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Benzopyrene Inhibits SH-SY5Y Neuroblastoma Cells

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Abstract

Benzopyrene (BaP) is polycyclic aromatic hydrocarbon (PAH), and is chemically modified in the animal body to form a number of metabolites that may elicit a various toxicity. However, the effect of BaP on neuroblastoma differentiation has remained unclear. We have studied the effect of BaP on neurite outgrowth using human SH-SY5Y neuroblastoma cells induced to differentiate by all-trans-retinoic acid (RA). Whereas BaP, at a concentration of 1 μM, had no significant effect on the viability of differentiating SH-SY5Y cells, the neurite outgrowth of differentiating SH-SY5Y cells 48 h after BaP treatment was significantly inhibited. Treatment of RA-stimulated differentiating SH-SY5Y cells with 0.1–3 μM BaP resulted in decreased level of cross-reactivities with tissue glutaminase (TGase) antibody in a dose-dependent manner. To investigate the involvement of AhR signaling in the inhibition of neurite outgrowth of differentiating neuroblastoma cells by BaP, we used AhR antagonists, α- Naphthoflavone and CH223191. Cotreatment of α- Naphthoflavone (1 μM) to BaP-treated SH-SY5Y cells recovered the expression level of TGase protein up to 200% of control. Like α- Naphthoflavone, another potent AhR antagonist, CH223191 (0.1 to 1 μM) also recovered the inhibition effects of BaP on neurite outgrowth and TGase expression level of neuroblastoma cells. These results suggested that AhR signaling should be involved in the inhibition process of BaP on RA-induced differentiation of SH-SY5Y neuroblastoma cells.

Keywords Benzopyrene; SH-SY5Y neuroblastoma cell; Neurite outgrowth; Aryl hydrocarbon receptor (AhR)

Introduction

Benzopyrene (BaP) is a common polycyclic aromatic hydrocarbon (PAH) that is a byproduct of cigarette smoking and incomplete combustion of gasoline and wood [1-3]. BaP exerts potent carcinogenic activity in several animal species including human beings [4]. BaP is metabolically activated by cytochrome P-450 system to a number of metabolites that may elicit toxicity [2]. BaP-7,8-diol-9,10-epoxide (BPDE), the ultimate metabolite, can bind to cellular DNA forming DNA adducts, and is known to be the initial stage in BaP-induced carcinogenesis [5,6].

The aryl hydrocarbon receptor (AhR) is a cytosolic ligand-activated transcription factor that dimerizes with AhR-nuclear translocator (ARNT) to activate the transcription of target genes including *Cyp1a1* gene. In the first step of metabolism of B[a]P to BPDE, B[a]P binds to AhR, and BaP-AhR complex translocates to nuclei to form a heterodimer wih ARNT [7]. This complex then binds to the xenobiotic-responsive element (XRE) in the upstream region of the *Cyp1a1* gene, inducing its expression [8,9]. The AhR is considered to mediate carcinogenic effects of BaP.

A growing body of evidence supports the hypothesis that the AhR plays an important role in several physiological areas, such as cell growth [10,11], apoptosis [12,13], migration [14], vascular development [15], and immune responses [10]. AhR is also likely to influence cell differentiation via direct mechanisms not requiring P450-mediated xenobiotic metabolism [15-17]. AhR activation by environmental PAH or HAH could result in crosstalk with developmental pathways [18].

Human SH-SY5Y neuroblastoma cells are used as a well-established cell line for studying neuronal properties and neurotoxicity in culture, as they display terminal neuronal differentiation [19]. Neuronal differentiation *in vitro* is accompanied by profound morphological alterations, including the formation of neurites. Neurite outgrowth plays key roles in neuronal development and regeneration. Therefore, it is regarded as a representative marker for assessment of neurotoxic effects.

In the present study, we investigated the effect of benzpyrene on RA-induced differentiation of human SH-SY5Y neuroblastoma cell line. We found that BaP inhibited the neurite outgrowth and the expression of TGase protein of neuroblastoma cells. To investigate the involvement of AhR signaling in the inhibition of neuroblastoma differentiation by BaP, we used AhR antagonists, suggesting that AhR signaling play important roles in neuroblastoma differentiation.

Materials and Methods

Chemicals

Benzopyrene (BaP) and All-trans-retinoic acid (RA) were supplied by Sigma–Aldrich (Sigma Chemical Co., St. Louis, Missouri, USA). α-Naphthoflavon (α-NF) and CH223191 were purchased from EMD (EMD Chemicals. Inc., 480 S. Democrat Road, Gibbstown, United States)

Cell viability

The methylthiazoltetrazolium (MTT) assay was performed to measure the cytotoxicity of BaP on differentiating SH-SY5Ycells. Cells were plated in 24-well plates at a density of 110^5 cells/mL. After incubation for 24 h, BaP (0.3 to 5 μM) was treated for 48 h in the presence or absence of 10 μM RA. Following exposure to BaP, the cells were incubated for 4 h with 0.5 mg/mL MTT at 37. The reaction was stopped by removing the medium and adding acid isopropanol. The absorbance was measured at 595 nm of wavelength using a microplate reader (Bio-Rad, Laboratories, Hercules, CA).

Cell culture

The human SH-SY5Y neuroblastoma cells were purchased from Korean Cell Line Bank. Cells were maintained in complete culture medium consisting of Ham F12 and Eagle minimum essential medium (Invitrogen Co., Carlsbad, California) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, Utah) plus 100 units/mL penicillin, 100 mg/mL streptomycin, and nonessential amino acids in a humidified incubator with 5% CO_2 and 95% air at 37. The medium was refreshed every 2 days.

Cell morphological analysis

SH-SY5Y cells were plated in 24-well plates at a density of 110^5 cells/mL. After incubation for 24 h, the medium was refreshed, and 10 μM RA and various concentrations of BaP were added simultaneously. After incubation for 2 days, the cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 minutes, washed with PBS, stained with Coomassie Brilliant Blue R-250 for 20 minutes, and washed with PBS. The morphological changes in the cells were observed under a phase-contrast microscope. Those cells which had processes longer than twice of the diameter of cell body were considered as neurite-bearing cells [20]. The percentage of the cells with neurites in a particular culture was determined by counting at least 300 cells in each sample.

Western blot analysis

For Western blot analysis, the SH-SY5Y cells were plated in100-mm plates at a density of 110^5 cells/mL (10 mL). After incubation for 2 days, the medium was refreshed and incubated in the presence and absence of RA and BaP. After incubation for indicated times, the cells were washed with ice-cold PBS and lysed with lysis buffer (50 mM HEPES pH7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl2, 5 mM EDTA) containing protease inhibitor cocktail (Sigma Chemical Co.) at 4 for 30 minutes. Total proteins were obtained after centrifugation at 14,000g for 30 minutes at 4 . The protein concentrations were determined by Bradford assay (Pierce, Rockford, Illinois). All proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel and electrophoretically transferred to nitrocellulose membrane (Millipore Co., Bedford, Mass.). After blocking at room temperature for 90 minutes in TBS-Tween containing 5% skim milk, the membranes were incubated with primary antibodies of anti-TGase (Santa Crus, California) at a dilution of 1:1,000 in 1% BSA-TBST buffer for 2 h at room temperature, followed by incubation with horseradish peroxidase-conjugated secondary antibody (Zymed, San Francisco, California). After washing 3 times with TBS-Tween, the bands were detected with a standard ECL detection system (LabFrontier Co., Seoul, Korea).

Statistical analysis

Results were expressed as mean values ±standard error (mean ±S.E.). Statistical analysis was performed by Student's t-test. A level of $p<0.05$ was considered to be significant.

Results

Cytotoxic effect of BaP on differentiating SH-SY5Y neuroblastoma cells

To investigate the effects of BaP on neurite outgrowth of differentiating SH-SY5Y neuroblastoma cells, the suitable subacute BaP concentrations were determined. After treatment of SH-SY5Y cells with RA (10 μM) for 48 h in the presence of various concentrations of BaP, the viability of SH-SY5Y cells was determined. As shown in Figure 1, BaP concentrations less than 3 μM had no significant effect on the viability of differentiating SH-SY5Y cells.

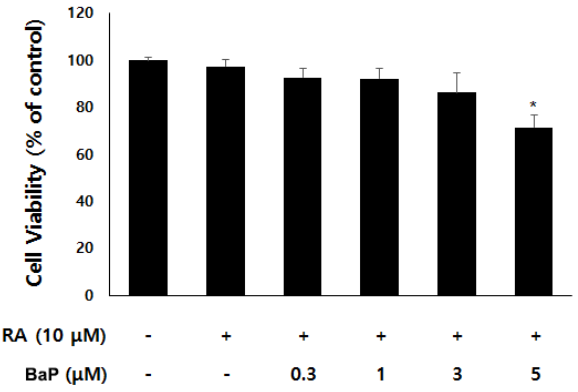

Figure 1: Effect of BaP on the cell viability of differentiating SH-SY5Y cells. The cells were treated with BaP for 48 h in the presence of RA (10 μM). Then, the viability of SH-SY5Y cells was determined by MTT assay. The percentage of viable cells was calculated by defining the viability of cells without RA and BaP treatment as 100%. Data are expressed as the meanstandard error obtained from four independent experiments. *P<0.05 when compared to RA-treated cells.

Effects of BaP on neurite outgrowth and expression of TGase in differentiating SH-SY5Y cells

The neurotoxicity of BaP on human SH-SY5Y neuroblastoma cells was initially assessed by examining whether BaP could inhibit neurite outgrowth of differentiating SH-SY5Y cells. BaP was used at various concentrations up to 1 μM, which had no significant effect on the viability of differentiating SH-SY5Y cells (Figure 1). As shown in Figure 2, BaP had no significant effect at low concentration (0.1 μM) on neurite outgrowth in differentiating SH-SY5Y cells. However, the neurite outgrowth of RA-treated SH-SY5Y cells treated with 1 μM BaP was significantly inhibited.

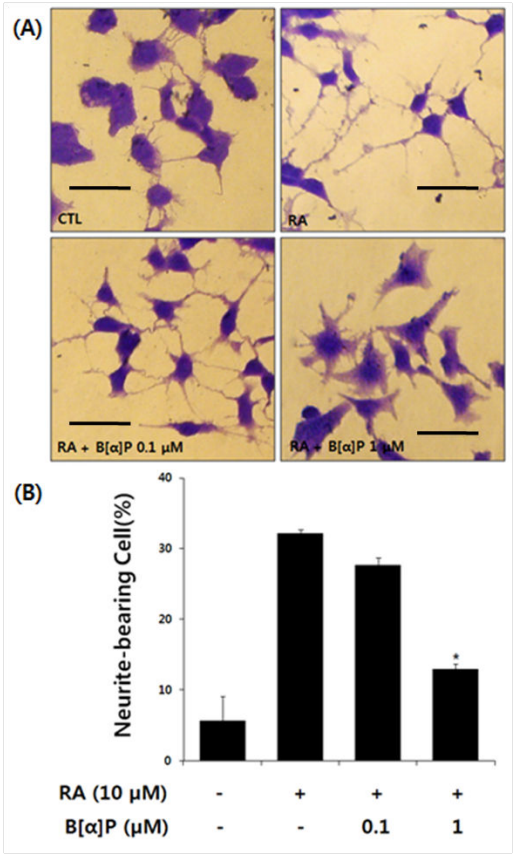

Figure 2: Benzoapyrene inhibits neurite outgrowth in differentiating SH-SY5Y cells. (A) The cells were induced to differentiate for 48 h with RA (10 μM) in the presence or absence of BaP (0.1 and 1 μM). Cells were fixed in 4% paraformaldehyde and stained with Coomassie Brilliant Blue (CBB). Shown are images of typical fields of cells viewed by inverted light microscope. (B) Neurite outgrowth was quantified by counting the number of cells exhibiting neurites that were two times longer than the cell body diameter in length. The proportion of cells with neurites was expressed as a percentage of the total number of cells. Approximately 300 cells were counted in each sample. The data are expressed as the mean±standard error of four independent experiments. *P<0.05 when compared to RA-treated cells. Scale bar =100 μm.

The molecular basis of the inhibitory effects of BaP on neurite outgrowth of SH-SY5Y cells was studied further by probing Western blots of cell extracts with antibodies against Transglutaminase (TGase). TGase is one of a set of proteins that are strongly up-regulated in differentiating SH-SY5Y cells in response to retinoic acid, and is known to be necessary for neuronal differentiation of SH-SY5Y cells [21,22]. TGase, therefore, is widely used as a common indicator for RA-induced differentiation of neuronal cells. In order to determine the effects of BaP on TGase expression in differentiating SH-SY5Y cells, cells were treated with 10 μM RA and various concentrations of BaP for 48 h. As shown in Figure 3, BaP-treated cell extracts exhibited decreased cross-reactivities with TGase antibody when compared to control extracts in a dose-dependent manner.

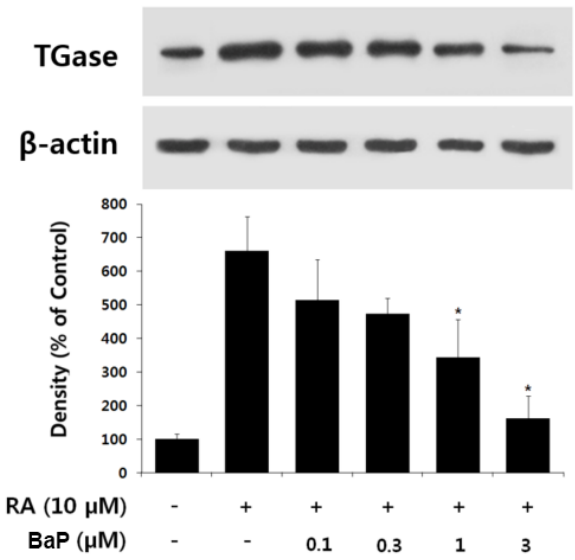

Figure 3: Western blotting analysis of TGase in differentiating SH-SY5Y cells. The cells were treated with various concentrations of BaP and RA (10 μM) for 48 h. The Western blot data represents one of three experiments. Densitometric analysis was performed to determine the intensity of TGase bands. Values are normalized to β-actin band and expressed as the percentage of cells treated with retinoic acid (RA) only. Data are mean±standard error of three independent experiments. *P<0.05 when compared to RA-treated cells.

Effect of α-NF on neurite outgrowth and expression of TGase in differentiating SH-SY5Y cells treated with BaP

To investigate the involvement of AhR in the inhibition of neuroblastoma differentiation by BaP, we determined the effect of α-Naphthoflavone (α-NF) on neurite outgrowth and TGase expression in differentiating SH-SY5Y cells treated with BaP. a-Naphthoflavone (α-NF) also known as 7,8-benzoflavone and 2-phenyl-benzo(h)chromen-4-one, is a synthetic flavone derivative. α-NF, well known as AhR antagonist, inhibits the induction of cytochrome P450 1A1 gene expression by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). It has been suggested that the AhR antagonist activity is due to the formation of a-NF-cytosolic AhR complexes that fail to undergo transformation [23]. It was also reported that a-NF attenuated BaP-7,8-dione-mediated DNA strand breaks [24]. As shown in Figure 4, the addition of 1 μM BaP in RA-treated SH-SY5Y cells decreased the percentage of neurite bearing cells, compared to RA only treated cells. We treated SH-SY5Y cells with three concentrations of α-NF from 0.1 μM to 1 μM one hour prior to BaP and RA treatment. The percentage of neurite bearing cells was recovered by treatment of α-NF (1 μM) up to 35 % (Figure 4).

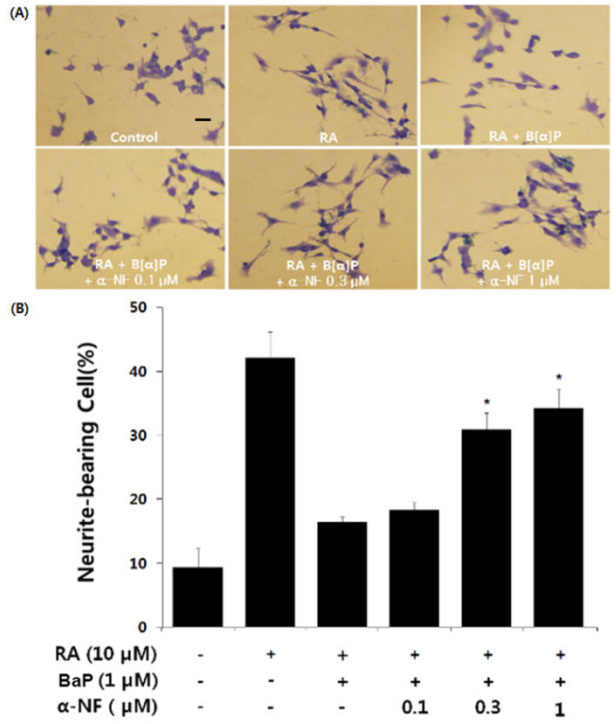

Figure 4: Effect of α-Naphthoflavon (α-NF) on neurite outgrowth of differentiating SH-SY5Y cells treated with BaP. (A) The cells were treated with α-NF 1 hour prior to BaP and RA treatment. Cells were harvested 48 h after BaP and RA (10 μM) treatment, fixed in 4% paraformaldehyde, and stained with Coomassie Brilliant Blue. Shown are images of typical fields of cells viewed by inverted light microscope. (B) Neurite outgrowth was quantified by counting the number of cells exhibiting neurites that were two times longer than the cell body diameter in length. The proportion of cells with neurites was expressed as a percentage of the total number of cells. Approximately 300 cells were counted in each sample. The data are expressed as the mean±standard error of four independent experiments. *P<0.05 when compared to RA and BaP-treated cells. Scale bar = 100 μm.

The expression of TGase protein shows the degree of differentiation of SH-SY5Y neuroblastoma cells. As shown in Figure 5, the TGase level of cells treated with only RA (10 μM) went up to about 330% of the control. And then, the addition of 1 μM BaP to RA-treated SH-SY5Y cells decreased the level of TGase expression to about the level of the control group. However, cotreatment of α-NF (1 μM) to BaP-treated SH-SY5Y cells recovered the level of TGase protein by up to 200% of the control. These results suggested that AhR might be involved in the inhibition of SH-SY5Y neuroblastoma differentiation by BaP.

Effect of CH223191 on neurite outgrowth and expression of TGase in differentitating BaP-treated SH-SY5Y cells

To confirm the effect of AhR antagonist on BaP-mediated inhibition of SH-SY5Y differentiation, we used another potent AhR antagonist, CH-223191. It competes with AhR agonists (such as TCDD and BaP) for binding to the AhR, and inhibits the ability of AhR agonists to activate the AhR signal transduction [25].

As shown in Figure 6, up to more than 38% of cells treated with only RA (10 μM) were neurite bearing cells. The neurite outgrowth of RA-treated SH-SY5Y cells was significantly inhibited by 1 μM BaP treatment. The AhR antagonist, CH223191, was used at various concentrations up to 3 μM one hour prior to BaP and RA treatment. The percentage of neurite bearing cells was recovered by treatment of CH223191 (1 μM) up to 26% (Figure 6).

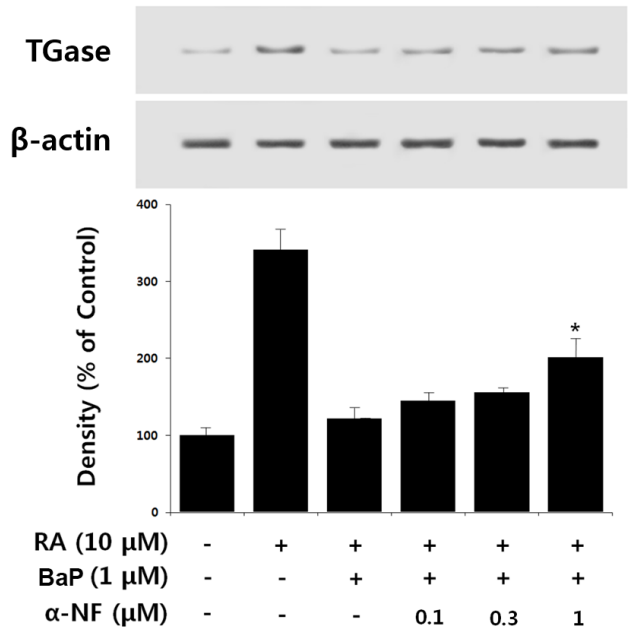

Figure 5: Effect of α-Naphthoflavon (α-NF) on TGase expression in differentiating SH-SY5Y cells treated with BaP. The cell were pretreated with α-NF (0.1~1 μM) for 1 h and treated with RA (10 μM) and BaP (1 μM) for 48 h. The Western blot data represents one of three experiments. Densitometric analysis was performed to determine the intensity of TGase bands. Values are normalized to β-actin band. The relative levels of TGase were compared and expressed as the percentage of the control. Data are mean±standard error of three independent experiments. *P<0.05 when compared to RA and BaP-treated cells.

As shown in Figure 7, the TGase level goes up to about 460% of the control in the treatment of 10 μM RA to SH-SY5Y neuroblastoma. If 1 μM of BaP was added to the RA-treated SH-SY5Y neuroblastoma, the TGase level goes down again to the semblance of about 200% control. However, cotreatment of threeconcentrations of CH223191 (0.1 to 1 μM) to BaP-treated SH-SY5Y cells recovered the level of TGase protein up to 360% of the control in a dose dependent manner (Figure 7). These results also confirmed that AhR should be involved in the inhibition process of BaP on SH-SY5Y neuroblastoma differentiation by RA.

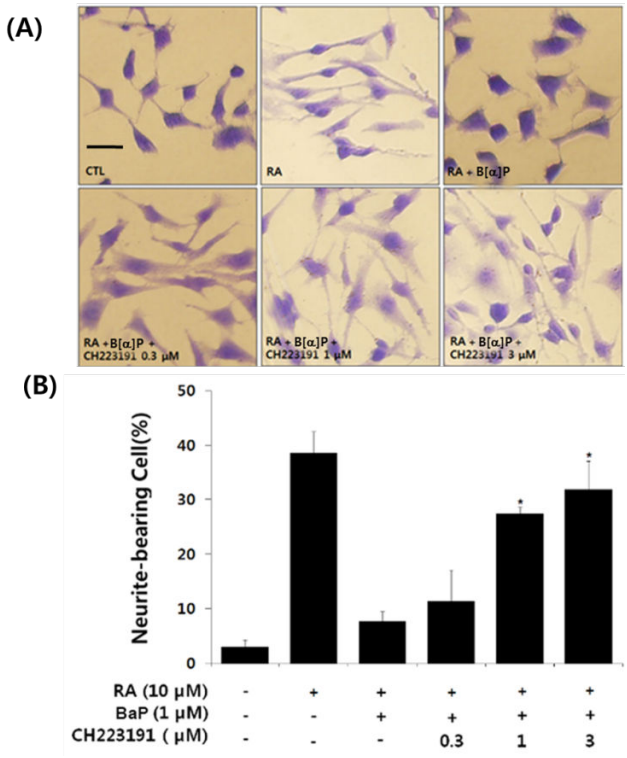

Figure 6: Effect of CH223191 on neurite outgrowth of differentiating SH-SY5Y cells treated with BaP. (A) The cell were treated with CH223191 one hour prior to RA (10 μM) and BaP (1 μM) treatment. Cells were harvested 48 h after BaP treatment, fixed in 4% paraformaldehyde, and stained with Coomassie Brilliant Blue. Shown are images of typical fields of cells viewed by inverted light microscope. (B) Neurite outgrowth was quantified by counting the number of cells exhibiting neurites that were two times longer than the cell body diameter in length. The proportion of cells with neurites was expressed as a percentage of the total number of cells. Approximately 300 cells were counted in each sample condition. The data are expressed as the mean±standard error of four independent experiments. *P<0.05 when compared to RA and BaP-treated cells. Scale bar = 100 μm.

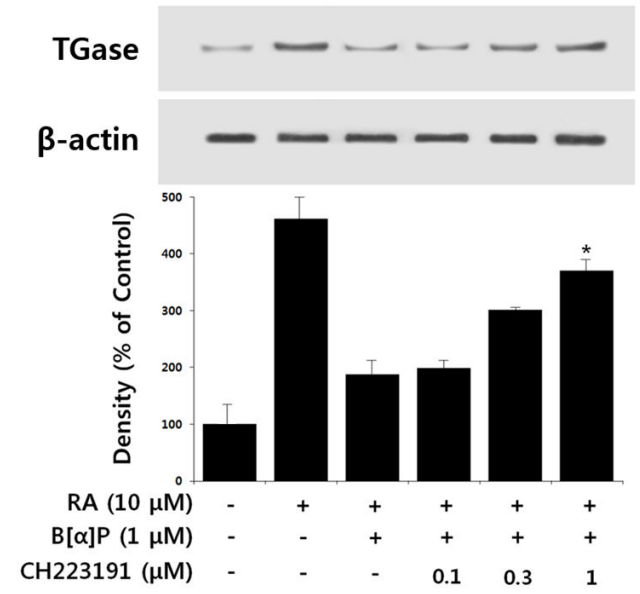

Figure 7: Effect of CH223191 on TGase expression in differentiating SH-SY5Y cells treated with BaP. The cells were pretreated with CH223191 (0.1~1 μM) for 1 hour and treated with RA (10 μM) and BaP (1 μM) for 48 h. The Western blot data represents one of three experiments. Densitometric analysis was performed to determine the intensity of TGase bands. Values are normalized to β-actin band and expressed as the percentage of cells treated with retinoic acid (RA) only. Data are mean±standard error of three independent experiments.

Discussion

BaP is a five-ring polycyclic aromatic hydrocarbon and known as a mutagen and a carcinogen [26]. Although a great deal of study has been focused on carcinogenic properties of BP, it is increasingly evident that BaP could contribute to neurodevelopmental disorders seen with *in vivo* exposures [27] (Grandjean and Landrigan, 2006). Low, nontoxic levels of BP could impair neurodifferentiation in PC 12 cells undergoing nerve growth factor-induced differentiation [28]. Our results also showed thatBaP could inhibit neurodifferentiation in differentiating SH-SY5Y cells stimulated by RA.

It is well known that the AhR plays an essential role in the induction of cytochrome P4501A (*CYP1A1*) and the conversion of BaP into its active form [7]. First, BaP binds to the AhR in the cytosol, this induces a conformational change in the receptor leading to the dissociation of Hsp90, and AhR-BaP complex is translocated to the nucleus, and forms AhR-BaP-ARNT complex. This complex mediates the transcription of XRE-dependent genes including *CYP1A1*. BaP is metabolically activated to benzopyrene diol epoxide (BPDE) derivative, which binds to macromolecules (DNA, RNA, and protein) to produce macromolecular adducts [29-31]. Therefore, the AhR is considered to mediate toxic effects of BaP. We also observed that BaP inhibited the neurite outgrowth of differentiating SH-SY5Y cells and decreased the level of cross-reactivities with TGase antibody. However, Cotreatment of AhR antagonists, α-Naphthoflavon and CH223191, to BaP-treated SH-SY5Y cells recovered the expression level of TGase protein, conforming the involvement of AhR in the inhibition effect of BaP on neurite outgrowth of RA-induced differentiating SH-SY5Y neuroblastoma cells.

The AhR is a transcription factor belonging to the sensory factors of the bHLH-PAS family. Activation of AhR is initiated by ligand binding, which leads to translocation of AhR into the nucleus where it dimerizes with its partner protein ARNT [32]. The AhR/ARNT heterodimer binds to specific regulatory DNA sequences known as xenobiotic response elements (XREs), which lead to increased gene expression of a variety of genes involved in metabolism of xenobiotic compounds as well as of many endogenous substances [33].

The AhR has been studied for several decades largely because of its critical role in xenobiotic-induced toxicity and carcinogenesis. Although the molecular mechanisms leading to AhR activation in responding to xenobiotics are well understood, AhR signaling pathways in the absence of exogenous ligands remain largely unknown. In recent years, many investigators are trying to understand the physiological role of AhR under normal cellular conditions. Increasing experimental evidence suggests that the AhR has important regulatory roles in normal physiology, such as cell proliferation and differentiation, immune system homeostasis and tumor development [34]. Recent findings suggest that the AhR is a multi-functional protein and has crosstalk with several other important signal transduction pathways [35].

In conclusion, the present study shows that BaP inhibits the neurite outgrowth in differentiating human SH-SY5Y neuroblastoma cells. However, AhR antagonists recovered the inhibition of neuroblastoma differentiation by BaP. These results suggest that AhR signaling might be involved in the inhibition effect of BaP on the differentiation of neuroblastoma cells. In conclusion, our studies have demonstrated that BaP inhibits neurite outgrowth in differentiating human SH-SY5Y neuroblastoma cells via an AhR-dependent mechanism.

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