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Peroxisome proliferator-activated receptor-γ (PPARγ) agonist is neuroprotective and stimulates PGC-1α expression and CREB phosphorylation in human dopaminergic neurons

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ABSTRACT

Mitochondrial dysfunction has been linked to several neurodegenerative diseases such as Parkinson's disease (PD). Peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) is a master gene for mitochondrial biogenesis and has been shown to be neuroprotective in models of PD. In this work we have studied the mechanisms by which peroxisome proliferator-activated receptor-γ (PPARγ) selective agonist *N*-(2-benzoylphenyl)-*O*-[2-(methyl-2-pyridinylamino)ethyl]-L-tyrosine hydrate (GW1929) acts on human dopaminergic neurons in culture. Data showed that GW1929 increased the viability of human dopaminergic neurons and protected them against oxidative stress induced by H_2O_2 and the mitochondrial toxin Rotenone. The enhanced resilience of the neurons was attributed to increased levels of mitochondrial antioxidants and of PGC-1α. GW1929 treatment further increased cell respiration, mitochondrial biogenesis and sirtuin-1 (SIRT1) expression in the human dopaminergic neurons. Phosphorylation of cAMP responsive element-binding protein (CREB) was also robustly increased in GW1929-treated cells. Together these results show that the PPARγ agonist GW1929 influences CREB signaling and PGC-1α activities in the human dopaminergic neurons contributing to an increased cell viability. This supports the view that drugs acting on the PPARγ-PGC-1α signaling in neurons may have beneficial effects in PD and possible also in other brain disorders.

1. Introduction

Neurodegenerative diseases are characterized by the progressive loss of specific classes of neurons. The underlying mechanisms for selective neuronal vulnerability in brain diseases are not fully understood but are linked to alterations in cell signaling, organelle dysfunctions, enhanced oxidative stress, and brain inflammation (Lin and Beal, 2006; Saxena and Caroni, 2011; Garden and La Spada, 2012). Mitochondria are the major source of energy in the cell and neuronal survival depends on proper mitochondrial function (Lin and Beal, 2006; Rugarli and Langer, 2012). Mitochondrial dysfunctions contribute to the pathogenesis of Parkinson's disease (PD) leading to the degeneration of dopaminergic neurons in substantia nigra pars compacta (Abou-Sleiman et al., 2006; Henchcliffe and Beal, 2008). Studies of familial forms of PD have also identified several genes that are linked to mitochondria and to the regulation

Abbreviations: AC6, adenylyl cyclase 6; CRE, cAMP responsive element; CREB, cAMP responsive element-binding protein; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; GW1929, *N*-(2-benzoylphenyl)-*O*-[2-(methyl-2-pyridinylamino)ethyl]-L-tyrosine hydrate; H89, *N*-[2-[[3-(4-Bromophenyl)-2-propenyl]amino]ethyl]-5-isoquinolinesulfonamide dihydrochloride; HGB, human globulin; HRP, horseradish peroxidase; mtDNA, mitochondrial DNA; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ND1, NADH-ubiquinone oxidoreductase chain 1; NRF1, nuclear respiratory factor 1; OCR, oxygen consumption rate; PD, Parkinson's disease; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator-1α; PKA, protein kinase A; PPARγ, peroxisome proliferator-activated receptor-γ; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; SIRT1, sirtuin-1; SOD2, superoxide dismutase 2; SQ22536, 9-(tetrahydro-2-furanyl)-9*H*-purin-6-amine; TBS, Tris-buffered saline; TFAM, mitochondrial transcription factor A; Trx2, thioredoxin reductase 2.

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of cell viability and metabolism by this organelle (Schapira, 2007).

Peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) is a transcriptional co-activator that regulates mitochondrial biogenesis and respiration as well as the cell defense system against reactive oxygen species (ROS) (Wu et al., 1999; Puigserver and Spiegelman, 2003; St-Pierre et al., 2006). Recent studies have shown that PGC-1α plays a key role in cell metabolism together with the NAD^+-dependent deacetylase, sirtuin-1 (SIRT1) that can activate PGC-1α (Rodgers et al., 2005; Revollo and Li, 2013). PGC-1α is expressed in the nervous system and plays a protective role in different models of brain diseases (St-Pierre et al., 2006; Mudo et al., 2012). The mechanisms involved in the neuroprotective effects of PGC-1α are, however, not fully understood (Lindholm et al., 2012).

Peroxisome proliferator-activated receptor γ (PPARγ) is a member of the nuclear receptor superfamily of ligand-inducible transcription factors and regulates genes involved in glucose, amino acid, and lipid metabolism (Chen et al., 2012; Ahmadian et al., 2013). Drugs acting on PPARγ, including the thiazolidinediones rosiglitazone and pioglitazone, are widely used in the treatment of type-2 diabetes (Yki-Jarvinen, 2004). Recent studies further suggest that the PPARγ agonists can influence energy homeostasis (Chiang et al., 2012) and reduce reactive oxygen species (ROS) in the brain (Aleshin and Reiser, 2013). It has therefore been suggested that PPARγ agonists may have neuroprotective potential in the treatment of various neurodegenerative diseases (Patrone et al., 2014).

To clarify the mechanisms by which PPARγ agonists act on neurons, we have in this work studied the effects of the PPARγ agonist *N*-(2-benzoylphenyl)-*O*-[2-(methyl-2-pyridinylamino) ethyl]-L-tyrosine hydrate (GW1929) using cultured human dopaminergic neurons. Data showed that GW1929 increased the viability of human dopaminergic neurons and protected them against oxidative stress. GW1929 acted by increasing PGC-1α levels and by enhancing cell respiration and mitochondrial biogenesis in human dopaminergic neurons. There was also an increase in phosphorylation of cAMP responsive element-binding protein (CREB) by GW1929 that can contribute to neuroprotection.

2. Materials and methods

2.1. Cell culture

Human mesencephalic cells called MESC2.10 were cultured as previously described (Lotharius et al., 2002; Makela et al., 2014). In brief, cells were cultured in poly-D-lysine (Sigma–Aldrich, St. Louis, MO, USA) coated flasks (75 cm^2) in Dulbecco's Modified Eagle's Medium (DMEM)/F12 (Gibco, Life Technologies, Carlsbad, CA, USA) supplemented with B27 (Gibco) and Penicillin/streptomycin and 20 ng/ml of human basic fibroblast growth factor (Peprotech, Rocky Hill, NJ, USA). To differentiate cells into dopaminergic neurons, the MESC2.10 cells were plated on poly-D-lysine/laminin (Sigma) coated wells at a density of 30,000 cells/cm^2. The following day the medium was changed to a medium containing DMEM/F12 supplemented with B27 (Gibco), and 1 μg/ml tetracycline (Sigma) and lacking fibroblast growth factor. Cells were differentiated for 2–6 days and fresh medium was added every second day. Differentiated cells expressed markers for dopaminergic neurons (Makela et al., 2014), such as tyrosine hydroxylase (TH) and dopamine transporter (DAT) (Fig. 1A–B). The dopaminergic neurons were treated further for 24 h with 0.1–1 μM of the PPARγ agonist GW1929 (Sigma). In some experiments 100 μM 9-(tetrahydro-2-furanyl)-9*H*-purin-6-amine (SQ22536; Tocris Bioscience, Bristol, UK) was used to inhibit adenylyl cyclase, and 10 μM *N*-[2-[[3-(4-Bromophenyl)-2-propenyl]amino]ethyl]-5-isoquinolinesulfonamide dihydrochloride (H89; Cell Signaling Technology, Danvers, MA, USA) was used to inhibit protein kinase A (PKA). 10 μM forskolin (Sigma) was used to stimulate cyclic AMP. Subsequently the cells were analyzed further as indicated below.

2.2. Immunoblotting and immunostaining

Immunoblotting was done essentially as described (Korhonen et al., 2001; Mäkelä et al., 2014). Cell lysates were made using RIPA buffer [150 mM NaCl, 1 mM EDTA, 1% (v/v) NP-40, 0.25% (w/v) sodium deoxycholate, 50 mM Tris-HCl, pH7.4] and 1% (w/v) sodium dodecyl sulfate (SDS). 30 μg of protein was run on a SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and proteins were transferred to nitrocellulose membrane (Amersham Biosciences, Buckinghamshire, UK). The membranes were blocked in 5% non-fat milk-Tris-buffered saline (TBS)-0.1% (v/v) Tween 20 (TBS-T) for 1 h at RT and primary antibodies were incubated overnight at +4 °C. Antibodies used were: anti-CREB (diluted 1:1000, Cell Signaling Technology), anti-p-CREB (Ser133) (1:1000, Cell Signaling Technology), anti-nuclear respiratory factor 1 (NRF1; 1:1000, Abcam, Cambridge, UK), anti-PGC-1α (1:2000, Calbiochem, San Diego, CA, USA), anti-SIRT1 (1:1000, Cell Signaling Technology), anti-superoxide dismutase 2 (SOD2; 1:5000, AbFrontier, Seoul, Korea), anti-mitochondrial transcription factor A (TFAM; 1:1000, Abcam), anti-thioredoxin reductase 2 (Trx2; 1:1000, AbFrontier), anti-TH (1:1000, Cell Signaling Technology) and β-Actin (1:5000, Sigma). The following day the membranes were washed with TBS-T and secondary horseradish peroxidase (HRP)-conjugated antibodies (1:2500, Jackson ImmunoResearch, West Grove, PA, USA) were added for 1 h at RT. Super Signal West Pico chemiluminescent substrate (Thermo Scientific, Waltham, MA, USA) was used for detection, and quantification wad done using Image J software.

Immunostaining was done as described (Hyrskyluoto et al., 2014) by fixing cells differentiated for 6 days using 4% paraformaldehyde at RT for 20 min. Cells were first incubated for 1 h in phosphate based saline (PBS) containing 0.1% Triton-X-100 and 5% bovine serum albumin (Sigma) at RT, followed by an overnight incubation at 4 °C in presence of anti-DAT antibodies (1:200; Novus Biological, Littleton CO, USA). Cells were washed and incubated with secondary Alexa 594 antibody (1:500; Invitrogen Molecular Probes) for 1 h, washed further and mounted with Mowiol mounting media (Sigma). Images were captured using Leica fluorescent microscope DM4500B. Controls without primary antibodies showed no staining.

2.3. Immunoprecipitation

Cells were stimulated with 1 μM GW1929 for 24 h and lysed in RIPA buffer. Immunoprecipitation was carried out using 500 μg protein and 1.5 μg of PGC-1α antibody (Calbiochem). Samples were rotated in a shaker for 12 h at +4 °C and 50 μl of protein G agarose (Roche, Basel, Switzerland) was added for an additional 6 h. The beads were washed three times with RIPA buffer and the extracts were run on 8% SDS-PAGE. Proteins were transferred to a nitrocellulose membrane, blocked in 5% BSA-TBS-T for 1 h in RT and probed with anti-α-acetylated lysine antibody (1:1000, Cell Signaling Technology) in 5% BSA-TBS-T overnight +4 °C. The membrane was then washed with TBS-T and HRP-conjugated secondary antibody (1:2500) was added for 1 h. Detection of bands was done as above. The membrane was stripped using 0.1 M glycine pH 2 for 20 min and reprobed with anti-PGC-1α antibody (1:2000, Calbiochem) in 5% BSA-TBS-T. Quantifications were done using ImageJ software.