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Lithium ions increase hepatic glycogen synthase stability through a proteasome-related mechanism

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Abstract

Incubation of rat hepatocytes with LiCl resulted in an overall increase in the activity ratio of glycogen synthase (GS), concomitantly with a decrease in active GS kinase-3 levels. GS total activity was also increased in a dose- and time-dependent manner. This latter effect correlated with the amount of immunoreactive enzyme determined by immunoblotting. Cycloheximide and actinomycin-D did not modify LiCl action on GS activity. Lithium ions did not induce any changes in GS mRNA levels. Furthermore, the increase in the total amount of GS induced by LiCl was further augmented after addition of a specific, calpain and proteasome inhibitor. Our results indicate that LiCl increases hepatocyte GS activity through increasing both the activation state of the enzyme and its cellular content. This latter increase is mediated through a modification of the proteasome-regulated proteolytic pathway of the enzyme.

Keywords: Rat hepatocytes; Lithium ions; Glycogen synthase; Protein stability

Lithium ions show several remarkable effects on hepatic glycogen metabolism. Thus, LiCl activates hepatic glycogen synthase (GS) in systems such as isolated hepatocytes [1,2], primary cultured hepatocytes [3], and whole animals [4]. This activating action is triggered in the presence and absence of glucose. However, glycogen phosphorylase (GP) is activated by LiCl only in the absence of glucose [2]. The combination of these effects induces a clear increase in glycogen synthesis when hepatocytes are incubated with LiCl in the presence of glucose but not in the presence of lactate and pyruvate [2,5,6].

GS-induced glycogen synthesis is achieved by both activation and changes in the intracellular location of the enzyme, since these two actions are required for proper accumulation of glycogen in hepatocytes [7]. In this regard, glucose simultaneously prompts the activation and the translocation of the enzyme [8]. Hepatic GS is activated by dephosphorylation, and several protein kinases and phosphatases are involved in this mechanism [9]. Glycogen synthase kinase-3 (GSK3) is one of these protein kinases [10]. LiCl is a known inhibitor of GSK3 [11] and this property may explain the basis of lithium-induced GS activation. Experimental evidence suggests that glycogen synthesis requires both the activation of GS and its translocation, since effectors that activate GS without causing its translocation fail to stimulate glycogen synthesis [12]. Furthermore, in skeletal muscle cells transfect with a constitutively activated GSK3 isoform, LiCl also increases glycogen deposition, although this effect is less intense than in wild-type cells [13]. Taken together, these observations stress the importance of establishing whether LiCl stimulates glycogen synthesis not only through a direct activation of GS, but also through concomitant translocation of the enzyme.

We studied the mechanisms through which LiCl activates GS and, hence, stimulates hepatic glycogen synthesis. Our results indicate that the LiCl-induced hepatocyte GS

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activation is not related to a concomitant translocation of the enzyme. The LiCl action was concomitant with a significant increase in its total amount. Regarding the increase in total GS protein, LiCl did not affect total GS mRNA content. Furthermore, when actinomycin-D, a specific DNA transcription inhibitor, and cycloheximide, a specific RNA translation inhibitor, were added to LiCl in the incubation media, no modifications were observed on total protein or mRNA content of GS. In contrast, the results of the incubation with LiCl in the presence of ALLN,[1] a specific inhibitor of the calpain and proteasome pathway, suggests that the mechanism of action of LiCl on the total GS content is related to an increase in the protein stability.

Materials and methods

Preparation and incubation of hepatocyte homogenates

Hepatocyte suspensions were prepared from male Sprague–Dawley rats fasted for 24 h, as previously described [14]. Cells were resuspended in Krebs–Ringer bicarbonate buffer (pH 7.4). Samples (4 mL) of these suspensions, containing 4–5 × 10^6 cells/mL, were incubated at 37 °C with gassing and continuous shaking (100 strokes/min). LiCl and glucose were dissolved in water at a concentration of 1 M. Variable volumes of these solutions were added to the cell suspensions to obtain the desired final concentration. Actinomycin-D, a specific DNA transcription inhibitor [15], was used at a final concentration of 0.5 μg/mL, whereas cycloheximide, a specific RNA translation inhibitor [15], was used at a final concentration of 2 μg/mL. ALLN (*N*-acetyl-Leu-Leu-Norleu-al), a calpain and proteasome pathway inhibitor, was used at a final concentration of 5 μg/mL. At the end of the incubations, cells were centrifuged (3000*g* for 20 s) and pellets were immediately homogenized in 320 μL of ice-cold buffer containing 10 mM Tris/HCl (pH 7.0), 0.6 M sucrose, 150 mM KF, 15 mM ethylene diamine tetraacetic acid (EDTA), 25 μg/mL leupeptin, 0.5 mM phenylmethanesulphonyl fluoride, and 50 mM β-mercaptoethanol, using a Polytron homogenizer with a PT-7 rotor at setting 6 for 20 s. Homogenates were centrifuged at 9200*g* for 15 min at 4 °C. Pellets were then recovered and resuspended in 640 μL of the above mentioned buffer. Samples of supernatants and pellets were taken and used immediately for the analyses described.

Assay methods

The GS activity ratio in the presence and the absence of glucose 6-phosphate (G 6-P) and GS total activity were measured at 30 °C as described in [16]. One unit of enzyme activity was established as the amount of enzyme that incorporates 1 μmol of [^{14}C]glucose from UDP-[^{14}C]glucose into glycogen/min. Glycogen was measured as described in [7]. G 6-P was measured enzymatically as in [17]. Protein concentration was measured by the Biuret protein assay as described in [18], or by the Bradford method [19] using bovine serum albumin as standard.

Immunochemical techniques

Western blot analyses were performed on cell homogenates as described above. These homogenates were also centrifuged and pellets were resuspended as described above. Samples were then taken and immediately heated at 100 °C for 5 min in the presence of 25 mM-Tris/HCl (pH 6.8), 2.5% (v/v) β-mercaptoethanol, and 1% (w/v) sodium dodecyl sulfate (SDS). They were then electrophoresed in 10% polyacrylamide slab gels in the presence of SDS and transferred to membranes (Immobilon PVDF, Millipore, Billerica, USA) as described previously [7]. For GS detection, we used a polyclonal antibody, L1, previously characterised in our laboratory [3]. To detect total GSK3β, a mouse monoclonal IgG antibody raised against full-length GSK3β of Xenopus origin was used (Santa Cruz Biotechnology, Inc., CA). The phosphorylated form of GSK3β was detected using a polyclonal antibody that detects this kinase only when it is phosphorylated in Ser9. The antibody was produced against a synthetic phospho-Ser9 peptide (KLH coupled) derived from human GSK3β (Cell Signaling Technology, Inc., Danvers, USA). Enzymes were quantified by scanning the developed films.

Preparation of total RNA from hepatocytes and Northern blot analysis of GS mRNA

Samples (4 mL) of hepatocyte suspensions were incubated for a range of times in the presence (Li) or absence (control) of 30 mM LiCl. At the end of incubations, 1 mL of each hepatocyte suspension was centrifuged (10,000*g* for 15 s) and the pellet was rinsed with ice-cold phosphate-buffered saline (PBS, pH 7.4). The pellet was then carefully resuspended in 1 mL of RNAzol solution. After this extraction, total RNA was precipitated in isopropanol, rinsed with 70% ethanol and resuspended in 0.5% SDS and 1 mM EDTA solution in diethylpyrocarbonate-treated water. RNA concentration was calculated by measuring absorbance at 260 nm and at 280 nm to determine protein contamination. Only samples with A_{260}/A_{280} ratios higher than 1.9 were used.

For Northern blot analysis, total RNA samples (20 μg) were loaded in a vertical and denaturing 1.5 mm-wide gel containing formaldehyde. Samples were then electrophoresed for 3 h at 85 V, and were transferred to nitrocellulose filters. Filters were hybridized with an antisense ^{32}P-labelled riboprobe, prepared with a Riboprobe system (Promega, Madison, USA) from a 2-kb *Eco*RI fragment of rat liver glycogen synthase cDNA [20,21]. After hybridization at 65 °C in the presence of 50% formamide, membranes were washed to a final stringency of 15 mM NaCl, 0.15 mM sodium citrate, 0.1% SDS at 65 °C and subjected to autoradiography.

Statistical analysis

Data are expressed as means ± SEM. Differences between the experimental groups were evaluated using the unpaired Student's *t*-test for several independent observations. A $P < 0.05$ was considered significant.

Suppliers

All of the reagents were of analytical grade and came from Merck (Darmstadt, Germany) and Sigma (Saint Louis, MO, USA).

Results

Effects of LiCl on GS activity

Hepatocytes from 24 h-fasted rats were incubated for 1 h with or without 30 mM LiCl. Incubation with LiCl induced an increase in the total amount of immunoreactive GS protein in homogenates from the hepatocytes (Fig. 1A). This increase was also observed in the supernatants and the pellets from these homogenates. Moreover, these results were corroborated when GS total activity (+G 6-P) was measured in hepatocytes incubated with or without LiCl. After 1 h of incubation with 30 mM LiCl, GS total activity increased by 17% in supernatant and 38% in pellet homogenates (Table 1).

On the other hand, LiCl treatment induced an increase in the GS activity ratio both in the supernatants and pellets in all the conditions tested (Fig. 1B). Furthermore, the effect

[1] *Abbreviations used:* G 6-P, glucose 6-phosphate; ALLN, *N*-acetyl-Leu-Leu-Norleu-al.