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Lithium ion reversibly inhibits inducer-stimulated adipose conversion of 3T3-L1 cells

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Adipose conversion of 3T3-L1 cells by inducers (dexamethasone, 1-methyl-3-isobutylxanthine and insulin) was inhibited by LiCl at concentrations from 2 to 20 mM. The effect of LiCl was reversible and the inhibited cells were converted to adipocytes when stimulated after the removal of LiCl. Inhibition by LiCl of adipose conversion was accompanied with a blockage of the enhanced [^{3}H]thymidine incorporation and cellular proliferation that occurred before the adipocyte phenotype was expressed. Of the cations tested, only Li^+ had these effects.

Li^+; Adipose conversion; Glycerophosphate dehydrogenase; (3T3-L1 cell)

1. INTRODUCTION

When the growth of cultured fibroblasts is arrested, a certain population spontaneously converts into cells showing the morphological appearance of adipocytes [1]. 3T3-L1 is a line of Swiss 3T3 fibroblasts with a high frequency of such conversion [2-5], and it is useful as a model of adipocyte differentiation. The spontaneous conversion of 3T3-L1 takes a month after confluence is reached, but the addition of inducers such as DEX, MIX and insulin greatly accelerates this process [4,6-8]. An inhibitor of poly(ADP-ribose) synthetase, nicotinamide, prevents this conversion of 3T3-L1 [9].

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Abbreviations: DEX, dexamethasone; MIX, 1-methyl-3-isobutylxanthine; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; GPDH, glycerophosphate dehydrogenase

Li^+ affects proliferation and differentiation of a variety of cells. The earliest observations of these effects include the leukocytosis that occurred in manic-depressive patients receiving Li^+ as therapy [10]. Li^+ increases the number of pluripotential stem cells (CFU-S) [11], stimulates the proliferation of granulocyte-macrophage-committed stem cells (CFU-GM) [12], and reduces erythroid-committed stem cells (BFU-E, CFU-E) [13]. An experiment, that used Friend erythroleukemia as a model of erythroid-committed stem cells, suggested that Li^+ inhibits both the growth of the cells and their differentiation induced by dimethyl sulfoxide [14]. The growth of murine mammary epithelium in both organ culture [15] and primary cell culture [16] is stimulated by Li^+. However, Li^+ does not enhance the expression of a differentiated phenotype (casein secretion) of these mammary cells [15,17]. Li^+ was mitogenic with BALB/c 3T3 [18] and kidney epithelial cells [19] but not with another kind of epithelial cells, MDCK [18]. Li^+ inhibits neurite outgrowth of PC12 cells induced by nerve growth factor, a model of the critical step of neuronal differentiation [20]. We describe here

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the reversible inhibition by Li^+ of the adipose conversion of 3T3-L1.

2. MATERIALS AND METHODS

DMEM from Nissui Pharmaceutical Co. (Tokyo), FCS from M.A. Bioproducts (Walkersville), and dishes from Nunc (Roskilde) were used for cell cultures. Insulin, DEX, dihydroxyacetone phosphate (Sigma, St. Louis), MIX (Aldrich, Milwaukee), monensin, A23187 (Calbiochem-Behring, La Jolla), ouabain (Merck, Darmstadt), [*methyl*-^{3}H]thymidine (25 mCi/mmol; Amersham, Buckinghamshire) and LiCl (99% pure; Wako, Osaka) were used. To stain lipid droplets in differentiated 3T3-L1 cells, Oil Red O from Chroma (Stuttgart) was used. For dye-binding assays of protein, a kit from Bio-Rad (Richmond) was used. All other reagents were of the highest quality commercially available.

2.1. *Cell culture and adipose conversion*

3T3-L1 cells provided by Dr H. Green (Harvard Medical School, MA) were maintained as described [21]. For adipose conversion, cultures at confluence were treated with DMEM containing 10% FCS, 250 nM DEX, 0.5 mM MIX, 10 μg/ml insulin (the induction medium), and LiCl or other reagents at the concentrations indicated in the figure legends. Two days later, the medium was changed to DMEM containing 10% FCS and cells were allowed to differentiate.

2.2. *Glycerophosphate dehydrogenase assay*

Cultures in 35-mm dishes were washed three times with phosphate-buffered saline and harvested into a buffer containing 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 1 mM 2-mercaptoethanol. Cells were disrupted by sonication and the supernatant from the cell lysate was used for enzyme assays as in [22] after being centrifuged at 15 000 × g for 10 min and at 105 000 × g for 1 h.

2.3. *[^{3}H]Thymidine incorporation*

The culture in a 96-well plate was incubated in 30 μl DMEM containing 1 μCi [^{3}H]thymidine for 1 h at 37°C in a CO_2 incubator. Cells were fixed and made permeable by treatment with methanol for 10 min. Then they were washed three times with water, twice with 5% cold trichloroacetic acid, and twice again with water. The washed cells were lysed with 0.15 ml of 0.3 N NaOH and the radioactivity was counted.

3. RESULTS AND DISCUSSION

When growth-arrested 3T3-L1 cells at confluence were stimulated by the induction medium, the highly expanded cells started to contract and the incorporation of [^{3}H]thymidine was stimulated (fig.1). When the cells were removed from the induction medium at the end of day 2, most cells divided into two (fig.1) to fill open spaces resulting from the contraction of cells. The phenotypes of adipocytes such as GPDH activity and lipid accumulation were detected by day 4 and reach maximum levels by day 6 (not shown). This inducer-stimulated adipose conversion could be produced in any 3T3-L1 culture within 2 weeks of

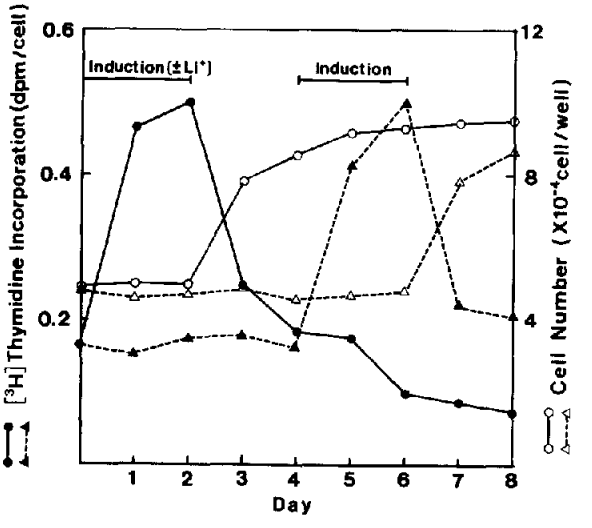

Fig.1. Effect of LiCl on the inducer-stimulated [^{3}H]-thymidine incorporation and proliferation of confluent 3T3-L1 cells. A series of confluent 3T3-L1 cultures (●,○) in 96-well plates were treated with the induction medium during days 1–2 and days 5–6. Another series of cultures (▲,△) were treated with the induction medium containing 20 mM LiCl during days 1–2 and with the induction medium alone during days 5–6. During days 3–4 and days 7–8, all cultures were in DMEM containing 10% FCS. [^{3}H]Thymidine incorporation (●,▲) and cell number (○,△) of the cultures in both series were monitored every day.

its reaching confluence. If LiCl (20 mM) was present during the induction period (days 1 and 2), however, incorporation of [^{3}H]thymidine was not stimulated and cells did not proliferate (fig.1). When the effects of LiCl on the expression of GPDH activity and lipid accumulation were tested on cultures treated in the same way (fig.2B), these phenotypes of adipocytes were expressed as little as in the control culture without stimulation (fig.2A) even at the end of day 8. The inhibition by LiCl of the stimulated [^{3}H]thymidine incorporation and cellular proliferation was reversible, because both incorporation and proliferation in cultures treated first with the induction medium containing LiCl were stimulated by treatment with induction medium without LiCl (fig.1). The results shown in fig.2 indicate that the effect of LiCl on the expression of adipocyte phenotypes is reversible as well. When the cultures treated first with the induction medium containing LiCl were treated again with the induction medium without it (fig.2D), both GPDH activity and lipid accumulation were expressed at the end of day 8 to the same extent as in a control culture with stimulation (fig.2C).

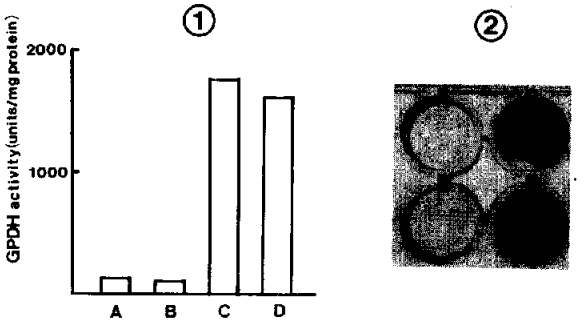

Fig.2. Reversible inhibition of the inducer-stimulated adipose conversion of 3T3-L1 cells by LiCl. Confluent 3T3-L1 cultures in 35-mm dishes for the assay of GPDH activity (1), or in a 24-well plate for the Oil Red O staining (2), were left without any treatment (A), treated with induction medium containing 20 mM LiCl during days 1–2 (B), treated with the induction medium without LiCl during days 3–4 (C), or treated with the induction medium containing 20 mM LiCl during days 1–2 and with the induction medium alone during days 3–4 (D). During other periods not specified above, all cultures were in DMEM containing 10% FCS. GPDH activity and the amount of protein in the cultures in the 35-mm dishes were assayed at the end of day 8 (1). Cultures in the 24-well plate were stained with Oil Red O at the end of day 8 (2). The means of the specific GPDH activity from two independent cultures are shown. One unit of GPDH activity corresponds to 1 nmol/min NADH consumption.

We used the expression of GPDH activity as an index, and estimated the LiCl concentration needed to inhibit inducer-stimulated adipose conversion (fig.3). Inhibition was detectable with 2 mM LiCl and maximum at concentrations of 20 mM and higher. The dose response was similar when lipid accumulation was used as the index. This effective concentration range of LiCl was comparable to those in other experimental systems where Li^+ affects cellular proliferation or differentiation [11–20]. In an experiment summarized in table 1, the specificity of Li^+ was examined by comparison of the effects of chloride salts of other monovalent cations. The addition of 20 mM NaCl, KCl, RbCl, or CsCl to the induction medium did not reduce the specific activity of GPDH in cultures at the end of day 6. When CsCl or RbCl was added, 20 mM was somewhat cytotoxic to 3T3-L1 cells and the apparent staining density of

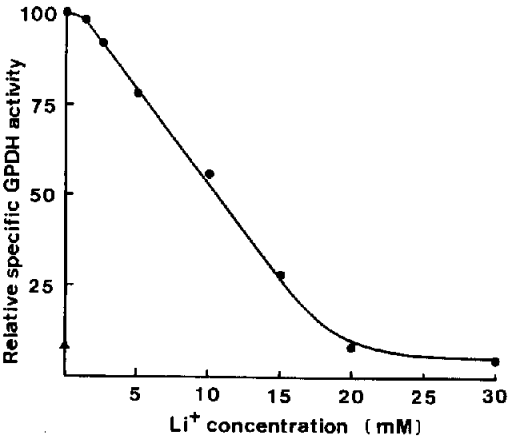

Fig.3. Concentration dependence of the LiCl effect on the inducer-stimulated expression of GPDH activity. Confluent 3T3-L1 cultures in 35-mm dishes were treated with the induction medium containing the indicated concentration of LiCl during days 1–2 and the medium was changed to DMEM containing 10% FCS at the end of day 2. GPDH activities and protein levels were assayed at the end of day 6. Means of two independent cultures are presented (●). The mean specific activity of cultures without LiCl was 2103 nmol/min per mg protein. The mean value from cultures without stimulation is given for reference (▲).

Table 1

Effect of monovalent cations on the inducer-stimulated adipose conversion of 3T3-L1 cells

Salts	Relative specific GPDH activity
None	100
NaCl	100
KCl	101
RbCl	124
CsCl	109
LiCl	11

Confluent 3T3-L1 cultures in 35-mm dishes were stimulated for 2 days with the induction medium containing 20 mM of the salts indicated. The medium was changed to DMEM containing 10% FCS at the end of day 2, and GPDH activity and protein levels were assayed at the end of day 6. Means from two independent cultures are given. The mean specific activity of GPDH in the control cultures (no salt) was 2473 nmol/min per mg protein

accumulated lipid with Oil Red O was lower than that of the control induction. Even in these cultures, however, the relative specific activity of GPDH was the same as or higher than that of the control induction. This may be due to the selective toxicity of these cations to 3T3-L1 cells that did not differentiate.

Li^+ is mitogenic with BALB/c 3T3 fibroblasts [18]. We observed that the incorporation of [^{3}H]-thymidine into 3T3-L1 cells before confluence was stimulated 1.3-fold by LiCl at 5–20 mM. When the cells were confluent however, LiCl alone did not have any effect. Instead, Li^+ inhibited the inducer-stimulated [^{3}H]thymidine incorporation and cellular proliferation. This suggests that Li^+ affects adipose conversion through disturbance of the cellular system(s) that transmits signals of the inducers.

Li^+ affects cAMP-dependent events in some kinds of cells [23,24]. An inhibitor of cAMP phosphodiesterase, MIX, accelerates the adipose conversion of 3T3-L1 cells [7], so the effect of Li^+ may be due to reduction of the intracellular cAMP level. Evidence has accumulated that shows that growth factors such as insulin and EGF stimulate fluxes of monovalent cations [25]. Rozengurt et al. [26] postulated that the increased Na^+ influx stimulates Na^+,K^+-ATPase and thus stimulates the growth of quiescent cells. If insulin stimulates the

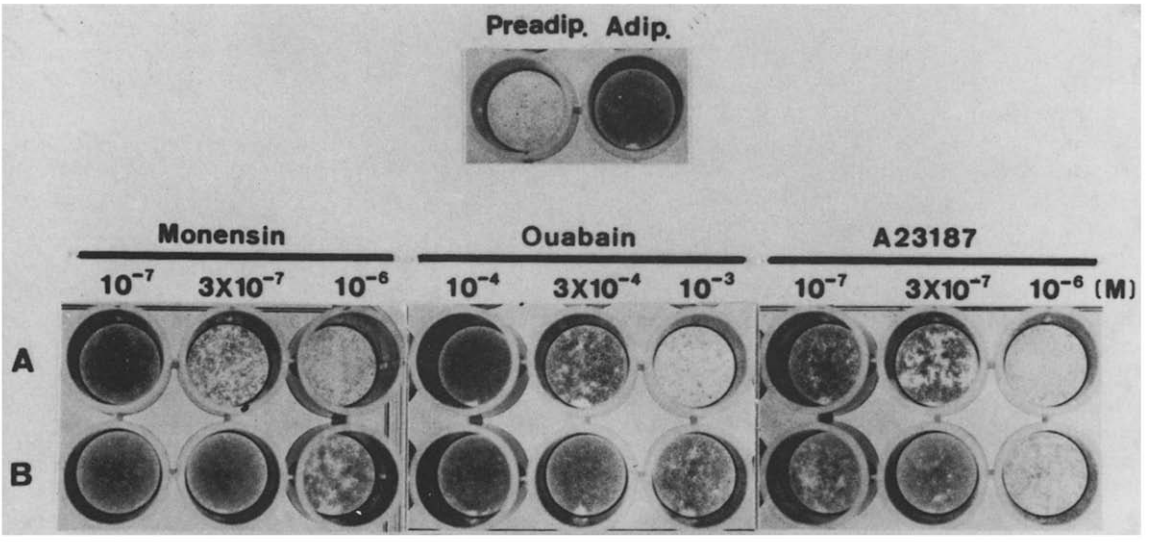

Fig.4. Effects of monensin, ouabain and A23187 on the inducer-stimulated adipose conversion of 3T3-L1 cells. Confluent 3T3-L1 cultures in 24-well plates were treated with induction medium containing indicated concentrations of drugs during days 1–2. In treatment A, the cultures were kept in DMEM containing 10% FCS for an additional 6 days. In treatment B, the cultures were treated again with induction medium without any drugs during day 3 and 4 and were kept in DMEM containing 10% FCS for an additional 4 days. At the end of day 8, all cultures were stained with Oil Red O. Preadip., confluent 3T3 L1 cells; Adip., 3T3-L1 adipocytes.

adipose conversion of 3T3-L1 cells through such a mechanism, the influx of Li^+ instead of Na^+ may abrogate the activity of insulin. It is relevant to this hypothesis that ouabain at concentrations of 0.3 mM and higher had an effect similar to that of Li^+ on the inducer-stimulated adipose conversion of 3T3-L1 cells (fig.4). Intra- and extracellular concentrations of Na^+, K^+, Ca^{2+} and Mg^{2+} may profoundly affect the activities of the inducers. Perhaps Li^+ inhibits inducer-stimulated adipose conversion by affecting the movement and redistribution of these cations. In support of this hypothesis, ionophores such as monensin and A23187 showed a tendency to inhibit the inducer-stimulated adipose conversion of 3T3-L1 cells (fig.4) at low concentrations at which they were not cytotoxic.

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