Biochem. J. (1990) 270, 119–123 (Printed in Great Britain)

Down's syndrome fibroblasts exhibit enhanced inositol uptake

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The inositol metabolism of Down's syndrome (DS, trisomy 21) skin fibroblasts was examined. We report that DS cells accumulated [^{3}H]inositol 2–3-fold faster than did other aneuploid or diploid controls. In contrast, trisomy 21 did not affect the uptake of choline, serine or glucose. Kinetic analysis demonstrated an increased maximal velocity of high-affinity, Na^+-dependent, inositol transport, consistent with the expression of higher numbers of transporters by DS cells. Enhanced uptake was accompanied by a proportional increase in the incorporation of radiolabelled inositol into phospholipid. We suggest that an imbalance of inositol metabolism may contribute to plasma membrane abnormalities characteristic of DS cells.

INTRODUCTION

Down's syndrome (DS) is the most common viable aneuploidy in humans and is the major genetic cause of mental retardation [1]. In addition to a distinctive pattern of morphological defects, DS is associated with an unusual prevalence of particular disease states, including muscle hypotonia, leukaemias, immune deficiency, cataracts and neuropathological changes resembling Alzheimer's disease [2]. On the cellular level, reports of altered neuronal electrical properties [3,4], decreased Na^+/K^+-ATPase activity [5,6] and exaggerated responses to certain drugs [7–10] point to dysfunction at the plasma membrane. Trisomy of chromosome 21 has been recognized as the basis of DS for 30 years. More recently, rare cases have been reported which suggest that trisomy of only the distal region of the long arm of chromosome 21 is sufficient to generate DS pathology [2]. Nevertheless, the mechanisms by which the extra genetic material distorts normal development and function remain unclear. Presumably a 1.5-fold overexpression of chromosome 21 gene products, proportional to the change in gene dosage, is fundamentally responsible [11,12]. However, secondary effects influencing the expression of genes on other chromosomes may also be involved, as may non-specific effects common to any chromosomal imbalance. A clearer understanding of how trisomy 21 disrupts the DS cell would not only elucidate the pathogenesis of DS but also shed new light on the role of key biochemical pathways in the development and function of normal cells.

Inositol is an essential cellular nutrient with pivotal roles in membrane structure, signal transduction and development [13–15]. We have used a large collection of human skin fibroblast strains to investigate the impact of trisomy 21 on inositol metabolism. Here we report that DS fibroblasts accumulate inositol markedly faster than normal diploid, non-DS aneuploid or Alzheimer's disease cells.

MATERIALS AND METHODS

Cell culture

Human skin fibroblast strains were obtained from three sources. From the Human Genetic Mutant Cell Repository (Camden, NJ, U.S.A.), 24 strains were purchased: GM3440, GM0275A, GM0288A, GM3529, GM05659, GM00408B, GM00041B, GM03348B, GM01381B, GM2767B, GM4617, GM2067A, GM4592A, GM4928, GM1413, GM04616, GM04614, GM0230B, GM00137B, GM0692, GM1399, GM1359, GM7408 and GM3184. From the NIH Aging Cell Repository, three strains were purchased: AG0364A, AG4401 and AG4402. Four strains were a gift from the laboratory of Diane Arthur (University of Minnesota): FS1, FS2148, PLESSAL7324 and KEES7387. All strains were karyotyped at the source. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum at 37 °C in a humidified 5% CO_2 atmosphere. On the day before an experiment, confluent cultures in 75 cm^2 flasks were split into 24-well plates at a density of 25000 cells per well.

Uptake studies

Cells were rinsed twice with Dulbecco's phosphate-buffered saline (PBS; 137 mM-NaCl, 8.1 mM-Na_2HPO_4, 2.7 mM-KCl, 1.5 mM-KH_2PO_4, 0.9 mM-$CaCl_2$, 0.5 mM-$MgCl_2$, pH 7.4) at 37 °C, then incubated in 250 μl of Hanks' balanced salt solution (137 mM-NaCl, 5.4 mM-KCl, 4.2 mM-$NaHCO_3$, 1.26 mM-$CaCl_2$, 0.49 mM-$MgCl_2$, 0.44 mM-KH_2PO_4, 0.41 mM-$MgSO_4$, 0.34 mM-Na_2HPO_4, 5.5 mM-glucose, pH 7.4) at 37 °C, containing 0.1 μM-*myo*-[^{3}H]inositol (Amersham; 80 Ci/mmol). At the times indicated the radiolabelled medium was removed by aspiration from duplicate wells and the cells were rinsed with 3 × 500 μl of ice-cold PBS. Cells were then dissolved in 500 μl of 1 M-NaOH. Aliquots were neutralized and transferred to vials containing scintillation fluid for counting of radioactivity. Protein determinations of replicate wells were determined by the bicinchoninic acid procedure [16]. For kinetic studies, uptake during 50 min incubations was determined over the inositol concentration range 0.1–700 μM. Uptake at 0 °C was considered to be non-specific and was subtracted from total uptake.

For determinations of [^{3}H]inositol incorporation into phospholipid, cells were scraped into 750 μl of methanol/HCl (100:1, v/v), and then into an additional 750 μl of PBS. Lipids were extracted from the 1.5 ml scraped cell suspension by addition of 750 μl of chloroform, followed by mixing and centrifugation for 10 min at 1000 *g*. The upper aqueous phase was removed and the lower lipid phase was evaporated to dryness, then redissolved in scintillation fluid for counting.

Abbreviations used: DS, Down's syndrome; PBS, phosphate-buffered saline.

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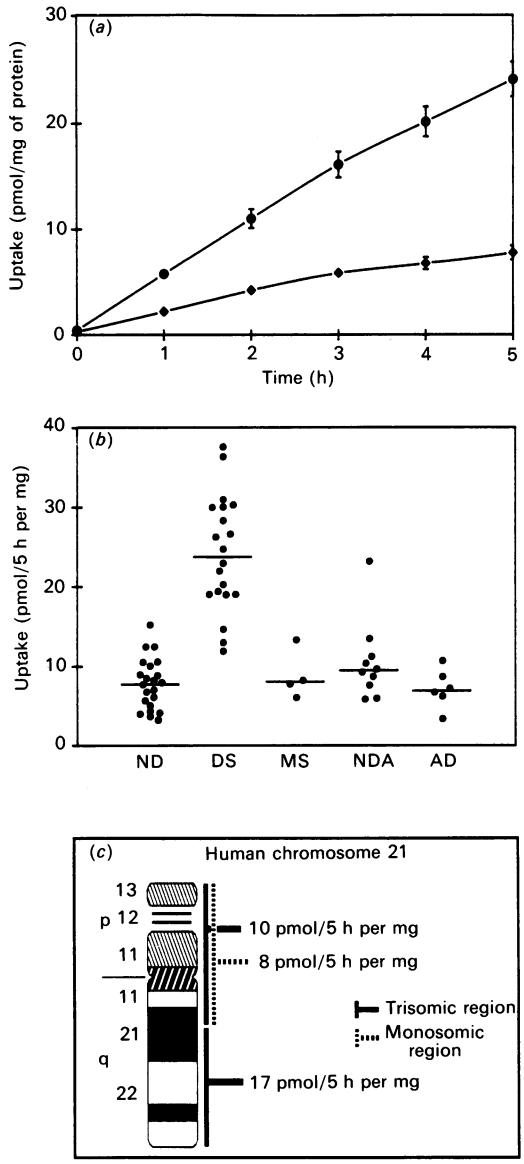

Fig. 1. Inositol uptake by DS and control fibroblasts

(*a*) Time course of [^{3}H]inositol uptake. Points represent means ± S.E.M. (when larger than the point) of 20 experiments (10 strains) for DS (●) and 22 experiments (11 strains) for normal diploids (◆). (*b*) Scatter plot of inositol uptake by normal diploid (ND), DS, monosomy 21 (MS, two strains), non-DS aneuploid (NDA, five strains) and Alzheimer's disease (AD, four strains) fibroblasts. Each point represents the final point (duplicate determinations) of a 5 h time course. All strains were tested twice and are represented by two points. Horizontal lines mark the median of each group. All experiments paired two strains from different groups. The average number of passages in culture for cells from each group was 14 for DS, 14 for normals, 16 for monosomy 21, 13 for non-DS aneuploids and 9 for Alzheimer's disease. The outlier in the NDA group represents a very rapidly growing trisomy 18 strain. At later passages this strain transported both inositol and glucose at a rate similar to that in normal cells. (*c*) Mean uptake (two experiments) by fibroblast strains aneuploid for discrete segments of chromosome 21.

RESULTS

Inositol uptake was measured from 0–5 h using *myo*-[^{3}H]inositol at 0.1 μM. DS fibroblasts accumulated 3-fold more inositol than normal diploid cells (Fig. 1*a*). In each of 14 independent experiments which paired DS and normal cells, inositol uptake by DS cells exceeded that by normals.

To gain insight into the genetic basis of enhanced inositol uptake by DS cells, inositol uptake by a number of other genetically aberrant human fibroblast strains was examined (Fig. 1*b*). Monosomy 21 cells accumulated inositol at a rate equivalent to normal diploid cells, not 50% slower as would be predicted for a simple chromosome 21 gene dosage effect. Non-DS aneuploid controls also did not exhibit enhanced inositol uptake. This group included fibroblast strains trisomic for chromosomes 15, 18 and 20, as well as a strain trisomic for only the pter→q21 region of chromosome 21 (outside the region associated with the DS phenotype) and a strain monosomic for this same region. The testing of strains trisomic for only portions of chromosome 21 allowed for further localization of the chromosomal region associated with enhanced inositol uptake (Fig. 1*c*). The non-DS strain trisomic for bands pter→q21 of chromosome 21 exhibited near-normal inositol uptake. In contrast, a DS strain trisomic for only the q21→qter region accumulated inositol at a rate similar to that in cells with complete trisomy 21. In addition, because of intriguing genetic, biochemical and neuropathological links between DS and Alzheimer's disease [17–19], we examined inositol uptake by three Alzheimer fibroblast strains. However, inositol uptake by Alzheimer's disease cells was similar to that by normal diploid cells (Fig. 1*b*). Thus constitutive enhancement of fibroblast inositol uptake was associated specifically with trisomy of the distal region of the long arm of chromosome 21.

To rule out the possibility that some generalized anomaly of transport across the plasma membrane might be operating in the DS fibroblast, the rates of uptake of choline, L-serine and D-glucose were examined. Choline and L-serine were chosen as controls because, like inositol, they are phospholipid precursors, whereas D-glucose represents a carbohydrate isomer of inositol. Experiments were done concurrently with, and used the same cells as, experiments demonstrating differential uptake of inositol. However, we found no significant differences in the uptake time courses of these nutrients by DS as compared with normal diploid cells (Fig. 2).

Kinetic analysis of fibroblast inositol uptake indicated the presence of high- and low-affinity uptake systems (Fig. 3). Similar two-system kinetics for inositol uptake have been documented in a number of other cell types [20–23]. Apparent K_m values for the high-affinity components were near the serum inositol concentration of 30 μM in both DS and normal diploid cells. In contrast, the maximum velocity, $V_{max.}$, of the high-affinity component of uptake was more than twice as high in DS cells as in normals. Differences between DS and normal kinetics (both K_m and $V_{max.}$) in the low-affinity portion of the curve were less dramatic and could not account for the magnitude of the difference in uptake between DS and normal cells seen at inositol concentrations near and below serum levels. Furthermore, data from individual experiments did not consistently exhibit low-affinity components but were sometimes better fitted by models assuming a single high-affinity component plus a simple diffusion component. Therefore the clearest interpretation of these data is that enhanced inositol uptake by DS cells stems from increased maximal velocity of high-affinity inositol transport.

Fibroblast high-affinity inositol uptake was further characterized with regard to dependence on extracellular Na^+ and the effect of inhibitors. Removing Na^+ from the buffer progressively reduced inositol uptake to background levels (similar to uptake at 0 °C) and attenuated the difference between DS and normal uptake (Fig. 4*a*). Isomers of inositol inhibited uptake of [^{3}H]inositol in the rank order scyllitol ≥ inositol > *scyllo*-inosose > *epi*-inositol > D-glucose (Fig. 4*b*). Phloridzin and ouabain, the Na^+/K^+-ATPase poison, were also potent

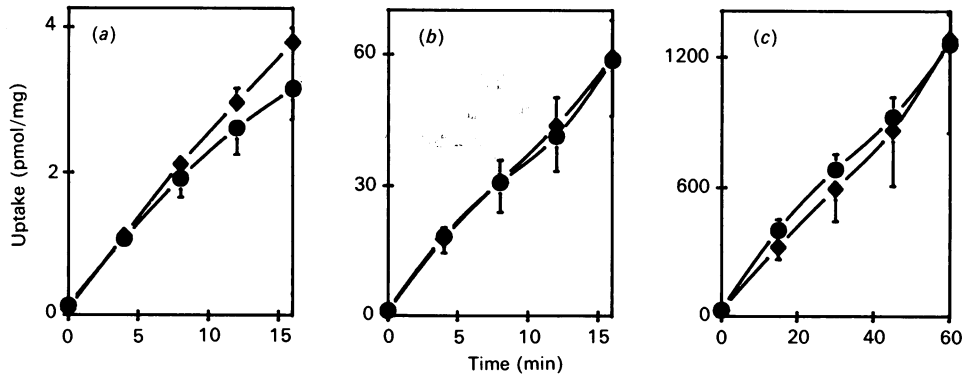

Fig. 2. Uptake of choline (*a*), L-serine (*b*) and D-glucose (*c*) by DS and normal diploid fibroblasts

Time courses were determined as described in the Materials and methods section, except that PBS was used as the incubation buffer. Uptake of [^{3}H]choline (80 Ci/mmol; 0.1 μM) was performed at 25 °C. Points represent means ± S.E.M. of five experiments testing two DS (●) and two normal diploid (◆) fibroblast strains. Uptake of L-[^{3}H]serine (7 Ci/mmol; 0.1 μM) was performed at 25 °C, with six experiments testing two DS and two normal diploid strains. Uptake of D-[^{14}C]glucose (0.3 Ci/mmol; 20 μM) was performed at 37 °C, with six experiments testing three DS and three normal diploid strains.

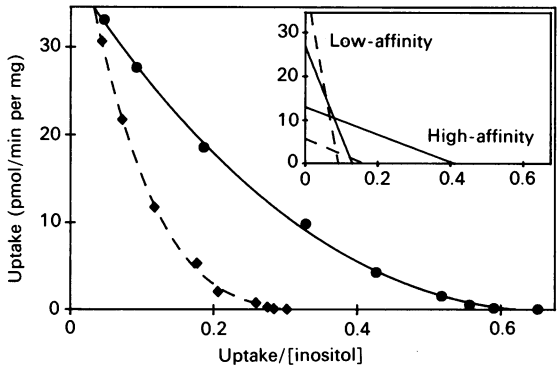

Fig. 3. Kinetics of inositol uptake by DS and normal diploid fibroblasts, presented as Eadie–Hofstee plots

Points are the means of 11 experiments testing four DS (●) and three normal diploid (◆) strains. Non-linearity suggests the presence of more than one uptake system. Non-linear least-squares regression (Marquardt–Levenberg method; RS/1 software) to a two-system model [35] gave the best fit to the averaged data. This yielded high-affinity kinetic parameters of K_m = 31.5 μM, $V_{max.}$ = 13.0 pmol/min per mg for DS; and K_m = 35.4 μM, $V_{max.}$ = 5.8 pmol/min per mg for normals. Low-affinity parameters were K_m = 205 μM, $V_{max.}$ = 26.8 pmol/min per mg for DS; and K_m = 448 μM, $V_{max.}$ = 41.3 pmol/min per mg for normals. The inset shows the linear components calculated for each curve.

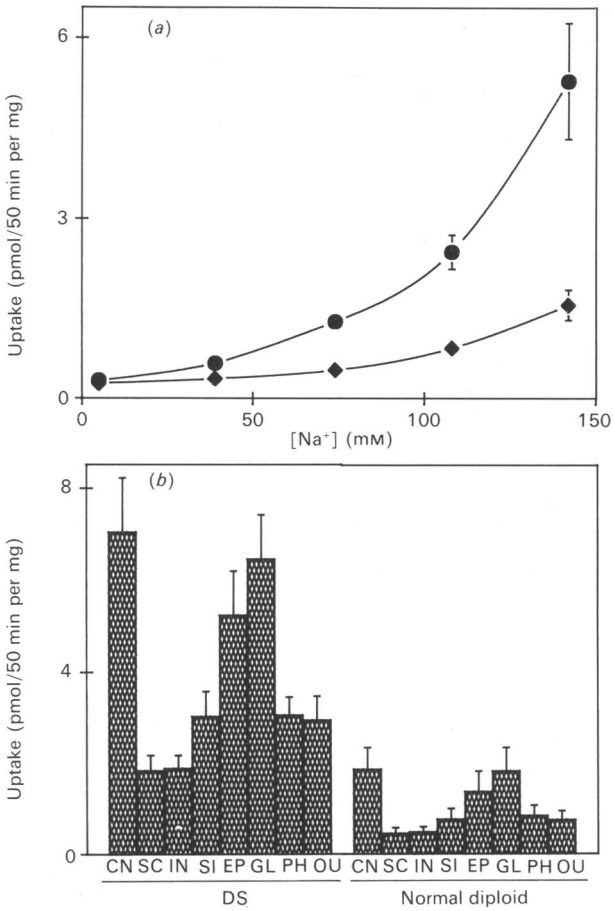

Fig. 4. Na$^+$-dependence and response to inhibitors of inositol uptake by DS and normal diploid fibroblasts

(*a*) Uptake of 0.1 μM-[^{3}H]inositol in PBS was determined with LiCl progressively replacing NaCl to maintain osmolarity. Points are the means ± S.E.M. of six experiments testing two DS (●) and three normal (◆) strains. Isomolar replacement of NaCl with choline chloride gave comparable results. (*b*) Inhibition of 0.1 μM-[^{3}H]-inositol uptake by inhibitors at 100 μM. Inhibitors were present throughout incubations in PBS. Ouabain was also present during a 10 min preincubation. Data are the means ± S.E.M. of three experiments testing one DS and one normal diploid strain. CN, control; SC, scyllitol; IN, inositol; SI, *scyllo*-inosose; EP, *epi*-inositol; GL, glucose; PH, phloridzin; OU, ouabain.

inhibitors. DS and normal cells showed the same pattern of inhibition.

Enhanced uptake of [^{3}H]inositol by DS cells was accompanied by a proportional increase in the rate of incorporation of [^{3}H]inositol into lipid (Fig. 5). The fact that the fraction of the total accumulated [^{3}H]inositol incorporated into lipid was similar in DS and normal cells reinforces the conclusion that the primary difference between DS and normal inositol metabolism is at the point of inositol uptake. If the enhanced uptake were driven by a difference in the metabolism of inositol within the DS cell, one would predict that the relative distribution of [^{3}H]inositol into aqueous and lipid fractions would be affected. An increase in the levels or turnover of inositol lipids in DS membranes is an important potential consequence of enhanced inositol uptake. However, the demonstrated increase in incorporation of [^{3}H]inositol into phospholipid does not necessarily prove that the

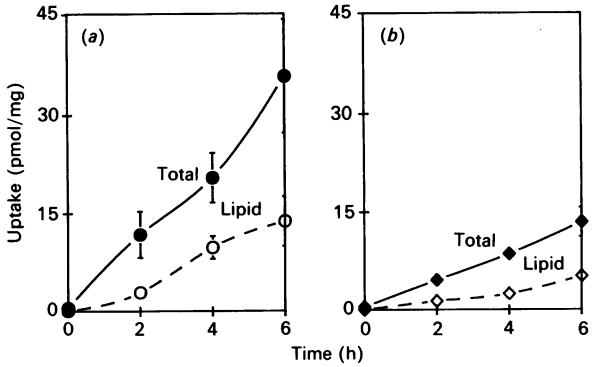

Fig. 5. Incorporation of [^{3}H]inositol into lipid by DS (*a*) and normal diploid (*b*) fibroblasts

Time courses of total uptake were performed as described in the text. At each time point, [^{3}H]inositol incorporation into lipid was determined by acidified methanol and chloroform extraction of replicate wells. Data are the means ± S.E.M. of four experiments testing three DS and two normal diploid strains.

absolute rate or extent of inositol lipid synthesis is increased in DS cells. Determination of the effect of increased uptake on actual mass levels of inositol in DS cells is prerequisite to such conclusions.

DISCUSSION

We report that DS fibroblasts accumulate inositol markedly faster than do normal diploid cells. Other aneuploid and Alzheimer's disease cells did not show enhanced inositol uptake, suggesting that the effect is a specific consequence of trisomy of the long arm of chromosome 21. Moreover, the effect appears to be specific to the transport of inositol, as choline, serine and glucose uptake were not altered in DS cells. Kinetic analysis showed an increased maximal velocity of high-affinity inositol uptake, with no corresponding change in apparent K_m. The response to inhibitors and to removal of extracellular Na^+ was also not changed in DS cells. These results suggest that DS fibroblasts express increased numbers of qualitatively equivalent high-affinity inositol transporters. An increase in the turnover number of the transporter in DS cells remains a less likely alternative.

Our findings might easily be explained as a simple dosage effect of an inositol transporter gene mapping to the long arm of chromosome 21. However, we found an increase in transport velocity beyond the 1.5-fold increase expected from simple gene dosage alone [11,12]. Moreover, no decrease in the rate of uptake by monosomy 21 cells was observed. Thus an order of complexity beyond a simple gene dosage effect may be operating here. For example, the overexpression of some other gene in trisomy on the long arm of chromosome 21 may secondarily stimulate inositol uptake.

We have characterized the high-affinity inositol transporter of human skin fibroblasts with regard to kinetics, Na^+-dependence and response to several inhibitors. The properties we describe are similar, in various aspects, to those previously described for inositol uptake by several other cell types from kidney [24], intestine [25], nerve [26,27], choroid plexus [28] and many cultured cell lines [20–22]. This suggests that many tissues may express the same or a similar high-affinity, Na^+-dependent, inositol transporter. In contrast, some tissues, including liver [29] and muscle [30], exhibit only low-affinity, non-active, inositol transport. It will now be important to test more rigorously for the identity of inositol transporters in different tissues while determining which other tissues may exhibit enhanced inositol uptake in the trisomy 21 state. In addition, it will be important to investigate how the distribution of inositol may be altered in the DS individual. The potential for effects on the development and function of the DS brain deserve particular attention. Mouse models of DS may prove useful in such studies [31].

Enhanced inositol uptake may influence the levels or turnover of inositol lipids in DS cell membranes. Previous reports have demonstrated altered neuronal plasma membrane properties, particularly decreased Na^+/K^+-ATPase activity and conduction velocity [32–34], resulting from persistent changes in free inositol levels. Thus, in the DS cell, imbalanced inositol metabolism may contribute to documented plasma membrane abnormalities, including altered electrophysiological properties of cultured neurons [3,4], and decreased platelet Na^+/K^+-ATPase activity [5,6]. Broader implications seem likely, particularly if the transduction of signals across the plasma membrane is affected. Clearly, further studies of the genetic basis of enhanced inositol uptake and its role in the pathogenesis of DS are warranted.

We thank V. E. Anderson, J. W. Eaton and J. R. Sheppard for helpful advice and contributions. This work was supported by NIH grants HG-00143 and MH-14647.

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Received 25 April 1990/1 June 1990; accepted 20 June 1990