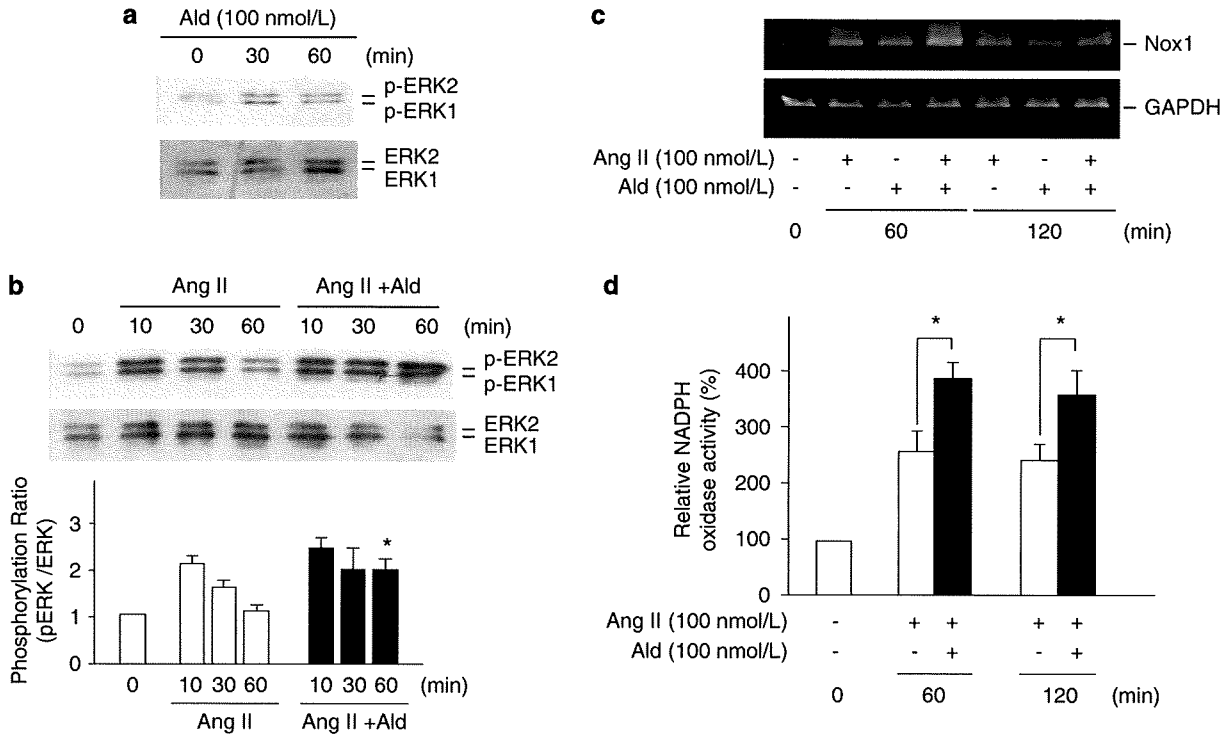

Figure 6 (**a**) Effect of aldosterone (Ald, 100 nmol l^{-1}) on ERK1/2 phosphorylation. (**b**) Additional effect of Ald (100 nmol l^{-1}) on ERK1/2 phosphorylation when co-treated with angiotensin II (Ang II). The phosphorylation ratio is shown as described in Figure 3a. *$P<0.01$ *vs.* Ang II alone. (**c**) Additional effect of Ald (100 nmol l^{-1}) on Nox1 expression when co-treated with Ang II. Rat aortic smooth muscle cells (RASMCs) were treated with Ang II (100 nmol l^{-1}), Ald (100 nmol l^{-1}) or both for the indicated times. (**d**) Additional effect of Ald on NADPH oxidase activity when co-treated with Ang II. RASMCs were treated with Ang II (100 nmol l^{-1}) or Ang II+Ald (100 nmol l^{-1}) for the indicated times. NADPH oxidase activity is presented as the fold increase over the value obtained from static control cells at time 0. *$P<0.01$.

cells were co-treated with Ang II. Aldosterone (100 nmol l^{-1}) significantly increased ERK phosphorylation in RASMCs (Figure 6a). When co-treated with Ang II (100 nmol l^{-1}), ERK phosphorylation tended to increase at 10 and 30 min after stimulation, but was not significant statistically. However, 60 min after stimulation, it significantly increased in RASMC co-treated with Ang II and aldosterone compared with Ang II alone (Figure 6b). The Nox1 expression (Figure 6c) and NADPH oxidase activation (Figure 6d) were also enhanced by co-treatment with aldosterone compared with Ang II alone.

MR antagonists inhibited CS-induced NADPH oxidase activity

We examined whether CS-induced production of aldosterone contributes to NADPH oxidase activity using MR antagonists. Both eplerenone and spironolactone attenuated the CS-induced activity (Figures 7a and b). Similarly, Nox1 mRNA expression induced by CS was suppressed by eplerenone (Figure 7c).

We also examined the effect of co-treatment with eplerenone and ARBs on CS-induced NADPH oxidase activity. Olmesartan and valsartan suppressed the CS-induced activity to a similar extent as eplerenone; however, co-treatment with eplerenone had an additive suppressive effect on ARBs (Figure 7d).

DISCUSSION

In this study, we showed for the first time that CS increased the expression of CYP11B2 in VSMCs. Although the levels of CYP11B2 were much lower in VSMCs than in the adrenal gland and CS could not increase the concentration of aldosterone above the level detectable by enzyme immunoassay, a marked increase in aldosterone concentrations was observed in CS-exposed cells in the presence of the substrate of aldosterone, 11-deoxycorticosterone. CS as well as exogenous aldosterone enhanced NADPH oxidase activity, which has an important role in ROS production in the vascular wall and, furthermore, the MR antagonists, spironolactone and eplerenone, significantly suppressed the CS-induced increase in NADPH oxidase activity. These results suggest that local aldosterone synthesis by CS contributes to the activation of NADPH oxidase through MR in VSMCs. Although the role of locally produced aldosterone in the vascular wall is controversial because of its small quantity, recent reports have indicated that vascular aldosterone potentiates Ang II-induced hypertrophy[10] or proliferation[23] of cultured VSMCs. The CS-induced upregulation of CYP11B2 expression and the inhibitory effects of the MR antagonist on CS-induced NADPH oxidase activity shown in this study suggest that a locally produced but very small amount of aldosterone contributes to CS-induced NADPH oxidation in the vascular wall.

NADPH oxidase is a multisubunit complex consisting of membrane-bound and cytosolic components, and in VSMCs, the expression of $p22^{phox}$, Nox1, Nox2 ($gp91^{phox}$), Nox4 (membrane-bound components) and $p47^{phox}$[6] (cytosolic components) has been reported.[24] In our RASMCs, the expression of Nox2 and $p47^{phox}$ was undetectable or quite low as previously described.[25–27] CS significantly increased the expression of Nox1 but not of the other subunits. Although several different mechanisms were proposed regarding the regulation of NADPH oxidase activity in VSMCs,

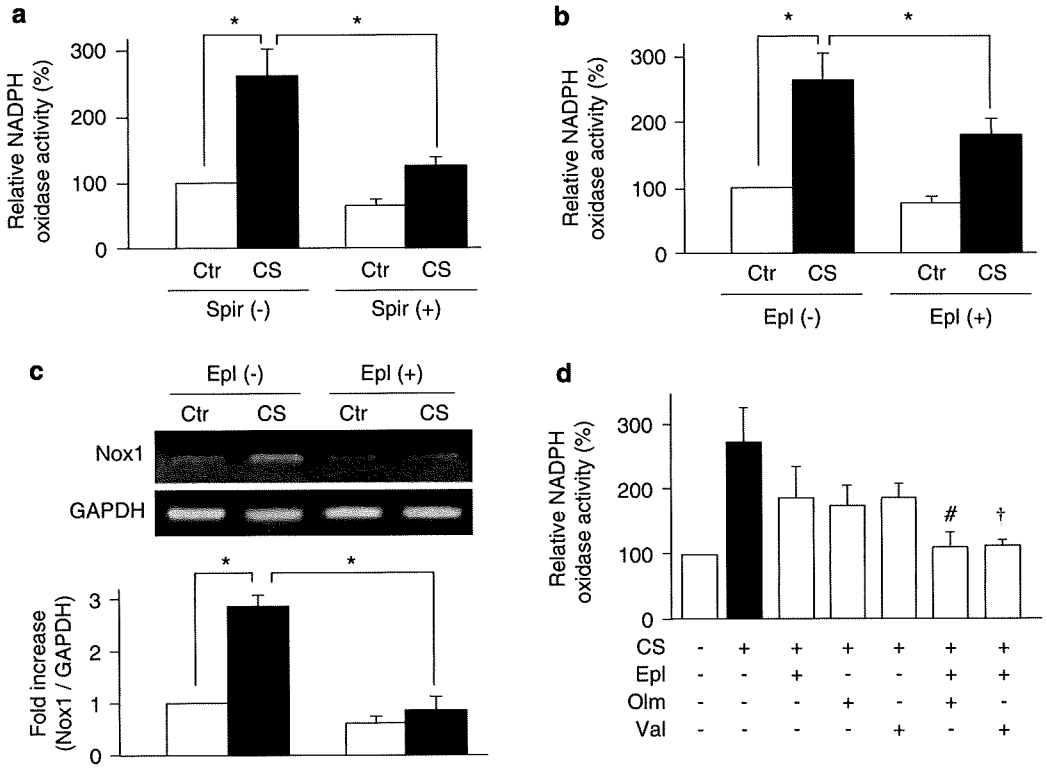

Figure 7 (**a**) Effect of spironolactone (Spir) on cyclic stretch (CS)-induced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity. Rat aortic smooth muscle cells (RASMCs) were exposed to CS (10%, 0.5 Hz) for 2 h in the absence or presence of Spir (20 μmol l^{-1}). NADPH oxidase activity is presented as the fold increase over the value obtained from static control cells (Ctr) in the absence of Spir. *$P<0.01$. (**b**) Effect of eplerenone (Epl) on CS-induced NADPH oxidase activity. RASMCs were exposed to CS (10%, 0.5 Hz) for 2 h in the absence or presence of Epl (10 μmol l^{-1}). NADPH oxidase activity is presented as the fold increase over the value obtained from static control cells (Ctr) in the absence of Epl. *$P<0.01$. (**c**) Effect of Epl on CS-induced Nox1 expression. RASMCs were maintained as described in (**a**). The normalized mRNA level is given as the fold increase over the values obtained from the static control cells (Ctr) without Epl. *$P<0.01$. (**d**) Synergistic effects of olmesartan (Olm) and valsartan (Val) with eplerenone (Epl) on CS-induced NADPH oxidase activity. RASMCs were exposed to CS (10%, 0.5 Hz) for 2 h in the presence of Epl (10 μmol l^{-1}) and/or Olm (100 nmol l^{-1}) and Val (100 nmol l^{-1}). NADPH oxidase activity is presented as the fold increase over the value obtained from static control cells (Ctr). *$P<0.05$ *vs.* CS. #$P<0.01$ *vs.* Olm alone. †$P<0.01$ *vs.* Val alone.

Nox1 is the only subunit of NADPH oxidase whose expression is reported to be inducible by various stimuli, such as Ang II, serum, platelet-derived growth factor[24] and prostaglandin $F_{2\alpha}$.[28] Our data suggest that CS stimulates NADPH oxidase activity by inducing the expression of Nox1.

Recent reports suggest that mitogen-activated protein kinases contribute to aldosterone production.[18,19] Our results show that the phosphorylation of ERK1/2, but not of JNK or p38, is enhanced by CS. The ERK inhibitor, U0126, almost completely suppressed the CS-induced expression of CYP11B2, indicating that CS induces expression through the phosphorylation of ERK1/2. We also show that Ang II enhanced ERK1/2 phosphorylation, NADPH oxidase activity and Nox1 expression in RASMCs as well as CS. Ang II also increased CYP11B2 expression in RASMCs, although the effect was relatively small (Figure 5c). However, olmesartan could only partially suppress CS-induced ERK1/2 phosphorylation and CYP11B2 expression, whereas the effect of U0126 was almost complete. Therefore, the CS-mediated expression of CYP11B2 induced through the phosphorylation of ERK1/2 seemed to be partially mediated by Ang II, but Ang II-independent pathways may also exist. Although the mechanism remains unclear, Ang II-independent aldosterone production has been suggested. In half of the patients with essential hypertension treated with angiotensin-converting enzyme inhibitors, despite sufficient suppression of the plasma Ang II concentration, aldosterone secretion was not suppressed,[29] a phenomenon usually called the 'aldosterone breakthrough.' In hypertensive rats, an aldosterone antagonist has been shown to facilitate the cardioprotective effects of angiotensin receptor blockers.[30] Our results suggest one mechanism for Ang II-independent aldosterone production in VSMCs.

The above findings led us to investigate whether an aldosterone antagonist is able to suppress CS-stimulated NADPH oxidase activity independent of Ang II. Figure 6d clearly shows that eplerenone suppressed the CS-induced activation of NADPH oxidase to a similar extent as ARBs and, furthermore, that co-treatment with eplerenone and ARBs potentiated the suppressive effect. A recent study using an animal model found that eplerenone reduced atherosclerosis by attenuating oxidative stress partially independent of Ang II.[31] Consistent with this report, our results show the additional effects of eplerenone with ARBs.

Taken together, our data suggest that CS enhances CYP11B2 expression through ERK1/2 phosphorylation dependent on or independent of Ang II and that synthesized aldosterone contributes to ROS generation in the vascular wall (Figure 8). Our data show that aldosterone itself activated ERK1/2 in VSMCs as reported in a recent study.[32] Furthermore, it has also been reported that aldosterone induces angiotensin-converting enzyme (ACE) gene expression, which is required for Ang II production in cultured rat cardiocytes.[33] Under conditions with excessive stretch stimuli such as hypertension,

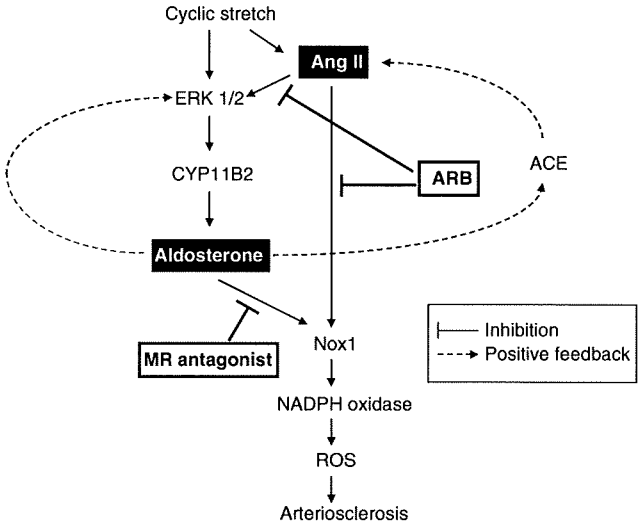

Figure 8 Hypothetic signaling pathway of cyclic stretch-mediated ROS generation through aldosterone.

aldosterone induction in the cardiovascular system may activate these complicated positive feedback loops, resulting in the enhancement of ROS generation and subsequent local vascular wall injury. The suppressive effect of eplerenone on CS-induced NADPH oxidase activation even in the presence of ARBs shown in this study suggests the usefulness of MR antagonists not only in the treatment of high blood pressure but also in the prevention and/or treatment of atherosclerotic complications.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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Journal of Pharmacological Sciences

Full Paper

Dictyostelium Differentiation-Inducing Factor-1 Binds to Mitochondrial Malate Dehydrogenase and Inhibits Its Activity

Tomoko Matsuda[1], Fumi Takahashi-Yanaga[1,*], Tatsuya Yoshihara[1], Katsumi Maenaka[2], Yutaka Watanabe[4], Yoshikazu Miwa[1], Sachio Morimoto[1], Yuzuru Kubohara[5], Masato Hirata[3], and Toshiyuki Sasaguri[1]

[1]*Department of Clinical Pharmacology, Faculty of Medical Sciences,* [2]*Medical Institute of Bioregulation,*
[3]*Laboratory of Molecular and Cellular Biochemistry, Faculty of Dental Sciences, Kyushu University, Fukuoka 812-8582, Japan*
[4]*Department of Applied Chemistry, Faculty of Engineering, Ehime University, Matsuyama 790-8577, Japan*
[5]*Biosignal Research Center, Institute for Molecular and Cellular Regulation, Gunma University, Maebashi 371-8512, Japan*

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Abstract. We have reported that the differentiation-inducing factors (DIFs) DIF-1 and DIF-3, morphogens secreted from *Dictyostelium discoideum*, inhibit proliferation of several cancer cells via suppression of the Wnt/β-catenin signaling pathway. However, the target molecules of DIFs involved in the anti-proliferative effects are still unknown. In the present study, DIF-1–tethered resins were synthesized to explore the target molecules of DIFs, and mitochondrial malate dehydrogenase (mMDH) was identified as one of the target molecules. In the in vitro assay, DIF-1 and other analogs including 2-MIDIF-1, DIF-3, and 6-MIDIF-3 were found to be capable of binding to mMDH but not to cytoplasmic MDH. However, only DIF-1 and 2-MIDIF-1 inhibited the enzymatic activity of mMDH. The effects of DIF analogs on ATP content and cell proliferation were then analyzed using HeLa cells. DIF-1 and 2-MIDIF-1 were found to lower the ATP content and both chemicals inhibited HeLa cell proliferation, suggesting that inhibition of mMDH activity affected cell energy production, probably leading to the inhibition of proliferation. These results suggest that the inhibition of mMDH activity by DIF-1 and 2-MIDIF-1 could be one of the mechanisms to induce anti-proliferative effects, independent of the inhibition of the Wnt/β-catenin signaling pathway.

Keywords: differentiation-inducing factor, mitochondrial malate dehydrogenase (mMDH), energy production, ATP content, cell proliferation

Introduction

Differentiation-inducing factors (DIFs), first identified in *Dictyostelium discoideum* as putative morphogens required for stalk cell differentiation (1 – 4), also affect mammalian cells (5 – 8). We reported that DIF-1 and DIF-3 [1-(3,5-dichloro-2,6-dihydroxy-4-methoxyphenyl) hexan-1-one and 1-(3-chloro-2,6-dihydroxy-4-methoxyphenyl)hexan-1-one, respectively] inhibited mammalian cell proliferation by suppressing the Wnt/β-catenin signaling pathway via the activation of glycogen synthase kinase-3β (GSK-3β) (9 – 13). Since the constant activation of the Wnt/β-catenin signaling pathway has frequently been demonstrated in several types of human malignant neoplasms (14, 15), it could be suggested that DIFs are potent antitumor agents and identification of the target molecule(s) for DIFs may offer ideas for the design of new anticancer drugs. However, the target molecule(s) of DIFs involved in the anti-proliferative effect and/or the inhibition of the Wnt/β-catenin signaling pathway are unknown. Shimizu et al. first reported that calmodulin-dependent cyclic nucleotide phosphodiesterase could be a pharmacological target molecule for DIF-1, whereas a specific inhibitor for phosphodiesterase 1 failed to mimic

*Corresponding author. yanaga@clipharm.med.kyushu-u.ac.jp
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the anti-proliferative effect of DIF-1 (16).

In the present study, the target molecules of DIFs were explored. For this purpose, DIF-1–tethered beads for affinity chromatography were synthesized. Upon further investigation, we found that the mitochondrial isoform of malate dehydrogenase (mMDH) was one of the target molecules of DIFs.

Malate dehydrogenase (MDH; EC 1.1.1.37) is an essential enzyme in the tricarboxylic acid cycle and malate-aspartate shuttle (17, 18), oxidizing malate to oxaloacetate and reducing oxaloacetate to malate, respectively. Two isoenzymes of MDH are found in animal tissues: one is in the mitochondria (mMDH, 314 amino acids) and the other is in the cytoplasm (cMDH, 332 amino acids) (19). The major role of mMDH is to oxidize malate to oxaloacetate in the tricarboxylic acid cycle, and that of cMDH is to reduce oxaloacetate to malate in the malate-aspartate shuttle. Since both the tricarboxylic acid cycle and malate-aspartate shuttle play essential roles in the energy production in cells, we analyzed the effect of DIF-1 and other DIF analogs on the activities of MDH in relation to ATP production and cell proliferation using HeLa cells.

Materials and Methods

Materials

DIF-1 and DIF-3 (1-(3,5-dichloro-2,6-dihydroxy-4-methoxyphenyl)hexan-1-one and 1-(3-chloro-2,6-dihydroxy-4-methoxyphenyl)hexan-1-one, respectively) were synthesized according to the procedure of Masento et al. (20). The other DIF analogs (2-MIDIF-1, 2-methoxy isomer of DIF-1, and 6-MIDIF-3, 6-methoxy isomer of DIF-3) were synthesized by Toyama Chemical Co. (Tokyo). Mitochondrial MDH (M2634) and cMDH (M7383) prepared from porcine heart were purchased from Sigma (St. Louis, MO, USA).

Preparation of DIF-1–tethered resin

To a solution of DIF-1 (20 mg, 0.065 mmol) and 2,6-lutidine (11 μL, 0.078 mmol) in CH_2Cl_2 (200 μL) was added 2-chlorethanesulfonyl chloride (8 μL, 0.078 mmol), and the resulting mixture was stirred for 2 h at room temperature. After work-up, silica-gel chromatography (AcOEt/hexane 1:4) gave mono-*O*-ethenesulfonylated DIF-1 (11 mg, 42%) together with DIF-1 (58% recovered). AffiGel 10 (chemical capacity: 0.01 mmol/gel mL; Bio-Rad, Herucles, CA, USA,) was modified by treatment with 2-aminoethanethiol in isopropanol. The modified resin (1 mL) was treated with sulfonyl-DIF-1 (8.0 mg, 0.02 mmol) and triethylamine (3.3 μL, 0.024 mmol) and ethanol (0.5 mL) for 1 h. The resin was obtained by filtration of the mixture and washing with ethanol. It was then similarly treated with *N*-phenylmaleimide (3.5 mg, 0.02 mmol), triethylamine (3.3 μL, 0.024 mmol), and ethanol (0.5 mL) for 3 h in order to cap the residual SH function. After filtration, the DIF-1–tethered affinity resin was obtained.

Cell culture

The human cervical carcinoma cell line HeLa was grown in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum, 100 U/mL penicillin G, and 0.1 mg/mL streptomycin.

Affinity chromatography using DIF-1–tethered resins

Cytoplasmic proteins were prepared from cells cultured in 100-mm dishes using NE-PER™ nuclear and cytoplasmic extraction reagents (Pierce, Rockford, IL, USA). Just before use, 10 μL of DIF-1–tethered-beads were washed with 1 mL of buffer A containing 10 mM Tris-HCl pH 7.4, 1 mM EDTA, 50 mM KCl, and 10% glycerol and then resuspended with 200 μL of the same buffer. The beads were incubated in the presence or absence of 100 μM of DIF-1 at 4°C for 1 h, followed by the addition of the cell extract (1 mg of cytoplasmic proteins) and incubation for an additional 2 h. Proteins bound to the beads were extracted in Laemmli's sample buffer after extensive washing with buffer A.

Identification of binding protein

The extracted protein from the beads was separated by 10% SDS-PAGE, followed by silver staining. The gel pieces with bands only observed in the absence of DIF-1 were treated with trypsin, and the resulting peptides were analyzed by liquid chromatography/mass/mass spectrometry (LC-MS/MS) as described in the previous report (21). Mass spectroscopy data were analyzed by the MASCOT search engine (Matrix Science, Boston, MA, USA) using the International Protein Index (European Bioinformatics Institute, Hinxton, UK) as references.

Quartz crystal microbalance (QCM) assay

To measure the affinity of DIF and its analogs for MDH, a QCM assay was performed as described previously (22) with minor modifications. Briefly, 2 μL of 4 mg/mL mMDH, cMDH or bovine serum albumin (BSA) was fixed on the sensor-tip of the QCM and placed in the chamber filled with 2 mL of phosphate-buffered saline. After the frequency of the sensor-tip was stable, ethanol (vehicle), DIF-1 or the analogs (2 μL; final concentration of 30 μM) were injected into the chamber. The frequency of the chip was measured at 25°C with constant stirring.

Measurement of enzymatic activities of mMDH and cMDH

Activities of mMDH or cMDH were assayed either in

the forward direction (malate + NAD^+ → oxaloacetate + NADH) or reverse direction. Mitochondrial MDH (10.1 mg/mL) or cMDH (7.4 mg/mL) were diluted 1,000-times with a solution containing 0.1 mg/mL BSA and 1 μM dithiothreitol and were pre-incubated with or without DIF-1 (final concentration: 30 μM) for 10 min on ice. Then, 10 μL of the enzyme solution and 40 μL of β-NAD^+ (final concentration: 10 mM) was mixed in a 96-well plate, followed by monitoring at the wavelength of 340 nm using a Flex Station3 micro-plate reader (Molecular Devices, Sunnyvale, CA, USA). Three minutes later, 50 μL of malate (final concentration: 500 mM) was added to the each well as substrate. One unit was defined as the activity that can increase NADH by 1.0 μmol per min.

Intracellular ATP measurement and cell proliferation assay

HeLa cells (2.0×10^4 cells/well) were seeded into 24-well plates and treated with or without 30 μM DIF-1 or the analogs for given periods. Cells were harvested by the trypsin/EDTA treatment and enumerated using a Coulter Counter (Beckman Coulter, Fullerton, CA, USA). Intracellular ATP content was determined with a luciferin–luciferase method using an ATP assay kit (TOYO INK, Tokyo). Luciferase activity was determined with a luminometer (Lumat LB 9507; Berthold Technologies, Bad Wildbad, Germany).

Results

DIF-1 modification and immobilization

Ligand-immobilized beads were used as a tool to identify the target molecules of DIF-1. Therefore, we first synthesized the DIF-1 analog, DIF-1-sulfonate shown in Fig. 1A (see also Fig. 5A for the structure of DIF-1), which could be connected to reactive beads. Assays of cell proliferation was performed using HeLa cells, since we reported that DIF-1 and DIF-3 inhibited mammalian cell proliferation. As shown in Fig. 1B, DIF-1-sulfonate clearly exhibited inhibitory effects on cell proliferation, albeit with a weaker potency than DIF-1, encouraging us to further synthesize DIF-1–tethered beads using the analog.

DIF-1 binds to mMDH and the effects on the enzyme activity

HeLa cell extracts were mixed with immobilized-DIF-1 and affinity chromatography was performed as described in Materials and Methods. To identify the proteins that bind specifically to DIF-1, the cell extracts were incubated with the immobilized beads in the presence or absence of DIF-1 at 4°C for 2 h. Binding proteins were separated by 10% SDS-PAGE and two silver-

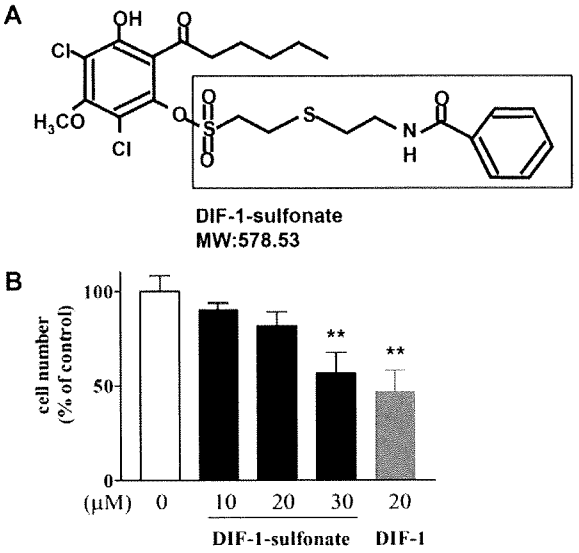

Fig. 1. Chemical structure and effects of DIF-1-sulfonate. A) Structure of DIF-1-sulfonate. The sulfonate group acting as a bridge for connection to the beads is marked by a rectangle. B) Effect of DIF-1-sulfonate on the cell proliferation. HeLa cells were treated with the indicated chemicals for 48 h and enumerated. The reported data are each the mean ± S.D. of three independent experiments (n = 3). $^{**}P < 0.01$ vs. control (by Student's *t*-test).

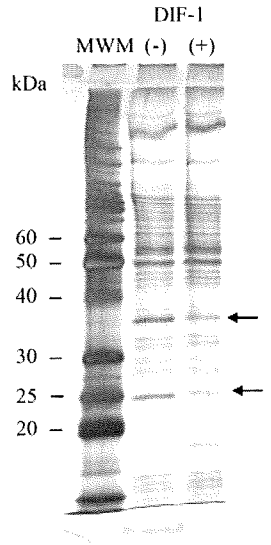

Fig. 2. DIF-1–tethered resin-binding proteins in HeLa cells. The extract from HeLa cells was loaded onto DIF-1–tethered resin with or without excess amount of DIF-1. The bound protein was analyzed by SDS-PAGE followed by silver staining. Arrows indicate two bands that specifically bound to DIF-1–tethered resin.

stained bands were identified as the proteins that bind to DIF-1–tethered beads, but not in the presence of the soluble ligand (Fig. 2). The upper band was identified as mMDH by mass spectrometry, while the lower band was

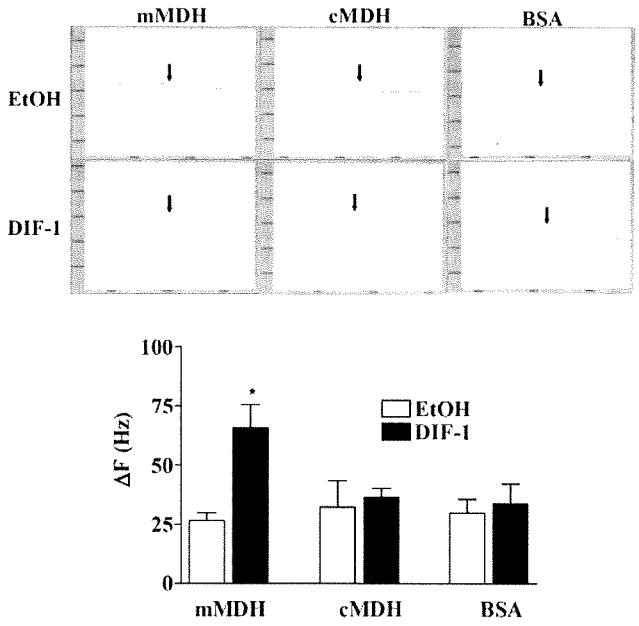

Fig. 3. DIF-1 binds with mMDH. QCM assay was performed as described in Materials and Methods. A difference of frequency was measured before and after injection. The arrows in the upper figure indicate the injection points of vehicle (ethanol: EtOH) or DIF-1. The lower graph shows the difference in frequency between before and after injection. The reported data are each the mean ± S.D. of three independent experiments. $*P < 0.05$ vs. control (by Student's *t*-test).

not identified. To confirm whether DIF-1 binds directly to mMDH in vitro, we performed QCM analysis. As shown in Fig. 3, the frequency was significantly reduced by adding DIF-1 when mMDH was fixed on the sensor tip. No significant changes were observed when cMDH, the cytosolic isoenzyme of MDH or BSA was fixed on the tip, indicating that DIF-1 directly binds to mMDH but not to cMDH. Subsequently, the effect of DIF-1 on the activities of mMDH and cMDH were analyzed. Mitochondrial MDH or cMDH, obtained from a commercial source was assayed for NAD^+-dependent oxidation (forward direction). DIF-1 inhibited mMDH activity in a dose-dependent manner (Fig. 4A), whereas cMDH activity was not affected (Fig. 4B). The result agreed well with the result shown in Fig. 3, indicating that DIF-1 inhibited mMDH activity by direct binding.

Effects of DIF-1 analogs on mMDH

The interactions between mMDH and DIF analogs (2-MIDIF-1, DIF-3, and 6-MIDIF-3) were analyzed by the QCM assay. As shown in Fig. 5A, all DIF analogs examined were revealed to directly bind to mMDH and the positive signals with 2-MIDIF-1 was highest among the analogs examined, suggesting that 2-MIDIF-1 had the highest affinity. The effects of DIF analogs on the enzyme activity were then analyzed (Fig. 5B). Interestingly, of the 3 analogs examined, only 2-MIDIF-1 was inhibitory, although the effect of 2-MIDIF-1 appeared weaker than that of DIF-1 (Figs. 4A and 5B).

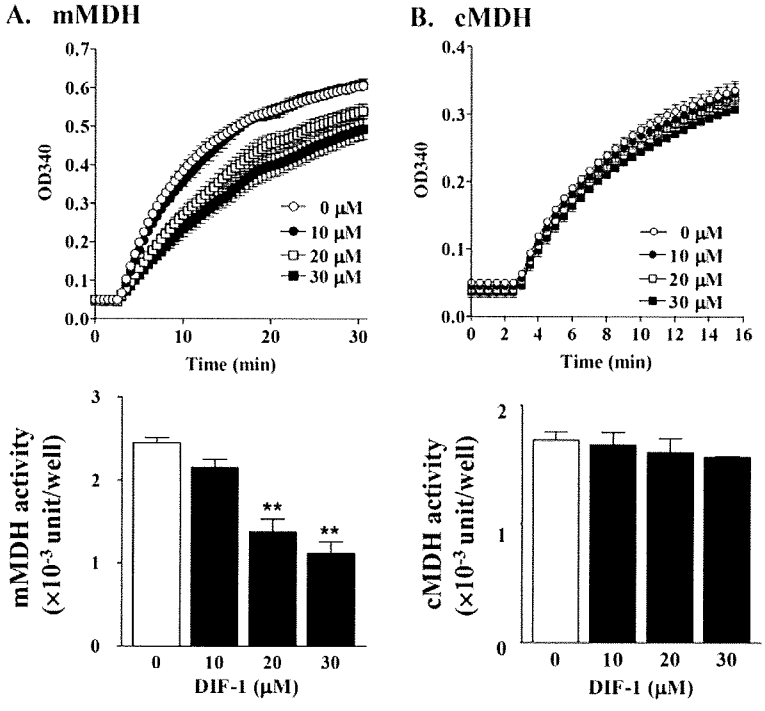

Fig. 4. Effects of DIF-1 on MDH activity. The mMDH (A) and cMDH (B) activities were assayed in the forward direction using different concentrations of DIF-1. Upper panels show the change of absorption and lower panels show the graph for MDH activity calculated from the upper panels as described in Materials and Methods. Each value is the mean ± S.E.M. of three independent experiments done in triplicate. $**P < 0.01$ vs. control (by Student's *t*-test).

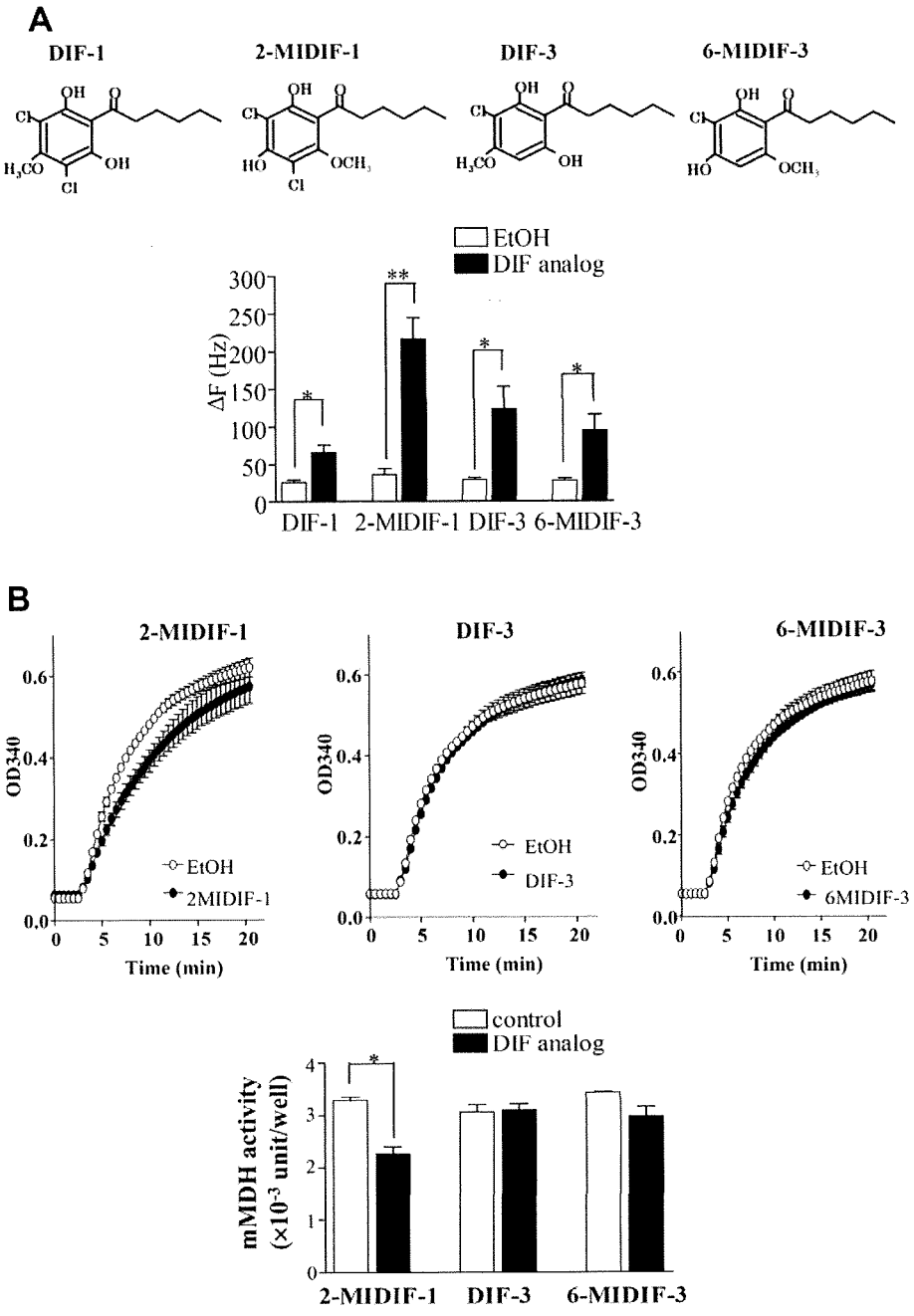

Fig. 5. Effect of DIF analogs on mMDH. A) Upper panel shows the chemical structure of DIF analogs and lower panel shows the binding property of DIF analogs to mMDH. Difference of frequency was measured before and after injection. Data are shown as the difference of frequency between before and after injection. Each value is the mean ± S.E.M of three independent experiments. $*P < 0.05$ vs. control, $**P < 0.01$ vs. control (by Student's *t*-test). B) Effect of DIF analogs on mMDH activity. mMDH activity was measured in the forward direction. Upper panels show the change of absorption and lower panel shows the graph for mMDH activity calculated from the upper panel as described in Materials and Methods. Each value is the mean ± S.E.M. of three independent experiments done in triplicate. $*P < 0.05$ vs. control (by Student's *t*-test).

Effects of DIF-1 and DIF analogs on ATP content and cell growth

To examine the relationship between the inhibition of mMDH activity, cell energy production, and cell proliferation, the effects of DIF analogs on intracellular ATP content and cell proliferation were examined using HeLa cells. The cellular content of ATP was gradually increased as the time of culture increased up to 72 h, and DIF-3 and 6-MIDIF-3, ineffective in the mMDH assay, did not impact the results (Fig. 6A). On the other hand, DIF-1 and 2-MIDIF-1 lowered the ATP content in the same order of potency as that for the inhibition of mMDH activity.

Cell proliferation was also analyzed. As shown in Fig. 6B, DIF-1 and DIF-3 were the most potent inhibitors of the cell proliferation, while 6-MIDIF-3 was ineffective. In the case of 2-MIDIF-1, the inhibition of cell proliferation became evident at 72 h, when the cellular content of ATP was apparently lowered to a similar level as that with DIF-1. However, the ATP content of HeLa cells after 72-h treatment with 2-MIDIF-1 was still approximately 70% of that at time 0, suggesting that 2-MIDIF-

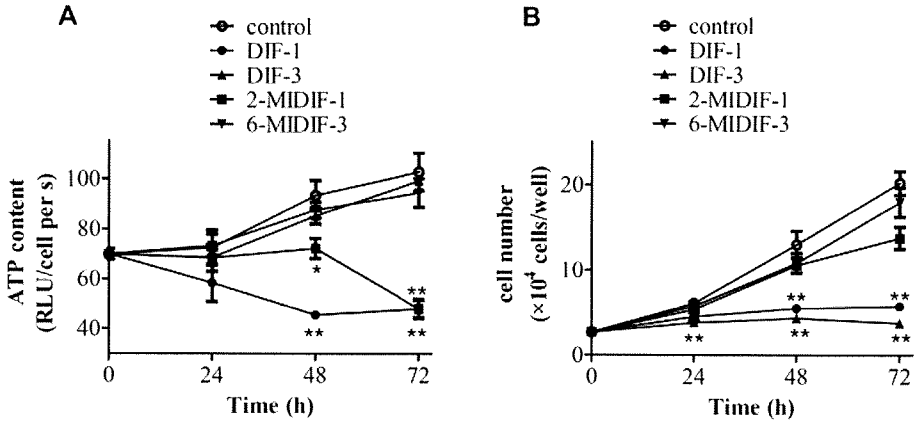

Fig. 6. Effects of DIF analogs on intracellular ATP content and cell growth. HeLa cells (2.0×10^4 cells/well) were seeded into 24-well plates and treated with or without 30 μM of DIF analogs for the given periods. Cells were harvested by trypsin/EDTA treatment, followed by the measurement of the ATP content (A) and cell number (B). The reported data are each the mean ± S.E.M. of three independent experiments, each performed in duplicate. $^*P < 0.05$ vs. control, $^{**}P < 0.01$ vs. control (by Student's t-test).

1–treated HeLa cells did not deplete ATP. This result was well correlated with the fact that 2-MIDIF-1 showed a weak inhibitory effect on cell proliferation.

Discussion

Previously, we have been reported that DIF-1 and DIF-3 inhibit cell proliferation via the suppression of the Wnt/β-catenin signaling pathway in human cancer cells (9 – 13). Although the purpose of the study was to find the target molecule of DIFs related with the Wnt/β-catenin signaling pathway, unfortunately we could not identify such a protein from mass spectrometry. Mitochondrial MDH, one of the DIFs target proteins found in this study, is not a component of the Wnt/β-catenin signaling pathway and this enzyme was inhibited by only DIF-1 and not by DIF-3, whereas both chemicals showed an inhibitory effect on the Wnt/β-catenin signaling pathway. These observations suggest that other target molecules related with the Wnt/β-catenin signaling pathway must exist. Further studies are required to identify the target molecules of DIF analogs.

We analyzed the changes in cellular content of ATP and cell proliferation in HeLa cells treated with DIF analogs. DIF-1 lowered the ATP content and inhibited cell proliferation more robustly than 2-MIDIF-1. This might have resulted from the fact that DIF-1 exhibited stronger inhibition of mMDH activity than 2-MIDIF-1 and suggest that lowering the ATP content caused by the inhibition of mMDH is pharmacologically relevant to the inhibition of cell proliferation.

Alternatively, a recently reported finding that DIF-1, but not 2-MIDIF-1, was able to promote glucose uptake and consumption could explain the inhibition results (23). It is possible that DIF-1–treated cells deplete glucose in the medium during the culture period. Furthermore, it is well known that glycolysis rather than the tricarboxylic acid cycle is the main energy production system in a number of cancer cell types (24 – 26), suggesting that inhibition of mMDH activity might not have a strong effect on ATP production and inhibition of glycolysis might be more effective to reduce ATP production in HeLa cells. Consequently, depletion of glucose in the culture medium by DIF-1 likely resulted in the decrease of the ATP content in the HeLa cells. This notion also might explain the delay of 2-MIDIF-1 on the ATP content. At the initial stage of the culture, 2-MIDIF-1–treated cells produced ATP mainly using glycolysis, uptaking glucose from the culture medium, but at the later stage, consumption of glucose triggered oxidization of fatty acids to yield acetyl-CoA for the tricarboxylic acid cycle, and gluconeogenesis from amino acids followed by glycolysis, thus producing an overall lower level of ATP.

In this report, we showed that mMDH, one of the essential enzymes in the tricarboxylic acid cycle and malate-aspartate shuttle for main energy production in normal cells, is one of the target molecules of DIFs. Indeed, all DIFs analogs examined in this study could bind to mMDH. However, among the DIF analogs examined, only DIF-1 and 2-MIDIF-1 inhibit mMDH activity, suggesting that the chlorine at the 5 position of the DIF ring is essential for inhibition. Although we have reported that both DIF-1 and DIF-3 inhibited proliferation of human cancer cells, this finding may suggest that DIF-3 is more suitable as a candidate for a new anti-cancer drug than DIF-1, since DIF-3 lacks the chlorine at the 5 position of the DIF ring and does not affect mMDH activity.

Acknowledgments

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