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Research Paper

Differential activation of catalase expression and activity by PPAR agonists: Implications for astrocyte protection in anti-glioma therapy

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ABSTRACT

Glioma survival is dismal, in part, due to an imbalance in antioxidant expression and activity. Peroxisome proliferator-activated receptor (PPAR) agonists have antineoplastic properties which present new redox-dependent targets for glioma anticancer therapies. Herein, we demonstrate that treatment of primary cultures of normal rat astrocytes with PPAR agonists increased the expression of catalase mRNA protein, and enzymatic activity. In contrast, these same agonists had no effect on catalase expression and activity in malignant rat glioma cells. The increase in steady-state catalase mRNA observed in normal rat astrocytes was due, in part, to *de novo* mRNA synthesis as opposed to increased catalase mRNA stability. Moreover, pioglitazone-mediated induction of catalase activity in normal rat astrocytes was completely blocked by transfection with a PPARγ-dominant negative plasmid. These data suggest that defects in PPAR-mediated signaling and gene expression may represent a block to normal catalase expression and induction in malignant glioma. The ability of PPAR agonists to differentially increase catalase expression and activity in normal astrocytes but not glioma cells suggests that these compounds might represent novel adjuvant therapeutic agents for the treatment of gliomas.

Introduction

Novel therapeutic approaches that selectively protect normal brain cells and/or sensitize glioma cells to anti-cancer therapies are urgently needed. Roughly 70,000 new cases of primary brain tumors will be diagnosed in the US, the majority being grade 4 astrocytoma (glioblastoma) [1]. Despite multimodality therapy including surgery, radiation therapy and chemotherapy with temozolomide, median survival remains only approximately 15 months [2] and the 5-year survival rate is 1% [3]. This outcome reflects both glioma cell resistance to therapy and the risk of radiation-induced normal brain injury, which limits the total dose that can be safely administered to the tumor [4]. Recent data suggest that progressive cognitive impairment occurs in ~50% of brain tumor patients who are long-term survivors after treatment with partial or whole-brain irradiation [5,6]. This negative prognosis is, in part, due to radiation-induced oxidative tissue injury.

The central nervous system (CNS) is inherently susceptible to oxidative stress. This is evidenced by the CNS being: (1) highly active in oxidative metabolism, leading to a relatively high rate of reactive oxygen species (ROS) production [7]; (2) relatively low in the specific activity of the key antioxidant enzymes superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) [8] in oligodendrocytes, neurons and endothelial cells [9,10]; and (3) rich in readily oxidizable polyunsaturated fatty acids (PUFAs) such as docosahexaenoic acid and eicosapentaenoic acid [11].

These issues suggest that a lipid signaling-based therapeutic strategy may be beneficial in treating CNS cancers. PUFAs and oxidized lipids are ligands for peroxisome proliferator-activated

Abbreviations: 9cRA, 9 *cis* retinoic acid; ActD, actinomycin D; BCNU, 1,3-bis (2-chloroethyl)-1-nitrosourea; CNS, central nervous system; DCF, dichlorofluorescein; GPx, glutathione peroxidase; GSH, glutathione; Pio, Pioglitazone; PPAR, Peroxisome proliferator-activated receptor; PPARγ-d.n, PPARγ-dominant negative; PPRE, PPAR-response elements; PUFAs, polyunsaturated fatty acids; ROS, reactive oxygen species; Rosi, Rosiglitazone; RXR, retinoid X receptor; shRNA, short hairpin RNA; SOD, superoxide dismutase; TZDs, thiazolidinediones

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receptors (PPARs) [12,13], which are members of the nuclear hormone receptor (NHR) superfamily. PPARs are ligand-activated transcription factors that heterodimerize with the retinoid X receptor (RXR), bind to PPAR-response elements (PPRE), and thereby regulate gene expression [14]. Three isotypes of PPARs have been identified and designated α, β/δ, and γ [15]. PPARα is activated by fibrates, a class of cholesterol-lowering drugs used in the treatment of dyslipidemia, and functions by enhancing β-oxidation in the liver [16]. PPARγ agonists, such as thiazolidinediones (TZDs), are best known for controlling hyperglycemia and treating type 2 diabetes mellitus, in part, through the regulation of adipocyte differentiation and lipid storage/metabolism [17]. PPARβ/δ is expressed in tissues controlling lipid metabolism and acts as a lipid sensor, thus representing a molecular target for treating metabolic syndrome [18]. Data supports the concept that PPARγ mediates anti-proliferative and pro-apoptotic activities in tumor cells [19].

Studies have shown that activation of PPARγ has antineoplastic effects through redox-dependent mechanisms. The PPARγ agonists Rosiglitazone (Rosi) and Pioglitazone (Pio) decreased cell viability in both human (U87 and A172) and rat (C6) malignant glioma cells whereas normal rat astrocytes were not affected [19,20]. This cytotoxic effect was spared by N-acetylcysteine suggesting reactive oxygen species (ROS) are playing an integral role. PPARγ agonists decreased glutathione (GSH) levels in C6 rat glioma cells and increased dichlorofluorescein (DCF) fluorescence in both the primary rat astrocytes and C6 rat glioma cells [20]. Moreover, Pio treatment significantly reduced tumor volume in a rat glioma model by decreasing proliferation, suppressing MMP9 induction, and inhibiting tumor cell invasion [19].

The cytotoxic effect of PPARγ agonists on glioma cells is partially mediated by enhanced redox reactions. Yet, normal rat astrocytes, which are the most prevalent cell type in the CNS, comprising more than 50% of brain volume and outnumbering neurons approximately 9:1 [21], appear to be resistant to PPARγ-mediated cytotoxicity [22]. Catalase gene expression and enzymatic activity was the only antioxidant enzyme significantly increased by PUFAs [23]. In light of this, it is hypothesized that catalase is responsible for the protective effects following PPAR agonist treatment of primary cultures of normal rat astrocytes. To investigate this putative role of PPARs in catalase regulation, we treated primary cultures of normal rat astrocytes and C6 rat glioma cells with PPAR agonists. We report herein that PPAR agonists increased catalase gene expression and enzymatic activity in primary cultures of normal rat astrocytes. In contrast, PPAR agonists failed to increase catalase expression in glioma cells. To gain insight into a potential mechanism, Cos-1 cells were transfected with a PPARγ-dominant negative (PPARγ-d.n.) plasmid, revealing that PPARγ plays a significant role in the regulation of catalase expression. The ability of PPAR agonists to selectively upregulate catalase expression in normal astrocytes but not glioma cells suggests that these compounds might represent novel adjuvant therapeutic agents for the treatment of gliomas.

Methods

Materials

Tissue culture materials were obtained from the following manufacturers: Dulbecco's Modified Eagle's Medium (DMEM/F12), Minimum Essential Medium (MEM), trypsin/EDTA penicillin and streptomycin (Gibco, Grand Island, NY); fetal bovine serum (Hyclone; Logan, Utah); L-glutamine and gentamycin (Amersham; Arlington Heights, IL). Pioglitazone, Troglitazone, WY-14,643, and 9 *cis* Retinoic Acid (9cRA) were purchased from BioMol (Plymouth Meeting, PA); Rosiglitazone was purchased from Cayman Chemical (Ann Arbor, MI). The PPARγ agonists CP-086,325-02 (CP086; Darglitazone) and CP-096,125 (CP096) were kindly donated by Michael Gibbs at Pfizer (Groton, CT). The PPARα specific agonist GW3276 and GR-259662 were kind gifts from Tim Willson at Glaxo Wellcome (Research Triangle Park, NC).

Cell culture

Primary rat astrocytes were isolated from 1 to 2 day old Sprague-Dawley rat pups [23]. The cells were cultured in MEM containing 10% FBS, 2 mM L-glutamine, 6 g/L glucose (33 mM) and 50 μg/mL gentamycin. The cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2. Astrocytes were purified from other glial cells by shaking the flasks before each media change. The media was changed every fourth day and the cells were used within a week of reaching confluence. Immunofluorescence staining with GFAP was carried out to characterize the cell purity (> 98% astrocytes).

The rat malignant glioma cell line C6 was grown in DMEM/F12 containing 10% FBS, 50 IU/mL penicillin, and 50 IU/mL streptomycin. Cells were maintained at 37 °C as a monolayer in 75 cm^2 tissue culture flasks in a humidified atmosphere containing 5% CO_2. Stock cultures were passaged twice weekly by trypsinization. For experiments, the cultures were between passage 12 and 20, and the incubations started when the cells were roughly 40% confluent.

Cloning of promoter deletion constructs

Total genomic DNA from primary rat astrocytes or rat brain microvessel endothelial cells (RBMECs) was isolated as previously reported [24]. Rat catalase promoter deletion constructs were generated by PCR using the following primers: -1046, 5′-ACAGCCCACAGCCCATAATC-3′ (3641–3660); -938, 5′-ATTGATTAAAATGAAAAATAAGCGAC-3′ (3751–3776); and -207, 5′-CTCCTTCCAA TCCTGTCCC-3′ (4481–4499). The number represents the location of the rat catalase promoter construct with respect to the translational start site. The common downstream primer, 5′-CAGATGAAGCAGTGGAAGGA-3′ (4719–4738), was used with all the primers listed above. The numbers inside the parentheses indicate the location of primers with reference to the published sequence beginning with the first nucleotide of exon 1 [GenBank accession # M25669].

PCR was performed using Taq DNA polymerase (Perkin-Elmer; Emeryville CA). The PCR conditions consisted of 94 °C for 4 min, followed by 35 cycles of 94 °C for 1 min, 58 °C for 50 s, and 72 °C for 1.5 min, followed by a final extension at 72 °C for 5 min. The expected sizes of the PCR products were verified by 1 kb and 100 bp DNA markers (Promega; Madison, WI) on a 1% TAE gel. The PCR products were ligated into the pCR2.1 TA-TOPO cloning vector and transformed into competent cells as described by the manufacturer (Invitrogen; Carlsbad, CA). DNA was isolated using the gel extraction kit (Qiagen), digested with EcoRI (Holden Cancer Center; University of Iowa) and the identity was verified by sequencing (DNA Core Facility; University of Iowa). The constructs were engineered by subcloning the promoter deletion fragments from pCR2.1 (Invitrogen) into a luciferase-based plasmid pGL3-basic (Promega). The constructs were verified by sequencing (DNA Core Facility; The University of Iowa).

The tandem tripeat of the catalase PPRE (DR1x3) was synthesized using an oligonucleotide consisting of TAATCAAGGTGAAAGTTGAGAAG with KpnI and XhoI restriction sites on its 5′- and 3′-ends, respectively. The oligonucleotide was restricted with KpnI and XhoI, gel isolated, and cloned into pGL3-basic. The DR1x3 was verified by sequencing.