer cell line, SUIT-2, was also transfected with the PPRE-luciferase construct and demonstrated similar increases in luciferase activity following rosiglitazone or 15d-PGJ_2 treatment (data not shown). Based on these results, we conclude that AsPC-1 cells contain functional PPARγ

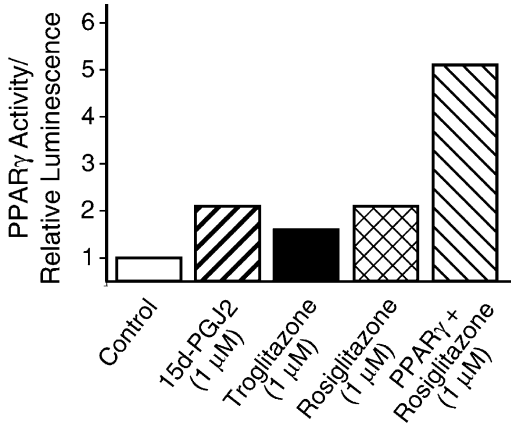

Fig. 1. AsPC-1 cells contain functional PPARγ protein. AsPC-1 cells were transfected with a 3× PPRE/luciferase vector (2 μg/60 mm plate) and *Renilla* luciferase using FuGene as the transfectant. The following day, cells were treated with either 1:1000 dilution of DMSO (control) or PPARγ ligands at a final concentration of 1 μM. Twenty-four hours later, cells were dissolved in lysis buffer and firefly luciferase and *Renilla* luciferase activities were determined. Data are reported as normalized to transfected vehicle treated cells. A PPARγ construct was co-transfected in one group of cells to serve as a positive control in the presence of rosiglitazone (1 μM).

protein, which can be activated by high affinity ligands such as rosiglitazone.

Rosiglitazone increases PTEN expression through PPARγ activation

In other cancers, activation of PPARγ inhibits the transcription of factors related to tumor progression, including NF-κB [26], COX-2 [27], and MMP-9 [28]. The ability of PPARγ ligands to induce cell cycle arrest, apoptosis, and differentiation in tumor cells is linked to the inhibition of these factors. Other tumor suppressors, such as PTEN, may induce similar anti-tumor effects when its levels are increased [29]. To investigate whether PPARγ activation increases expression of PTEN protein in pancreatic cancer cells, AsPC-1 cells were treated with rosiglitazone and Western blotting was performed. We observed a rapid and sustained increase in PTEN levels following treatment with rosiglitazone in AsPC-1 cells (Fig. 2A). To confirm that the effect of rosiglitazone on PTEN levels was due to activation of PPARγ, GW9662, a selective inhibitor of PPARγ [30], was added concurrently with rosiglitazone. Inhibition of PPARγ by GW9662 almost completely blocked the effect of rosiglitazone on PTEN expression (Fig. 2B), suggesting that rosiglitazone increases PTEN levels through activation of PPARγ.

The regulation of PTEN expression is poorly understood, although increased transcription is believed to be the most important factor [31]. The tumor suppressor p53 has been reported to bind to a site within the PTEN promoter region [32], although recent evidence suggests that p53 binding is only a weak modifier of PTEN transcription [33]. We demonstrate that PTEN protein levels are increased by PPARγ activation providing a novel mechanism for inhibiting PI3K activity.

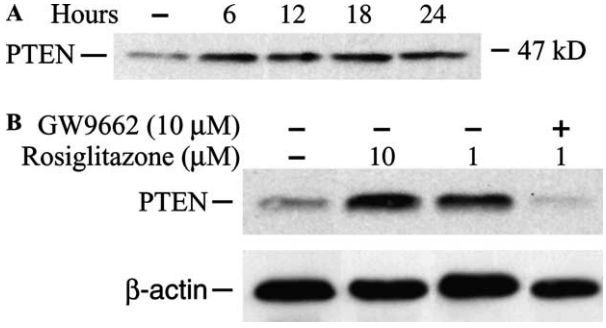

Fig. 2. Rosiglitazone increases PTEN levels in AsPC-1 cells through activation of PPARγ. (A) AsPC-1 cells were treated with rosiglitazone (10 μM) and whole cell lysates were obtained at the time points indicated. Lysates were resolved on a 10% SDS–PAGE and immunoblotted for PTEN protein. (B) AsPC-1 cells were treated with rosiglitazone (1 or 10 μM) in the presence or absence of the selective PPARγ inhibitor GW9662 (10 μM) for 24 h. Western blotting was performed for PTEN protein expression. The membrane was stripped and re-probed for β-actin to ensure equal loading of protein.

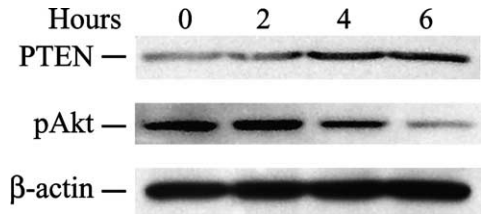

Fig. 3. Rosiglitazone increases PTEN levels and inhibits phosphorylation of Akt in AsPC-1 cells. AsPC-1 cells were treated with rosiglitazone (10 μM) for the indicated times and whole cell protein was extracted. Protein lysates were immunoblotted for PTEN and phosphorylated Akt on the same membrane. The membrane was stripped and re-probed for β-actin to ensure equal loading of protein.

Rosiglitazone-induced PTEN expression inhibits the PI3K pathway

PTEN is a phosphatase that inhibits the activity of PI3K, but non-functional PTEN protein has been described in some cancers [34]. Additionally, Akt2 is overexpressed in pancreatic cancers [5], thus increased expression of functional PTEN would be required to inhibit the phosphorylation of Akt. To establish whether the increased amount of PTEN protein is able to inhibit PI3K activity, Western blotting was performed to detect phosphorylated Akt in rosiglitazone-treated AsPC-1 cells. Phosphorylated Akt is present in untreated cells; levels decreased after 4 h of treatment with rosiglitazone. This reduction in phosphorylated Akt correlated with an increase in PTEN expression that occurred at the same time point (Fig. 3). Thus, in AsPC-1 cells, activation of PPARγ increases PTEN levels and inhibits phosphorylation of Akt. Inhibition of Akt may attenuate the invasive properties of pancreatic cancer cells and reduce their resistance to apoptosis. Since PPARγ ligands are currently in clinical use and are relatively free of side effects [35], the ability to use these non-toxic agents to inhibit the PI3K pathway is important. Activation of PPARγ may provide a unique approach to pancreatic cancer treatment through increased expression of the tumor suppressor PTEN.

In conclusion, we demonstrate that selective activation of PPARγ increases the expression of PTEN protein in pancreatic cancer cells. This increased expression of PTEN inhibits the phosphorylation of Akt and subsequently may inhibit the ability of Akt to promote pancreatic cancer invasion and resistance to apoptosis. Non-toxic PPARγ ligands, such as rosiglitazone, may represent novel therapies for the treatment or prevention of pancreatic cancer.

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