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Ability of higenamine and related compounds to enhance glucose uptake in L6 cells

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ABSTRACT

β2-adrenergic receptor (β2AR) agonists are employed as bronchodilators to treat pulmonary disorders, but are attracting attention for their modulation of glucose handling and energy expenditure. Higenamine is a tetrahydroisoquinoline present in several plant species and has β2AR agonist activity, but the involvement of each functional groups in β2AR agonist activity and its effectiveness compared with endogenous catecholamines (dopamine, epinephrine, and norepinephrine) has rarely been studied. Glucose uptake of muscle cells are known to be induced through β2AR activation. Here, the ability to enhance glucose uptake of higenamine was compared with that of several methylated derivatives of higenamine or endogenous catecholamines. We found that: (i) the functional groups of higenamine except for the 4'-hydroxy group are required to enhance glucose uptake; (ii) higenamine shows a comparable ability to enhance glucose uptake with that of epinephrine and norepinephrine; (iii) the *S*-isomer shows a greater ability to enhance glucose uptake compared with that of the *R*-isomer.

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1. Introduction

The β2-adrenergic receptor (β2AR) is a G protein-coupled receptor expressed widely in the pancreas, liver, skeletal muscle, and adipose tissue. β2AR activity is involved in regulation of relaxation of airway smooth muscle,[1] hepatic glucose production,[2] glucose uptake in muscle,[3,4] lipolysis,[5] and other functions.[6,7]

The synthetic agonists of β2AR are utilized widely as bronchodilators to treat pulmonary disorders such as asthma and chronic obstructive pulmonary disease because they can relax muscle.[8,9] In addition, because of the increasing prevalence of type-2 diabetes mellitus (T2DM) and obesity worldwide, β2AR agonists are attracting attention for their modulation of glucose handling and energy expenditure.[10] Short-term treatment with clenbuterol,[11] as well as chronic treatment with salbutamol combined with exercise,[12] has been demonstrated to improve glucose tolerance in a murine model of T2DM . Treatment with the synthetic selective β2AR agonist formoterol has been shown to increase the metabolic rate and fat oxidation in overweight humans.[13]

Apart from synthetic β2AR agonists, higenamine (**1**), a tetrahydroisoquinoline present in several plant species,[14–18] has been reported to have β2AR agonist activity.[14] Owing to this activity, **1** has been shown to: protect against cardiac injury and myocyte apoptosis;[19] induce relaxation in isolated tracheal rings and protect from experimentally induced asthma in guinea pigs.[14,20] Acute oral intake of **1** increases the concentration of circulating free fatty acids and energy expenditure in humans, which may be beneficial for obesity treatment.[21] Additionally, several biologic effects, like anti-inflammatory activity, immunomodulatory effects, or anti-thrombotic effect, for which the detailed mechanism of action has not been elucidated are also reported.[22] These beneficial bioactivities has made higenamine an attractive target for further study.

Previously, we reported the isolation of higenamine 4'-*O*-β-D-glucoside (**2**) from lotus plumule, and later confirmed that it is a mixture of (*S*)- and (*R*)-isomers.[23,24] Higenamine (**1**) itself has been studied widely for its β2AR agonist activity and related biologic effects, but the derivatives of **1** have not. Glucoside derivatives of **1** are rare,[25] and the influence of a glucose moiety on β2AR agonist activity is not clear. Methylation is a common biosynthetic pathway,[26] and an asymmetric carbon atom in **1** has been noted. However, the β2AR agonist activity of methylated derivatives have not been investigated, and a comparison in the activity of (*R*)-(+)-higenamine (**1R**) with (*S*)-(−)-higenamine (**1S**) is only investigated for the anti-aggregation of platelets which represents α-adrenergic receptor antagonist activity.[27] Moreover, the agonist activity of **1** compared with its endogenous ligands has not been documented.

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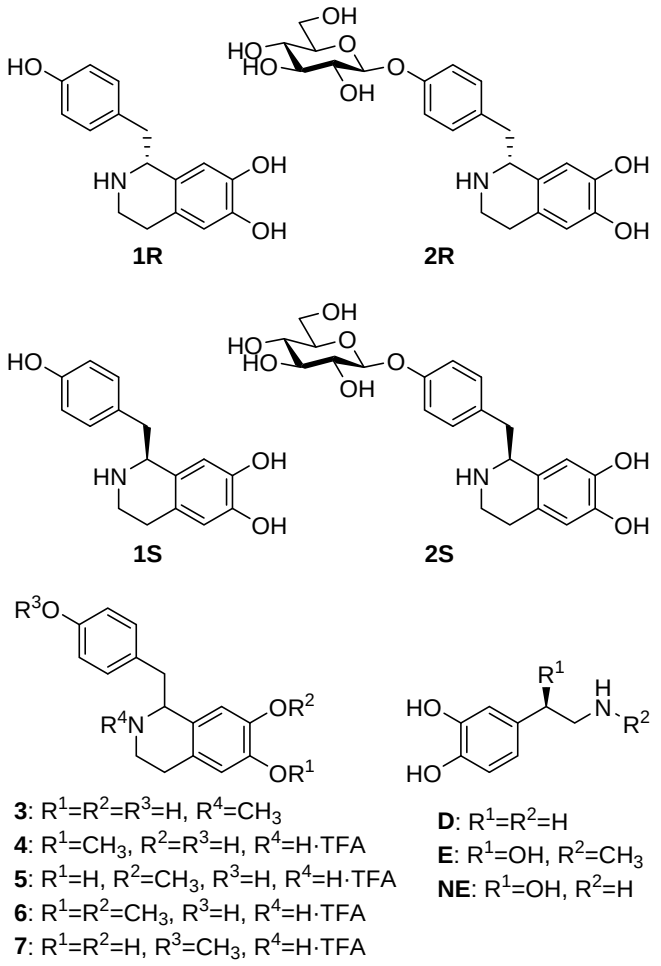


Figure 1. Structures of the compounds **1**-**7**, dopamine (**D**), L-epinephrine (**E**), and L-norepinephrine (**NE**).

Here, we synthesized the methyl derivatives of **1**. Along with (*R*)-(+)- and (*S*)-(−)-higenamine (**1R**, **1S**), their glucoside derivatives (**2R**, **2S**), and several endogenous catecholamines (Figure 1), enhancement of glucose uptake in immortalized rat skeletal muscle cells (L6 cells) was tested and compared.

2. Materials and methods

2.1. General

L-epinephrine bitartrate, L-norepinephrine bitartate, and dopamine hydrochloride were purchased from Tokyo Chemical Industry (Tokyo, Japan). Other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan) unless specified otherwise. Fluorescence was measured using a Synergy™ MX microplate reader (BioTek Instruments, Winooski, VT, USA). An AMX 500 (Bruker, Billerica, MA, USA) or JNM-EX 270 (Jeol Ltd., Tokyo, Japan) system was used to obtain nuclear magnetic resonance (NMR) spectra. *Tert*-butanol or residual solvent was used as an internal standard (D_2O: ^1H NMR 1.24 ppm, ^{13}C NMR: 30.29 ppm; methanol-d_4: ^1H NMR 3.30 ppm, ^{13}C NMR: 49.00 ppm). A LCT Premier spectrometer (Waters, Milford, MA, USA) was used to obtain electrospray ionization mass spectra (ESI MS).

2.2. Syntheses

*2.2.1. N-methylhigenamine (**3**)*

Higenamine (**1**, 44.8 mg, 0.16 mmol) was dissolved in methanol (4.5 mL) and formaldehyde solution (36–38%, 1.1 mL). Sodium tetrahydroborate (48.8 mg, 1.2 mmol) was added and the mixture stirred for 24 h. The reaction mixture was concentrated and purified by preparative silica-gel thin-layer chromatography (TLC) (chloroform/methanol = 4/1 with 1% acetic acid) and then by high-performance liquid chromatography (HPLC) with an InertSustain C18 column (20 × 250 mm; GL Sciences, Tokyo, Japan) and 30% aqueous methanol with 0.1% trifluoroacetic acid (TFA) as the mobile phase to obtain **3** (12.6 mg, 0.044 mmol, 28%) as a colorless oil.

^1H-NMR (270 MHz, methanol-d_4, rt): 2.92 (3H, s), 2.95–3.11 (3H, m), 3.20–3.40 (2H, m), 3.64–3.75 (1H, m), 4.46–4.50 (1H, m), 6.16 (1H, s), 6.65 (1H, s), 6.76 (2H, d, J = 6.8 Hz), 7.00 (2H, d, J = 6.8 Hz) ppm; ^{13}C-NMR (125 MHz, methanol-d_4, rt): 22.19, 40.95, 42.10, 46.62, 66.85, 116.01, 116.27, 116.94, 121.47, 121.59, 126.87, 131.99, 145.81, 147.37, 158.32 ppm; HR ESI MS (positive): $[M+H]^+$ found *m*/*z* 286.1458, $C_{17}H_{20}NO_3^+$ requires *m*/*z* 286.1443; IR (neat): 1410, 1565, 3387 cm^{-1}.

*2.2.2. N-carbobenzoxyhigenamine (**8**)*

Higenamine (**1**, 323.8 mg, 1.19 mmol) was dissolved in a mixture of 1,4-dioxane (8 mL) and water (4 mL). Carbobenzoxy chloride (0.34 mL, 2.4 mmol) was added and the mixture stirred. The solution was adjusted to pH 8 by saturated sodium hydrogen carbonate solution and stirred for 14 h at room temperature. The reaction mixture was diluted by water and extracted by ethyl acetate. The organic layer was washed with brine, dried over sodium sulfate, and concentrated to obtain **8** (458.8 mg, 1.13 mmol, 94%). The higenamine moiety with a carbobenzoxy group on the cyclic amine contained isomers (rotamers resulting from the 4-hydroxybenzyl group or diastereomers resulting from the stereochemistry of the protective group on the cyclic amine), so detailed data were not obtained for compound **8**.

*2.2.3. 6-O-methyl, 7-O-methyl, and 6,7-di-O-methylhigenamine (**4**-**6**)*

Compound **8** (458.8 mg, 1.13 mmol) was dissolved in *N*,*N*-dimethylformamide (11 mL) and iodomethane (0.14 mL, 2.26 mmol). Potassium carbonate (313 mg, 2.26 mmol) was added and the mixture stirred for 48 h under an argon atmosphere. The reaction mixture was diluted by water and extracted by ethyl acetate. The organic layer was washed with brine, dried over sodium sulfate, and concentrated. The residue was purified by silica-gel column chromatography (hexane/acetone = 6/1 to 2/1) to obtain methylated fractions (353.3 mg). The fraction was dissolved in ethyl acetate (5 mL), 10% palladium on carbon was added, and the mixture stirred for 48 h under a hydrogen atmosphere. The reaction mixture was passed through a Celite® pad, concentrated and purified by preparative TLC (chloroform/methanol = 3/1 with 1% acetic acid) and then by HPLC with a Cosmosil πNAP column (10 × 250 mm; Nacalai Tesque, Kyoto, Japan) and 30% aqueous methanol with 0.1% TFA as the mobile phase to obtain **4** (23.0 mg, 0.058 mmol, 5%), **5** (13.2 mg, 0.033 mmol, 3%) and **6** (68.8 mg, 0.167 mmol, 15%) as brownish oils.

6-*O*-methylhigenamine (**4**): ^1H-NMR (500 MHz, methanol-d_4, rt): 2.95–3.08 (3H, m), 3.23–3.37 (2H, m), 3.44–3.50 (1H, m), 3.84 (3H, s), 4.59 (1H, m), 6.63 (1H, s), 6.76 (1H, s), 6.79 (2H, d, J = 7.5 Hz), 7.12 (2H, d, J = 7.5 Hz) ppm; ^{13}C-NMR (125 MHz, methanol-d_4, rt): 26.02, 40.52, 40.93, 56.51, 57.94, 112.75, 114.30, 117.09, 123.77, 125.16, 127.07, 131.78, 146.92, 149.39, 158.34 ppm; HR ESI MS (positive): $[M+H]^+$ found *m*/*z* 286.1454, $C_{17}H_{20}NO_3^+$ requires *m*/*z* 286.1443; IR (neat): 1455, 1518, 1671, 2847, 3014, 3126 cm^{-1};

7-*O*-methylhigenamine (**5**): ^{1}H-NMR (500 MHz, methanol-d_4, rt): 2.94–2.98 (2H, m), 3.05–3.09 (1H, m), 3.28–3.33 (2H, m), 3.47–3.53 (1H, m), 3.66 (3H, s), 4.61 (1H, t, J = 8.1 Hz), 6.44 (1H, s), 6.63 (1H, s), 6.80 (2H, d, J = 8.1 Hz), 7.10 (2H, d, J = 8.1 Hz) ppm; ^{13}C-NMR (125 MHz, methanol-d_4, rt): 25.76, 40.29, 40.65, 56.41, 57.87, 111.34, 116.26, 117.00, 123.53, 125.05, 127.40, 132.02, 147.95, 148.07, 158.31 ppm; HR ESI MS (positive): $[M+H]^+$ found *m/z* 286.1468, $C_{17}H_{20}NO_3^+$ requires *m/z* 286.1443; IR (neat): 1448, 1518, 1675, 2847, 3012, 3141 cm^{-1};

6,7-di-O-methylhigenamine (**6**): ^{1}H-NMR (500 MHz, methanol-d_4, rt): 3.02–3.12 (3H, m), 3.27–3.36 (2H, m), 3.50–3.57 (1H, m), 3.63 (3H, s), 3.81 (3H, s), 4.65 (1H, t, J = 8.1 Hz), 6.45 (1H, s), 6.79 (1H, s), 6.80 (2H, d, J = 8.1 Hz), 7.11 (2H, d, J = 8.1 Hz) ppm; ^{13}C-NMR (125 MHz, methanol-d_4, rt): 25.95, 40.21, 40.59, 56.45, 56.55, 57.74, 111.58, 113.01, 117.02, 124.79, 125.04, 127.35, 132.05, 149.29, 150.63, 158.35 ppm; HR ESI MS (positive): $[M+H]^+$ found *m/z* 300.1615, $C_{18}H_{22}NO_3^+$ requires *m/z* 300.1600; IR (neat): 1455, 1614, 2837, 3013, 3141 cm^{-1}.

2.2.4. 4'-O-methylhigenamine (7)

2-(4-methoxyphenyl)acetaldehyde (185 mg, 1.24 mmol) and dopamine hydrochloride (195 mg, 1.03 mmol) were dissolved in a mixture of acetonitrile (5 mL) and 0.1 M phosphate buffer (pH6, 5 mL). After stirring for 24 h at 50°C, the reaction was diluted by water and 10% sodium hydrogencarbonate solution was added to adjust the pH to 8. The resulting solution was extracted by ethyl acetate, and the organic layer was washed with brine and dried over sodium sulfate. After concentration, the residue was purified by silica-gel column chromatography (chloroform/methanol = 4/1 with 1% acetic acid) to obtain compound **7** (46.0 mg, 0.16 mmol, 13%) as a brownish oil.

^{1}H-NMR (500 MHz, methanol-d_4, rt): 2.87–3.04 (3H, m), 3.23–3.28 (1H, m), 3.36–3.39 (1H, m), 3.43–3.48 (1H, m), 3.79 (3H, s), 4.59 (1H, t, J = 5.0 Hz), 6.58 (1H, s), 6.62 (1H, s), 6.93 (2H, d, J = 5.0 Hz), 7.21 (2H, d, J = 5.0 Hz) ppm; ^{13}C-NMR (125 MHz, methanol-d_4, rt): 25.82, 40.55, 40.97, 55.86, 57.96, 114.40, 115.73, 116.34, 123.82, 123.82, 128.47, 131.79, 145.88, 147.00, 160.89 ppm; HR ESI MS (positive): $[M+H]^+$ found *m/z* 286.1452, $C_{17}H_{20}NO_3^+$ requires *m/z* 286.1443; IR (neat) 1457, 1515, 1615, 1671, 2837, 3115 cm^{-1}.

2.3. Cell culture

L6 cells (JCRB9081; Lot 10102000) were provided by the JCRB Cell Bank through the National Institute of Biomedical Innovation (Tokyo, Japan). L6 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum at 37°C in a humidified atmosphere of 10% CO2. L6 Cells were reseeded in 48-well plates for the glucose-uptake assay. After L6 cells had reached confluence, the medium was switched to DMEM containing 2% horse serum for differentiation. The medium was replaced every second day, and experiments were undertaken 6–8 days after initiating differentiation.

2.4. Assay to measure enhancement of glucose uptake

The glucose-uptake assay was carried out as reported previously.[23] Briefly, after serum starvation, cells were incubated with or without the sample, or insulin (100 nM) as the positive control, and diluted in serum-free DMEM for 4 h. L6 cells were rinsed twice with Krebs–Ringer phosphate-HEPES (KRPH) buffer (20 mM HEPES, 136 mM NaCl, 4.7 mM KCl, 1 mM NaH_2PO_4, 1 mM $MgSO_4$, and 1 mM $CaCl_2$, pH 7.4) and incubated in KRPH buffer containing 1 mM 2-deoxyglucose for 20 min. After washing L6 cells thrice with KRPH buffer, an aqueous solution of 0.05 M NaOH was added to lyse L6 cells. The amount of 2-deoxyglucose contained in each lysate was measured by an enzymatic method.[28]

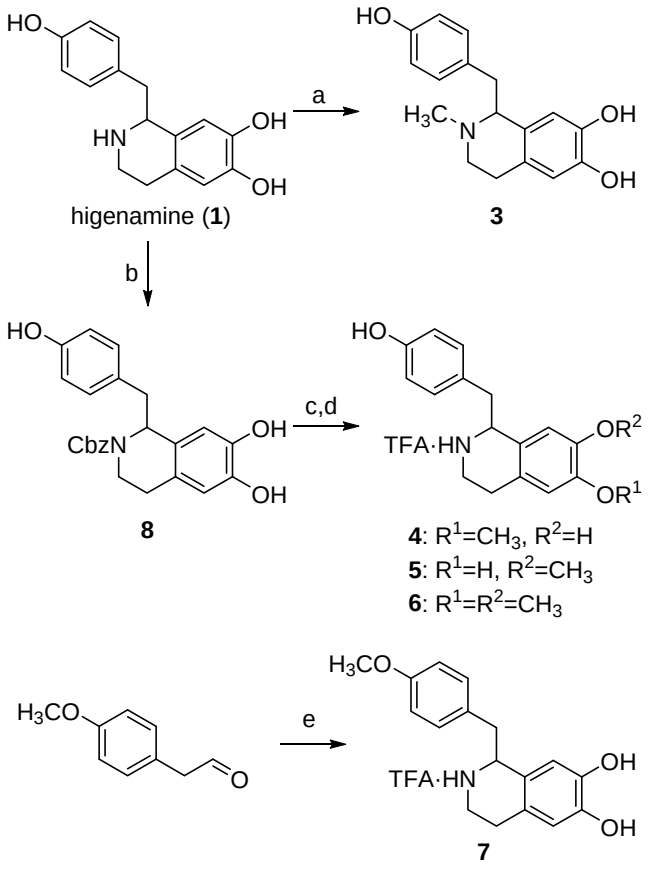
2.5. Statistical analyses and curve fitting

Experiments were repeated thrice and the representative values are shown as graphs. EZR software (http://www.jichi.ac.jp/saitama-sct/SaitamaHP.files/statmedEN.html) was used for statistical tests.[29] Dunnett's test was used to assess significant differences. $p < 0.01$ was considered significant. ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used for curve fitting to create graphs.[30]

3. Results and discussion

3.1. Preparation of higenamine and its derivatives

The racemic higenamine (**1**) was prepared by a reported procedure.[31] Prepared **1** was reacted with formaldehyde in the presence of sodium hydroborate to afford *N*-methyl derivative **3**. After protection of the cyclic amine by the carbobenzoxy group, methylation of **8** and subsequent removal of the carbobenzoxy group gave three methylated products: 6-*O*, 7-*O*, and 6,7-di-*O*-methylhigenamine (**4**–**6**). Unfortunately, 4'-*O*-methylhigenamine (**7**) was not obtained by this procedure. Thus, compound **7** was prepared through direct conjugation of 2-(4-methoxyphenyl)acetaldehyde with dopamine hydrochloride in the presence of the phosphate anion (Scheme 1).[31] Compounds **1R**, **1S**, **2R**, and **2S** were synthesized as reported previously.[24]



Scheme 1. Synthesis of compounds **3**–**7**. a) formaldehyde, $NaBH_4$, MeOH; b) CbzCl, 1,4-dioxane, water, $NaHCO_3$; c) MeI, K_2CO_3, DMF; d) 10% Pd-C, H_2, EtOAc and then HPLC purification; e) dopamine hydrochloride, MeCN, 0.1 M phosphate buffer (pH6.0), 50°C and then HPLC purification. See materials and method section for HPLC condition.

3.2. Enhancement of glucose uptake by the methyl derivatives of higenamine

The synthetic methyl derivatives **3**-**7** were compared with regard to their enhancement of glucose uptake with that of **1** (Figure 2). Higenamine (**1**, racemate) at >5 μM has more than twofold-enhanced glucose uptake compared with that of control. As expected from the previous finding of **2** (racemate) as a β2AR agonist,[23] 4'-*O*-methylhigenamine (**7**) retained its ability to enhance glucose uptake, which was comparable with that of **1**. Other tested methyl derivatives showed reduced ability to enhance glucose uptake. The ability of *N*-methylhigenamine (**3**) and 7-*O*-methylhigenamine (**5**) to enhance glucose uptake was largely diminished, with a 1.2-fold increase being seen at 100 μM. The ability of 6-*O*-methylhigenamine (**4**) to enhance glucose uptake was higher than that of **3** or **5**, with a gradual increase in glucose uptake being observed from 0.5 μM to 100 μM. However, the highest activity of **4** was a 1.6-fold increase in glucose uptake compared with that of control, which was 40% that of **1**. The dimethylated product **6** showed the same tendency as **5**.

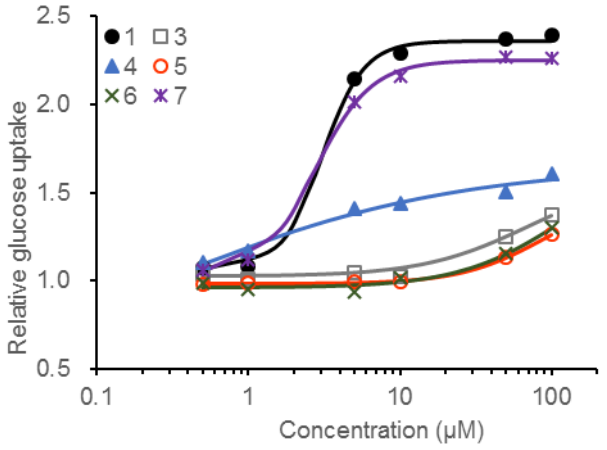


Figure 2. Enhancement of glucose uptake by methyl derivatives (**3**-**7**) and higenamine (**1**).

The crystal structure of β2AR bound to an epinephrine molecule was reported first by Ring et al.[32] In the three-dimensional model, each of the catechol hydroxyl groups and an amino group formed a hydrogen bond between the amino-acid residues of β2AR (hydroxyl group: S207, or S204 and N293; amino group: N312).[32] Higenamine (**1**) possesses a similar structure to that of epinephrine and docking study of **1** with β2AR (PDB:4LDO) resulted in analogous binding manner to epinephrine (see supplementary material).[33] Hence, the decrease in activity through methylation of a catechol hydroxyl group or cyclic amine of **1** is a fair result due to a disruption of those hydrogen bonds, together with the steric hindrance of the methyl group. The weak ability of **4** to enhance glucose uptake suggests the lower importance of the 6-hydroxyl group, or the existence of a relatively larger space for the methyl group to reside at the position. The decreased maximum enhancement of glucose uptake by **4** suggests that a 6-*O*-methyl group interferes with binding to β2AR, thereby resulting in partial agonist activity.

In contrast to other functional groups, the 4'-hydroxybenzyl moiety in **1** has no corresponding part with the epinephrine molecule. Presumably, methylation of the 4'-hydroxy group has no effect on the formation of hydrogen bonds and, thus, the activity of **7** is retained.

These results may also suggest why: (i) the metabolic derivatives of **1** have not been reported to be β2AR agonists; (ii) higenamine 4'-*O*-β-D-glucoside (**2**) has been reported to be an agonist.[23]

3.3. Comparison of enhancement of glucose uptake with endogenous catecholamines

The enhancement of glucose uptake of **1** was compared with that of dopamine, epinephrine, and norepinephrine (Figure 3). Compound **1** does not possess a hydroxyl group at the benzyl position beside the catechol moiety, which is included in hydrogen-bond formation with amino-acid residues of β2AR (D113 and N312). Nevertheless, it showed comparable enhancement of glucose uptake to that of epinephrine or norepinephrine, and higher enhancement of glucose uptake compared with that of dopamine. This result indicates involvement of the 4'-hydroxybenzyl moiety for the interaction of **1** and β2AR, but not through hydrogen bonding by the 4'-hydroxy group, for enhancement of glucose uptake to a level comparable with that of epinephrine or norepinephrine. This result also suggests that the stereochemistry of **1** influences enhancement of glucose uptake because it affects the stereoscopic position of the 4'-hydroxybenzyl moiety. Thus, we tested stereoisomers of **1** and **2** for enhancement of glucose uptake.

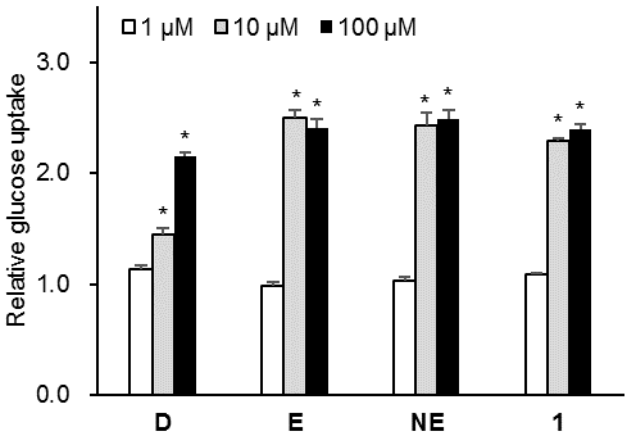


Figure 3. Enhancement of glucose uptake by catecholamines and higenamine (**1**). **D**: dopamine, **e**: L-epinephrine, **NE**: L-norepinephrine. *p<0.01 to control. Compound **1**, **E**, and **NE** showed maximum increase in glucose uptake above 10 μM and no further increases were observed at 100 μM.

3.4. Comparison of the enhancement of glucose uptake of R and S-isomers

The enhancement of glucose uptake of (*R*)-(+)-, (*S*)-(−)-higenamine (**1R**, **1S**), and the 4'-*O*-β-D-glucoside (**2R**, **2S**) are shown in Figure 4. In both compounds, the *S*-isomer exhibited enhancement of glucose uptake at a seven-fold lower concentration compared with that of the *R*-isomer (EC_{50}: **1R** 4.5 μM; **1S** 0.60 μM; **2R** 9.0 μM; **2S** 1.3 μM), indicating the importance of stereochemistry for this enhancement. This trend was similar to that for the anti-aggregation of platelets,[27] and increased survival in lipopolysaccharide-treated mice.[34] In addition, a difference in the maximum enhancement of glucose uptake was seen between **2R** and **2S**. Hence, the glucose moiety may interact with β2AR to hamper full activation of the receptor in **2R**, but less in **2S**.

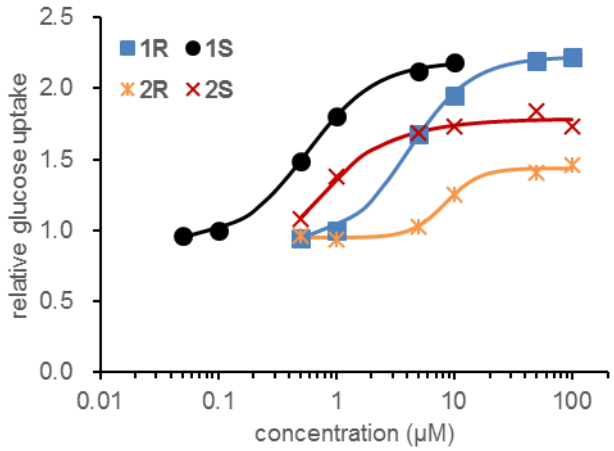


Figure 4. Enhancement of glucose uptake of stereoisomers **1R**, **1S**, **2R**, and **2S**.

4. Conclusions

Higenamine (**1**) and its related compounds were assessed for their ability to enhance glucose uptake in L6 cells, which is related directly to β2AR activation. We found that: (i) the functional groups of **1** except for the 4'-hydroxy group are required enhance glucose uptake; (ii) higenamine (**1**) shows a comparable ability to enhance glucose uptake with that of epinephrine and norepinephrine; (iii) the *S*-isomer shows a greater ability to enhance glucose uptake compared with that of the *R*-isomer. These will support the use of higenamine (**1**), or a plant that contains **1**, as candidate agents for clinical use to treat several health problems like diabetes and obesity which beneficial effect through β2AR activation are reported.

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