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Application of Stable Isotope Tracer Combined with Mass Spectrometric Detection for Studying *myo*-Inositol Uptake by Cultured Neurons from Fetal Mouse: Effect of Trisomy 16

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A gas chromatographic (GC)/mass spectrometric method for studying *myo*-inositol uptake by neurons *in vitro* is described. Cultured cortical neurons from fetuses of diploid and trisomy 16 mouse (animal model for Down syndrome) were incubated with a physiological concentration of hexadeuterated *myo*-inositol for 2–40 min. Washed cells were lysed and *scyllo*-inositol (internal standard) was added to the intracellular material which contained labeled *myo*-inositol taken up by the cells as well as the endogenous, unlabeled *myo*-inositol. The samples were evaporated to dryness and the analytes were converted into acetate derivatives. The components were separated by capillary GC, and the *m/z* 379 ion for labeled *myo*-inositol and the *m/z* 373 ion for *myo*-inositol and *scyllo*-inositol generated by chemical ionization in an ion trap mass spectrometer were monitored. Quantitation of the deuterium-labeled *myo*-inositol taken up by the neuron along with endogenous *myo*-inositol was achieved for 2–40 min of incubation. The labeled *myo*-inositol uptake was linear for up to 20 min and was Na^+ dependent in these neurons. This non-radioisotope method was used to demonstrate a significant (40%) increase in the rate of *myo*-inositol uptake by cortical neurons from the trisomy 16 mouse relative to control neurons. An increased *myo*-inositol uptake is consistent with evidence that the *myo*-inositol transporter gene is on both human chromosome 21 and mouse chromosome 16, and that *myo*-inositol concentrations are elevated in cerebrospinal fluid from adult Down syndrome individuals and brains from the fetal trisomy 16 mouse.

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INTRODUCTION

Cells accumulate *myo*-inositol [Fig. 1(a)] for membrane-phospholipid synthesis, signal transduction and osmoregulation. *myo*-Inositol is incorporated continuously into membrane lipids to generate phosphatidylinositol and subsequently phosphatidylinositol 4,5-bisphosphate. The latter phosphoinositide is a precursor for two major second messengers, *myo*-inositol 1,4,5-trisphosphate and diacylglycerol, released in a signal transduction cascade. For this signal-related metabolism and for osmotic volume regulation, cells maintain a high level of *myo*-inositol by active transport. A derangement in the homeostasis of *myo*-inositol is known to perturb certain physiological properties of neurons such as electric activity.[1,2]

We have reported a gas chromatographic/mass spectrometric (GC/MS) method to quantitate *myo*-inositol[3] and other polyols[4] in human cerebrospinal fluid and plasma. Using this method, we showed ~50% increases in the cerebrospinal fluid concentration and the cerebrospinal fluid to plasma concentration ratio of *myo*-inositol in Down syndrome adults.[5] The increased accumulation was unrelated to dementia. Additionally, we found a similar increase in *myo*-inositol in the brain of the trisomy 16 mouse,[6] an animal model of Down syndrome. We proposed that the increase was due to enhanced transport or synthesis of *myo*-inositol. An Na^+/*myo*-inositol cotransporter gene (SLC5A3) maps to the long arm of human chromosome 21,[7] consistent with this hypothesis.

Thus, in Down syndrome neurons may exhibit increased *myo*-inositol uptake and, consequently, altered dynamics of phosphoinositide-mediated signal transduction. We plan to investigate this in neurons from the trisomy 16 mouse using a stable isotope of *myo*-inositol. In this paper, we describe the mass spectrometric measurement of [$^2H_6$]*myo*-inositol [Fig. 1(b)] uptake by cultured cortical neurons from the trisomy 16 mouse and diploid controls. *scyllo*-Inositol [Fig. 1(c)] was used as an internal standard to quantitate the intracellular content of [$^2H_6$]*myo*-inositol and *myo*-inositol. Use of a stable isotope combined with mass spectrometric detection allowed simultaneous determination of the mass of the tracer transported into the cell and also the mass of the intracellular, native molecule.

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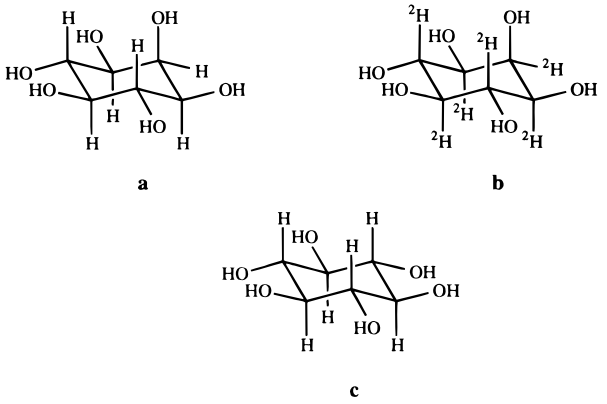

Figure 1. Structures of (a) *myo*-inositol, (b) [$^2H_6$]*myo*-inositol and (c) *scyllo*-inositol.

EXPERIMENTAL

Materials

Hexadeuterated (1,2,3,4,5,6-^{2}HC) *myo*-inositol, [$^2H_6$]*myo*-inositol [Fig. 1(b)], was purchased from C/D/N Isotopes (Pointe-Claire, Quebec, Canada). GC/MS analysis indicated that the product was practically free of unlabeled *myo*-inositol and >98% enriched in [$^2H_6$]-species. Also, the labeled *myo*-inositol contained <1% of other isomers of inositol. *myo*-Inositol and *scyllo*-inositol were obtained from Sigma Chemical (St. Louis, MO, USA). Pyridine and acetic anhydride were purchased from Alltech (Deerfield, IL, USA) and 4-dimethylaminopyridine from Fluka (New York, NY, USA). Pyridine reagent consisting of 1 mg ml^{-1} 4-dimethylaminopyridine in pyridine was prepared and stored under dry nitrogen. Solvents were obtained from Burdick and Jackson (Muskegon, NY, USA).

Animals

Trisomy 16 mice were obtained by breeding normal (C57BL/6) females to males with heterozygous Robertsonian translocations of chromosome 16 (Rb(16.17)32 Lub/Rb(11.16)2H).[8] The trisomic condition is a fetal lethal. At day 16 of gestation, trisomic fetuses were selected based on a characteristic anasarca.[9] Aneuploidy was confirmed by chromosome spreads.[10] Normal diploid fetal siblings were used as controls.

Pregnant mice, 16–17 days gestation, were anesthetized with CO_2 and killed by cervical dislocation. The fetuses were removed and placed in cold calcium-free dissection medium, consisting of 33 mM glucose, 22 mM sucrose, 5% Puck's saline (Colorado Serum, Denver, CO, USA) and 10 mM HEPES, at pH 7.4. The fetuses were killed by decapitation and the brains removed to fresh dissection medium. Pieces of cerebral cortex were dissected and minced with iridectomy scissors.

Cell cultures

Cortical pieces were incubated for 30 min at 37 °C in dissection medium containing 0.05% (v/v) trypsin (Biofluids, Rockville, MD, USA). The trypsin was removed by washing the cortical tissue twice in culture medium: minimal essential medium supplemented with 10% (v/v) fetal bovine serum (Hyclone, Logan, UT, USA), 5% (v/v) horse serum (Hyclone), 1% (v/v) glutamine (Biofluids) and 40 ng ml^{-1} 7S-nerve growth factor (Collaborative Research, Bedford, MA, USA). Dissociated cells were obtained by triturating the cortical tissue by passage through the narrow opening of a fire-polished Pasteur pipette. The cells were plated at a density of 5×10^5 on to 35 mm plastic culture plates (Nunc, Roskilde, Denmark) freshly coated with 0.05% (w/v) poly-L-lysine (Sigma Chemical). Cultures were maintained at 37 °C in an atmosphere of 5% CO_2–95% O_2 and the culture medium was replenished twice per week. These conditions produced healthy cultures of neurons showing some migration and slight clustering of the neurons after 1 week, and also a sparse growth of astrocytes.

Uptake experiments

After 8 days in culture, the medium was removed and the cortical cells were washed twice at 37 °C with Dulbecco's phosphate-buffered saline (PBS) (Cellgro, Herndon, VA, USA) containing 100 μM glucose. The cells were incubated for 2, 5, 10, 20 or 40 min in 500 μl of uptake buffer, Dulbecco's PBS containing 100 μM glucose and 135 μM [$^2H_6$]*myo*-inositol, at 37 °C on an orbital shaker. Each time point was repeated in quadruplicate or quintuplicate. Uptake was stopped by the immediate addition of 500 μl of ice-cold Dulbecco's PBS and removal of the medium. Cells were washed with cold PBS and then 500 μl of 0.2 M NaOH were added and the cell suspension was scraped with a Costar (Cambridge, MA, USA) cell lifter. This cell fraction was homogenized with a probe sonicator and stored at −80 °C. Aliquots were taken and assayed for protein content using a micro BCA kit (Pierce Chemical, Rockford, IL, USA).

The Na^+-dependent uptake study was performed in either medium containing NaCl (137 mM) or choline chloride (137 mM) in place of NaCl. The common components of these media were HEPES buffer (10 mM), the pH (7.4) of which was adjusted using KOH (4.4 mmol l^{-1} buffer), $CaCl_2$ (900 μM), $MgCl_2$ (500 μM) and glucose (100 μM). Cultured cortical neurons (six plates for each of trisomy 16 and diploid control) were washed (37 °C) in the medium (NaCl or choline chloride) and then incubated in the respective medium containing [$^2H_6$]*myo*-inositol for 10 min at 37 °C. Samples were processed as described above.

GC/MS system

The GC/MS system consisted of a Finnigan MAT (San Jose, CA, USA) ITS40 ion trap mass spectrometer interfaced to a Varian gas chromatograph equipped with a CTC A200S autosampler. The ion trap mass spectrometer was equipped with an automatic reaction control chemical ionization facility.

Capillary GC analysis was performed using a 30 m × 0.25 mm i.d. fused-silica column bonded with 50% phenyl–50% methyl polysiloxane, 0.25 μm film thick-

ness (Rtx-50; Restek, Bellefonte, PA, USA). The helium linear velocity through the column was 30 cm s^{-1}. Adsorptive surfaces of GC inlets and column were deactivated by injecting (split) 0.5 μl of 1,3-diphenyl-1,1,3,3-tetramethyldisilazane (Fluka) at 250 °C. Samples were injected splitless (0.6 min) with the injector temperature at 250 °C and the oven temperature initially at 150 °C. After 0.5 min, the oven temperature was ramped at 10 °C min^{-1} to 190 °C and then at 2 °C min^{-1} to 230 °C; finally the column was heated to 290 °C, held at this temperature for 9 min and then returned to the initial temperature. The temperature of the ion trap manifold was 225 °C and that of the transfer line was 270 °C.

Chemical ionization was performed with acetonitrile vapor serving as the reagent gas, as described previously.[11] Chemical ionization reaction parameters were optimized for acetonitrile. We used an ionization time (maximum time allowed for the ionization of reagent gas) of 1500 μs, a reaction time (maximum time allowed for the reagent ions to react with sample molecules) of 50 ms, an ionization level (value of the smallest mass stored in the trap during the ionization of the reagent gas) of 20 u, a reaction level (smallest mass stored during the reaction period) of 20 u and a reagent ion ejection level (mass value that is greater than or equal to the mass of the largest reagent ion produced by the reagent gas) of 55 u. Other operational parameters were as described in the manual of the ion trap systems. Acetonitrile vapor was introduced into the ion trap source in the same way as calibration gas. The metering valve was adjusted to produce the reagent ions: protonated acetonitrile at *m/z* 42 (100% abundance), molecular ion at *m/z* 41 (10%) and an adduct ion at *m/z* 54 (14%). Mass spectra were acquired over the mass range from 365 to 385. In the post-run, we constructed ion chromatograms for [$^2H_6$]*myo*-inositol (*m/z* 379), *myo*-inositol (*m/z* 373) and the internal standard, *scyllo*-inositol (*m/z* 373). Before each analysis, a standard mixture of acetates of labeled and protio *myo*-inositols and *scyllo*-inositol standard was injected into the GC/MS system and the detection reproducibility of the mass spectrometer was checked from the ion current ratio of each form of *myo*-inositol to the internal standard.

Assay

A stock solution consisting of a mixture of *myo*-inositol and [$^2H_6$]*myo*-inositol, 1 μg μl^{-1} each, was prepared in deionized water containing 10% (v/v) methanol. This solution was diluted to obtain 20 ng μl^{-1} of each analyte. The solutions for the calibration graph, 5, 2.5, 1.25, 0.625, 0.3125, 0.156, 0.078, 0.039 and 0.0195 ng μl^{-1} of *myo*-inositol/[$^2H_6$]*myo*-inositol, were prepared by further dilutions. A stock standard solution of *scyllo*-inositol (1 μg μl^{-1}) was prepared as above and was diluted to a concentration of 1 ng μl^{-1} to give a working internal standard solution.

A 100 μl volume of cell extract or calibration solution was mixed with 100 μl of internal standard solution (1 ng μl^{-1}) in a glass tube, 1 ml methanol was added, mixed and evaporated to complete dryness at 60 °C using a Savant evaporator (Savant Instruments, Farmingdale, NY, USA). The residue was treated with 100 μl of pyridine reagent and an equal volume of acetic anhydride, flushed with dry nitrogen and the reaction mixture was heated at 80 °C for 30 min. After cooling, most of the unreacted reagent was evaporated by blowing nitrogen into the tube. The derivatized product was dissolved in 4 ml of hexane–ethyl acetate (80:20, v/v), the solution was vortex mixed with 1 ml of 5% sodium hydrogencarbonate solution for 5 min and the layers were separated by centrifugation (3 min at 1000 *g*). The organic layer was transferred into another tube and evaporated in the Savant evaporator at 40 °C. The residue was reconstituted in 100 μl of ethyl acetate and 1 μl was injected into the GC/MS system.

Data analysis

Analyte to internal standard peak-area ratios for [$^2H_6$]*myo*-inositol and *myo*-inositol were determined for each concentration of the calibration graph. Regression analysis was performed of log (concentration) (x) *vs.* log (peak-area ratio) (y) for both labeled and unlabeled *myo*-inositol. A mean ± SD correlation coefficient (r) of 0.9996 ± 0.0003 and a slope of 1.030 ± 0.037 were obtained from four calibration graphs for [$^2H_6$]*myo*-inositol. The concentrations of *myo*-inositol and [$^2H_6$]*myo*-inositol in cell extracts were calculated from the log–log plot equation, $\log y = \log a + b \log x$. The concentrations were then expressed in nmol mg^{-1} protein. The mean concentration of [$^2H_6$]*myo*-inositol in the trisomy 16 mouse cortical neurons was compared with the respective mean of the diploid mouse cortical neurons by a two-tailed *t*-test. Data were analyzed using a statistics program (Instat; GraphPad Soft-ware, San Diego, CA, USA).

RESULTS AND DISCUSSION

We have reported a GC/MS technique for the quantitation of *myo*-inositol in human cerebrospinal fluid and plasma.[3] In that assay, *myo*-inositol and [$^2H_6$]*myo*-inositol (internal standard) were converted into hexaacetate derivatives and their highly abundant fragment ions generated by a chemical ionization reaction were monitored. The fragment ions at *m/z* 373 for *myo*-inositol and *m/z* 379 for [$^2H_6$]*myo*-inositol were formed by the loss of one CH_3COO residue from the molecular ions. In the present study, [$^2H_6$]*myo*-inositol was the tracer and quantitation of this and also the intracellular, unlabeled *myo*-inositol was required. *scyllo*-Inositol was found to be a suitable internal standard as the level of this stereoisomer in the cultured neurons is negligible and as chemical ionization of its acetate derivative yielded the fragment ion at *m/z* 373 similar to *myo*-inositol. The internal standard and *myo*-inositol were well resolved by capillary GC. The retention times were 21 min 11 s for *myo*-inositol, 21 min 1 s for [$^2H_6$]*myo*-inositol and 22 min 15 s for *scyllo*-inositol.

The MS detection of *myo*-inositol/[$^2H_6$]*myo*-inositol was found to be linear between 5 ng μl^{-1} and 19.5 pg μl^{-1} (calibration graph solution) when measured using

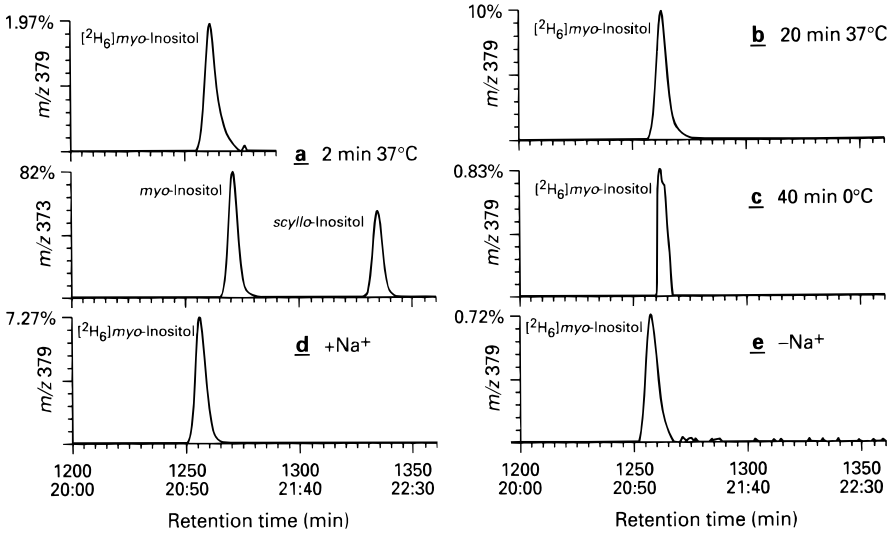

Figure 2. Capillary GC separation and MS detection of [$^2H_6$]*myo*-inositol/*myo*-inositol in cultured cortical neurons (a–e). [$^2H_6$]*myo*-Inositol accumulation with time (a–c). The ion chromatogram of *myo*-inositol and *scyllo*-inositol (internal standard) is shown for the 2 min uptake experiment only (a). Chromatogram (d) (+Na^+) is for [$^2H_6$]*myo*-inositol uptake in the presence of NaCl and (e) (−Na^+) is for the uptake when NaCl was replaced with choline chloride.

scyllo-inositol as an internal standard. Quantitation of intracellular free [$^2H_6$]*myo*-inositol was reproducible. Cell extracts from six plates incubated for 10 min with the tracer were combined and analyzed in eight replicates. A mean ± SD concentration of 2.240 ± 0.063 mmol per mg protein with an RSD of 2.8% was obtained for [$^2H_6$]*myo*-inositol in this pooled sample. A high reproducibility of this GC/MS technique for the determination of *myo*-inositol in other biological matrices was reported previously.[4]

In a group of ten healthy human subjects, the cerebrospinal fluid concentration of *myo*-inositol was found to be 135 ± 23 μM (mean ± SD).[5] The same physiological concentration of [$^2H_6$]*myo*-inositol was used in the present uptake study. After incubation for 2–40 min, cortical neurons were washed to remove the extracellular tracer and then lysed. Intracellular labeled and native *myo*-inositols were acetylated along with the added internal standard for GC/MS analysis. Figure 2(a) shows an ion chromatogram for [$^2H_6$]*myo*-inositol (*m/z* 379) that was taken up by the neurons following a 2 min exposure. The peak represents 269 fmol of labeled *myo*-inositol in 1 μl of the cell extract. The same ion chromatogram also shows the simultaneously acquired *m/z* 373 ion for the native *myo*-inositol and for *scyllo*-inositol (internal standard). At 20 min, increased labeled *myo*-inositol is evident from the chromatogram [Fig. 2(b)]. A few uptake experiments were carried out at 0 °C to determine uptake unrelated to the *myo*-inositol transporter. Figure 2(c) reflects a low rate of entry of [$^2H_6$]*myo*-inositol into cortical neurons at 0 °C. Nonspecific binding or passive diffusion into the cell at this low temperature may account for the [$^2H_6$]*myo*-inositol signal.

Quantitative data on [$^2H_6$]*myo*-inositol uptake by cortical neurons from diploid and trisomic fetal mice during 2–40 min of incubation are shown in Table 1. Intracellular concentrations of native *myo*-inositol were determined simultaneously and these are also shown. The amount of [$^2H_6$]*myo*-inositol taken up by the trisomy 16 mouse neurons was significantly higher for 2, 10 and 20 min incubations when compared with amounts in diploid neurons for the respective incubations. No significant increase in [$^2H_6$]*myo*-inositol was noted for the trisomic neurons at 40 min. The protein content of the neurons from the trisomy 16 mouse did not differ significantly from that of the diploid neurons.

Figure 3 is a plot of the time course of [$^2H_6$]*myo*-inositol concentrations in cortical neurons from the trisomy 16 mouse and diploid controls. [$^2H_6$]*myo*-Inositol uptake was linear up to 20 min both for trisomic ($r = 0.998$) and diploid ($r = 0.986$) neurons. The uptake rate was different for diploid and trisomic cortical neurons as indicated by the significant ($P < 0.05$) difference in slope of the regression lines. The

Table 1. Intracellular concentrations of [$^2H_6$]*myo*-inositol and *myo*-inositol during incubation of cortical neurons with [$^2H_6$]*myo*-inositol

Time (min)	Diploid[a] No. of plates	[$^2H_6$]*myo*-Inositol	*myo*-Inositol	Trisomy 16[a,b] No. of plates	[$^2H_6$]*myo*-Inositol	*myo*-Inositol
2	5	0.70 ± 0.06	38.7 ± 1.1	5	1.04 ± 0.05**	35.5 ± 0.4
5	4	1.80 ± 0.11	37.1 ± 1.3	5	2.07 ± 0.06	36.2 ± 0.8
10	5	2.40 ± 0.10	37.0 ± 0.5	5	3.31 ± 0.04***	37.8 ± 1.9
20	5	4.09 ± 0.22	43.6 ± 4.0	4	5.63 ± 0.36*	38.0 ± 1.9
40	4	9.16 ± 0.36	35.7 ± 1.5	5	9.07 ± 0.39	33.9 ± 0.6

[a] Concentrations (mean ± SEM) in nmol mg^{-1} protein.
[b] * $P < 0.01$; ** $P < 0.005$; *** $P < 0.0001$.

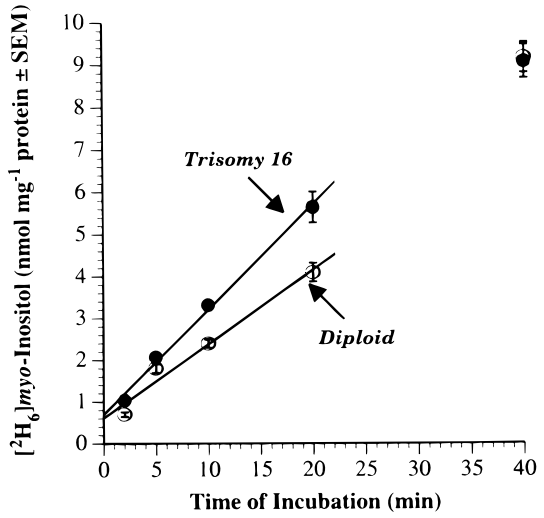

Figure 3. Time course of [$^2H_6$]*myo*-inositol uptake by cortical neurons from the trisomy 16 mouse and diploid controls. Data points are means ± SEM of four or five determinations.

[$^2H_6$]*myo*-Inositol uptake rate was 177 ± 11 pmol min^{-1} for diploid and 250 ± 12 pmol min^{-1} for trisomic neurons. The trisomic to diploid *myo*-inositol uptake rate ratio was 1.4.

High-affinity *myo*-inositol transport is Na^+ dependent and this has been observed in a variety of cell lines.[12] In the present study, isosmotic replacement of NaCl by choline chloride in the medium resulted in a dramatic reduction in [$^2H_6$]*myo*-inositol uptake by both the trisomy 16 and diploid cortical neurons (Fig. 4). The uptake was reduced by 89% for diploid and 88% for trisomic neurons. Also, the relative peak heights of *m/z* 379 ion in the chromatograms [Fig. 2(d) and (e)] reflect the magnitude of the difference in [$^2H_6$]*myo*-inositol uptake due to Na^+.

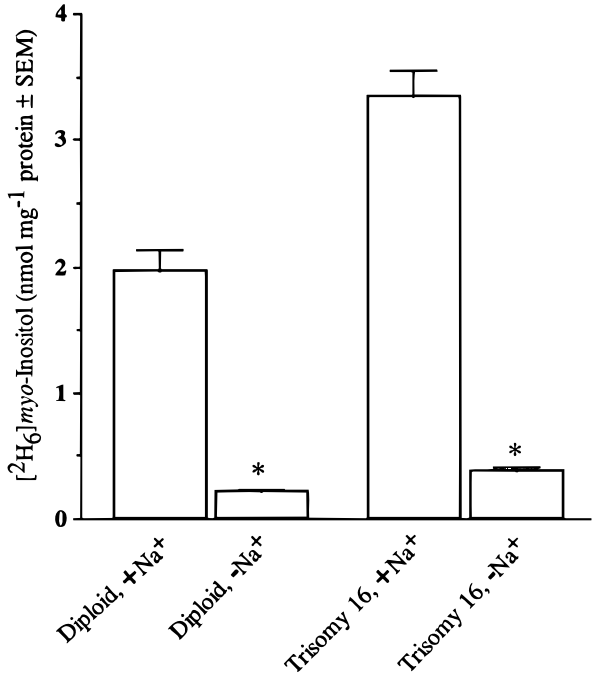

Figure 4. Effect of Na^+ depletion on [$^2H_6$]*myo*-inositol uptake by cultured cortical neurons. The uptake was for 10 min in a medium containing NaCl (+Na^+) or isosmolar choline chloride (−Na^+). Each vertical column and line represent mean and SEM respectively of six separate determinations. * $P < 0.0001$.

Cells, including neurons, transport *myo*-inositol from the extracellular fluid using a Na^+/*myo*-inositol cotransporter, which is dependent on Na^+/K^+-ATPase (Fig. 5). The transporter enables cells to accumulate millimolar levels of *myo*-inositol. The concentration of *myo*-inositol in cerebrospinal fluid is about six times that of plasma[4] and the transporter maintains this gradient. With a K_m of ~30 μM for *myo*-inositol, the transporter is saturated in the central nervous system,[13,14] where extracellular *myo*-inositol concentration is ~130 μM. In the stable isotope technique, the MS measurement provided directly the mass of the molecule transported into the cell at physiological concentrations of tracer. The validity of the technique was demonstrated by the linear uptake (with a minimum scattering of data) and also by our ability to detect a differential uptake when the Na^+/*myo*-inositol cotransporter expression was altered in the trisomy 16 neurons (Fig. 3). Thus, the method is useful for studying the kinetics of accumulation and turnover of *myo*-inositol. These kinetics include K_m and V_{max} characteristics of the transporter and the efflux mechanism under a variety of physiological conditions. It also should be possible to study the effect of altered cell uptake of *myo*-inositol on its incorporation into phosphoinositides and release of the second messenger *myo*-inositol 1,4,5-trisphosphate in a signal transduction cascade.

When the Na^+/*myo*-inositol cotransporter is saturated, as in the present study, the rate of transport is directly proportional to the number of active transport-protein molecules expressed on the cell surface. The Na^+/*myo*-inositol cotransporter gene is likely to be overexpressed in neurons of the trisomy 16 mouse.[6] Although additional data are required, the 1.4 times greater [$^2H_6$]*myo*-inositol uptake suggests a gene dosage effect of the transporter in cortical neurons from the trisomy 16 mouse. Additional information such as the V_{max} for the transporter is required to determine the

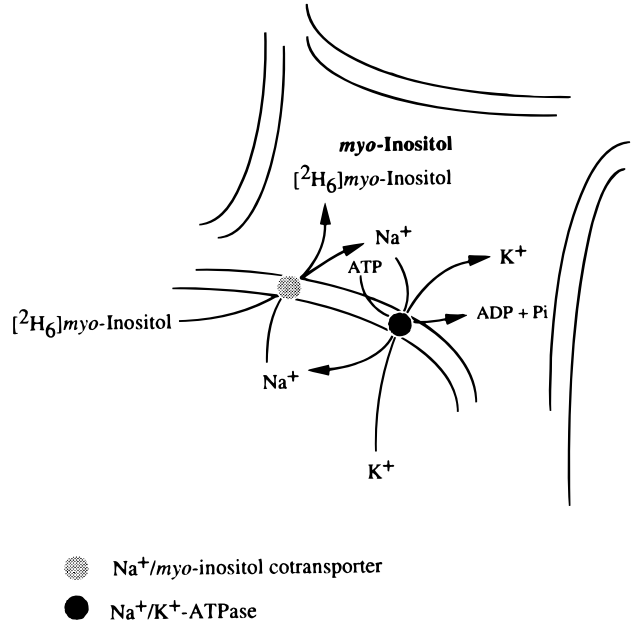

Figure 5. Schematic illustration of cellular uptake of [$^2H_6$]*myo*-inositol mediated by Na^+/*myo*-inositol cotransporter.

increase in number of functional Na^+/*myo*-inositol cotransporter expressed in the trisomy 16 mouse neurons. We are currently probing the dynamics of *myo*-inositol and phosphoinositide metabolism in Down syndrome and its animal model by this stable isotope/mass spectrometric approach.

CONCLUSIONS

The feasibility of studying *myo*-inositol uptake in cultured neurons by a non-radioisotope method has been demonstrated. Data were obtained at a physiologically relevant concentration of the substrate. The sensitivity and specificity of GC/MS detection allowed the measurement of the concentration of the stable isotope tracer in neurons. Because dilution of the tracer by the endogenous pool can be estimated, this methodology should be useful in determining the turnover of phosphoinositides. In general, the use of stable isotope tracers and their detection by MS allow the identification of both qualitative and quantitative changes in the physiologically active molecules in diseases *in vitro* and in human subjects.

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