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Hydrogen sulfide potently promotes neuronal differentiation of adipose tissue-derived stem cells involving nitric oxide-mediated signaling cascade with the aid of cAMP-elevating agents

Shinri Fujimoto, Azusa Satoh, Takehito Suzuki, Yoko Miyazaki, Kazuaki Tanaka, Makoto Usami, Tatsuya Takizawa *

Graduate School of Veterinary Medicine, Azabu University, Fuchinobe, Chuo-ku, Sagamihara, 252–5201, Japan

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ABSTRACT

Neuronal differentiation of adipose tissue-derived stem cells (ASCs) is potently promoted by valproic acid (VPA) through a gaseous signaling molecule, nitric oxide (NO). Here, we investigated the involvement of hydrogen sulfide (H_2S), another gaseous signaling molecule, in neuronal differentiation of ASCs. VPA-promoted neuronal differentiation of ASCs was accompanied by increased intracellular H_2S and sulfane sulfur with increased mRNA expression of enzymes synthesizing sulfane sulfur including cystathionine β-synthase (CBS), of which inhibition reduced the differentiation efficiency. H_2S donors, GYY4137 (GYY) or NaHS, potently promoted neuronal differentiation of ASCs when cAMP-elevating agents, dibutyryl cyclic adenosine monophosphate and isobutyl methyl-xanthine, were added as neuronal induction medium (NIM). Neuronal differentiation of ASCs promoted by NaHS or GYY was accompanied by Ca^{2+} entry and increased mRNA expression of voltage-gated Ca^{2+} channels. NaHS or GYY also increased mRNA expression of enzymes of the NO-citrulline cycle including inducible NO synthase (iNOS). It was concluded from these results that H_2S potently promoted differentiation of ASCs into neuronal cells expressing functional voltage-gated Ca^{2+} channels with the aid of cAMP-elevating agents, involving NO-mediated signaling cascade. These effects of H_2S were also considered as a partial mechanism for the VPA-promoted neuronal differentiation of ASCs.

1. Introduction

Adipose tissue-derived stem cells (ASCs) are multipotent cells capable of differentiating into neurogenic linage, being isolated from the subcutaneous fat in various animals including humans, dogs, mice and rats [1–5]. The great advantage of ASCs over stem cells derived from other tissues is that they can be prepared in large amounts by liposuction [6], which causes only slight wounds to their donors. It is therefore expected that neuronal cells differentiated from ASCs can be applied widely in biomedical fields, such as regenerative therapy of neurological disorder.

It has been shown that neuronal differentiation of ASCs is promoted by valproic acid (VPA), an antiepileptic and anticonvulsant drug, in dogs, rats and humans [2,5,7]. VPA-promoted neuronal differentiation of ASCs is mediated by nitric oxide (NO), a gaseous signaling molecule, through the iNOS–NO–sGC signaling pathway [8,9], and involves some protein signaling pathways [7]. VPA up-regulated mRNA expression of enzymes of the NO-citrulline cycle including inducible NO synthase (*iNOS*), which produces a large amount of NO, with the acetylation of their associated histone [8,9]. The promoting effect of VAP on neuronal differentiation of ASCs is synergistically enhanced by subsequent treatment with neuronal induction medium (NIM) containing

Abbreviations: AOAA, aminooxy acetic acid; ASC, adipose tissue-derived stem cell; Asl, arginosuccinate lyase; Ass, arginosuccinate synthase; Cacna1b, calcium voltage-gated channel subunit alpha 1b; Cacna1h, calcium voltage-gated channel subunit alpha 1h; CAT, cysteine aminotransferase; CBS, cystathionine β-synthase; CSE, cystathionine γ-lyase; DAF, Diaminofluorescein; DAPI, 4′, 6-diamidino-2-phenylindole; dbcAMP, dibutyryl cyclic adenosine monophosphate; eNOS, endothelial nitric oxide synthase; GYY, GYY4137; iNOS, inducible nitric oxide synthase; HPRT, hypoxanthine-guanine phosphoribosyl-transferase; IBMX, isobutyl methyl-xanthine; Mpst, mercaptopyruvate sulfurtransferase; NeFM, neurofilament medium polypeptide; NIM, neuronal induction medium; nNOS, neuronal nitric oxide synthase; VPA, valproic acid.

* Corresponding author. Graduate School of Veterinary Medicine, Azabu University, Fuchinobe, Chuo-ku, Sagamihara, Kanagawa, 252–5201, Japan.
E-mail address: takizawa@azabu-u.ac.jp (T. Takizawa).

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cAMP-elevating agents [2,5]. VPA-promoted differentiation of ASCs produce neuronal cells that selectively express functional N-type voltage-gated Ca^{2+} channels, and NIM seems to enhance the mRNA translation of molecules required for the differentiation [10].

Besides NO, it is possible that hydrogen sulfide (H_2S), a gaseous signaling molecule produced through degradation of sulfane sulfur synthesized by key enzymes, such as cysteine aminotransferase (CAT), cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE) and mercaptopyruvate sulfurtransferase (MPST) in animals, is also involved in the neuronal differentiation of ASCs. This is because NO interacts with H_2S both at their production and downstream of their signaling [11]. For example, NO production is regulated by H_2S in various ways; H_2S up- or down-regulates the expression of NO synthases (NOSs), in venular vessels and cardiomyocytes [12,13], and can directly inhibit the activity of NOSs [14]. It has also been indicated that H_2S promotes differentiation of keratinocytes, neural stem cells and regulatory T cells [16–18], while inhibits differentiation of ligament cells [19].

It is important, on the other hand, to establish methodology of stem cell differentiation for possible applications in regenerative therapy. In the present study, we investigate the involvement of H_2S in neuronal differentiation of ASCs for the establishment of effective differentiation method. First, H_2S production was examined in VPA-promoted neuronal differentiation of ASCs. Then, effects of H_2S on the differentiation and on Ca^{2+} entry in ASCs were examined.

2. Materials and methods

2.1. Isolation and culture of rat ASCs

ASCs were isolated from the inguinal region of 8 to 9-week-old male Wistar rats (Crlj: WI, Charles River Japan, Yokohama, Japan) as described previously [5]. ASCs from multiple animals each as an independent unit were cultured separately in Dulbecco's modified Eagle's medium (DMEM, Nissui Pharmaceutical, Tokyo, Japan) containing 10% newborn bovine serum (Invitrogen, Carlsbad, CA), and were used after two passages of subculture. The guideline of the Committee for Animal experimentation at Azabu University was observed for all animal experiments.

2.2. Neuronal differentiation assay

ASCs were cultured in the presence of VPA (Wako Pure Chemical Industries, Osaka, Japan), GYY4137 (GYY, Dojindo Laboratories, Kumamoto, Japan) or NaHS (Wako) for 2–6 days, and were subsequently incubated in neuronal induction medium (NIM, serum-free DMEM supplemented with 100 μM dibutyryl cyclic adenosine monophosphate (dbcAMP, Wako) and 125 μM isobutyl methyl-xanthine (IBMX, Wako)) for 2 h without VPA or H_2S donors. For inhibition of H_2S synthesis, aminooxyacetic acid (AOAA, Wako) was added to the culture medium together with VPA or GYY during the 3-day culture period. Neuronal differentiation was assessed by the expression of a neuronal marker, neurofilament medium polypeptide (NeFM), which was detected by immunocytochemistry. GYY and NaHS were dissolved in sterile water (final concentration 0.1%), and other chemicals were dissolved in dimethyl sulfoxide (final concentration 0.1%) before the addition to the culture medium.

2.3. Immunocytochemistry

NeFM was detected by using a rabbit polyclonal anti-NeFM (40–1259, 1: 400; Proteus, BioSciences, Ramona, CA, U.S.A.) as a primary antibody as described previously [8,9]. Cell nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI, ImmunoBioScience, Mukilteo, WA, U.S.A.). NeFM-positive cells were counted manually with the multi-point tool of the ImageJ software [20] for at least 300 cells per culture dish.

2.4. Detection of intracellular H_2S and sulfane sulfar

Intracellular H_2S and sulfane sulfar were detected with fluorescent indicators, HSip-1 DA (Dojindo) and SSP4 (Dojindo), respectively, according to the manufacturer's instruction. After the culture on glass coverslips in culture dishes, ASCs were incubated with 10 μM HSip-1 DA for 30 min at 37 °C or with 20 μM SSP4 for 15 min at 37 °C before the treatment with the anti-NeFM antibody. Fluorescent signal of H_2S and sulfane sulfar was detected with an inverted fluorescence microscope (AX80 microscope, Olympus Corp., Tokyo, Japan) at 515 nm.

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

Total RNA was extracted and analyzed as described previously [8,9]. The amount of mRNA was normalized to that of hypoxanthine-guanine phosphoribosyl-transferase (*Hprt*), and was expressed as a fold change compared to untreated groups. Primers were purchased from a supplier (Fasmac, Atsugi, Japan) (Table 1).

2.6. Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed using the ChIP Reagents (Nippon Gene, Tokyo, Japan) as described previously [8,9]. The immunoprecipitated DNA cross-linked with acetylated histone H3K9 (AcH3K9) was eluted and amplified by PCR using primers designed for promoter regions of the target genes (Table 1).

2.7. Detection of intracellular Ca^{2+}

Intracellular Ca^{2+} was detected with a fluorescent indicator, Fluo 4-AM (CS-22, Dojindo), as described previously [10]. After the culture on glass cover slips in culture dishes, ASCs were treated with 4.6 μM of Fluo 4-AM in the loading buffer, and were incubated for 60 min at 37 °C. Ca^{2+} fluorescent signal was observed in the recording buffer with an inverted fluorescent microscope (BZ-X700, Keyence, Osaka, Japan) at 515 nm.

2.8. Detection of intracellular NO

Intracellular NO was detected with a fluorescence NO-specific molecular probe, Diaminofluorescein-FM diacetate (DAF-FM, Goryo Chemical Inc., Sapporo, Japan), as described previously [8,9]. After the culture on glass coverslips placed in culture dishes, ASCs were incubated with 5 μM DAF-FM in serum-free DMEM for 60 min at 37 °C. NO fluorescent signal was detected with an inverted fluorescence microscope (AX80 microscope) at 515 nm.

2.9. Statistical analysis

Culture dishes were treated as an experimental unit. Statistical significance of differences among experimental groups were examined by the Tukey-Kramer test after one-way analysis of variance at a probability level of 5%. When variances were not homogeneous among the groups, values were log transformed before analysis.

3. Results

3.1. VPA-promoted neuronal differentiation of ASCs is accompanied by increased production of H_2S

VPA with NIM promoted differentiation of ASCs into neuronal cells expressing a neuronal maker NeFM (Fig. 1A). This promoted differentiation was accompanied by increased intracellular H_2S and sulfane sulfar, which was detected by fluorescent molecular probes Hsip-1 DA and SSP4, respectively (Fig. 1A). It was noted that although at lower incidences, NeFM positive cells by VPA or NIM treatment alone were also positive for H_2S and sulfane sulfur. When gene expression of key

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

Gene		Primer sequence (5′-3′)	Product Length (bp)	Anealing Temp.(°C)	Cycle
Cbs[a,b]	F	5′-GCATCTGCGTGTCCAAGAGC-3′	229	60	35
	R	5′-GGCATGCTCTCCCACATCCT-3′			
Cbs[c]	F	5′-ACCACGCCCATTTTAACGCC-3′	134	56	30
	R	5′-TCAGGCTCCTGCGTTCAGAG-3′			
Cse[a,b]	F	5′-CGAGGCCTGAAGACACTGCA-3′	133	58	34
	R	5′-CATGCTGAGGGTGAGACGGT-3′			
Cat[a,b]	F	5′-TCCGGAGCTGTGCTTCTCAG-3′	141	60	35
	R	5′-TGCCATTGTACCATCGCCCT-3′			
Mpst[a,b]	F	5′-CGCCGCCTTCTTTGACATCG-3′	80	58	35
	R	5′-CGAAATGCGTGGCACTAGGC-3′			
Mpst[c]	F	5′-GGCTGGGCTGGAACTTCCTAT-3′	167	56	30
	R	5′-AAAAGGTGACGGCACCAAACC-3′			
Nefm[b]	F	5′-AGGCTGAGTCCCCAGTGAAA-3′	220	58	n.a.
	R	5′-TCCACCTCCCCATTGATAGC-3′			
Cacna1b[b]	F	5′-GTTGTGAGCGCAGTAGACGTC-3′	282	60	n.a.
	R	5′-CTGCAACCTCCCTCTCTGG-3′			
Cacna1h[b]	F	5′-GCCCAGAGAAGGAACAAGGA-3′	186	60	n.a.
	R	5′-CCAACAACCTCAATGCCAAA-3′			
nNOS[a]	F	5′-CCACCCTGCACCATCTTCCA-3′	221	60	35
	R	5′-TTCCCGTCCATCCAGATGCC-3′			
iNOS[a,b]	F	5′-CACCACCCTCCTTGTTCAAC-3′	132	60	35
	R	5′-TTGGAGCGAGTTGTGGATTG-3′			
eNOS[a]	F	5′-ACTTTGGTGTTTGGCTGCCG-3′	117	60	35
	R	5′-CCGCCTTTTCCAGGGATCCT-3′			
Ass[b]	F	5′-GCAGGGACCATCCTTTACCA-3′	191	60	n.a.
	R	5′-ACCTTTCCTTCCACCCGTTC-3′			
Asl[b]	F	5′-GCGCTGACACGAGATTTAGAGA-3′	213	60	n.a.
	R	5′-GCACAGAGAAGCCCAAAACA-3′			
Hprt[a,b]	F	5′-AATGTCTGTTGCTGCGTC-3′	92	58	31
	R	5′-TGTCTGTCTCACAAGGGAAG-3′			

F, Forward; R, Reverse.
n.a., not applicable.
[a] , Reverse transcription (RT)-PCR.
[b] , real-time RT-PCR.
[c] , Chip PCR.

enzymes responsible for H_2S production through sulfane sulfur from L-cysteine was examined, mRNA expression of *Cbs*, *Cat* and *Mpst* were increased to 2- to 4-fold by VPA with or without NIM treatment, while that of *Cse* was decreased to less than half (Fig. 1B and C). The ChIP assay indicated increased acetylation of histone H3K9 associated with *Cbs* and *Mpst* genes, suggesting up-regulated mRNA expression of the genes at the transcription level (Fig. 1D). It was considered from these results that both VPA and NIM treatments increase the amount of cellular H_2S and sulfane sulfur which is associated with the enzymatic synthesis of sulfane sulfur and neuronal differentiation of ASCs.

3.2. H_2S production is involved in VPA-promoted neuronal differentiation of ASCs

AOAA, an inhibitor of CBS, suppressed VPA-promoted neuronal differentiation of ASCs with decreased cellular H_2S and sulfane sulfur in a concentration dependent manner when added to the culture medium (Fig. 2A and B). GYY, a slow releasing H_2S donor, alone increased the incidence of NeFM-positive ASCs to only about 20% even after 6 days culture (Fig. 2C). However, GYY with subsequent NIM treatment promoted the differentiation, increasing the incidence of NeFM-positive ASCs to a level comparable to that by VPA with NIM (Fig. 2D and E). In addition, the effects of GYY and NIM were suppressed by AOAA, indicating the importance of continued availability of H_2S provided by de novo production in the GYY-promoted differentiation as well (Fig. 2D and E). It was thus indicated that H_2S was involved in VPA-promoted neuronal differentiation of ASCs, and that H_2S potently promoted the differentiation with the aid of NIM.

3.3. H_2S with NIM treatment promotes neuronal differentiation of ASCs being accompanied by Ca^{2+} entry

H_2S-promoted neuronal differentiation of ASCs was confirmed by using another H_2S donor NaHS, which was added to the medium once a day for 3 days because of its fast releasing property. With subsequent NIM treatment, NaHS promoted neuronal differentiation of ASCs, increasing NeFM-positive cells in a concentration dependent manner (Fig. 3A and B). The mRNA expression of *NeFM* in ASCs was also increased by NaHS in a concentration dependent manner, which was roughly proportional to the incidence of NeFM-positive cells, suggesting its effects at the gene transcription level (Fig. 3C). Both of the H_2S donors, i.e., NaHS and GYY, with NIM, increased intracellular Ca^{2+} in ASCs as detected with a fluorescent indicator (Fig. 3D). In addition, NaHS with NIM increased mRNA expression of voltage-gated Ca^{2+} channels, *Cacna1b* and *Cacna1h*, which are increased in VPA-promoted neuronal differentiation [10], in ASCs (Fig. 3E and F). It was considered from these results that H_2S promoted neuronal differentiation of ASCs with the aid of NIM treatment, which was accompanied by Ca^{2+} entry through voltage-gated channels.

3.4. H_2S with NIM treatment up-regulates mRNA expression of enzymes of the NO-citrulline cycle

Effects of H_2S on NO production in neuronal differentiation of ASCs was examined. NaHS or GYY with NIM increased intracellular NO in ASCs as detected with a fluorescent indicator (Fig. 4A). Analysis of mRNA expression of the key enzymes of NO production in the NO-citrulline cycle showed that NaHS or GYY with NIM increased mRNA expression of inducible NO synthase (iNOS) alone in neuronal differentiation of ASCs; mRNAs of neuronal NO synthase (nNOS) and

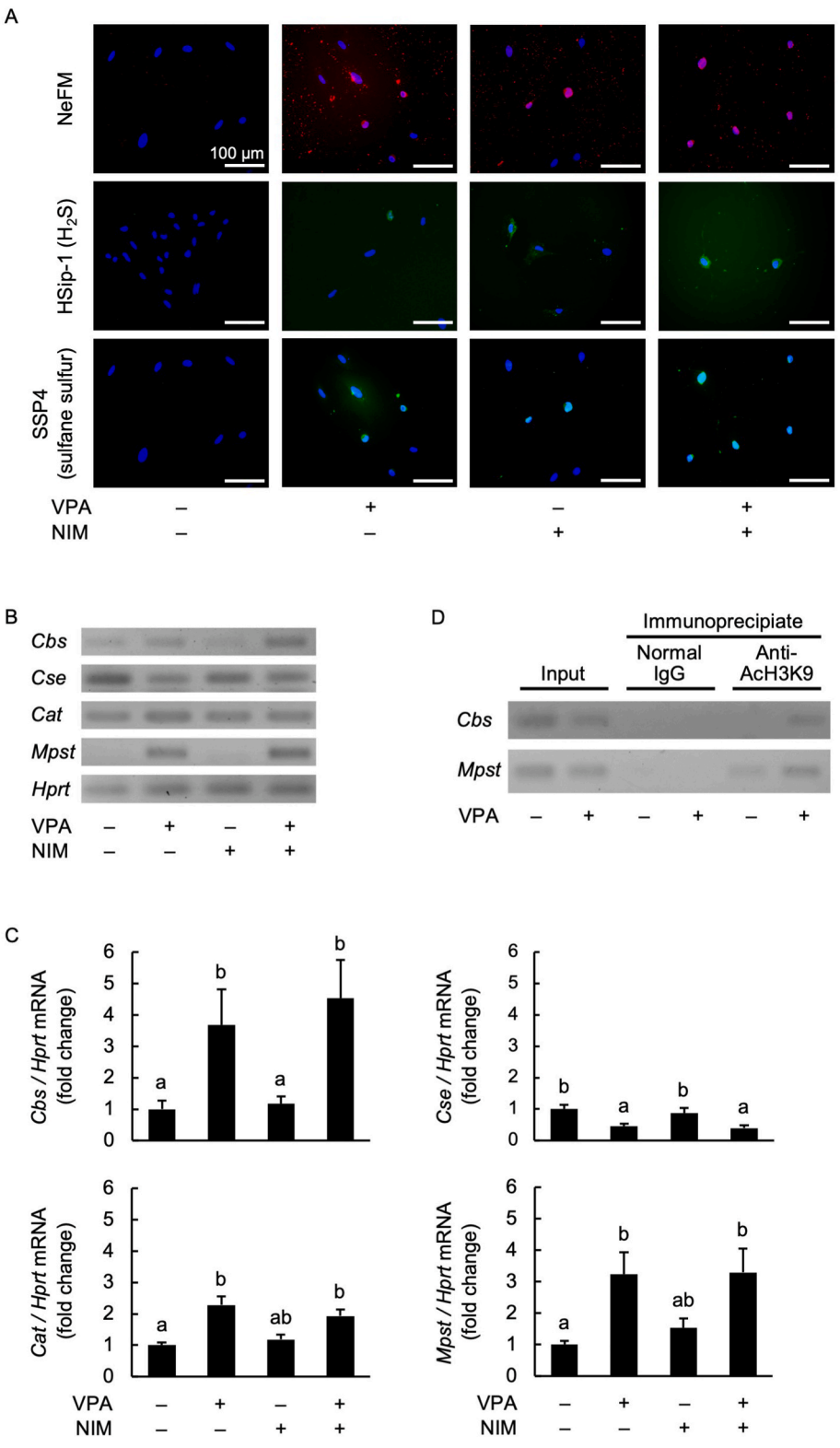

Fig. 1. VPA-promoted neuronal differentiation of ASCs is accompanied by increased production of H_2S. ASCs were cultured in the presence of VPA (2 mM) for 3 days with subsequent NIM treatment for 2 h. Mean and SEM are shown. Data were obtained from a single experiment after several preliminary experiments. **A**, Appearance of ASCs cultured in the presence of VPA. NeFM (red), cell nuclei (blue), H_2S (green) and sulfane sulfur (green) are shown. NeFM was detected by immunocytochemistry. Cell nuclei were stained with DAPI. H_2S and sulfane sulfur were detected with fluorescent probes, HSip-1 and SSP4, respectively. **B and C**, mRNA expression of enzymes producing H_2S. mRNAs of *Cbs*, *Cse*, *Cat* and *Mpst* were detected by RT-PCR. The amount of mRNA was determined by real-time RT-PCR analysis and normalized by *Hprt* mRNA. Different alphabetical letters indicate significant differences among groups at $p < 0.05$; e.g., groups labelled with letters 'a' and 'b' alone are different from each other, but groups with letters 'ab' are not different from groups labelled with either 'a' or 'b', $n = 8–12$. **D**, Acetylation of histone associated with genes encoding key enzymes producing H_2S before NIM treatment. Genes were analyzed by the ChIP assay.

endothelial NO synthase (eNOS) were not detected (Fig. 4B and C). NaHS or GYY with NIM also increased mRNA expression of arginosuccinate synthase (*Ass*), but not of arginosuccinate lyase (*Asl*), of the NO-citrulline cycle (Fig. 4D and E). It was thus considered that H_2S increased NO in neuronal differentiation of ASCs through up-regulation of some enzymes of the NO-citrulline cycle.

4. Discussion

The present results show for the first time that H_2S promotes neuronal differentiation of ASCs with the aid of NIM containing cAMP-elevating agents. This may indicate that H_2S is a signal transducer of VPA to NO signaling in neuronal differentiation of ASCs because effects of H_2S on mRNA expression of molecules involved in the differentiation

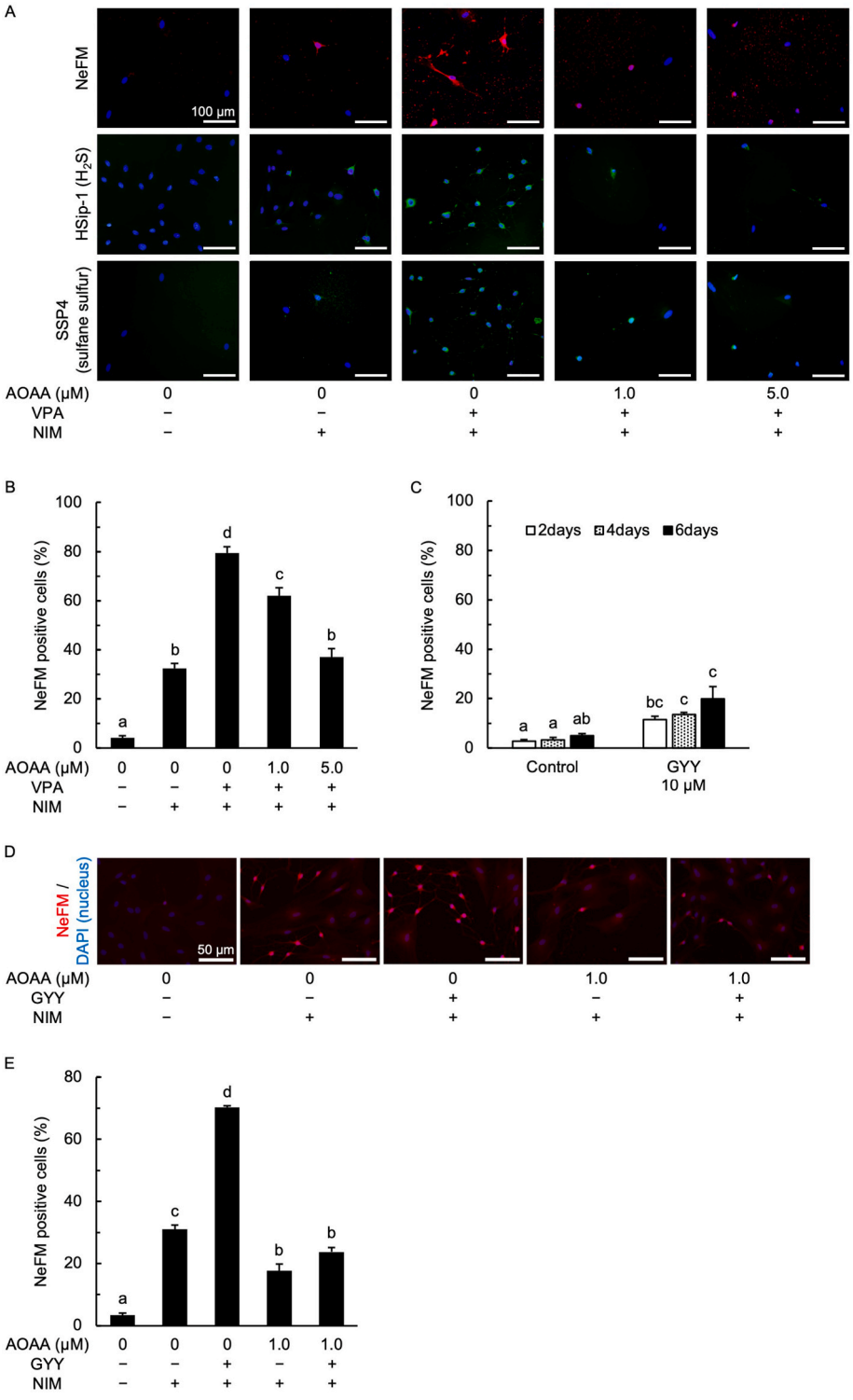

Fig. 2. H_2S production is involved in VPA-promoted neuronal differentiation of ASCs.
ASCs were cultured in the presence or absence of AOAA, VPA (2 mM) and GYY (10 μM) with or without subsequent NIM treatment for 2 h. NeFM was detected by immunocytochemistry and are shown in red. Cell nuclei were stained with DAPI and are shown in blue. NeFM-positive cells were counted for at least 300 cells per culture dish. Different alphabetical letters indicate significant differences among groups at $p < 0.05$; e.g., groups labelled with letters 'a' and 'b' alone are different from each other, but groups with letters 'ab' are not different from groups labelled with either 'a' or 'b'. Mean and SEM are shown. Data were obtained from a single experiment after several preliminary experiments. **A,** Appearance of ASCs cultured in the presence of AOAA and VPA for 3 days with NIM treatment. **B**, Incidence of NeFM-positive ASCs cultured in the presence of AOAA and VPA for 3 days with NIM treatment. n = 4–6. **C**, Incidence of NeFM-positive ASCs cultured in the presence of GYY or VPA for 2–6 days without NIM treatment. n = 4. **D**, appearance of ASCs cultured in the presence of AOAA and GYY for 3 days with NIM treatment. **E**, Incidence of NeFM-positive ASCs cultured in the presence of AOAA and GYY for 3 days with NIM treatment. n = 4 or 5.

through NO-signaling, were similar to those by VPA; i.e., mRNA expression of *NeFM*, *Cacna1b*, *Cacna1h*, *iNOS* and *Ass* are increased in common by H_2S and VPA [8–10]. In this context, it is supposed that the up-regulation of *iNOS* by H_2S indicate a signaling cascade from H_2S to NO to execute various functions required for the differentiation.

The role of NIM, or cAMP-elevating agents, in H_2S-promoted neuronal differentiation of ASCs is not known in detail at present. However, NIM seems to increase production of cellular H_2S and sulfane sulfur not at the transcriptional level of related enzymes, which is supported by suppressive effects of AOAA in H_2S donor-promoted neuronal differentiation of ASCs with NIM treatment; AOAA seems to inhibit NIM-induced production of H_2S and sulfane sulfur even in the presence of GYY. In addition, because effects of H_2S and VPA in neuronal differentiation of ASCs are essentially the same as described above, it is probable that NIM works as in the VPA-promoted differentiation; i.e., NIM seems to enhance the mRNA translation of molecules required in neuronal differentiation of ASCs, and to be involved in neurite outgrowth [10]. On the other hand, since cAMP signaling regulates adipocyte differentiation transcriptionally [21], NIM may also work transcriptionally in the H_2S-promoted differentiation.

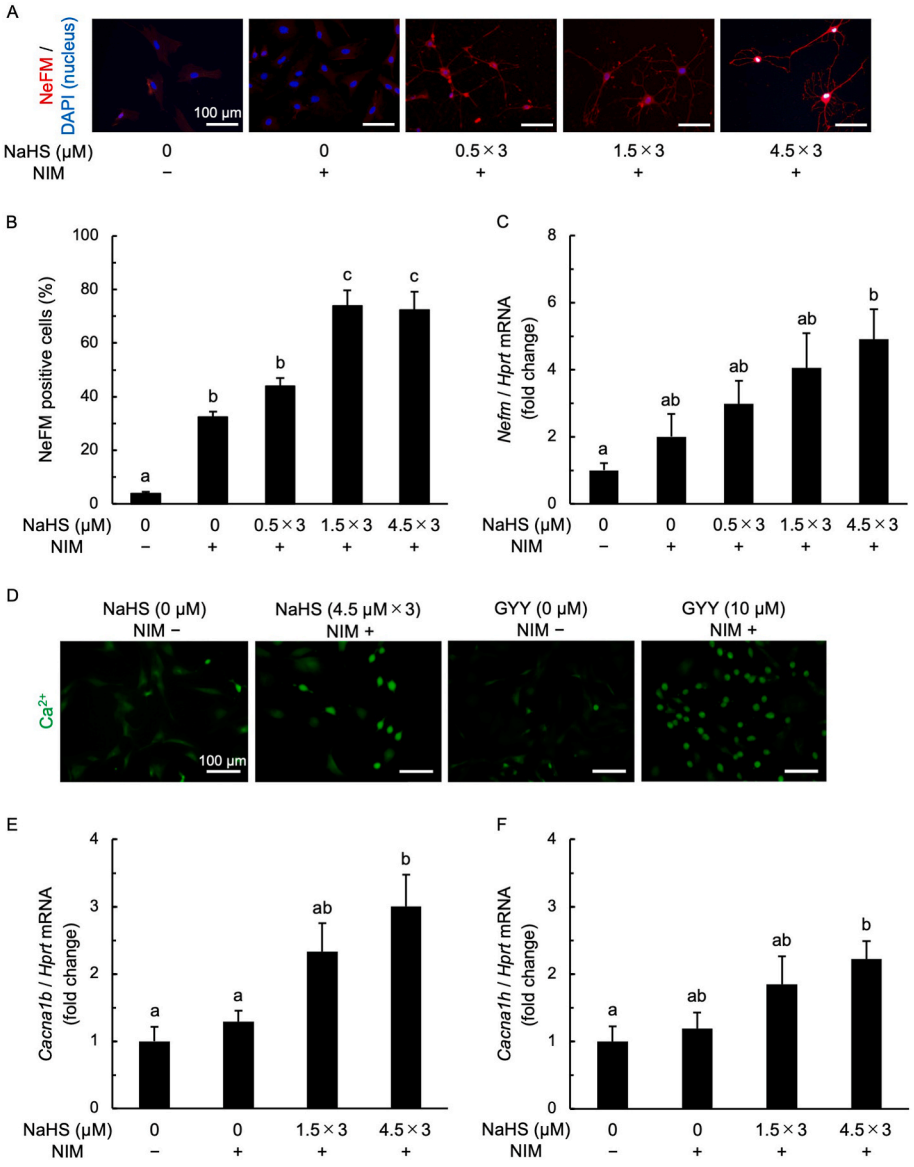

Fig. 3. H_2S with NIM treatment promotes neuronal differentiation of ASCs being accompanied by Ca^{2+} entry.

ASCs were cultured in the presence of NaHS or GYY for 3 days with subsequent NIM treatment for 2 h. Different alphabetical letters indicate significant differences among groups at $p < 0.05$; e.g., groups labelled with letters 'a' and 'b' alone are different from each other, but groups with letters 'ab' are not different from groups labelled with either 'a' or 'b'. Mean and SEM are shown. The amount of mRNA was determined by real-time RT-PCR analysis and normalized by *Hprt* mRNA. Data were obtained from a single experiment after several preliminary experiments. **A**, Appearance of ASCs cultured in the presence of NaHS. NeFM was detected by immunocytochemistry and are shown in red. Cell nuclei were stained with DAPI and are shown in blue. **B**, Incidence of NeFM-positive ASCs cultured in the presence of NaHS. NeFM-positive cells were counted for at least 300 cells per culture dish. n = 5. **C**, Amount of *Nefm* mRNA in ASCs. n = 5 or 6. **D**, Images of intracellular Ca^{2+} in ASCs. Ca^{2+} was detected with a fluorescent indicator and is shown in green. **E**, Amount of *Cacna1b* mRNA in ASCs. n = 4–9. **F**, Amount of *Cacna1h* mRNA in ASCs. n = 5 or 6.

There seems to be mechanisms other than H_2S signal transduction in VPA-promoted neuronal differentiation of ASCs. This is because without NIM, VPA promotes the differentiation more effectively than H_2S, increasing the incidence of differentiated cells to as high as 60% [10], and because the extent of increase in mRNA expression of *NeFM*, *Cacna1b*, *Cacna1h*, *iNOS* and *Ass* is smaller by H_2S than by VPA [8–10]. VPA may up-regulate more genes than H_2S, due to its direct inhibitory activity on histone deacetylases [22,23], or may be more potent than H_2S in that it also increases sulfane sulfar, a stored form of H_2S [24], to work continuously; continued availability of H_2S seems to be important for the differentiation as indicated by the suppressive effects of AOAA on the GYY-promoted differentiation.

In the regulation of the NO-citrulline cycle, it is noted that mRNA expression of *iNOS* and *Ass* is up-regulated by H_2S as in the previous study, where their activity seems to be a rate-limiting step in the NO production in VPA-promoted neuronal differentiation of ASCs [8]. It is thus expected that enzymes encoded by these genes works as a target for regulatory factors in neuronal differentiation of ASCs, which would increase variety of techniques in the application of the differentiated cells in common.

Although not identified in the present study, the type of channels responsible for the Ca^{2+} entry in ASCs treated with H_2S donors is supposed to be Cav2.2 encoded by *Cacna1b*. This is because VPA induces functional expression of Cav2.2 selectively [10], where H_2S is produced as shown in the present study; if functional expression of other types of Ca^{2+} channels is induced by H_2S, VPA should also have induced it. However, there remains a possibility that other Ca^{2+} channels are expressed without VPA, which would enable regenerative therapy of diseases associated with the channels. Likewise, NO signaling pathways are supposed to work in H_2S-promoted differentiation of ASCs as in the VPA-promoted differentiation, because VPA increases cellular H_2S as a part of its promoting effects.

From the standpoint of clinical applications, H_2S-promoted neuronal differentiation of ASCs has some technical merits than VPA-promoted one. For example, although depending on its donors, H_2S does not remain in the differentiated cells, because it is an endogenous gaseous signaling molecule with extremely short half-life *in vivo* [25]. As mentioned above, H_2S seems to alter gene expression less extensively than VPA, avoiding gene expression unnecessary for the differentiation of ASCs. While controlled exposure to H_2S may be technically more difficult than that to VPA, neuronal differentiation of ASCs can be regulated in two steps, i.e., by H_2S and NIM, because H_2S requires subsequent NIM treatment to efficiently promote the differentiation; on the contrary, VPA can promote the differentiation by itself. The two-step

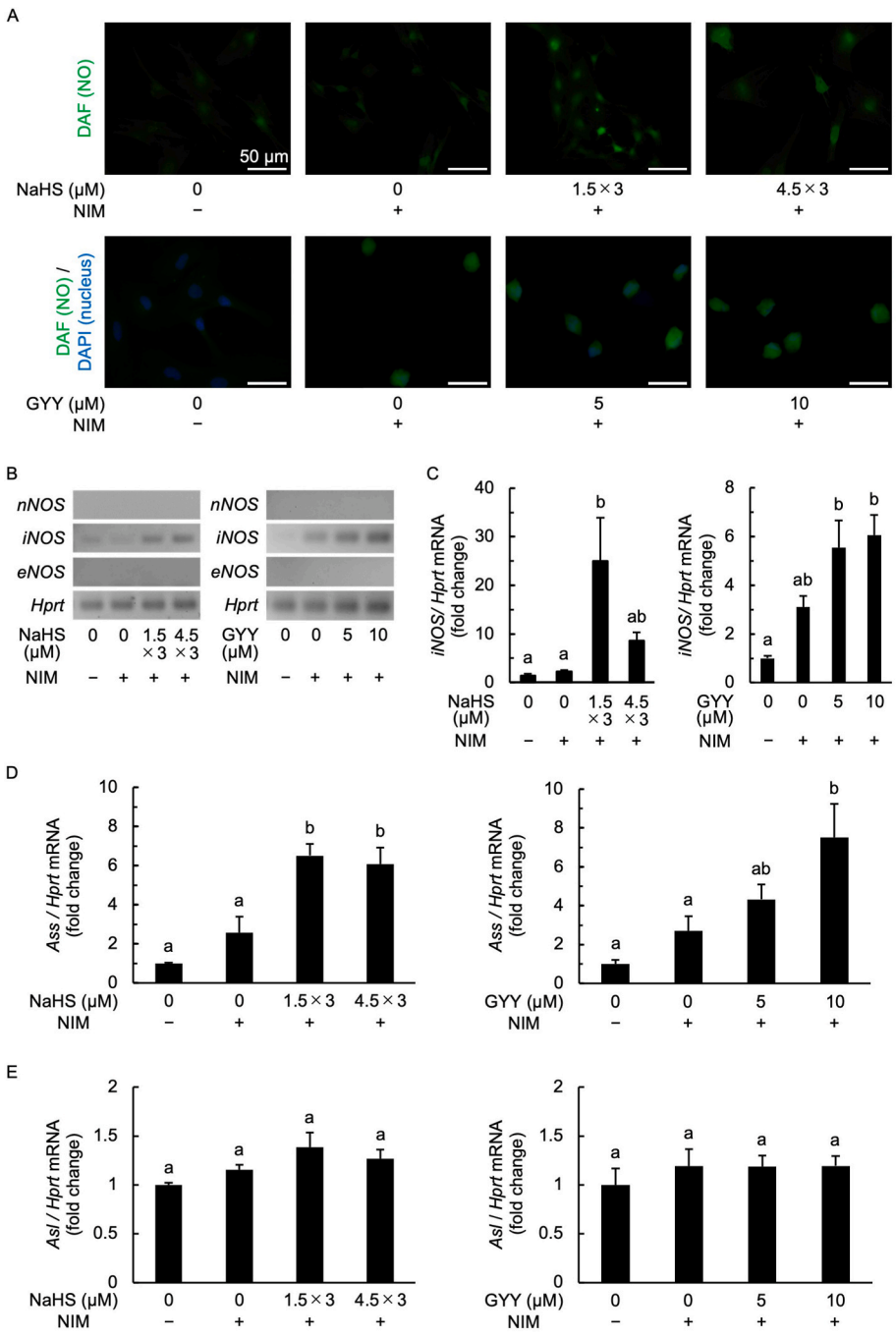

Fig. 4. H_2S with NIM treatment up-regulates mRNA expression of enzymes of the NO-citrulline cycle. ASCs were cultured in the presence of NaHS or GYY for 3 days with subsequent NIM treatment for 2 h. Different alphabetical letters indicate significant differences among groups at $p < 0.05$; e.g., groups labelled with letters 'a' and 'b' alone are different from each other, but groups with letters 'ab' are not different from groups labelled with either 'a' or 'b'. Mean and SEM are shown. The amount of mRNA was determined by real-time RT-PCR analysis and normalized by *Hprt* mRNA. Data were obtained from a single experiment after several preliminary experiments. **A**, Appearance of ASCs cultured in the presence of NaHS or GYY. NO was detected by a fluorescent probe and are shown in green. Cell nuclei were stained with DAPI and are shown in blue. **B,** mRNA expression of *nNOS*, *iNOS* and *eNOS* detected by RT-PCR. **C**, Amount of *iNOS* mRNA in ASCs. n = 5–11. **D**, Amount of *Ass* mRNA in ASCs. n = 4. **E**, Amount of *Asl* mRNA in ASCs. n = 4–6.

regulation enables more flexible technical use of differentiated ASCs.

In conclusion, H_2S potently promotes differentiation of ASCs into neuronal cells expressing functional voltage-gated Ca^{2+} channels with the aid of cAMP-elevating agents, involving NO-mediated signaling cascade. These effects of H_2S are also considered as a partial mechanism for the VPA-promoted neuronal differentiation of ASCs.

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Declaration of competing interest

The authors declare no conflicts of interest in regards to this manuscript.

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