Isoproterenol produces a rapid increase in sialidase activity in rat heart tissue and cardiomyocyte-derived H9c2 cells in culture

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Abstract The effects of isoproterenol on sialidase activity in rat cardiomyocytes were examined. Administration of isoproterenol to rats (0.2 or 2 mg/kg body weight) produced an increase in sialidase activity in total membrane fraction of heart tissue within 120 min (121 ± 13% of the control at 120 min after administration of 0.2 mg isoproterenol/kg, $n = 5$, $P < 0.05$). Sialidase activity in cardiomyocyte-derived H9c2 cells was also increased by treatment with isoproterenol (10 μM) for 60 min. The effect of isoproterenol on sialidase activity was amplified by the addition of 3-isobutyl-1-methylxanthine (IBMX). Sialidase activity in H9c2 cells was elevated by treatment with dibutyryl cAMP plus IBMX without isoproterenol. The content of *N*-acetylneuraminic acid in cells decreased by 22% after treatment with isoproterenol plus IBMX. These results suggest that sialidase activity in rat cardiomyocytes is regulated by β-adrenergic stimulators via a cAMP-dependent process. The increased activity of sialidase may account for the reduction of sialic acid content of cells.

Key words: Sialidase; Isoproterenol; 3-Isobutyl-1-methylxanthine; Sialic acid; *N*-Acetylneuraminic acid; Cardiomyocyte; H9c2 cell

1. Introduction

Sialidase is an exoglycosidase that cleaves α-linked sialic acid residues of sialoglycoconjugates [1]. This enzyme plays a crucial role in the catabolism of sialoglycoconjugates; the deficiency of sialidase activity in sialidosis or galactosialidosis causes accumulation of sialooligosaccharides, sialoglycoproteins, and gangliosides in tissues [2]. Sialidase has also been implicated in diverse cellular events including development [3–6], growth and differentiation [7–10], aging [11,12], and neoplastic transformation [13]. While sialidase activity undergoes specific changes during these biological processes, the regulatory mechanism of enzyme activity remains to be clarified.

Previously, it was reported that intravenous administration of β-adrenergic agonists to mice causes a significant decrease in sialic acid contents of parotid plasma membranes [14–17]. These findings led us to the assumption that β-adrenergic stimulation may cause an increase in sialidase activity, which accelerates the degradation of sialic acid compounds in cells. In the present study, we examined the effects of isoproterenol on sialidase activity in rat heart tissue and clonal cardiomyocyte-derived H9c2 cells in culture. We also investigated the influence of β-adrenergic stimulation on sialic acid contents of H9c2 cells.

2. Materials and methods

2.1. Materials

Seven-week-old Sprague–Dawley male rats were purchased from Japan SLC (Shizuoka, Japan). Rat cardiomyocyte-derived H9c2 cells were obtained from American Type Culture Collection (Manassas, VA, USA). Other reagents were purchased from the following companies: L-(−)-isoproterenol (ICN Biomedicals, Aurora, OH, USA), 1,2-diamino-4,5-methylenedioxybenzene (DMB, Dojindo Laboratories, Kumamoto, Japan), and 3-isobutyl-1-methylxanthine (IBMX) and 4-methylumbelliferyl-*N*-acetylneuraminic acid (4MU-Neu5Ac) (Sigma, St. Louis, MO, USA).

2.2. Administration of isoproterenol to animals

Isoproterenol was administered to rats as an intravenous bolus (0.02–2 mg/kg body weight) through the tail vein. At 20 or 120 min after isoproterenol administration, the animals were decapitated, and the heart tissues were excised. Subcellular fractionation of heart tissue was performed at 4°C. Tissue was homogenized with 8 volumes of 0.32 M sucrose containing 25 mM KCl, 3 mM $MgCl_2$, 2 mM $CaCl_2$ and 50 mM Tris–HCl (pH 7.4) using a Teflon-glass homogenizer. The homogenate was centrifuged at $650 \times g$ for 10 min. The pellet was homogenized with the same volume of buffer and centrifuged under the same condition. The supernatants were combined and centrifuged at $100\,000 \times g$ for 30 min. The resultant pellet was used as total membrane fraction.

2.3. Cell culture

Clonal H9c2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum under 5% CO_2 in air at 37°C [18]. For experiments, cells at 80–90% confluence were preincubated in serum-free DMEM at 37°C for 10 min, followed by treatment with 1 or 10 μM isoproterenol for 60 min under the serum-free condition. In some experiments, cells were treated with 100 μM IBMX for 30 min after a 10 min preincubation period, followed by treatment with the β-adrenergic agonist in the presence of IBMX. The treated cells were washed twice with cold phosphate-buffered saline (pH 7.4), scraped off the dish, and homogenized in 10 mM Tris–HCl, pH 7.4 using a Teflon-glass homogenizer.

2.4. Sialidase assay

Sialidase activity was measured using 4MU-Neu5Ac as the substrate [19]. The reaction mixture consisted of 0.2 mM 4MU-Neu5Ac, 0.1 M sodium acetate buffer (pH 4.8), and enzyme preparation (40–200 μg as protein) in a final volume of 100 μl. After incubation at 37°C for 1 h, the reaction was terminated by addition of 0.15 M

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Abbreviations: Neu5Ac, *N*-acetylneuraminic acid; DMB, 1,2-diamino-4,5-methylenedioxybenzene; IBMX, 3-isobutyl-1-methylxanthine; 4MU-Neu5Ac, 4-methylumbelliferyl-Neu5Ac; Neu5Prop, *N*-propanoylneuraminic acid

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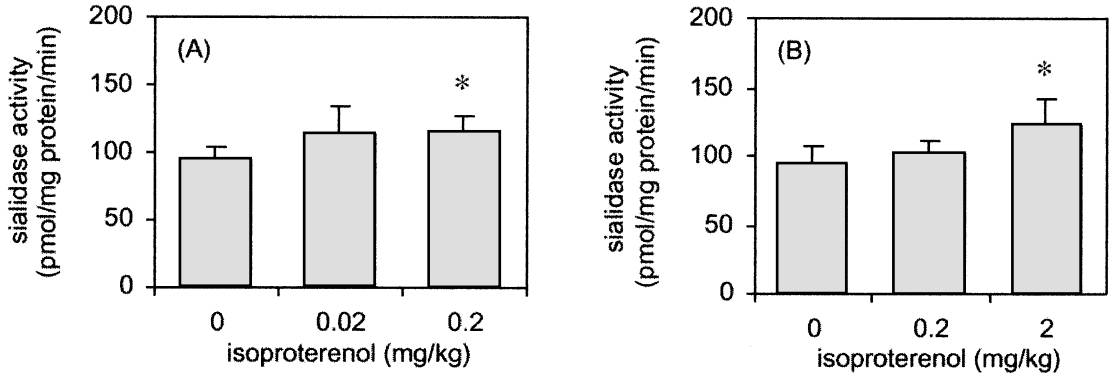

Fig. 1. Effect of isoproterenol on sialidase activity in total membrane fractions of rat heart tissue. Seven-week-old rats were intravenously administered a single dose of isoproterenol (0.02–2 mg/kg body weight). The heart tissue was excised at 120 min (A) or 20 min (B) after administration of isoproterenol. Sialidase activity in total membrane fraction was assayed at pH 4.8 using 4MU-Neu5Ac. Each column and attached bar represent the mean ± S.D. ($n = 5$ for each group). Statistical analysis was performed by Student's t-test. *$P < 0.05$.

glycine–NaOH buffer (pH 10.8, 2.9 ml). The released 4MU was quantitated by measuring fluorescence at excitation and emission wavelengths of 365 nm and 445 nm, respectively [20]. Preliminary experiments showed that this assay condition was optimal for measuring sialidase activity in rat heart tissue and H9c2 cells (data not shown).

Protein was determined by the method of Lowry et al. [21].

2.5. Sialic acid analysis

Sialic acids in H9c2 cells were quantitated using a fluorometric high performance liquid chromatographic (HPLC) method [22] with *N*-propanoylneuraminic acid (Neu5Prop) as an internal standard [23]. In brief, an aliquot of cell homogenate was treated in 50 mM sulfuric acid at 80°C for 1 h. A fixed amount of Neu5Prop was added to the hydrolysate, followed by incubation of the mixture with DMB reagent at 60°C for 2.5 h. The fluorescent derivatives of sialic acids were analyzed by HPLC equipped with an ODS column (Mightysil RP 18, 250×4.6 mm, Kanto Chemicals, Tokyo, Japan) and fluorescence detector. The excitation and emission wavelengths were 373 and 448 nm, respectively.

3. Results

Three different doses of isoproterenol were administered to rats, and sialidase activity in total membrane fractions of heart tissue was examined. At the lowest dose (i.e. 0.02 mg isoproterenol/kg body weight), sialidase activity tended to increase, but did not reach a statistically significant level at 120 min after isoproterenol administration. At the middle dose (0.2 mg/kg), sialidase activity increased and reached a 1.21-fold level of control at 120 min (Fig. 1A). The time required for the response of sialidase activity was shortened to 20 min using the highest dose of isoproterenol (i.e. 2 mg/kg) (Fig. 1B).

The effect of isoproterenol on sialidase activity was further examined using a clonal H9c2 cell line. Sialidase activity increased at 1 μM of isoproterenol, though statistical significance was not attained by incubation for 60 min. The enzyme activity significantly increased at 10 μM isoproterenol (Fig. 2). The response of sialidase activity to isoproterenol was magnified in the presence of IBMX (100 μM), a phosphodiesterase inhibitor (Fig. 3). Sialidase activity in H9c2 cells was also elevated by treatment with dibutyryl cAMP (1 mM) plus IBMX (100 μM) in the absence of β-agonist (Fig. 4).

Sialic acid analysis was performed using H9c2 cells treated with isoproterenol plus IBMX (Table 1). The Neu5Ac content was reduced to 78% of the control. No significant difference in the content of Neu5Gc was observed between the treated cells and control.

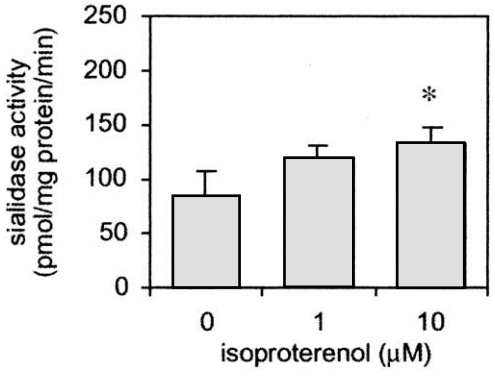

Fig. 2. Effect of isoproterenol on sialidase activity in H9c2 cells. Rat cardiomyocyte-derived H9c2 cells in culture were preincubated in serum-free DMEM at 37°C for 10 min, followed by treatment with 1 or 10 μM isoproterenol for 60 min. Cells were homogenized in 10 mM Tris–HCl, pH 7.4 and subjected to sialidase assay as described in Fig. 1. Each column and attached bar represent the mean ± S.D. ($n = 4$ for each group). *$P < 0.05$.

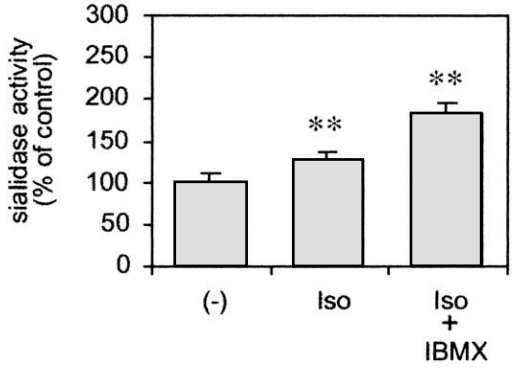

Fig. 3. Effect of IBMX on the isoproterenol-induced increase of sialidase activity in H9c2 cells. Rat cardiomyocyte-derived H9c2 cells in culture were preincubated in serum-free DMEM at 37°C for 10 min, followed by incubation with 100 μM IBMX. The cells were then treated with 10 μM isoproterenol for 60 min without changing the medium. Each column and attached bar represent the mean ± S.D. ($n = 4$ for each group). **$P < 0.01$.

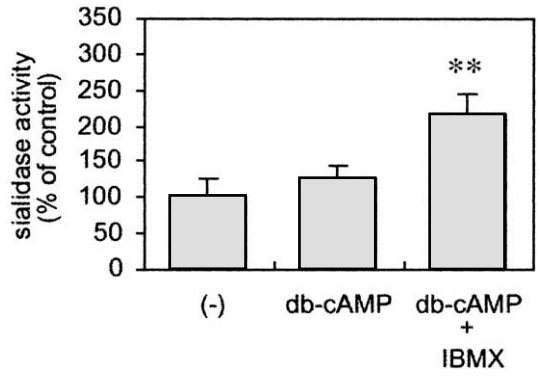

Fig. 4. Effects of dibutyryl cAMP (plus IBMX) on sialidase activity in H9c2 cells. H9c2 cells in culture were preincubated in serum-free DMEM for 10 min, followed by treatment with 1 mM dibutyryl cAMP for 60 min. Alternatively, H9c2 cells were incubated with 100 μM IBMX after preincubation in serum-free DMEM for 10 min, followed by treatment with the cyclic nucleotide for 60 min. Each column and attached bar represent the mean ± S.D. ($n = 4$ for each group). $^{**}P < 0.01$.

4. Discussion

There are several studies reporting changes of sialidase activity caused by exogenously added chemical agents to cells. For example, retinoic acid or lipopolysaccharides have been shown to increase sialidase activity in human leukemia HL-60 cells [24] or mouse T lymphocytes [25]. However, the responses of sialidase activity were relatively slow and required days to be seen. Thus, it is assumed that the observed changes may not be a direct outcome caused by the agents, but represent one of many complex differentiation processes in cells. In the present study, we examined the effects of the β-adrenergic agonist isoproterenol on sialidase activity in rat heart tissue and the clonal H9c2 cell line. H9c2 cells were originally established from embryonic rat ventricle and have been shown to preserve elements of electrical and hormonal signaling pathways found in adult cardiomyocytes [26,27]. The expression of β_1- and β_2-adrenergic receptors in functional states has also been demonstrated [28]. Isoproterenol was shown to produce a rapid increase in sialidase activity of rat cardiomyocytes under in vitro and in vivo experimental conditions within 1 h. This is the first report about pharmacological regulation of sialidase activity.

The increased sialidase activity in isoproterenol-treated H9c2 cells was strongly augmented by co-treatment of cells with IBMX, a phosphodiesterase inhibitor. It is known that β-adrenergic agonists raise the intracellular cAMP levels of target cells. This effect is amplified by treatment with IBMX [29]. It was thus suggested that the elevation of intracellular cAMP may be responsible for the increase in sialidase activity. This assumption was supported by the observation that the treatment of H9c2 cells with dibutyryl cAMP plus IBMX generated a similar effect on sialidase activity as that of isoproterenol.

Isoproterenol not only produced an increase in sialidase activity, but also brought a concomitant reduction of sialic acid contents in cells. It is known that most sialic acids are associated with plasma membranes in eukaryotic cells [30]. Evidence has also been provided suggesting the significance of plasma membrane-bound sialidase in the catabolism of sialoglycoconjugates in the membranes. For example, plasma membrane-bound sialidase may play an important role in the turnover of gangliosides in brain myelin [31] or neuroblastoma cells [32]. A positive correlation between the activity of plasma membrane sialidase and the turnover rate of radiolabeled sialic acid compounds on the cell surface was observed with Rous sarcoma virus-transformed chick embryonic fibroblasts [33]. It is thus reasonable to speculate that the increased activity of membrane-bound sialidase by β-agonists may account for the reduction of sialic acid contents in H9c2 cells. To clarify this issue, identification of sialidase isoenzymes responding to β-adrenergic stimulation would be required in a future study.

Table 1
Effect of isoproterenol plus IBMX on sialic acid contents of H9c2 cells

Treatment	Sialic acid content (ng/mg protein)	
	Neu5Ac	Neu5Gc
–	1700 ± 190	61 ± 7
Isoproterenol	1470 ± 80	51 ± 14
Isoproterenol+IBMX	1330 ± 140*	51 ± 15

Cells were treated with isoproterenol (10 μM) or isoproterenol (10 μM) plus IBMX (100 μM) as described in Fig. 3. Neu5Ac and *N*-glycolylneuraminic acid (Neu5Gc) of the cells were analyzed using the fluorometric HPLC method with Neu5Prop as the internal standard.
$^{*}P < 0.05$ ($n = 4$ for each group).

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