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Cancer drug troglitazone stimulates the growth and response of renal cells to hypoxia inducible factors

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ABSTRACT

Troglitazone has been used to suppress the growth of a number of tumors through apoptosis and autophagy. However, previous in vitro studies have employed very high concentrations of troglitazone ($\geq 10^{-5}$ M) in order to elicit growth inhibitory effects. In this report, when employing lower concentrations of troglitazone in defined medium, troglitazone was observed to stimulate the growth of primary renal proximal tubule (RPT) cells. Rosiglitazone, like troglitazone, is a thiazolidinedione (TZD) that is known to activate Peroxisome Proliferator Activated Receptor γ (PPARγ). Notably, rosiglitazone also stimulates RPT cell growth, as does γ-linolenic acids, another PPARγ agonist. The PPARγ antagonist GW9662 inhibited the growth stimulatory effect of troglitazone. In addition, troglitazone stimulated transcription by a PPAR Response Element/Luciferase construct. These results are consistent with the involvement of PPARγ as a mediator of the growth stimulatory effect of troglitazone. In a number of tumor cells, the expression of hypoxia inducible factor (HIF) is increased, promoting the expression of HIF inducible genes, and vascularization. Troglitazone was observed to stimulate transcription by a HIF/luciferase construct. These observations indicate that troglitazone not only promotes growth, also the survival of RPT cells under conditions of hypoxia.

1. Introduction

Thiazolidinediones (TZDs) are used in the treatment of type II diabetes, as well as a number of cancers, including the Renal Cell Carcinoma [1,2], presumably via their ability to activate Peroxisome proliferator activated receptor γ (PPARγ), a ligand-activated transcription factor [1]. PPARγ has been observed to play a very broad role in biological systems, stimulating adipocyte differentiation, improving the insulin-sensitivity of a number of insulin-sensitive tissues, and preventing inflammation in a context dependent manner [1]. The ability of TZDs to suppress a number of cancers has been attributed to their ability to inhibit growth, and stimulate apoptosis in a PPARγ dependent manner [3,4].

PPARγ is activated by a number of ligands, including TZDs, free fatty acids and eicosanoids such as 15-deoxy-$\Delta^{(12,14)}$-Prostaglandin J_2 (PGJ_2) [5]. Ligand binding to PPARγ results in a conformational change causing PPARγ to form a heterodimer with the Retinoid X Receptor (RXR), which increases the affinity of PPARγ for its response elements (PPREs), present on the promoters of target genes [6].

TZDs have been employed in a number of medical conditions. In addition their use to prevent tumor expansion, TZDS are employed in type II diabetes, lowering blood glucose, and increasing the sensitivity of a number of tissues to insulin [6]. TZDs also have been used to slow the progression of diabetic nephropathy [5]. Diabetic nephropathy is characterized by the progressive deterioration of glomerular function, and ultimately tubulo/interstitial disease [5]. While the effects of TZDs on glomerular disease have been extensively studied, their effects on the RPT and surrounding tissue are not well understood. The effects of TZDs and PPARγ in the RPT are also of interest, because the RPT is the site of origin of the Renal Cell Carcinoma (RCC). Previous reports concerning the effects of TZDs on RPT cells and RCC cells in vitro indicate that TZDs when applied at concentrations $\geq 10^{-5}$ M are growth inhibitory both in cultured RPT cells and RCC cells, and may even stimulate apoptosis [7]. However, studies with a number of animal models of diabetic nephropathy have indicated that TZDs not only reduce proteinuria, but in addition retard the development of the injury to the renal proximal tubule that occurs after the integrity of the glomerulus is compromised [5]. In order to clarify these issues, the effects of troglitazone and other PPARγ ligands on the growth of primary

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cultures of RPT cells have been examined. The primary RPT cell cultures closely resemble normal tubule epithelial cells *in vivo*, and continue to express PPARγ [8,9]. Hormonally defined medium was employed, which facilitated the observation of effects of PPARγ ligands at concentrations considerably lower than previously studied. The results indicate that TZDs and albumin-associated fatty acids stimulate growth, that HIF is activated, and that PPARγ is involved.

2. Materials and methods

2.1. Materials

Bovine insulin, human transferrin, Fatty Acid Free (FAF) Bovine Serum Albumin (BSA) (catalog number 0281), Human Serum Albumin (HSA) replete with FAs (catalog number 1653), and FAF HSA prepared from 1653 (catalog number 1887) were from Sigma Aldrich Chemical Corp (St. Louis, Mo.). Dulbecco's Modified Eagle's Medium (DME), Ham's F12 Medium (F12), and soybean trypsin inhibitor was from Invitrogen Corp. (Carlsbad, Calif.). The Prism 6 program was obtained from Graph Pad Software, Inc. (San Diego, Calif.). The OK cell line, and the HK-2 cell line were obtained form the American Type Culture Collection. The MDCK cell line was obtained from Milton H. Saier, Jr. (UCSD, San Diego, Calif.), while the mouse M1 collecting duct cell line was obtained from Alejandro Bertorello (Stockholm, Sweden). The rabbit kidney proximal tubule cell line RPT clone I8 was immortalized as described previously [10]. The 3-HRE-Luc plasmid [11] was obtained from Dr. Jacques Pouyssegur, CNRS, Nice, Fr., and the PPRE X3-TK-luc vector [12] was obtained from Addgene. pSV beta gal was from Promega.

2.2. Kidney cell cultures

Established cell lines were maintained in a basal medium (DME/F12) consisting of a 50:50 mixture of DMEM and Ham's F12 supplemented with 15 mM HEPES (pH 7.4), 20 mM sodium bicarbonate, 92 U/ml penicillin and 0.2 mg/ml streptomycin, as previously described [13]. Water used for medium and growth factor preparations was purified using a Milli-Q deionization system. Medium K-1, the growth medium for stock cultures of MDCK cells, M1 cells, OK cells and RPT clone 8 cells, consists of DME/F12 further supplemented with 5 μg/ml bovine insulin, 5 μg/ml human transferrin, 5×10^{-12} M triiodothyronine (T_3), 5×10^{-8} M hydrocortisone, 25 ng/ml PGE_1, and 5×10^{-8} M selenium. Primary RPT cell cultures were maintained in medium RK-1, which consists of DME/F12 supplemented with 5 μg/ml insulin, 5 μg/ml transferrin, 5×10^{-8} M hydrocortisone, 92 U/ml penicillin and 0.01% kanamycin (rather than streptomycin).

Primary cultures of rabbit kidney proximal tubule (RPT) cells were initiated from rabbit kidneys, after sacrificing rabbits following a protocol submitted to, and approved by the University at Buffalo IACUC, as previously described [8]. The RPT cell cultures were initiated in Medium R-K1. MDCK, M1, OK, HK-2 and RPT clone I8 cell cultures were subcultured using 0.53 mM EDTA/0.05% trypsin in PBS (EDTA/trypsin), followed by inhibition of trypsin action with soybean trypsin inhibitor, as previously described [14].

2.3. Growth studies

Growth studies were conducted with primary cultures in 35 mm dishes. In the growth studies with the established cell lines, the cultures were washed twice with PBS, detached using EDTA/trypsin, trypsin action inhibited using soybean trypsin inhibitor, and cells inoculated into culture dishes at 10^3 cells/dish, as previously described [14]. The medium in the growth studies was DME/F12 supplemented with 5 μg/ml bovine insulin, 5 μg/ml human transferrin, and other effectors. The cultures were maintained in a humidified 5% CO_2/95% air environment for 5 days. At the end of 5 days the cells were removed from the culture dish using EDTA/trypsin, and counted with a Coulter Counter as previously described [14].

The average cell number in each condition was calculated from 4 determinations, using the Prism 6 Program. The "fold control cell number" was calculated by dividing the average cell number observed in experimental cultures by the Control Cell Number (i.e. the cell number present in cultures maintained in DME/F12 supplemented with insulin and transferrin alone), unless otherwise specified. Differences between conditions were determined to be statistically significant by conducting T tests using Prism 6 software. Differences were deemed significant when $p < 0.05$.

2.4. Transient transfection studies

Primary cultures were cotransfected with PPRE X3-TK-luc (1 μg) (or 3HRE-Luc), and pSVβgal (0.2 μg) utilizing lipofectamine, and the next day incubated 2 h in DME/F12 supplemented with 5 μg/ml insulin and 5 μg/ml transferrin, followed by the addition of appropriate effectors and another 18 h incubation [15]. The monolayers were then solubilized in Reporter Lysis Buffer, centrifuged (13,600 × g; 4 °C), and luciferase activity was determined in a BioTek Plate Reader, using a luciferase assay buffer [15].

Each luciferase determination was normalized with respect to its β-galactosidase activity [15]. Averages of 4 determinations were calculated in each condition (±the Standard Error of the Mean (SEM)), and compared to the Control value (from untreated cultures). The statistical significance of observed differences was assessed as statistically significant ($p < 0.05$) by ANOVA, using the Newman–Keuls Multiple Comparison Test (Prism 6 software).

3. Results

3.1. Effect of PPARα and PPARγ agonists on growth

In order to initially determine whether PPAR affect RPT cell growth, the effects of troglitazone, a PPARγ agonist, and fenofibrate, a PPARα agonist on primary RPT cell growth were examined. Fig. 1A shows that troglitazone stimulates the growth of primary RPT cells, unlike the case with fenofibrate, as shown in Fig. 1B. A growth stimulatory effect of troglitazone was observed in 2 other RPT cell lines, including Opossum Kidney (OK) (Fig. 2A), and the human HK-2 cell line (Fig. 2B). In contrast, troglitazone did not have a significant effect on the growth the distal tubule epithelial cell line MDCK (Fig. 2C), derived from canine kidney, and the mouse M1 cortical collecting duct cell line (Fig. 2D).

3.2. Evidence for the involvement of PPARγ

The involvement of PPARγ in mediating these effects was evaluated further in primary RPT cells. First, the effects of rosiglitazone on primary RPT cell growth were examined. Rosiglitazone is a TZD that is highly specific for PPARγ. Fig. 3A shows that rosiglitazone was growth stimulatory, acting within the same concentration range that elicited the troglitazone stimulation. Secondly, the effect of GW9662, a highly specific PPAR γ antagonist, on the troglitazone stimulation was studied. Fig 3B shows that the growth stimulatory effect of 1 μM troglitazone was significantly inhibited by 5 μM GW9662. Finally, in transiently transfected primary RPT cells, the effect of 1 μM troglitazone and 1 μM rosiglitazone on the expression of a PPRE reporter construct (PPRE X3-TK-luc) was examined. PPRE X3-TK-Luc contains a PPAR regulatory element (PPRE) linked