Lithium chloride inhibits the expression and secretion of insulin-like growth factor-binding protein-1

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Abstract

Insulin-like growth factor-binding protein-1 (IGFBP-1) regulates IGF availability for glucose homeostasis. The IGFBP-1 promoter shares common regulatory response elements with phosphoenolpyruvate carboxykinase (PEPCK), the expression and activity of which is inhibited by lithium chloride, associated with an inhibition of glycogen synthase kinase (GSK)-3 activity, in the rat hepatoma cell line H4-II-E. We therefore determined the effect of lithium chloride on IGFBP-1 expression and secretion in H4-II-E cells. Lithium chloride inhibited IGFBP-1 secretion in a dose response and reversible manner by approx 80% during 5-h and 16-h incubations.

An inhibitory effect on IGFBP-1 mRNA expression was observed at 2 h. The inhibitory effect of lithium and insulin were not additive when used alone, but inhibition by lithium occurred when insulin action was blocked by activating AMP-activated protein kinase with 5-aminoimidazole-4-carboxamide-riboside (AICAR). These findings suggest that GSK-3 inhibition, or another pathway activated by lithium, may be involved in a pathway controlling IGFBP-1, inhibiting synthesis when insulin activity is absent or impaired.

Journal of Endocrinology (2001) **171**, R11–R15

Introduction

The insulin-like growth factor-binding proteins (IGFBPs) are a family of six peptides which have high affinity IGF binding but also IGF-independent activities (Baxter, 2000). IGFBP-1 is characterised by dynamic variability of levels in the circulation due to regulation of its hepatic secretion (Lee *et al.*, 1997). Insulin is a dominant inhibitor of hepatic IGFBP-1 transcription under normal physiological conditions. This inverse relationship allows endocrine IGFBP-1 to act as a glucose counterregulator, blocking availability of IGFs for glucose homeostasis. During conditions such as catabolic stress (Cotterill *et al.*, 1996) and intensive exercise (Hopkins *et al.*, 1994) the inverse correlation between insulin and IGFBP-1 may be lost. Hepatic insulin resistance and circulating stimulators of IGFBP-1 may have direct roles, and these factors may interact. Insulin is unable to attenuate interleukin 6-stimulated IGFBP-1 secretion in HepG2 human hepatoma cells (Samstien *et al.*, 1996). The AMP-activated protein kinase (AMPK) pathway is activated by environmental stresses such as hypoxia and intensive exercise. We have recently shown that an AMPK activator may also block the inhibitory effect of insulin on IGFBP-1 (Lewitt, 2001). Insulin inhibits IGFBP-1 via phosphatidylinositol

3-kinase (PI3K) and protein kinase B (PKB/Akt) (Cichy *et al.*, 1998) by inhibiting activation by the Forkhead transcription factor, FKHR (Kops *et al.*, 1999). An inhibitory effect of AMPK activation on insulin-induced glucose uptake is mediated downstream of PI3K in 3T3-L1 adipocytes (Salt *et al.*, 2000).

IGFBP-1 and PEPCK promoters share common regulatory response elements, however their patterns of regulation may differ. In H4-II-E cells, for example, AMPK activation stimulates IGFBP-1 (Lewitt, 2001), but inhibits PEPCK (Lochhead *et al.*, 2000). It has been reported that lithium chloride inhibits PEPCK gene expression (Bosch *et al.*, 1992). Lithium is a potent allosteric inhibitor of glycogen synthase kinase-3 (GSK-3) (Davies *et al.*, 2000). It is also reported to activate PKB/Akt (Chalecka-Franaszek & Chuang, 1999) and, in high concentrations, to inhibit casein kinase 2 (Davies *et al.*, 2000). In H4-II-E cells the action of lithium on PEPCK expression mimics that of selective inhibitors of GSK-3 and is independent of PKB/Akt and FKHR (Lochhead *et al.*, 2001).

The aim of these studies was to determine the effect of lithium in H4-II-E cells on IGFBP-1 secretion and expression in the presence and absence of AMPK-induced insulin resistance.

Figure 1 Lithium inhibits secretion of IGFBP-1 by H4-II-E cells. IGFBP-1 secretion was measured by immunoassay as described in the *Methods*. The effect of 10 mM LiCl was not additive to a maximally inhibitory concentration of insulin (I, 100 ng/ml). Results are the mean $\pm$ SE pooled from 3 experiments, each having 3 replicates per condition. *, $P < 0.001$, compared to control (C).

Materials and Methods

Lithium chloride (LiCl) and potassium chloride (KCl) were purchased from Merck (Darmstadt, Germany) and 5-aminoimidazole-4-carboxamide-riboside (AICAR), from Sigma (Sweden). Recombinant human insulin (Actrapid) was obtained from Novo Nordisk (Denmark). H4-II-E cell maintenance and IGFBP-1 immunoassay have been described previously (Lewitt, 2001). In brief, rat IGFBP-1 was measured using an antibody capture assay. Rat IGFBP-1 was used as the fixed antigen competing for rabbit polyclonal anti-rat IGFBP-1 antibody with pure rat IGFBP-1 standard or sample. The effective assay range was 0.05 to 10 ng. At 0.9 ng the between and within-assay imprecisions were 15% and 4.8% respectively. Experiments were in 96-well plates (Costar, Corning, NY) using confluent cells after 24-h serum-free. Incubations were in 200 μ l and, after a 1.5-h preincubation, medium plus effectors were replaced for a 5-h or 16-h collection. In experiments to determine recovery, 16-h conditioned medium was removed before two 5-h collection periods. Samples from one experiment were run in the same assay using 10–50 μ l medium. Results are the mean $\pm$ SE. Unless otherwise indicated, statistical significance, taken as $P < 0.05$, was determined by analysis of variance (SigmaStat, SPSS Science, Chicago, IL). Cell viability was measured by colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Mosmann, 1983). Native PAGE was according to a published method (Jones *et al.*, 1991). Concentrated samples containing 10 ng IGFBP-1 from control and

10 mM LiCl-treated cells were run on 10% gels, transferred to nitrocellulose, incubated with anti rat IGFBP-1, B4 1:1000 (Lewitt *et al.*, 1994), and detected with radioiodinated protein A (Sigma) and autoradiography. For studies of mRNA expression, cells were incubated in 12-well plates (Costar) with the effectors for 2 h and total RNA extracted using guanidine isothiocyanate-phenol-chloroform (Trizol, Life Technologies, Sweden). Northern blots were hybridized with a rIGFBP-1 cDNA probe (GenBank M89791, kindly provided by Guck T. Ooi), labelled with ^{32} P-dCTP by random prime labelling (Ready-To-Go, Amersham Pharmacia Biotech, Sweden).

Results

IGFBP-1 secretion by H4-II-E cells was inhibited in a dose response fashion by LiCl (Figure 1). KCl, used as another control, did not inhibit IGFBP-1. Under these culture conditions, lithium inhibited IGFBP-1 by 80%, to a similar extent as a maximally effective concentration of insulin, however the effects of insulin and lithium were not additive. LiCl not only inhibited levels of IGFBP-1 protein secretion, but also effectively inhibited IGFBP-1 mRNA expression (Figure 2).

In order to exclude a non-specific, toxic effect of lithium we assessed the reversibility of the inhibitory effect of lithium on IGFBP-1 after a more prolonged incubation period. As shown in a representative experiment in Figure 3, after a 16-h incubation, lithium remained an effective inhibitor of IGFBP-1, inhibiting secretion by more than

Figure 2 Inhibition of IGFBP-1 mRNA expression by LiCl in H4-II-E cells. Cells were exposed to effectors for 2 h. As controls, cells incubated without (C, $n=1$) and with KCl 10 mM (KCl, $n=2$). The effect of increasing concentrations of LiCl ($n=2$ for each) is compared with insulin 100 ng/ml (I, $n=2$).

Figure 3 Recovery of IGFBP-1 secretion after long-term inhibition by LiCl in H4-II-E cells. Cells were exposed to LiCl for a 16-h incubation. The conditioned medium was collected and cells incubated in the absence of lithium for two 5 h recovery periods. Results are the mean $\pm$ SE of 4-8 replicates per condition. *, $P<0.001$, †, $P<0.05$ (compared to no lithium within a given incubation period). Lithium had no effect on the pattern of secreted isoforms of IGFBP-1 (inset). Samples from control (C) and 10 mM LiCl (Li) treated cells, containing 10 ng IGFBP-1 were subjected to immunoblotting after non-denaturing PAGE.

80%. This contrasts to the inhibitory effect insulin, which diminishes with time (Lewitt *et al.*, 1996). Within hours of removal of lithium, lithium's inhibitory effect on IGFBP-1 was completely reversed. In a parallel experiment, cell viability was assessed after a 16-h exposure to 10 mM lithium. After a 4-h incubation with MTT, the quantity of formazan product in solubilised lithium-treated cells was $6.0 \pm 2.7\%$ greater than in control wells ($n=6$, $P<0.05$, *t*-test).

Lithium has an effect, albeit less potently, on protein kinases other than GSK3 (Davies *et al.*, 2000), including

casein kinase 2, which can phosphorylate IGFBP-1 (Ankrapp *et al.*, 1996). In Figure 3(inset) we show that the pattern of isoforms of IGFBP-1 seen on immunoblotting after native PAGE are not changed by 10 mM lithium chloride. There was also no change in intensity of the bands detected in these 10 ng samples.

AICAR, which activates AMPK, has been shown to block the inhibitory effect of insulin of IGFBP-1 secretion in these cells (Lewitt, 2001). We determined the combined effect of lithium and insulin in the presence of AMPK activation. In the presence of AMPK activation, an inhibitory effect of lithium was observed (Figure 4). This contrasts to the finding that, in the presence of insulin without AMPK activation, lithium is unable to further inhibit IGFBP-1 secretion. The tendency to a stimulation by insulin in the presence of AMPK activation and lithium inhibition was not statistically significant.

Discussion

In this paper we present the novel observation that lithium chloride acutely and reversibly inhibits IGFBP-1 expression and secretion, without affecting its phosphorylation status. Lithium chloride is an inhibitor of GSK-3 (Davies *et al.*, 2000), a protein kinase with a wide spectrum of action on gene transcription, protein translation and microtubule stability (Coghlan *et al.*, 2000). The observation, in the same cell line that we have used, that GSK-3 is involved in the regulation of PEPCK and glucose-6-phosphatase, leads to us to conclude that it is a likely candidate to be involved in a pathway leading to IGFBP-1 inhibition. The activator of such a pathway leading to changes in IGFBP-1 synthesis *in vivo* is speculative. Amino acids might play this role, since they have been shown to inhibit GSK-3 in muscle cells (Peyrollier *et al.*, 2000; Armstrong *et al.*, 2001) and are known to inhibit IGFBP-1 *in vivo* and *in vitro* (Pao *et al.*, 1993; Straus *et al.*, 1993).

IGFBP-1 expression often changes in parallels with other genes involved in glucose homeostasis. However GSK-3-dependent changes could regulate IGFBP-1 for roles other than glucoregulation. IGFBP-1 prevents apoptosis by an IGF-dependent mechanism (Butt *et al.*, 1999), and by IGF-independent pathways, such as interaction with integrin receptors to induce FAK dephosphorylation (Perks *et al.*, 1999). GSK-3 β participates in cell survival pathways. Disruption of the GSK-3 β results in hepatocyte apoptosis and embryonic lethality (Hoeftlich *et al.*, 2000). However acute inhibition of GSK3 promotes survival of primary neurones (Cross *et al.*, 2001). Any role of IGFBP-1 in these responses is purely speculative at this time.

The observation that the combination of maximally effective concentrations of insulin and lithium were not

Figure 4 Lithium inhibits IGFBP-1 in the presence AICAR, which blocks the inhibitory effect of insulin. IGFBP-1 concentrations were measured by immunoassay as described in the *Methods*. Results are the mean $\pm$ SE pooled from 3 experiments, each having 3 replicates per condition. *, $P < 0.01$ (compared to no additions); †, $P < 0.01$ (compared to no AICAR); ‡, $P < 0.001$.

additive in suppressing IGFBP-1 suggests an alternative mechanism of lithium action, involving a common pathway with insulin. It is possible that, like insulin (Cichy *et al.*, 1998), lithium inhibits IGFBP-1 via PKB/Akt and that the inhibitory effect of insulin on IGFBP-1 secretion is upstream of PKB/Akt. In support of this hypothesis, lithium has been shown to be an activator of PKB/Akt, although in another cell type, neurons (Chalecka-Franaszek & Chuang, 1999). Another possible explanation for this phenomenon is that insulin activity itself may inhibit a lithium-activated pathway leading to IGFBP-1 inhibition.

In conclusion, we have identified a novel inhibitor IGFBP-1 production. Furthermore we report that lithium is still able to inhibit IGFBP-1 secretion when the inhibitory effect of insulin on IGFBP-1 is blocked with the AMPK-activator, AICAR. This leads us to speculate on the potential role for activating this pathway in insulin resistant states. Unravelling these pathways of action on IGFBP-1 is the focus of our current investigations.

Acknowledgements

The authors thank Inga-Lena Wivall-Helleryd for technical assistance and Professors Kerstin Hall and Suad Efendic for their helpful discussions. This research was supported by the Swedish Medical Research Council (04224), the Novo Nordisk Foundation, and the Swedish Diabetes Association.

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