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Valproic acid promotes mature neuronal differentiation of adipose tissue-derived stem cells through iNOS–NO–sGC signaling pathway

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ABSTRACT

Valproic acid (VPA) remarkably promotes the differentiation of adipose tissue-derived stem cells (ASCs) to mature neuronal cells, enabling neuronal induction within only three days. Here, we investigated the involvement of NO-signaling in the VPA-promoted neuronal differentiation of ASCs as a possible mechanism. Cultured rat ASCs were differentiated to matured neuronal cells rich in dendrites and expressing βIII-tubulin protein, a neuronal marker, by treatments with VPA at 2 mM for 3 days and subsequently with the neuronal induction medium (NIM) containing cAMP-elevating agents for 2 h. Increased intracellular NO was detected in neuronal cells differentiated from ASCs treated with VPA by a fluorescence NO-specific probe, diaminofluorescein-FM diacetate. However, a NO donor (NOC18) increased the incidence of neuronal cells only to a lesser extent than VPA, indicating the insufficiency of exogenous NO. RT-PCR analysis of ASCs treated with VPA showed increased mRNA expression of inducible nitric oxide synthase (*iNOS*) with the acetylation of its associated histone H3K9. iNOS inhibitors (1400 W and dexamethasone) or a soluble guanylate cyclase (sGC) inhibitor (ODQ) decreased the incidence of neuronal cells differentiated from ASCs treated with VPA. These inhibitors also decreased the mRNA expression of mature neuronal markers, neurofilament medium polypeptide (*NeFM*) and microtubule-associated protein 2 (*MAP2*), as well as βIII-tubulin (*TUBB3*), to various extents. It was considered from these results that VPA promoted mature neuronal differentiation of ASCs through the iNOS–NO–sGC signaling pathway. This provided insights into the regulated neuronal differentiation of ASCs in clinical applications.

1. Introduction

It has been indicated that nitric oxide (NO), endogenously produced by nitric oxide synthase (NOS), participates in diverse biological phenomena including neuronal differentiation as a signaling molecule. For example, inhibition of NOS reduced differentiation of stem cells to neurons and increased differentiation to non-neuronal cells in *in vivo* developing brain [1] and cultured neural stem cells [2]. There are three major isoforms of NOS, i.e., neuronal (nNOS or NOS1), inducible (iNOS or NOS2), and endothelial (eNOS or NOS3). These isoforms were originally named according to their tissue expression and inducibility, but all of them are constitutively expressed in various tissues.

Adipose tissue-derived stem cells (ASCs) are multipotent cells that can differentiate into neurogenic linage cells, being isolated from the subcutaneous adipose tissues in humans [3], dogs [4], mice [5] and rats [6,7]. ASCs have an advantage over stem cells derived from other tissues in that they can be prepared in large amounts with less injury to their donors by liposuction [8]. It is therefore expected that neuronal cells differentiated from ASCs are practically applicable in regenerative therapy of neurological disorder.

We previously showed that valproic acid (VPA) significantly promoted the mature neuronal differentiation of rat ASCs when evaluated by the expression of neuronal markers and dendrites [7]. The treatment of ASCs with VPA (2 mM) for only 3 days and with subsequent neuronal induction medium (NIM) for 2 h, increased the incidence of ASCs expressing the mRNAs of mature neuronal markers, neurofilament medium polypeptide (*NeFM*) and microtubule-associated protein 2 (*MAP2*), to as high as about 80%. This VPA-promoted neuronal differentiation was characterized in that the neuronal cells expressing βIII-tubulin, an immature neuronal marker, at both protein and mRNA levels as well as the mature neuronal markers.

In the present study, we investigated the involvement of NO-signaling in the VPA-promoted neuronal differentiation of ASCs as a possible mechanism. The presence of intracellular NO and effects of NO

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signaling inhibitors were examined in rat ASCs treated with VPA.

2. Material and methods

2.1. Isolation and culture of ASCs

ASCs were isolated from subcutaneous adipose tissues of the inguinal region of 8- or 9-weeks old Wistar rats (Crlj: WI, Charles River Japan, Kanagawa, Japan) as described previously [7]. The isolated ASCs were used after two passages of subculture in the growth medium, i.e., Dulbecco's modified Eagle's medium (DMEM, Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 10% newborn bovine serum (NBS, Invitrogen, Carlsbad, CA, U.S.A.). All animal experiments in the present study were carried out according to the guideline of the Committee for Animal Experimentation at Azabu University.

2.2. Induction of neuronal differentiation of ASCs

ASCs were differentiated into neuronal cells as described previously [7]. The subcultured ASCs were seeded on non-coated 35-mm plastic dish at a density of 1×10^4 cells/dish (TR4000, NIPPON Genetics Co., Ltd., Tokyo, Japan), and were cultured in the growth medium for the treatment with 2 mM VPA (Wako Pure Chemical Industries, Osaka, Japan) for 3 days. After the VPA treatment, ASCs were incubated for 2 h in the NIM, i.e., DMEM supplemented with 100 μM dibutyryl cyclic adenosine monophosphate (dbcAMP, Wako Pure Chemical Industries), and 125 μM isobutyl methyl-xanthine (IBMX, Wako Pure Chemical Industries). Neuronal differentiation was evaluated by immunocytochemistry and RT-PCR analysis of neuronal markers. In some experiments, *N*-(3-(aminomethyl)benzyl) acetamidine (1400 W, 10 μM) or dexamethasone (DXA, 5 μM) as NOS inhibitors, NOC18 (100 μM) as an NO donor, or 1H-[1, 2, 4] oxadiazolo [4, 3-a] quinoxalin-1-one (ODQ, 20 μM) as a soluble guanylate cyclase (sGC) inhibitor, all of which were purchased from Wako Pure Chemical Industries, were added to the culture medium during treatments with or without VPA. All the chemicals added to the culture medium were dissolved in dimethyl sulfoxide at a final concentration of 0.1%.

2.3. Immunocytochemistry

Cells were fixed with 3.7% (w/v) formaldehyde in PBS for 15 min at room temperature (RT). After permeabilized with 0.2% Triton X-100 for 10 min, the cells were incubated with a mouse *anti*-βIII-tubulin antibody (1:200; ab74978, Abcam Plc., Cambridge, U.K.) and a rabbit *anti*-iNOS antibody (1: 100; sc-650, Santa Cruz Biotechnology, Santa Cruz, Dallas, TX, U.S.A.) for 1 h at RT. After washed with PBS, the cells were incubated with secondary antibodies (FITC-conjugated goat anti-mouse IgG and Cy3-conjugated goat anti-rabbit IgG, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, U.S.A.) for 30 min at RT. The cells were then washed with PBS and counterstained with 4', 6-diamidino-2-phenylindole (DAPI, ImmunoBioSience Corp, Mukilteo, WA, U.S.A.) for nuclear staining before fluorescence microscopic observation. In randomly selected views, total cells, βIII-tubulin positive, iNOS positive or double positive cells were counted manually in at least 300 cells per dish.

2.4. Detection of intracellular NO

Intracellular NO was detected with a green fluorescence NO-specific molecular probe, Diaminofluorescein-FM diacetate (DAF-FM, Goryo Chemical Inc., Sapporo, Japan). ASCs were seeded on glass coverslips in culture dishes, and were cultured with or without VPA for three days. After the culture, the cells were incubated with DAF-FM in serum-free DMEM for 60 min at 37 °C. NO in the ASCs was detected by inverted fluorescence microscopy at 515 nm with the Olympus BX60 microscope (Olympus Corp., Tokyo, Japan).

Table 1
Primers used in the polymerase chain reaction (PCR) analysis.

Gene		Primer sequence	Product length (base pair)
TUBB3[a]	Forward	5′-GGCCTCCTCTCACAAGTATGT-3′	167
	Reverse	5′-CGCCCTCTGTATAGTGC-3′	
NeFM[a]	Forward	5′-AGGAGCGCCACAACCACGACC-3′	156
	Reverse	5′-ATGCGGCGATCTCGATGTCCA-3′	
MAP2[a]	Forward	5′-ACCTTCCTCCATCCTCCCTC-3′	151
	Reverse	5′-AGTAGGTGTTGAGGTGCCGC-3′	
HPRT[a, b]	Forward	5′-AATGTCTGTTGCTGCGTC-3′	92
	Reverse	5′-TGTCTGTCTACAAGGGAAG-3′	
nNOS[a]	Forward	5′-CCCGTTTCCTCAAGGTCAAG-3′	266
	Reverse	5′-CTTGTTCACCTCCTCCAGCC-3′	
iNOS[a]	Forward	5′-AAGCCCCGCTACTACTCCAT-3′	475
	Reverse	5′-TCTGCAGGATGTCTTGAACG-3′	
eNOS[a]	Forward	5′-GGACCCACTGGTATCCTCTTGG-3′	312
	Reverse	5′-ATATCTCGGGCAGCAGCCTTGG-3′	
iNOS[b]	Forward	5′-CACCACCCTCCTTGTTCAAC-3′	132
	Reverse	5′-CAATCCACAACTCGCTCCAA-3	
iNOS[c]	Forward	5′-GTCCATCGCGAATGAGCTA-3′	100
	Reverse	5′-TATACCCATCCACGCTCTGC-3′	

Genes are listed in the order of appearance in the experimental results.
[a] Reverse transcription (RT)-PCR.
[b] real-time RT-PCR.
[c] PCR in the chromatin immunoprecipitation assy. *iNOS*, inducible nitric oxide synthase; *nNOS*, neuronal nitric oxide synthase, *eNOS*, endothelial nitric oxide synthase; *TUBB3*, βIII-tubulin; *NeFM*, neurofilament medium polypeptide; *MAP2*, microtubule-associated protein 2; *HPRT*, hypoxanthine phosphoribosyltransferase.

2.5. Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted with the IOSGEN (Nippon Gene Co., Ltd., Tokyo, Japan), and was reverse-transcribed for single-strand cDNA with oligo (dT) primer and the Superscript III reverse transcriptase (Invitrogen). PCR was performed with the Taq DNA polymerase (KAPA Biosystems, Woburn, MA, U.S.A.). Each PCR cycle was: denaturation at 98 °C for 10 s, annealing at 58–60 °C for 30 s, and elongation at 72 °C for 30 s. Semi-quantitative PCR was performed for *iNOS* at 28 cycles and for *HPRT* at 31 cycles. The reaction products were electrophoresed on a 2.0% agarose gel and visualized with ethidium bromide. Real-time RT-PCR of the mRNAs for *iNOS* and hypoxanthine phosphoribosyl transferase (*HPRT*) was performed using the ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, U.S.A.), and the results were analyzed using the ABI PRISM 7500 Dissociation Curve Software v 1.0 (Applied Biosystems). The relative amount of mRNAs was normalized to that of *HPRT*, a housekeeping gene. Primers listed in Table 1 were purchased from Fasmac Co., Ltd. (Atsugi, Japan).

2.6. Chromatin immunoprecipitation (ChIP) assay

DNA/protein fragments were prepared from the ASCs treated with VPA, and were immunoprecipitated by the cross-linked ChIP [9] using the ChIP Reagents (Nippon Gene Co., Ltd., Tokyo, Japan) with an anti-acetylated histone H3K9 antibody (NB21-1074, Novus Biologicals, Littleton, CO, U.S.A.) or a control IgG (Santa Cruz Biotechnology Inc., CA, U.S.A.) according to the manufacturer's protocol. DNA was extracted from the immunoprecipitate, and *iNOS* gene was amplified by PCR with a primer in Table 1 as described above.

2.7. Statistical analysis

Results are expressed as mean ± S.E. Statistical significance of the differences between the experimental groups was examined by the one-way analysis of variance with the Tukey-Kramer multiple comparison test at a probability level of 5%.

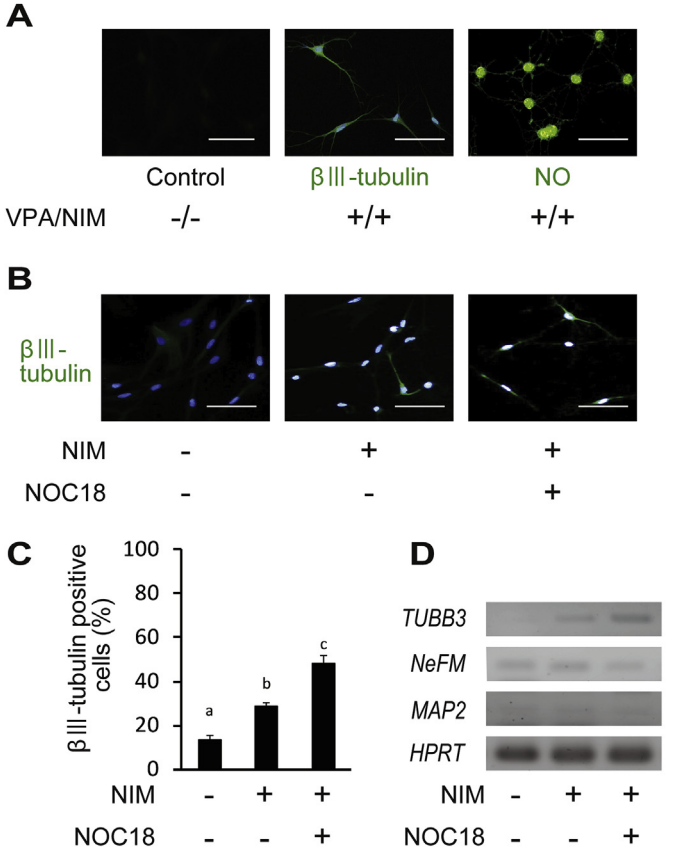

Fig. 1. Detection of intracellular nitric oxide (NO) and effects of exogenous NO on the neuronal differentiation of adipose tissue-derived stem cells (ASCs). Neuronal differentiation of cultured rat ASCs was induced by the treatment with VPA (2 mM) for 3 days and then with NIM for 2 h. (A) Detection of NO in neuronal cells differentiated from ASCs. βIII-tubulin and NO were detected as green fluorescence by immunocytochemistry and by an NO-specific probe, diaminofluorescein-FM diacetate, respectively. Scale bar = 100 μm. (B, C, D) Effects of exogenous NO on the neuronal differentiation of ASCs. Cultured rat ASCs were treated with NOC18 instead of VPA for 3 days and then treated with NIM for 2 h. (B) Detection of βIII-tubulin in differentiated ASCs as green by immunocytochemistry. The nucleus was stained as blue with 4′,6-diamidino-2-phenylindole. Scale bar = 100 μm. (C) Incidence of βIII-tubulin positive cells differentiated from ASCs as detected by immunocytochemistry. Mean ± S.E. of four experiments is shown. Values without any common alphabetical letters are significantly different from each other at $p < 0.05$. (D) mRNA expression of neuronal markers in differentiated ASCs. The mRNAs of *TUBB3*, *NeFM*, *MAP2* and *HPRT* were detected by RT-PCR. *TUBB3*, βIII-tubulin; *NeFM*, neurofilament medium polypeptide; *MAP2*, microtubule-associated protein 2; *HPRT*, hypoxanthine phosphoribosyltransferase.

3. Results

3.1. Detection of intracellular NO and effects of exogenous NO on the neuronal differentiation of ASCs

The VPA and NIM treatment induced the differentiation of ASCs into mature neuronal cells rich in dendrites and expressing βIII-tubulin protein, a neuronal marker (Fig. 1A), as previously reported [7]. The same treatment increased intracellular NO, which suggested the involvement of NO in the neuronal differentiation of ASCs (Fig. 1A).

On the basis of the above results, the effects of NO on the neuronal differentiation of ASCs were examined by using a slow NO donor, NOC18 [10,11], instead of VPA. The addition of NOC18 to the culture medium for 3 days prior to the NIM treatment increased βIII-tubulin and induced much less branched dendrites, while the NIM treatment alone increased βIII-tubulin to a lesser extent (Fig. 1B). NOC18 increased the incidence of βIII-tubulin positive cells to about 50% (Fig. 1C), which was higher than that by the NIM treatment alone but was lower than that (about 86%) by the VPA and NIM treatment in the previous study [7]. The NIM and NOC18 treatment increased the expression of *TUBB3* (Fig. 1D), but not *NeFM* and *MAP2,* whose expression had been increased in the neuronal cells differentiated from ASCs treated with VPA [7]. These results indicate that exogenous NO is insufficient to promote the mature neuronal differentiation of ASCs.

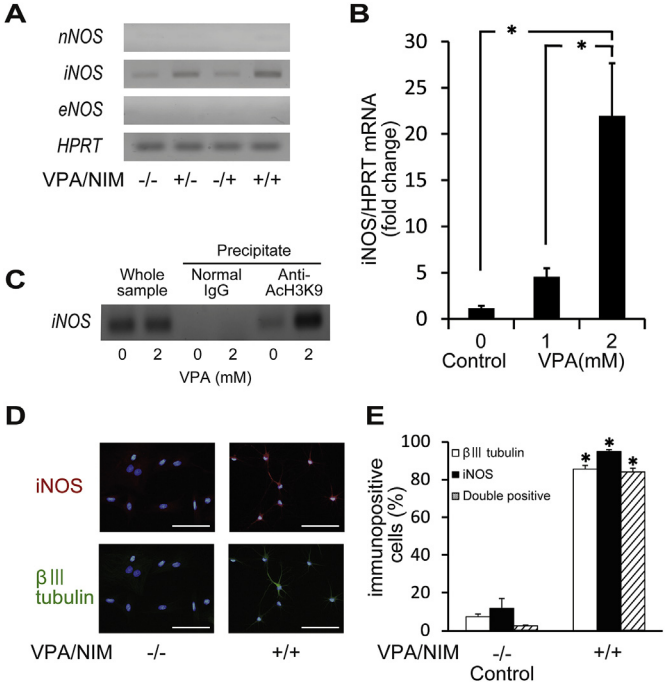

Fig. 2. Expression of NOS mRNA in neuronal differentiation of ASCs treated with VPA. Neuronal differentiation of cultured rat ASCs was induced by the treatment with VPA (2 mM) for 3 days and then with NIM for 2 h. (A) mRNA expression of NOSs in ASCs with or without neuronal induction by VPA and NIM. The mRNAs of *nNOS*, *iNOS*, *eNOS* and *HPRT* were detected by reverse transcription-polymerase chain reaction (RT-PCR). (B) Effects of VPA on the expression of *iNOS* mRNA in ASCs before the treatment with NIM. mRNA was quantified by real-time RT-PCR. Mean ± S.E. of four experiments is shown. (C) Chromatin immunoprecipitation (ChIP) assay of acetylated histone H3K9 associated with *iNOS*. Acetylated histone H3K9 was precipitated with specific antibody, and associated *iNOS* gene was detected by PCR. (D) Detection of iNOS (red) and βIII-tubulin (green) in differentiated ASCs by immunocytochemistry. The nucleus was stained as blue with 4′, 6-diamidino-2-phenylindole. Scale bar = 100 μm. (E) Incidence of βIII-tubulin positive and iNOS positive cells differentiated from ASCs as detected by immunocytochemistry. Mean ± S.E. of four experiments is shown. Asterisks indicate significant differences from the control at $p < 0.05$. *nNOS*, neuronal NOS; *iNOS*, inducible NOS; *eNOS*, endothelial NOS. Other abbreviations are the same to Fig. 1.

3.2. Expression of NOS mRNA in neuronal differentiation of ASCs treated with VPA

Only *iNOS* mRNA, not *nNOS* nor *eNOS* mRNAs, was detected in the ASCs treated with VPA when examined by RT-PCR, (Fig. 2A). The VPA treatment greatly increased the amount of *iNOS* mRNA at 2 mM in a concentration-dependent manner (Fig. 2B). The ChIP assay with RT-PCR showed that the VPA treatment caused acetylation of histone H3 at the K9 region associated with the *iNOS* gene, suggesting the increased expression of *iNOS* mRNA at the transcription level through histone acetylation (Fig. 2C).

iNOS was detected also at the protein level by immunocytochemistry in the neuronal cells expressing βIII-tubulin protein (Fig. 2D). In addition, the VPA and NIM treatment greatly increased the

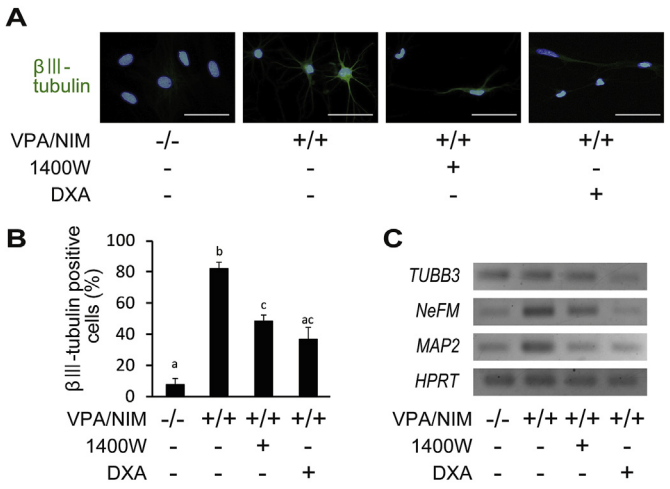

Fig. 3. Effects of iNOS inhibitors on the neuronal differentiation of ASCs treated with VPA and NIM. Neuronal differentiation of cultured rat ASCs was induced by the treatment with VPA (2 mM) for 3 days and then with NIM for 2 h. iNOS inhibitors, 1400 W (10 μM) and dexamethasone (DXA, 5 μM), were added to the culture medium during the VPA treatment. (A) Detection of βIII-tubulin in differentiated ASCs as green by immunocytochemistry. The nucleus was stained as blue with 4′, 6-diamidino-2-phenylindole. Scale bar = 100 μm. (B) Incidence of βIII-tubulin positive cells differentiated from ASCs as detected by immunocytochemistry. Mean ± S.E. of four experiments is shown. Values without any common alphabetical letters are significantly different from each other at $p < 0.05$. (C) mRNA expression of neuronal markers in differentiated ASCs. The mRNAs of *TUBB3*, *NeFM*, *MAP2* and *HPRT* were detected by RT-PCR. The abbreviations are the same to Fig. 1.

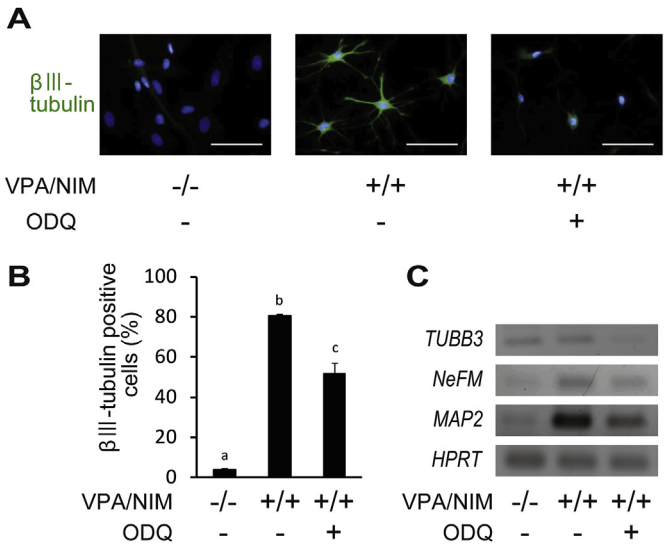

Fig. 4. Effects of a soluble guanylate cyclase (sGC) inhibitor on the neuronal differentiation of ASCs treated with VPA and NIM. Neuronal differentiation of cultured rat ASCs was induced by the treatment with VPA (2 mM) for 3 days and then with NIM for 2 h. An sGC inhibitor, 1H-[1, 2, 4] oxadiazolo [4, 3-a] quinoxalin-1-one (ODQ, 20 μM), was added to the culture medium during the VPA treatment. (A) Detection of βIII-tubulin in differentiated ASCs as green by immunocytochemistry. The nucleus was stained as blue with 4′, 6-diamidino-2-phenylindole. Scale bar = 100 μm. (B) Incidence of βIII-tubulin positive cells differentiated from ASCs as detected by immunocytochemistry. Mean ± S.E. of three or four experiments is shown. Values without any common alphabetical letters are significantly different from each other at $p < 0.05$. (C) mRNA expression of neuronal markers in differentiated ASCs. The mRNAs of *TUBB3*, *NeFM*, *MAP2* and *HPRT* were detected by RT-PCR. The abbreviations are the same to Fig. 1.

incidence of βIII-tubulin positive ASCs more than 10-fold, almost all of which are also iNOS positive, confirming the coexpression of iNOS and βIII-tubulin proteins in the neuronal cells (Fig. 2E). It was thus supposed that NO was produced by iNOS, whose expression was increased through the acetylation of histone H3, in the neuronal differentiation of ASCs by the VPA and NIM treatment.

3.3. Effects of iNOS inhibitors on the neuronal differentiation of ASCs treated with VPA and NIM

The involvement of iNOS in the neuronal differentiation of ASCs was examined by using two different types of iNOS inhibitor, i.e., 1400 W, a competitive iNOS inhibitor [12], and DXA, an iNOS mRNA destabilizer [13]. The addition of either 1400 W or DXA to the culture medium during the VPA treatment induced the neuronal differentiation with the appearance of immature neuronal cells, which was poorly dendritic with decreased βIII-tubulin protein (Fig. 3A). 1400 W or DXA decreased βIII-tubulin positive cells to about half or lower of that in the ASCs treated with VPA and NIM, confirming the reduced neuronal differentiation quantitatively (Fig. 3B). It was considered from these results that iNOS was involved in the mature neuronal differentiation of ASCs treated with VPA.

The mRNAs of all the neuronal markers examined, i.e., *TUBB3*, *NeFM* and *MAP2*, were increased in the ASCs treated with VPA and NIM compared to those in non-treated ASCs (Fig. 3C). All of these mRNAs were decreased by 1400 W in the ASCs treated with VPA and NIM, indicating the involvement of NO produced by iNOS in their expression (Fig. 3C). On the other hand, DXA more severely decreased the expression of all the neuronal markers, in particular *TUBB3* and *NeFM*, suggesting the involvement of iNOS functions other than NO production in the expression of the neuronal markers (Fig. 3C). It was noted that there was no correlation between the incidence of βIII-tubulin positive cells and *TUBB3* mRNA expression through the experimental groups. It was therefore considered that *TUBB3* mRNA did not necessarily serve as a neuronal marker of differentiated ASCs.

3.4. Effects of an sGC inhibitor on the neuronal differentiation of ASCs treated with VPA and NIM

The involvement of sGC signaling in the neuronal differentiation of ASCs was examined by using an sGC inhibitor, ODQ that potently and selectively inhibited NO-stimulated guanylate cyclase activity in this signaling [14]. The addition of ODQ to the culture medium during the VPA treatment decreased βIII-tubulin, and induced only few dendrites, suggesting the involvement of sGC signaling in the neuronal differentiation (Fig. 4A). ODQ decreased βIII-tubulin positive cells to about one-third of that in the ASCs treated with VPA and NIM, confirming the reduced neuronal differentiation quantitatively (Fig. 4B). In addition, ODQ decreased the expression of all the neuronal markers examined, suggesting again the involvement of sGC signaling in the neuronal differentiation of ASCs treated with VPA and NIM (Fig. 4C).

4. Discussion

In the present study, we showed that VPA promoted mature neuronal differentiation of ASCs through the iNOS–NO–sGC signaling pathway; the VPA treatment induced iNOS, but not nNOS or eNOS, through the acetylation of histone H3K9, and inhibitors of iNOS and sGC reduced the mature neuronal differentiation. It is considered from the present results that the activated iNOS–NO–sGC signaling pathway increased the expression of neuronal genes, *TUBB3*, *NeFM*, and *MAP2*, with which the neuronal differentiation was triggered by the NIM treatment, because IBMX, a cAMP-elevating ingredient of the NIM, alone can induce the neuronal differentiation of ASCs after a longer treatment period [6].

It is intriguing that iNOS, but not nNOS, is involved in mature neuronal differentiation of ASCs. This is because iNOS is essential for mature neurogenesis under pathological conditions [15,16]. On the

other hand, nNOS is involved in immature neuronal differentiation during development [17]. The present findings may indicate that the mature neuronal differentiation of ASCs is a part of neuronal repair mechanisms in adults, which is reasonable for the application in regenerative therapy of neurological disorder.

There was no correlation between the incidence of βIII-tubulin positive cells and the *TUBB3* expression in ASCs treated with the NO inhibitors or donor in the present study. This is possibly due to the intensified immunofluorescence of tubulin during the polymerization to form dendrites in the βIII-tubulin positive cells, which is irrespective of the *TUBB3* mRNA expression changes, but is critical for neuronal differentiation. In this context, βIII-tubulin should be regarded as inducible tubulin, which is often expressed in various tissues in toxic microenvironments such as hypoxia and poor nutrient supply [18-20], rather than as an immature neuronal marker. This notion is supported by the co-expression with mature neuronal markers and the mature neuronal appearance of differentiated ASCs in the present study.

The present results provided insights into the regulation of VPA-promoted neuronal differentiation of ASCs in its clinical application. For example, the neuronal differentiation of ASCs could be regulated step by step by using modifiers in the iNOS–NO–sGC signaling pathway. On the basis of the interaction between cAMP and cGMP in the dendrite formation [21], mature neuronal differentiation of ASCs could be regulated by the treatment with NIM, which includes a cAMP-elevating agent as an active ingredient.

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