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Pro-MMP-2 activation by the PPARγ agonist, ciglitazone, induces cell invasion through the generation of ROS and the activation of ERK

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Abstract Peroxisome proliferator-activated receptor γ (PPARγ) is a nuclear receptor modulating a variety of biological functions including cancer cell proliferation and differentiation. However, the role of PPARγ and its ligands in tumor invasion is unclear. To evaluate a possible role for PPARγ ligands in tumor invasion, we examined whether PPARγ agonists including pioglitazone, troglitazone, rosiglitazone, and ciglitazone could affect the activity of matrix metalloproteinases (MMPs) in the HT1080 cell line, a well-studied and well-characterized cell line for MMP research. The gelatin zymography assay showed that ciglitazone activated pro-MMP-2 significantly. In addition, ciglitazone increased the expression of MMP-2, which was accompanied by an increase of membrane type 1-MMP (MT1-MMP) expression. The PPARγ antagonist, GW9662 attenuated the ciglitazone-induced PPARγ activation but it did not affect the pro-MMP2 activation by ciglitazone, suggesting that the action of ciglitazone on the pro-MMP-2 activation bypassed the PPARγ pathway. Antioxidants and various inhibitors of signal transduction were used to investigate the mechanism of ciglitazone-induced pro-MMP-2 activation. We found that the sustained production of reactive oxygen species (ROS) was required for pro-MMP-2 activation by ciglitazone. We also found that PB98059, an inhibitor of MEK-ERK, significantly blocked ciglitazone-induced pro-MMP-2 activation and that extracellular signal-regulated kinase (ERK) was hyperphosphorylated by ciglitazone. Moreover, cell invasion was significantly increased by ciglitazone in the HT1080 cell lines, whereas cell motility was not affected. This study suggests that ciglitazone-induced pro-MMP-2 activation increases PPARγ-independent tumor cell invasion through ROS production and ERK activation in some types of cancer cells.**

Keywords: Tumor invasion; Matrix metalloproteinase-2 activation; Peroxisome proliferator-activated receptor γ agonists; Ciglitazone; Reactive oxygen species production; Extracellular signal-regulated kinase

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Abbreviations: PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator response element; TZD, thiazolidinedione; CIG, ciglitazone; PGZ, pioglitazone; TGZ, troglitazone; RSG; rosiglitazone; MMP, matrix metalloproteinase; MT1-MMP, membrane-type 1-matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; ROS, reactive oxygen species; DCFH-DA, dichlorofluorescin diacetate; ERK, extracellular signal-regulated kinase

1. Introduction

The metastatic cascade consists of several sequential steps, including cells migration from the primary tumor, invasion of the local tissue, entry of the circulation, residence in a distant vascular bed, extravasation into the target organ, and proliferation as a secondary colony. Among these steps, tumor invasion is important [1]. The matrix metalloproteinases (MMPs) are the key family of proteolytic enzymes involved in the tumor invasion [2]. These enzymes are classified into collagenases, gelatinases, stromelysins, and membrane-type MMPs and can collectively degrade all components of the extracellular matrix [3]. Tissue inhibitors of metalloproteinases (TIMPs) complex with MMPs and regulate the activity of individual MMPs [4,5]. MMPs and TIMPs play a significant role in facilitating tumor invasion and metastasis not only by degrading the extracellular matrix but also by interacting with other biological molecules such as cell adhesion molecules, cytoskeletal proteins, and growth factors [6,7]. MMP-2 (gelatinase A, type IV collagenase) and MMP-9 (gelatinase B, type IV collagenase) are important for degrading type IV collagen which is a major component of the basement membrane. MMP-2 and MMP-9 are abundantly expressed in various malignant tumors and have been implicated in tumor invasion and metastasis [8,9]. MMP-2 is secreted as an inactive zymogen that can be activated by the action of highly expressed MT1-MMP [10].

Peroxisome proliferator-activated receptor γ (PPARγ), a member of the nuclear hormone receptor superfamily, is a ligand-dependent transcription factor that regulates gene expression by binding as a heterodimer with a retinoid X receptor (RXR) to specific peroxisome proliferator response elements (PPREs) in enhancer sites [11,12]. It is activated not only by its natural agonists such as 15-deoxy-Δ12,14 prostaglandin J2 and fatty-acid derivatives but also by the synthetic agonists of thiazolidinediones (TZDs) including ciglitazone (CIG), pioglitazone (PGZ), rosiglitazone (RSG), and troglitazone (TGZ) [13]. Studies using PPARγ ligands have implicated PPARγ in cell-growth inhibition in a wide variety of tumor cells [14–17]. This has raised considerable interest in the possible use of PPARγ ligands as tumor-preventative and tumor-therapeutic agents. Most studies have focused on the anti-growth effect of PPARγ ligands on tumor cells. Very little is known about the role of PPARγ ligands in cancer cell invasion. Here, we show that pro-MMP-2 is activated by the PPARγ agonist

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ciglitazone, with an increase in invasive activity in HT1080 cells. Interestingly, this does not involve PPARγ activation but rather the production of reactive oxygen species (ROS) followed by the activation of extracellular signal-regulated kinase (ERK).

2. Materials and methods

2.1. Cell culture and materials

HT1080 fibrosarcoma cells were grown in DMEM supplemented with 50 mg/l gentamicin (Invitrogen) and 10% fetal bovine serum in an atmosphere of 95% air and 5% CO_2. Experimental cultures were grown in serum-starved medium for 4 h and stimulated with the appropriate chemicals as indicated. Ciglitazone, rosiglitazone, troglitazone, pioglitazone and GW9662 were purchased from Cayman chemical (Ann Arbor, MI). AG1024, AG1295, AG1478, SU1498, genistein, indometacin, LY294002 and PD98059 were purchased from Calbiochem (La Jolla, CA). 2′,7′-Dichlorofluorescin diacetate (DCFH-DA) were purchased from Sigma (St Louis, MO). Antibodies against PPARγ and phospho-ERK were purchased from Cell Signaling Technology (Hitchin, Hertfordshire). Antibody against MMP-2 was from Chemicon International (Temecula, CA). Anti-MT1-MMP, anti-ERK and β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. Cell viability

3-[4,5-Dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide (MTT) (Roche Molecular Biochemicals) assay was performed. Cells were plated in 96 wells. After incubation, MTT was added for the final concentration to be 0.5 mg/ml. After incubation for 4 h, media were removed and 0.1 ml of DMSO was added. After dissolving the formazan, the absorbance was measured at 570 nm with ELISA reader and the reference absorbance was measured at 655 nm for the control.

2.3. Zymography

Enzymatic activities of MMP-2 and MMP-9 were assayed by gelatin zymography. Conditioned media from the cell culture were analyzed for gelatin degradation activity by SDS–PAGE under non-reducing conditions. 1 mg/ml gelatin was prepolymerized on a 10% polyacrylamide gel as a substrate. Electrophoresis was carried out at 4 °C. The gel was washed twice with washing buffer (50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 2.5% Triton X-100), followed by a brief rinsing in washing buffer without Triton X-100. Gelatinolytic activity was developed in an incubation buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$, 0.02% NaN_3, 1 μM $ZnCl_2$) at 37 °C for 16 h and visualized by staining the gel with Coomassie Blue R-250. A clear zone of gelatin digestion that appeared indicated the presence of MMPs.

2.4. Western blot analysis

Protein extracts (50 μg) were separated by SDS–PAGE on 10% polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were blocked with 5% skim milk or 3% bovine serum albumin and probed with an antibody specific for the proteins of interest. The membranes were then incubated with a horseradish-peroxidase-conjugated secondary antibody and developed with chemiluminescent ECL reagents (Amersham, Piscataway, NJ). To determine that the amount of protein in each lane was comparable, the membranes were stripped and probed with antibody against β-actin.

2.5. RT-PCR

Total RNA from cultured cells was isolated using TRI reagent (Molecular Research Center) and reverse-transcribed into cDNA using reverse transcriptase (Promega) and oligo(dT). cDNA aliquots corresponding to 1 μg RNA were semi-quantitatively analyzed by PCR. The following primers were used for PCR analysis: MT1-MMP, 5′-gaa taa cca agt gat gga tgg ata c-3′ and 5′-ttt gtt ccc ctt gta gaa gta agt g-3′; MMP-2, 5′-aga tct gca aac agg aca ttg tat t-3′ and 5′-ttc ttc ttc acc tca ttg tat ctc c-3′; TIMP-2, 5′-gag aca aag agg aga gaa agt ttg c-3′ and 5′-ttt atc tgc ttg atc tca tac tgg a-3′; and GAPDH, 5′-cca tgg aga agg ctg ggg-3′ and 5′-caa agt tgt cat gga tga cc-3′. PCR products were resolved on 1% agarose gels and visualized by ethidium bromide staining.

2.6. Transfection and luciferase assay

HT1080 cells were plated in 6-well plates. DNA constructs were transfected using LipofectAMINE 2000 reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). 2 μg pGL3-MT1-MMP reporter vector was co-transfected with 0.5 μg galactosidase [18] and 1 μg PPARγ expression vector was co-transfected with 1 μg (PPRE × 3)-tk-luciferase and 0.5 μg β-galactosidase expression vector. After 24 h of transfection, the luciferase activities were measured using the luminometer. β-galactosidase activities were measured using *O*-nitrophenyl-galactopyranoside as a substrate. (PPRE × 3)-tk-luciferase and PPARγ expression vector were kindly provided by Dr. Young Yang (Korea Research Institute of Bioscience and Biotechnology).

2.7. Measurement of ROS formation

Generation of ROS was assessed by using the oxidation sensitive fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma). HT1080 cells were pre-treated with 4 μM ciglitazone for various incubation times. Cells were incubated with 5 μM DCFH-DA for 30 min at 37 °C, harvested and followed by analysis in a confocal microscopy (Zeiss) and FACScan flow cytometry (Becton–Dickinson). For confocal microscopy analysis, cells were plated onto a Lab-Tek chamber slide (Nunc) and treated with ciglitazone.

2.8. Cell invasion and motility assay

Transwell chamber was used for each invasion assay. The lower and upper parts of Transwell (Corning Glass) were coated with 10 μl of type I collagen (0.5 mg/ml) and 20 μl of 1:2 mixture of Matrigel:DMEM, respectively. 5×10^4 cells were plated on the upper compartment of a Transwell chamber. The medium of the lower chambers also contained 0.1 mg/ml bovine serum albumin. After incubation for 18 h, cells that had penetrated through the filters were counted. The lower surface of the membrane was fixed with methanol and stained with hematoxylin and eosin. Random fields were counted under a light microscope.

To examine the effect of the agents on cell motility, cells were seeded into Transwell on membrane filters coated with 10 μl of type I collagen (0.5 mg/ml) at the bottom of the membrane. Migration in the absence or presence of agents was measured as described in the invasion assay. In addition to this, cell motility was measured using a wound-healing method. Cells were grown to near-confluency, and a wound was created with the blunt end of a tip. This was documented through time-lapse photography.

2.9. Statistical analysis

All data were represented as means ± S.D. for three or more independent experiment. Statistical significance was evaluated using *t*-test for unpaired data using SPSS software (Norusis SPSS Inc, Release 6.0).

3. Results

3.1. Pro-MMP-2 activation by ciglitazone in HT1080 cells

We examined the effect of PPARγ agonists on MMP activation in HT1080 cells (Fig. 1). After treatment with each of the PPARγ agonists, pioglitazone, troglitazone, rosiglitazone, and ciglitazone, cell viability was tested by MMT assays and MMP activity was assayed by gelatin zymography. As shown in Fig. 1A, treatment with non-cytotoxic concentrations of ciglitazone significantly induced pro-MMP-2 activation in a dose-dependent manner without affecting pro-MMP-9 activity. Ciglitazone-induced pro-MMP-2 activation was also time-