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COX-2 EXPRESSION IN CHEMICAL INDUCED NEURO-DIFFERENTIATION OF NG108-15 CELL

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ABSTRACT

The pathogenesis of Alzheimer's disease is complicated and related to COX-2. The NG108-15 cell line is a neuronal cell line. COX-2 expression in chemical induced neuronal differentiation of NG108-15 cells was investigated. Neuritogenesis of NG108-15 cells was induced by treatment with 100 mM dibutyryl cAMP (Bt_2cAMP) and 100 µM 12-o-tetradecanoyl-phorbol 13-acetate (TPA) for 3 days. Cellular morphology alteration was monitored microscopically; the level of COX-2 mRNA and protein expression was determined by RT-PCR and western blotting, respectively. The differentiated NG108-15 cells exhibited neuron-like morphology. Twenty four hours after induction, there was a transient up-regulation of both COX-2 mRNA and protein level. There was a slight increase in COX-2 mRNA level on day 2 of NG108-15 cells, without a significant different in COX-2 protein level. COX-2 mRNA and protein in NG108-15 cells were a transient up-regulation after differentiation by Bt_2cAMP and TPA.

KEYWORDS : Alzheimer's disease, Cyclooxygenase 2 (COX-2), NG108-15 cells, Neurogenesis

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INTRODUCTION

AD is the most common cause of dementia among the elderly. Characteristics of AD are irreversible impairment of cognitive and memory functions and a physical deterioration.[1] The neuropathological features in AD are progressive deposits of β-amyloid plaques between nerve cells and neurofibrillary tangles within nerve cells, neuronal loss and inflammation of neurons.[2-3] The major component of amyloid plaques is amyloid β-peptide (Aβ) that derived from amyloid precursor protein (APP).[2] On the other hand, the role of APP was mediated neurite outgrowth promotion and was up-regulated during the development of nervous system.[4] However, the etiology of AD is complex and still largely unclear. Inflammatory mechanisms may, in part, mediate β-amyloid induced neuronal damage.

COX-2 (Cyclooxygenase 2) is one of the key enzymes involving in inflammation. It is transiently and rapidly induced in response to many inflammatory stimulus.[5] COX-2 mediates the synthesis of several pro-inflammatory mediators such prostaglandins PGG_2, PGH_2, PGI_2, PGD_2, PGE_2, $PGF_{2\alpha}$, and thromboxane A_2.[6] Up-regulation of COX-2 mRNA level was detected in Alzheimer's disease (AD) frontal cortex.[7] Autopsy of AD brain showed that COX-2 immunoreactivity could be detected between β-amyloid plaques in pyramidal neurons of the cerebral cortex and hippocampus.[8]

It has been reported that mouse neuroblastoma and rat glioma, NG108-15 cell line, exhibited many neuronal characteristics including APP production and neurite outgrowth.[9] NG108-15 cell line is a hybrid of mouse neuroblastoma (N18TG-2) and rat glioma (C6BU-1) cells.[10] This cell line has been employed as an *in vitro* model as these cells are able to release acetylcholine (ACh) and form cholinergic neuromuscular synapse.[11] There are some morphology differences between undifferentiated and differentiated NG108-15 cells was. Undifferentiated NG108-15 cells exhibited primary-cultured non-neuron like cells. The undifferentiated cells were flat, round in shape and displayed only a few neurites.[12] The deficiency of COX-2 expression was detected in original NG108-15 cells.[13] While differentiated NG108-15 cells showed neuron-like morphology and intercellular network formation, it has been reported that neurite outgrowth, APP production, and high levels of acetylcholine esterase (AChE) were detected in differentiated NG108-15 cells.[9, 11] Therefore, it is hypothesized that differentiation process may alter the COX-2 expression in NG108-15 cells.

The purpose of this study was to compare the levels of COX-2 mRNA and protein between undifferentiated and differentiated NG108-15 cells.

MATERIALS AND METHODS

Dibutyryl cAMP (Bt_2cAMP) and 12-*o*-tetradecanoyl-phorbol 13-acetate (TPA) were purchased from Sigma Chemical Co. (St Louis, Missouri, USA). All other chemicals were of the purest grade commercially available.

(i) *Cell culture*:
NG108-15 cells (ATCC® number HB-12317) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco BRL, Gaithersburg, Maryland, USA) supplemented with 2% HAT medium (10 mM hypoxanthine, 40 mM aminopterine, 1.6 mM thymidine; Gibco/Life Technologies, Eggenstein, Germany), and 4% fetal bovine serum (FBS) (Invitrogen, California, USA). Cells were incubated in a humidified incubator under an atmosphere containing 5% CO_2 at 37°C. The undifferentiated NG108-15 cells were cultured for 3 days.

(ii) *Differentiation induction*[14] :
To induce neuronal differentiation, culture medium was supplemented with 100 mM Bt_2cAMP and 100 μM TPA. The differentiated NG108-15 cells were cultured for 3 days.

(iii) *Reverse transcription polymerase chain reaction (RT-PCR)*[15] :

Total RNA was isolated from 2 x 10^6 cells RNA by adding 1 ml of Trizol® (Invitrogen, California, USA), followed by 0.5 ml of isopropanol to precipitate RNA, which was dissolved in 25 µl of DEPC-treated water. RNA concentration was measured based on absorbance at λ 260 nm. cDNA was synthesized using 2 µg of RNA, 200 U Superscript III (Invitrogen, California, USA) and 10 µM oligo-dT primer (Invitrogen, California, USA) in 20 µl of reaction mixture at 42°C for 60 min. PCR was carried out in a 10 µl of reaction mixture containing 1 µl of the first strand cDNA, 1 µM sense (5'-ATGTCTTCCAGCTCACGGTA-3' and antisense (5'-GAGCTACACAGTAGTCCTGA-3') COX-2 mRNA specific primers (Bio Basic Inc, Markham Ontario, Canada), 250 µM dNTPs, 2 U *Taq* DNA polymerase (Promega, Madison, USA), and 2.5 mM $MgCl_2$. COX-2 primers were search from PubMed (National center for biotechnology information, Maryland, USA) accession number S67722. Thermocycling was performed in GeneAmp® PCR System 9700 (Applied BioSystems, Foster, USA) as follows: 94°C for 10 min; 35 cycles of 94°C for 1 min, 56°C for 2 min, and 72°C for 2 min; and a final step of 72°C for 10 min. Amplicons (249 bp) were separated by electrophoresis in 6% polyacrylamide gel, stained with ethidium bromide and quantified using a gel documentation system (Quantity One Version 4.4; BIO-RAD, California, USA) To normalize gel loading, glyceraldehydes-3-phosphate dehydrogenase (GAPDH) mRNA was RT-PCR amplified (247 bp amplicon) using GAPDH-specific primers (5'-CAAGTTCAACGGCACAGTCA-3 and 5'-GGTTCACACCCATCACAAAC-3'). GAPDH primers were search from PubMed (National center for biotechnology information, Maryland, USA) accession number BC059110. The amplification reaction details were similar as previously mentioned for 18 cycles.

(iv) *Western blotting:*

After culture, the undifferentiated and differentiated NG108-15 10^6 cells were homogenized in ice-cold RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40 ([Octylphenoxy] Polyethoxyethanol), 0.1% SDS, 1% Sodium deoxycholate), incubated on ice for 30 min and then centrifuged at 21,255 g for 15 min at 4°C. Protein concentration was determined using Quant-iT™ protein assay kit (Invitrogen, California, USA). Protein samples then were heated at 100°C for 5 min and centrifuged at 15,616 g for 2 min. Samples (20 µg) were subjected to 10% SDS-PAGE, followed by protein transfer to PVDF membrane (Amersham Biosciences, New Jersey, USA). The membranes were blocked for non-specific in 5% milk in TBST before incubating with primary rabbit anti-mouse COX-2 polyclonal antibodies (Millipore corporation, Massachusetts, USA) at concentration of 1:300 for 3 hours at 25°C. The membranes were incubated with secondary goat anti-rabbit IgG antibodies (Santa Cruz Biotechnology, Inc., California, USA) at concentration of 1:5,000 for 1 hour at 25°C. To normalize the protein loading, membrane also was processed for immuno-detection of GAPDH as described above. Mouse anti-GAPDH monoclonal antibody (Millipore corporation, Massachusetts, USA) was obtained. Protein bands were detected using ECL method (Amersham Biosciences, New Jersey, USA) according to manufacturer's instructions and quantified using ImageJ system (Softpedia, Bucharest, Romania). Data are normalized are presented as mean ± SEM. Statistical ANOVA followed by a post-hoc LSD test and unpaired t-test were employed, with statistical significance when $p < 0.05$.

RESULTS

1. Morphological differences between undifferentiated and differentiated NG108-15 cells:

Undifferentiated NG108-15 cells were flat, round in shape and displayed only a few neurites (Figure 1 A, B and C). Upon exposure to 100 mM Bt_2cAMP and 100 µM TPA, cells underwent differentiation as manifested by presence of neuron-like morphology as shown by the extended neurites with abundant varicosities (Figure 1 D, E and F). The number of neurites increased rapidly and reached a maximum at day 3 post-exposure.

Morphology of undifferentiated and differentiated NG108-15 cells.

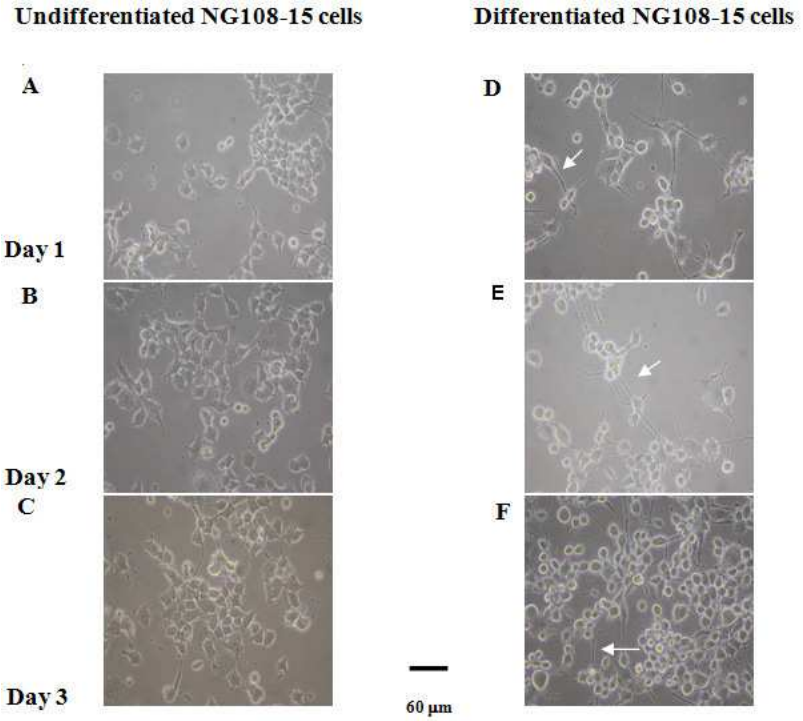

Figure 1

Morphology of undifferentiated and differentiated NG108-15 cells. Neurite outgrowth (arrow) is more obvious in differentiated cells than undifferentiated cells. Network formation is more pronounced in differentiated cells at days 2 and 3. Scale bar = 60 µm.

2. COX-2 expression in undifferentiated and differentiated NG108-15 cells:

The level of COX-2 expression was determined in both transcriptional and translational levels. The COX-2 mRNA and protein level, in both undifferentiated and differentiated NG108-15 cells, at day 1, 2 and 3, was determined. The level of COX-2 mRNA expression in differentiated NG108-15 cells, at 24 hours after exposure to Bt_2cAMP and TPA, was significantly higher than (1.6 fold) that of undifferentiated cell. The expression levels of COX-2 mRNA in undifferentiated NG108-15 cells increased slightly (1.2 fold) on day 2 of differentiation induction, before decreasing to the basal level (day 1) when subjected to differentiation induction at day 3 (Figure 2). In contrast, the COX-2 mRNA level in differentiated NG108-15 cells, upon chemical induction, continually decreased from day 1 to day 3. The reduction of COX-2 mRNA expression slightly decreased from day 1 to day 2, but dramatically decreased on day 3 (Figure 2).

COX-2 mRNA expression level in undifferentiated and differentiated NG108-15 cells.

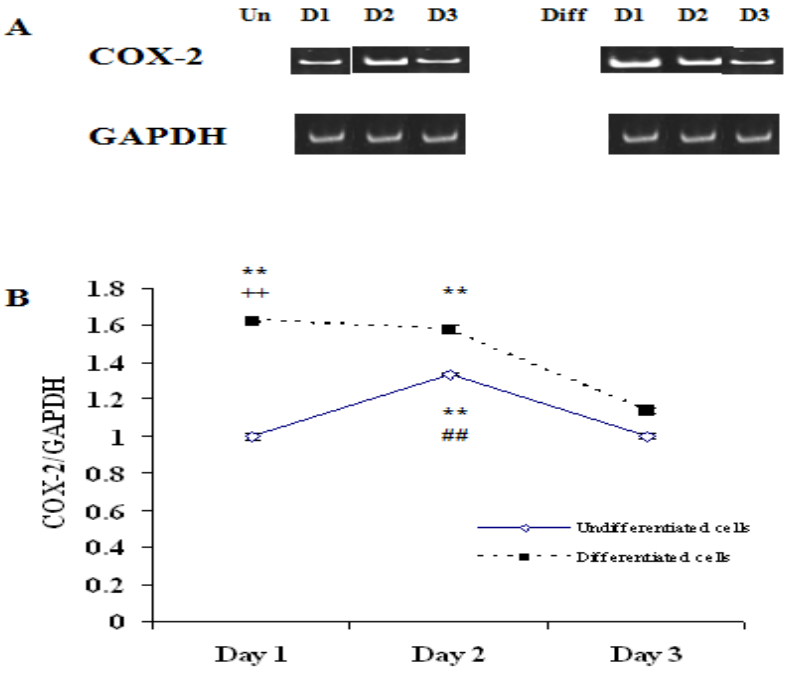

Figure 2

COX-2 mRNA expression level in undifferentiated and differentiated NG108-15 cells. Panel A, PAGE. Panel B, mRNA level of COX-2 relative to that of GAPDH; results are expressed as mean ± SEM of 3 separate experiments. ++ p < 0.01, compared with undifferentiated cells on the same day. ** p < 0.01, ## p < 0.01, compared with undifferentiated cells at day 1. Un, undifferentiated cells; Diff, differentiated cells; D1, day 1; D2, day 2; D3, day 3.

COX-2 protein of undifferentiated and differentiated NG108-15 cells.

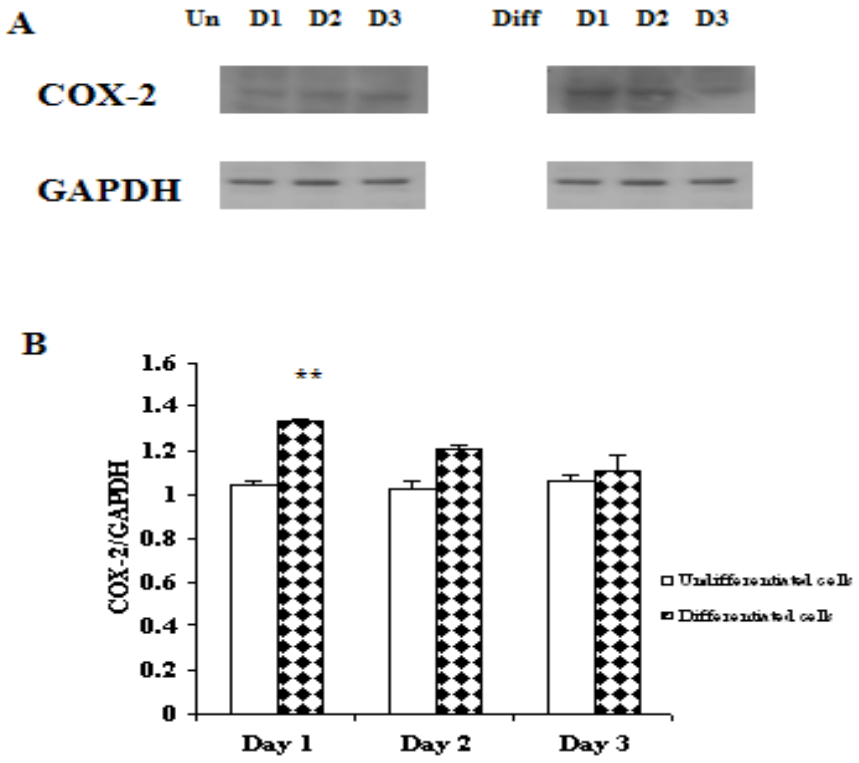

Figure 3

COX-2 content of undifferentiated and differentiated NG108-15 cells. Panel A, immunoreactive bands. Panel B, COX-2 band intensity relative to GAPDH; results are expressed as mean ± SEM of 3 separate experiments. ** p < 0.01, compared with undifferentiated cells on the same day. Un, undifferentiated; Diff, differentiated; D1, day 1; D2, day 2; D3, day 3.

The COX-2 protein expression, between undifferentiated and differentiated NG108-15 cells, was measured in similar experimental design that performed to determine mRNA expression. In undifferentiated cells, the level of COX-2 protein was not altered the 3-day period (Figure 3). However, the level of COX-2 protein in differentiated cells found significantly decreased in day 3. The COX-2 protein level between undifferentiated cells, on day 1, was significantly higher than that of in differentiated cells. However, the protein expression in these 2 cell types, in day 2 and day 3, was similar.

DISCUSSION

The morphology of undifferentiated NG108-15 cells showed flat, round in shape, resembling primary-cultured non-neuron-like cells. After differentiation by Bt_2cAMP and TPA, the cells exhibited neuron-like cells and synaptic formation. Bt_2cAMP, a cell-penetrating form of cAMP, and TPA are known agents that increase intracellular calcium ion content and activate protein kinase C, resulting in neuronal cell differentiation, as seen as neurite outgrowth and network formation,[14] as was observed in this study with NG108-15 cells. It has been reported that cAMP-induced neuritogenesis was dependent on MAPK/ERK kinase activity.[16] The characteristic of differentiated NG108-15 cells have been demonstrated in previous study such as formation of cholinergic synapse with cultured myotubes[17], presence of a wide range of voltage-dependent membrane currents[18], the high expression of choline acetyl transferase (CAT) activity[19], and the high expression of AChE and serotonin mRNAs[20-21]. However, there was no comparative information of COX-2 expression of AD related genes between undifferentiated and differentiated NG108-15 cells.

In this study, expression level of COX-2 mRNA and protein between undifferentiated and differentiated NG108-15 cells was not the same direction. Induction of neuritogenesis of NG108-15 cells by Bt_2cAMP and TPA resulted in a transient up-regulation of COX-2 mRNA level, together with a concomitant elevation in COX-2 immuno-reactive protein during the 3-day study period. It has been demonstrated that TPA induces COX-2 gene expression through ERK and nuclear factor-kappa B (NF-kB) activation in a mouse skin model.[22-23] Therefore, the up-regulation of COX-2 mRNA in differentiated cells may result from the TPA-activated ERK pathway-regulated NF-kB signaling cascade and NF-kB then binds to a specific binding site in the COX-2 promoter, resulting in the increase of COX-2 mRNA expression. The COX-2 protein up-regulation was in concordance with the mRNA level. However, expression of COX-2 mRNA in undifferentiated NG108-15 cells slightly increased on day 2 but COX-2 protein was unchanged. Nakanishi *et al.* demonstrated that COX-2 inhibitors suppressed the proliferation and differentiation of leukaemia cells both via COX-2 dependent and -independent pathway.[24] It is hypothesized that the up-regulation of COX-2 mRNA in undifferentiated cells at day 2 may relate to proliferation.

The up-regulation of COX-2 mRNA and protein in differentiated cells on day 1 was important in mediating inflammation response. Kim *et al.* reported that expression of COX-2 mRNA and protein peaked at 2 and 4 hr after stimulation with TPA in human breast epithelial cell line.[25] Induction of COX-2 expression may associate with activation of the NF-kB and MAPKs. The link between COX-2 and AD pathology has been demonstrated in previous study. The COX-2 mRNA up-regulation was found in AD frontal cortex.[3] The COX-2 immunoreactivity was prominent between senile plaques at cerebral cortex and hippocampus in autopsied AD brains.[4] Therefore, up-regulation levels of COX-2 mRNA and protein in differentiated cells on day 1 may be the same condition as early-stage of AD.

The down-regulation of COX-2 mRNA in differentiated cells on day 3 indicated that TPA induced inflammation response was decreased. A number of reports demonstrated that the use of non-steroid anti-inflammatory drugs (NSAIDs) reduced the risk of developing of AD.[26-27]

Moreover indomethacin is able to suppress the production of the secreted form of APP and Aβ. However, several clinical trials reported that the therapeutic effects of NSAIDs in Alzheimer's disease have largely failed.[28] The clinicians suggested that the lack of success may be using NSAIDs with late-stage Alzheimer's disease, wherein advanced neurodegeneration may be refractory to anti-inflammatory drug treatment. Therefore, the expression of COX-2 mRNA in differentiated cells on day 3 may not suit for selective COX-2 inhibitory agents in AD. However, this study was investigated the expression of COX-2 *in vitro* model by NG108-15 cells. Further investigations *in vivo* model are necessary to be clarify.

CONCLUSION

Induction of differentiation of NG108-15 cells by a combination of Bt_2cAMP and TPA treatment over a period of 3 days resulted in a transient elevation of COX-2 mRNA and protein expression levels. This *in vitro* model may be useful as a primary screening tool for potential COX-2 inhibitors that may have potential as AD therapeutic agents.

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