Inositol and inositol 1,4,5-trisphosphate content of Down syndrome fibroblasts exhibiting enhanced inositol uptake

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Fibroblasts from individuals with Down syndrome (DS; trisomy 21) exhibit increased inositol uptake. Here we examine the relationship between this increase in uptake and mass levels of free inositol and inositol 1,4,5-trisphosphate (IP$_3$) in DS fibroblasts. We report that human fibroblasts contain high levels of free inositol which are not significantly affected by the increase in inositol uptake associated with DS. In addition, increased uptake is accompanied by increased efflux of radiolabelled inositol from DS cells. Neither basal nor bradykinin-stimulated IP$_3$ levels in DS cells differ significantly from normal values. This work highlights the usefulness of the DS cell in uncovering the role of transport across the plasma membrane in cellular inositol homeostasis.

Inositol; Down syndrome; Inositol 1,4,5-trisphosphate; Transport; Human fibroblast

1. INTRODUCTION

Trisomy of all or part of chromosome 21 produces Down syndrome (DS), a major cause of mental retardation. We recently reported that skin fibroblasts from individuals with DS exhibit an increased rate of inositol accumulation due to a specific increase in the maximal velocity of high-affinity, Na$^+$-dependent inositol transport coincident with trisomy of the distal segment of the long arm of chromosome 21 [1]. These findings led us to suggest the possibility that altered regulation of high-affinity inositol transport may be a significant factor in the pathophysiology of DS. This possibility is supported by studies showing the expression of a similar high-affinity inositol transport system by many other tissues [2] including fetal brain [3]. However, the pathophysiologic significance of enhanced inositol uptake is ultimately dependent on how the levels and turnover of inositol and its metabolites are affected in the various cells and tissues of the DS individual.

Cells maintain high levels of free inositol by drawing from three potential sources: uptake from the media, de novo synthesis from glucose 6-phosphate, and recycling of inositol phosphates. The relative importance of each source is a point of controversy and certainly varies between different cell types. But the fact that the survival of most cells is dependent on an external supply of inositol [4] is evidence that transport across the

Abbreviations: DS, Down syndrome; IP$_3$, inositol 1,4,5-trisphosphate.

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plasma membrane plays a vital role. Catabolism of inositol is apparently limited to the kidney [5].

Changes in inositol homeostasis are associated with important functional and developmental consequences in certain cell types [6,7]. Neural cells in particular exhibit sensitivity to abnormally low or high inositol levels. For example, reduced inositol uptake by peripheral nerves from diabetic animals is accompanied by decreases in inositol content and inositol lipid metabolism, changes that are linked to deficits in Na$^+$,K$^+$-ATPase activity and conduction velocity [8]. In the brain, lowered inositol levels brought on by LiCl treatment are thought to underlie the ability of this drug to modulate neural activity [9]. Studies of both experimental animals and human subjects show that abnormally high inositol levels also impair nerve function. For example, induction of hyperinositolemia in rats results in decreased nerve conduction velocity [10]. Similarly, in patients with chronic renal failure elevation of plasma inositol is correlated with impaired nerve conduction [11]. More subtle electrophysiological effects have been reported to follow oral administration of inositol to patients [12,13]. The particular sensitivity of neural cells to inositol is also suggested by a study of cultured dorsal root ganglion cells in which morphologic abnormalities were observed following exposure to increasing concentrations of inositol [14]. Not all cell types, however, are similarly sensitive to altered inositol homeostasis. For example, renal epithelial cells increase high-affinity inositol uptake and intracellular inositol content as an adaptive response to osmotic stress [15]. This response is not associated with apparent changes in inositol lipid metabolism or signal transduction [16]. Inositol homeo-

stasis also appears to be a relevant factor in hematologic differentiation where differential expression of inositol transport is associated with changes in intracellular levels of free inositol and higher inositol phosphates characteristic of maturation down particular developmental pathways [17–19].

In this report we examine the relationship between enhanced inositol uptake and the overall mass levels of inositol in DS fibroblasts. In addition, receptor-mediated signal transduction via the inositol lipid cycle is evaluated through mass determinations of inositol 1,4,5-trisphosphate (IP_3). Our results indicate that notwithstanding enhanced inositol uptake, DS fibroblasts maintain near normal levels of inositol and IP_3.

2. MATERIALS AND METHODS

2.1. Cell culture

Human skin fibroblasts were obtained from the Human Genetic Mutant Cell Repository, Camden, NJ (GM02767B, GM04592, GM04616, GM04614, GM03348B, GM3440, GM00408B, GM05659 and GM0288A) and from the laboratory of Diane Arthur, University of Minnesota (PLESSAL7324). These cells comprised five trisomy 21 and five normal diploid strains. Cells were grown in Dulbecco's modified Eagle's Medium (DMEM, Gibco) supplemented with 5% fetal calf serum and 5% newborn calf serum in a humidified 5% CO_2 atmosphere.

2.2. Uptake and efflux studies

Cells were seeded into 24-well plates at a density of 25 000 cells/well and grown until near confluency (2–3 days). The uptake of *myo*-[³H]inositol (80 Ci/mmol, Amersham) during 2 h incubations was determined as described [1] except that DMEM containing 10 mM HEPES (pH 7.4) was used as the incubation medium. The final inositol concentration of this medium is ~40 μM. To assess efflux of inositol, cells were prelabelled for 2 h as above then washed with phosphate-buffered saline (PBS) and incubated in unlabelled media, 37°C. At the indicated times, this medium was removed by aspiration and the radioactivity remaining in the cells counted. The identity of the radioactivity appearing in the efflux medium was examined using thin-layer chromatography on silica gel plates (Whatman) developed in *n*-propanol/ethylacetate/water/25% ammonia (5:1:3:1), and visualized by autoradiography.

2.3. Determination of intracellular inositol

Intracellular free inositol was measured in perchloric acid extracts using a novel adaption of previously described methods based on the enzymatic oxidation of inositol [20–22]. Confluent fibroblast monolayers in 25 cm² flasks (Costar) were quickly washed 3 times with 12 ml of ice-cold PBS. Then 600 ml PBS/10 mM HEPES/0.0005% Phenol red was added followed by 120 μl of 10% perchloric acid. Flasks were kept on ice for 30 min with intermittent rocking. The supernatant (720 μl) was removed to plastic tubes and carefully neutralized with ~120 μl 1.5 M KOH/60 mM HEPES. Precipitated perchlorate salts were removed by centrifugation for 10 min at 2000 × g, 4°C. As a precautionary step, the supernatant was treated with 50 μl glucose oxidase reagent (70 U/ml glucose oxidase, 350 U/ml catalase in PBS) to eliminate the potential inference by glucose [21]. Tubes were incubated at 37°C for 90 min, and then heated at 100°C for 3 min to stop the reaction. Supernatants were stored at −70°C.

To 100 μl of sample in triplicate was added 50 μl of distilled water and 50 μl of a freshly prepared mixture of 25 mM NAD in 1 M K⁺ phosphate, pH 9; 20 mM ferrozine (Sigma) in 4 mM FeCl₃; and 63 μM phenazine methosulfate. The reaction was started by the addition of 5 mU of *myo*-inositol dehydrogenase (Sigma) in 10 μl PBS. Samples were vortexed and incubated at 37°C for 2 h. The reaction was stopped

by the addition of 10 μl of 10% perchloric acid. This treatment does not affect the colored ferrous-ferrozine complex [23] but eliminates any interference of phenol red absorbance. Supernatants were transferred to 96-well plates and the absorbance at 560 nm was determined on a plate-reader. Samples were read against the absorbance of inositol standards (0.5–10 nM) processed in the same way. When cellular samples were spiked with various known amounts of inositol prior to perchloric acid extraction, recovery averaged 91% ± 4 (n=5).

2.4. Determination of intracellular IP_3

Confluent cells in 24-well plates were incubated overnight in DMEM containing 1% newborn calf serum then exposed to 10 mM LiCl for 10 min prior to stimulation. Cells were washed and treated with 250 μl of DMEM/10 mM HEPES containing various concentrations of bradykinin. Incubations at 37°C were terminated by the addition of 10% perchloric acid and neutralized as above. IP_3 in the supernatants was measured by a radioreceptor binding assay [24] utilizing a specific IP_3 binding protein from bovine adrenal microsomes and [³H]IP_3 (Amersham).

3. RESULTS

To examine the relationship between enhanced inositol uptake by DS cells and cellular inositol content, these two parameters were measured concomitantly on replicate platings of the same cells (Fig. 1). In agreement with previous results [1] DS fibroblasts showed a dramatic (greater than 3-fold) increase in [³H]inositol accumulation. Surprisingly, however, the bulk free inositol content of DS fibroblasts was not significantly different from normals. These determinations of fibroblast inositol content are similar to previously determined levels in cultured endothelial [25] and epithelial cells [15,16]. However, other cell types, for example those of neural [26] or myeloid [17] origin contain much lower levels of inositol.

To investigate potential mechanisms which might contribute to the ability of DS fibroblasts to maintain normal levels of inositol under conditions of enhanced uptake, we examined the efflux of [³H]inositol from fibroblasts prelabelled for 2 h. DS cells showed a greatly increased initial rate of [³H]inositol efflux relative to that of normal diploid cells (Fig. 2A). For both DS and normal cells, the concentration of inositol in the efflux medium had little effect on the rate of efflux (Fig. 2B). The radioactivity appearing in the efflux medium
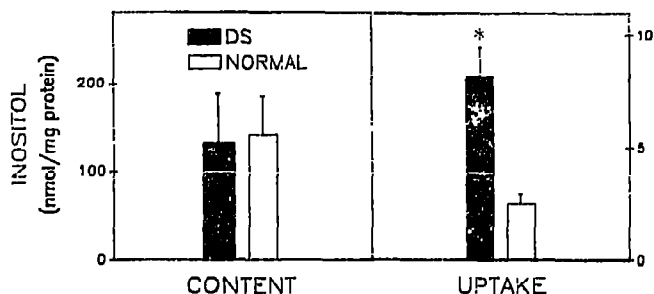

Fig. 1. Inositol content of DS and normal diploid fibroblasts and its relationship to inositol uptake. Values are the mean ± SE of four separate experiments (performed in triplicate) pairing DS and normal cells. *P < 0.01 compared to normal uptake, paired Student's t-test.
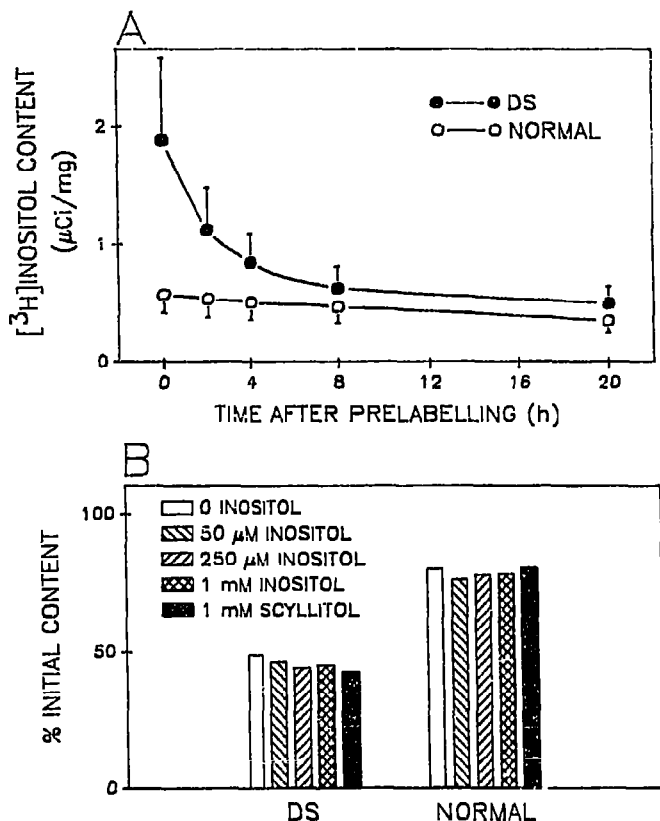
Fig. 2. Efflux of [³H]inositol from DS and normal diploid cells. (A) Time course of efflux following 2 h pulse with [³H]inositol. Data are the means ± SE of 3 separate experiments. (B) Effect of extracellular inositol on efflux. Cells were prelabelled as above then incubated in inositol-free DMEM supplemented with the indicated concentrations of inositol or its isomer scyllitol. Results are expressed as the percent of the initial radioactivity remaining in the cells after 3 h. Data are the mean of duplicate determinations from a single experiment representative of two similar experiments.

Fig. 3. IP₃ content of DS and normal diploid fibroblasts and its relationship to inositol uptake. (A) Time course of IP₃ production in response to 1 μM bradykinin. Data are the means of duplicate determinations from a single experiment. (B) Mean basal and bradykinin-stimulated (8 s) IP₃ levels from 5 experiments. (C) Inositol uptake by cells assayed for IP₃ content (panel B). *P < 0.02, paired Student's t-test.

migrated with [³H]inositol standards during thin-layer chromatography (data not shown).

To investigate a potential relationship between enhanced inositol uptake by DS cells and transmembrane signalling via IP₃ production, mass levels of IP₃ were measured in unstimulated and bradykinin-stimulated cells. Time courses of IP₃ production in cells exposed to 1 μM bradykinin showed peak stimulation between 5 and 10 s for both DS and normal cells (Fig. 3A). When IP₃ levels and inositol uptake were measured together in paired sets of DS and normal cells, neither basal nor bradykinin-stimulated IP₃ levels of DS cells were significantly different from those of normal cells (Fig. 3B), although the difference in uptake by DS and normal cells was more than 3-fold (Fig. 3C).

4. DISCUSSION

The present results demonstrate that increased inositol uptake by DS fibroblasts is not associated with significant changes in overall mass levels of free inositol
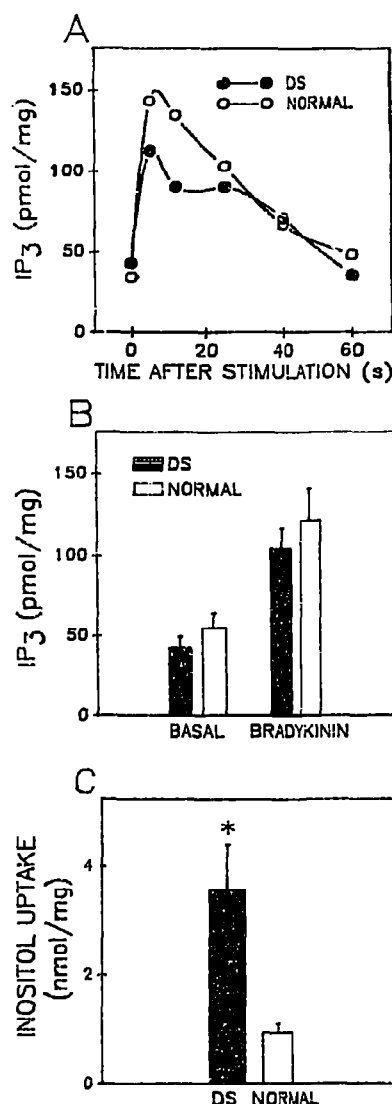
or its pivotal second messenger metabolite IP₃. Additional findings showing that the efflux of [³H]inositol from DS cells is also increased offer an attractive explanation of how DS fibroblasts are able to maintain normal inositol levels under conditions of enhanced uptake. However, without knowledge of the specific activity or actual mass levels of inositol exitting the cell it is difficult to judge with certainty the importance of this pathway. Thus, the mechanism and regulation of inositol efflux by DS cells deserve further investigation. It is also important to consider that transport across the plasma membrane may be a quantitatively minor (though not necessarily inconsequential) source of total

fibroblast inositol, and that this too may contribute to the lack of any detectable change in inositol levels in DS cells. The relatively high levels of inositol reported here for fibroblasts are consistent with this view.

In contrast to our findings with human skin fibroblasts, a close association between inositol transport activity and cellular inositol levels does exist in certain cell types. This has been demonstrated, for example, in cultured neuroblastoma [26], promyeloid [17], and renal cell lines [15]. However, studies of cultured lens epithelial cells [27] and in vitro preparations of aortic wall [28] show that in other tissues, like in fibroblasts, inositol uptake can be modulated without measurable changes in total inositol content. Clearly, there are important differences in the mechanisms through which different cell types maintain free inositol levels. These differences presumably reflect differential expression of inositol uptake, efflux, synthesis, and metabolism, as well as differences in the homeostatic mechanisms which regulate these processes.

The present results do not rule out the possibility that the levels or turnover of some small, discrete pool of inositol metabolites in the DS cell may be affected. Certainly the flux of a nutrient through the cell (independent of changes in mass levels) can itself be rate-limiting for metabolism [29]. Accordingly, our previous results have shown that enhanced uptake by DS cells is accompanied by an increased incorporation of [^{3}H]inositol into phospholipid [1]. Simmons and Winegrad [28,30] have demonstrated the existence of a discrete, rapidly turning over pool of phosphatidyl inositol in the rabbit aortic wall which is critically dependent on inositol transport. In this system, inhibition of normal inositol uptake results in impared N^+,K^+-ATPase activity through inhibition of a specific fraction of phosphatidyl inositol synthesis. These changes occur in the absence of changes in total tissue inositol. While the present study does not address the possibility that enhanced inositol uptake may influence this or other novel pathways of inositol metabolism in the DS fibroblast, it does confirm that any such influences, if present, operate apart from any significant effect on the best-characterized aspect of inositide metabolism, namely signal transduction via phosphatidylinositol 4,5-bisphosphate hydrolysis to IP$_3$.

At present we can only speculate as to the role of inositol homeostasis in DS. Further studies examining the transport, levels, and metabolism of inositol in other DS tissues are required. Such studies must bear in mind potential heterogeneity in the expression of transporters and other mechanisms regulating inositol levels in different tissues. It is noteworthy in this regard that neural and hematopoietic tissues, where the functional and developmental correlates of altered inositol uptake appear most pronounced, are the very tissues which exhibit the most significant pathological effects of trisomy 21 [31]. Thus we speculate that some imbalance of inositol metabolism may be related to the altered neural membrane properties [32,33] or the unusual prevalence of myeloproliferative disorders [34] which are characteristic of these tissues in DS. However, we are aware of no reports of inositol metabolism in any DS tissue or cell type other than skin fibroblasts. Weighing the pathophysiological significance of enhanced inositol uptake in DS will also require a clearer understanding of the role of transport in inositol homeostasis [6]. Even on the cellular level, while it is clear that changes in inositol transport influence plasma membrane properties in certain cell types, the relationship of transport to the complex regulatory network of inositol metabolites is only beginning to be understood [30,35,36]. At the very least, further studies of inositol metabolism in DS should offer new insights into these problems in cellular physiology. Along with such insights may come important clues to the pathophysiology of DS.

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