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Polychlorinated biphenyls impair dibutyryl cAMP-induced astrocytic differentiation in rat C6 glial cell line

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ABSTRACT

In the central nervous system, alteration of glial cell differentiation can affect brain functions. Polychlorinated biphenyls (PCBs) are persistent environmental chemical contaminants that exert neurotoxic effects in glial and neuronal cells. We examined the effects of a commercial mixture of PCBs, Aroclor1254 (A1254) on astrocytic differentiation of glial cells, using the rat C6 cell line as *in vitro* model. The exposure for 24 h to sub-toxic concentrations of A1254 (3 or 9 μM) impaired dibutyryl cAMP-induced astrocytic differentiation as showed by the decrease of glial fibrillary acidic protein (GFAP) protein levels and inhibition in change of cell morphology toward an astrocytic phenotype. The A1254 inhibition was restored by the addition of a protein kinase C (PKC) inhibitor, bisindolylmaleimide (bis), therefore indicating that PCBs disturbed the cAMP-induced astrocytic differentiation of C6 cells via the PKC pathway. The phosphorylation of signal transducer and activator of transcription 3 (STAT3) is essential for cAMP-induced transcription of GFAP promoter in C6 cells. Our results indicated that the exposure to A1254 (3 or 9 μM) for 24 h suppressed cAMP-induced STAT3 phosphorylation. Moreover, A1254 reduced cAMP-dependent phosphorylation of STAT3 requires inhibition of PKC activity. Together, our results suggest that PCBs induce perturbation in cAMP/PKA and PKC signaling pathway during astrocytic differentiation of glial cells.

1. Introduction

Astrocytes, the main class of neuroglia, are the most abundant cells in the central nervous system (CNS) providing an architecture for neurons and secreting growth factors and cytokines in the response to injury [1]. Besides, astrocytes are the major cell type that preferentially sequestrates metals and accumulates toxic agents [2], therefore suggesting a possible role in the control and/or modulation of neurotoxic effects.

Polychlorinated biphenyls (PCBs) are widespread and persistent environmental contaminants accumulating in food chain in polluted areas [3] that can affect nervous system development and functions [4]. PCBs were produced for use as non-flammable dielectrics in electronic parts, lubricants, plasticizers, vehicles for pesticide application, and pigment suspension agents in carbonless copy paper [5]. PCBs produce neurochemical alterations in several experimental models [6], behavioral changes in learning, motor activity and sexual behavior [7]. In addition, PCBs greatly affect cell viability, brain functions and have been associated with neurodegenerative disorders [8]. PCBs induce mitochondrial dysfunction and reactive oxygen species (ROS) production [9] and in turn, alterations of dopaminergic neurons [10],

Abbreviations: PCBs, polychlorinated biphenyls; dbcAMP, N^6,2′-*O*-dibutyryl cAMP; A1254, Aroclor 1254; DMSO, dimethyl sulfoxide; DMEM, Dulbecco's Modified Eagle's Medium; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; NMDA, N-methyl-D-aspartate; nNOS, neuronal nitric oxide; CNS, central nervous system; ROS, reactive oxygen species; GFAP, glial fibrillary acidic protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DAPI, 4′,6-diamidino-2-phenylindole; PKA, protein kinase A; PKC, protein kinase C; bis, 2-[1-(3-dimethylamino-propyl)indol-3-yl]-3-(indol-3-yl) maleimide; STAT3, signal transducer and activator of transcription 3; CRE, cAMP responsive element; CREB, cAMP-response element binding protein; TRE, CRE transcriptional response element.

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death of cerebellar granule cells via N-methyl-D-aspartate (NMDA) receptor activation [11] and of neuroblastoma cells via the involvement of neuronal nitric oxide (nNOS) [12]. Furthermore, chronic exposure to these pollutants can affect the development of the CNS [4] and neuronal plasticity [13]. Aroclor 1254 (A1254), a commercial mixture of PCBs [14], most commonly found in various foods and in human specimens at contaminated sites [15], has been widely utilized in studying PCBs toxicity [9–14].

Although several scientific studies have been conducted on the neurotoxicity triggered by PCBs, the effects of these pollutants on astrocytic differentiation has been poorly investigated. The rat C6 glial cell line [16] has been widely used as model for study of factors that modulate differentiation of glial cells [17]. The treatment of C6 cells with dibutyryl(db)-cAMP, leads to inhibition of cell growth and increased expression of the astrocytic marker glial fibrillary acidic protein (GFAP) correlated to change in cell morphology from an epithelial-like to a process-bearing morphology [18]. In this study, we determined A1254 cytotoxicity and the effects of sub-toxic concentrations on dbcAMP-induced astrocytic differentiation in C6 cells, by evaluation of GFAP levels and monitoring cell morphology.

Furthermore, since protein kinase C (PKC) signaling is involved in A1254 neurotoxicity [19–21], we also assessed the effects of a selective PKC inhibitor [21,22] on A1254-induced toxic effects. cAMP-induced GFAP expression in C6 cells also requires activation of signal transducer and activator of transcription 3 (STAT3) pathway [23,24], we also investigated the effect of A1254 on the activation status of STAT3.

2. Materials and methods

2.1. Materials

Aroclor 1254 (Cat. N. 48586, Lot N. LB58885) was purchased from Supelco (Italy, 99% purity) and dissolved in dimethyl sulfoxide (DMSO, cell culture tested, Sigma–Aldrich, Italy). The protein kinase C (PKC) inhibitor, 2-[1-(3-dimethylamino-propyl)indol-3-yl]-3-(indol-3-yl) maleimide (bis) was purchased from Cell Signaling Technology (Cat. N. 9841, Euroclone, Italy) and dissolved in DMSO. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, MTT and N^6,2′-*O*-dibutyryl cAMP (dbcAMP) were obtained from (Cat. N. D0260, Sigma–Aldrich, Italy).

2.2. Cell cultures and treatments

The C6 rat glial cell line [16] (American Type Culture Collection, ATTC CCL-107) was cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen, Life Technologies, Italy), 1.5 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin under humidified atmosphere of 5% CO_2 at 37 °C. Cells were sub-cultured twice a week by 3–5-fold dilution with culture medium. Treatments of sub-confluent cells were performed replacing the culture medium with those containing increasing concentrations of A1254 (0.05–90 μM) or protein kinase C inhibitor, bisindolylmaleimide [21,22] (0.05–12 μM). Astrocytic differentiation was induced using serum-free DMEM containing 1 mM dbcAMP. Co-exposure experiments were performed by adding simultaneously A1254 (3 or 9 μM) and/or bis (0.125 μM) during dbcAMP (1 mM) stimulation of C6 cells for 24 h. All the treatments were performed under serum-free conditions in presence of 0.1% (v/v) DMSO used as vehicle for A1254 and bis.

2.3. Viability assay

Cells were seeded onto 96-well plates (2×10^4 cells per well) and after the treatments, their viability was evaluated as mitochondrial activity using the MTT assay [25]. Briefly, the medium was removed and cells incubated with 100 μl MTT (0.5 mg/ml) for 1 h. After that, the solution was removed, formazan solubilized in 100 μl DMSO and the absorbance measured at 540 nm using a microplate reader (Labsystems Multiskan, MS). Results were expressed as percentage of cell survival vs. control cells cultured in serum-free medium with 0.1% (v/v) DMSO (vehicle) (which represent the 100% survival).

2.4. Immunocytochemistry and phase-contrast analysis

Cells grown on coverslips were treated and fixed by a 20 min exposure to cold 4% paraformaldehyde in PBS, and then subjected to immunocytochemistry and phase-contrast analysis. For immununocytochemistry, cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min. After blocking with 10% donkey serum for 1 h, the coverslips were incubated with a mouse anti-GFAP antibody (Cat. N. G3893, Sigma–Aldrich, Italy), thereafter, a fluorescein isothiocyanate (FITC)-secondary antibody (Jeckson-Li StarFish, Italy) was applied. After washing, coverslips were mounted with Vectashield medium containing 1.5 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, CA, USA) to visualize the nuclei. Immunofluorescence analysis was performed using a Leica DM LB microscope (Plan 20 × / 0.40 objective) connected to a Leica DFC 345 FX digital camera and images were captured using the Leica Application Suite 3.6 software (Version 3.6.0) (Switzerland). Phase-contrast images were captured using a Zeiss Axiovert 40 CFL inverted microscope (Carl Zeiss, Milan, Italy) (LD A-Plan 40 × /0.50 Ph 2 objective). The microscope was equipped with a 12.1-megapixel CCD digital videocamera (Canon, PowerShot G9, Italy) with a digital image software (Remote Capture DC, Canon). Images were imported into ImageJ software 1.43u, NIH.

2.5. Western blotting

Cells seeded in 6-well plates (2×10^5 per well) were subjected to different treatments and then lysed at 4 °C in 50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Igepal, 0.5% sodium deoxicolate, protease (Roche, Italy) and phosphatase inhibitor cocktails (Calbiochem, Italy). The total protein concentration was determined by a Bradford protein assay [26] using bovine serum albumin as a standard. Equal amounts of proteins (20 μg) were subjected to 12% SDS–PAGE performed as described by Laemmli [27]. The proteins were transferred to a nitrocellulose membrane (BA85; Schleicher & Schull) and incubated with a primary antibody: mouse anti-GFAP, anti-mouse STAT3 (Cat. N. 9139), anti-rabbit phospho-STAT3 (Ser727) (Cat N. 9134), anti-rabbit phospho-PKC substrates (Cat. N. 2261S) from Cell Signaling Technology (Euroclone, Italy) followed by incubation with an appropriated anti-mouse (Cat. N. 31430) or anti-rabbit (Cat. N. 31460) peroxidase-conjugated secondary antibody (Pierce, Thermo Scientific, Italy) in PBS containing 5% dry milk. The signals were visualized using an Enhanced Chemiluminescence (ECL) detection kit (Cat. N. RPN 2209, GE Healthcare, Italy). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Cat. N. AM4300, Ambion, Applied Biosystems, Italy) was used as protein loading control. Signal quantification was performed with ImageJ software 1.43u, NIH.

2.6. Statistical analysis

Statistical significance of treated samples against control cells (cultured in serum-free medium with vehicle 0.1% v/v DMSO) was determined by One-way analysis of Variance (ANOVA), followed by Dunnett's test. Each value represents the mean ± SEM of at least three independent experiments performed in triplicate ($^{*}p < 0.05$; $^{§}p < 0.01$; $^{\#}p < 0.001$).