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Signalling pathways and combinatory effects of insulin and amino acids in isolated rat hepatocytes

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Liver metabolism is influenced by hormones and nutrients. Amino acids such as glutamine or leucine induce an anabolic response, which resembles that of insulin in muscle and adipose tissue. In this work, the signalling pathways and the effects of insulin were compared to those of glutamine and leucine in isolated hepatocytes from normal and streptozotocin-diabetic rats. Glutamine increased cell volume and induced an anabolic response characterized by an activation of acetyl-CoA carboxylase (ACC), glycogen synthase (GS) and p70 ribosomal S6 kinase (p70S6K), the key enzymes in fatty acid, glycogen and protein synthesis, respectively. The effects of glutamine were independent of insulin and did not share its signalling components. Leucine, which is poorly metabolized by the liver and does not modify cell volume, activated ACC and p70S6K, and exerted a synergistic effect on the glutamine-induced activation of ACC and p70S6K. These amino acids did not affect insulin signalling. Insulin alone had no anabolic effect in hepatocytes, despite the activation of protein kinase B. Nevertheless, it enhanced the activation of ACC and p70S6K induced by leucine. However, insulin injected intravenously activated rat liver p70S6K. In hepatocytes from streptozotocin-diabetic animals, the metabolic responses to the amino acids and insulin were similar to those in normal hepatocytes. We conclude that glutamine, insulin and leucine exert different effects that are mediated by different signalling pathways, although their effects are combinatory. The anabolic effect of insulin in hepatocytes was strictly dependent on the permissive action of leucine.

Keywords: insulin; amino acids; p70S6K; acetyl-CoA carboxylase (ACC); diabetic rat.

In recent years, experimental evidence demonstrating the involvement of certain amino acids in the control of metabolism and signal transduction has accumulated (reviewed in [1]). A cross-talk between nutrients and cell signalling was first reported in studies of the regulation of hepatic autophagy, a process responsible for starvation-induced proteolysis [2]. Autophagy is inhibited by amino acids even in the absence of insulin. The mechanism involves phosphorylation of ribosomal protein S6 [3,4], which is implicated in the control of translation of certain mRNAs (reviewed in [5,6]). An amino-acid-dependent activation of the ribosomal protein S6 kinase (p70S6K), the Ser/Thr protein kinase responsible for S6-phosphorylation, was first reported in hepatocytes [7], and confirmed in several tissues and cultured cells where branched-chain amino acids like leucine play a predominant role in p70S6K activation [8–14]. Moreover, the branched-chain amino acids amplify the effect of insulin in activating p70S6K (reviewed in [15]).

Apart from their inhibition of proteolysis through p70S6K activation, amino acids also control fatty acid and glycogen metabolism. In rat hepatocytes, Na^+-cotransported amino acids like glutamine, alanine or proline stimulate lipogenesis by activation of acetyl-CoA carboxylase (ACC) and stimulate glycogen synthesis by activating glycogen synthase (GS) [16,17]. We consider the activation of ACC, GS and p70S6K as an anabolic response of the liver to these amino acids. When triggered by Na^+-cotransported amino acids, the anabolic response is initiated by the intracellular accumulation of glutamate and the subsequent increase in cell volume. In hepatocytes, the increase in glutamate stimulates a type 2A protein phosphatase (glutamate-activated protein phosphatase, GAPP), which activates ACC [18]. In addition, the anabolic response of hepatocytes can be mimicked by incubation of the cells in hypotonic media without amino acids. Therefore, swelling of liver cells can be regarded as an anabolic signal leading to the activation of GS, ACC and p70S6K.

The amino acid-induced anabolic response in liver is reminiscent of the action of insulin in muscle and adipose tissue, where the hormone also activates GS, ACC and p70S6K. This similarity is further supported by the inhibition of the effects of amino acids by wortmannin and LY294002, two unrelated inhibitors of phosphatidylinositol 3-kinase (PtdIns3K). This enzyme is known to mediate

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Abbreviations: ACC, acetyl-CoA carboxylase; GAPP, glutamate-activated protein phosphatase; GS, glycogen synthase; IR, insulin receptor; IRS-1, insulin receptor substrate-1; mTOR, mammalian target of rapamycin; p70S6K, p70 ribosomal protein S6 kinase; PDK1, 3-phosphoinositide-dependent protein kinase; PtdIns3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; PtdIns(3,5)P_2, phosphaditylinositol-3,5-bisphosphate

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most, if not all, short-term metabolic effects of insulin. A slight PtdIns3K activation by amino acids has indeed been observed in hepatocytes [7] and skeletal muscle [19], but not in other systems [8].

The binding of insulin to its receptor leads to the phosphorylation of the insulin receptor (IR) and the insulin receptor substrate-1 (IRS-1) and hence to the activation of PtdIns3K (reviewed in [20,21]). This leads to the production of phosphatidylinositol-(3,4,5)-P_3, a key second messenger, which recruits 3-phosphoinositide-dependent protein kinase (PDK1) to the plasma membrane. PDK1 phosphorylates and activates protein kinase B (PKB), also known as Akt. In skeletal muscle, PKB mediates the insulin-induced inactivation of GS kinase-3 by phosphorylation, thereby allowing GS to become dephosphorylated and activated. PtdIns3K activation is also known to result in p70S6K activation, a mechanism involving the mammalian target of rapamycin (mTOR) [22], which is inhibited by the immunosuppressant drug rapamycin. p70S6K can also be directly phosphorylated by PDK1 in a rapamycin-independent manner [23]. The mechanism of ACC activation by insulin, which is PtdIns3K-dependent, is still poorly understood [24,25].

The relationship between insulin and amino acid signalling on p70S6K activation has been studied in hepatoma cells [8] but not in normal liver cells. In this paper we compared the anabolic effects and signalling pathways of insulin and amino acids in isolated hepatocytes, a model that is close to the situation *in vivo*. The anabolic response was studied by measuring the changes in the activity of ACC, GS and p70S6K. We have chosen two distinct amino acids, namely glutamine and leucine, because they are transported into the hepatocytes by different systems and because their effects on cell volume differ, glutamine, but not leucine, inducing cell swelling. We found that the effects of cell swelling were not mediated by the insulin-signalling pathway and that the branched-chain amino acid leucine exerted a permissive effect on insulin action. Insulin alone did not induce an anabolic response in hepatocytes, although it did activate the first steps of its signalling pathway.

MATERIALS AND METHODS

Materials

The peptides corresponding to the protein kinase A inhibitor (PKI; TTYADFIASGRTGRRNAIHD), the PKB substrate (RPRAATF), and the p70S6K substrate (KKRNRTLSVA) were kindly provided by V. Stroobandt (Ludwig Institute, Brussels, Belgium). Antibodies against PKB, IRS-1, focal adhesion kinase ($p125^{FAK}$) and the p85 regulatory subunit of PtdIns3K, and the anti-phosphotyrosine Ig (clone 4G10) were from Upstate Biotechnology; the anti-p70S6K Ig was from Santa Cruz; and the anti-IR Ig was from Calbiochem.

Preparation and incubation of hepatocytes and perfusion of liver

Hepatocytes from overnight-fasted male Wistar rats (170–200 g of body weight) were prepared [26,27] and incubated at 37 °C for the indicated periods of time at a concentration of about 50 mg of cells per mL of Krebs–Henseleit bicarbonate medium in equilibrium with a 95% O_2/5% CO_2 gas phase. Incubations were carried in the presence of 20 mM glucose, and, when indicated, 100 nM insulin (Novo-Nordisk) and/or 10 mM amino acids, which are saturating concentrations; in the controls, an equivalent volume of 0.9% NaCl was added. At the end of the incubations, the cells were collected by centrifugation (2 s, microfuge) and the cell pellets were immediately frozen in liquid nitrogen. The cell pellets were homogenized in 0.5 mL of ice-cold lysis buffer (50 mM Hepes pH 7.5; 50 mM 2-glycerol phosphate pH 7.5; 150 mM NaCl; 2 mM EGTA; 2 mM EDTA; 0.2% (w/v) Triton X-100; 1% (v/v) Nonidet P-40, 1 mM sodium orthovanadate; 50 mM potassium fluoride; 5 mM sodium pyrophosphate; 1 mM dithiothreitol; 1 mM benzamidine; 0.5 $\mu g \cdot mL^{-1}$ leupeptin; 0.7 $\mu g \cdot mL^{-1}$ pepstatin; 0.5 $\mu g \cdot mL^{-1}$ aprotinin; 0.2 mM phenylmethanesulfonyl fluoride, 1 μM microcystin-LR). After centrifugation (20 000 ***g***, 15 min), the supernatants were stored at −80 °C.

Livers from overnight-fasted rats were perfused *in situ* at 37 °C as described previously [28] with Krebs–Henseleit bicarbonate medium containing 20 mM glucose and in equilibrium with a 95% O_2/5% CO_2 gas phase. The livers were perfused for 20 min to wash out blood and other foreign material and then further perfused with or without 100 nM insulin for 5 or 10 min and freeze-clamped in liquid nitrogen. The frozen tissues were homogenized (Ultra-Turrax) with 5 vol. (v/w) of the ice-cold lysis buffer and the supernatants (20 000 ***g***, 20 min) were stored at −80 °C.

Experiments on intact rats

Overnight-fasted rats were anaesthetized (Nembutal, 60 $mg \cdot kg^{-1}$, intraperitoneal) and injected intravenously with insulin (30 nmol per kg body weight, together with 1 mmol of glucose per kg body weight). After 5 min, the livers were freeze-clamped in liquid nitrogen and homogenized as described above. To induce diabetes, anaesthetized rats were injected intravenously with streptozotocin (Sigma, 52 mg per kg body weight). After 5–10 days, blood glucose concentrations were increased from 8.2 ± 1.3 to 32.3 ± 2.2 mM and 4.3 ± 0.7 to 12.8 ± 2.4 mM ($n = 4$) in fed and fasted rats, respectively. Hepatocytes from fasted diabetic rats were prepared and incubated as described above.

Immunoprecipitations

Each extract was incubated with antibody, prebound to 20 μL protein A– or G–Sepharose, in a total volume of 0.4–0.5 mL of lysis buffer for 2 h at 4 °C on a rotating wheel. PtdIns3K complexed to IRS-1 was immunoprecipitated from 150 to 200 μL aliquots of cell extracts with 3 μg of a rabbit polyclonal anti-(IRS-1) Ig. The complex was washed three times with phosphate-buffered saline containing 1 mM sodium orthovanadate and 1% (v/v) Nonidet P-40 in the first washing. PKB was immunoprecipitated from 100 μL of extract with 3 μg of mouse monoclonal anti-PKBα Ig [29], p70S6K from 70 μL of extracts with 1 μg of rabbit polyclonal anti p70S6K Ig [7], and $p125^{FAK}$ from 250 μL of extracts with 4 μg mouse monoclonal antibody [30].

Measurements of enzyme activities and proteins

ACC, GS, PtdIns3K, p70S6K [7] and PKB activities [29] were measured as described previously. $p125^{FAK}$ activation was measured after immunoprecipitation with anti-$p125^{FAK}$ Ig by two methods. In the first method, the tyrosine kinase activity was assayed using the copolymer poly GluTyr (Sigma, 4 : 1, mol/mol) as a substrate. The assay was performed in a final volume of 40 μL in the presence of 20 mM Tris/HCl pH 7.4, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 10 μg poly GluTyr, 4 μM Mg-[γ-^{32}P]ATP (Amersham Biosciences, 127 000 c.p.m.·pmol^{-1}). To stop the reaction, 30 μL of the assay mixture were spotted on 3ET31 paper (Whatman) and further processed as described [31]. In the second method, tyrosine phosphorylation of $p125^{FAK}$ was measured by immunoblotting with an anti-phosphotyrosine Ig (PY20, New England Biolabs). One unit of enzyme activity corresponds to 1 nmol (lipid and protein kinases) or 1 μmol (other enzymes) of product formed per min. Protein concentrations were measured according to Bradford [32] with bovine serum albumin as a standard.

Gel mobility shift assay of p70S6K

Hepatocyte extracts (10 μL) were boiled with Laemmli buffer [33] and then applied on a 7.5% SDS low-bis polyacrylamide gel (18 × 18 cm) containing 0.015% bisacrylamide. Electrophoresis was run for about 5 h at 4 °C starting with 220 V for 30 min and then running at 320–360 V. The proteins were blotted onto poly(vinylidene difluoride) transfer membrane (NEN) overnight at 4 °C and 25 V (150 mA). p70S6K was immunodetected with anti-p70S6K Ig.

RESULTS

Glutamine, but not insulin, activates ACC and GS in hepatocytes from control- and streptozotocin-diabetic rats

Incubation of freshly isolated hepatocytes with 10 mM glutamine induced a time-dependent activation of ACC and GS, in agreement with previous reports [7,16,17]. A maximal twofold to fourfold activation was observed after 20–30 min for GS and after 45–60 min for ACC (Fig. 1 A,C). Under the same conditions, 100 nM insulin did not elicit such an effect, nor did it modify the effect of 10 mM glutamine. This lack of effect of insulin in hepatocytes in suspension contrasts with the well-known activation of both enzymes in skeletal muscle, adipose tissue and hepatocytes in culture [24,34,35]. We could indeed confirm a significant activation of GS in skeletal muscle of rats injected with insulin for 5 min (from 234 ± 43 to 477 ± 31 mU·g^{-1} of tissue, means ± SEM, $n = 4$, $P < 0.01$).

The fact that the effects of glutamine were independent of insulin was reinforced by the results of experiments

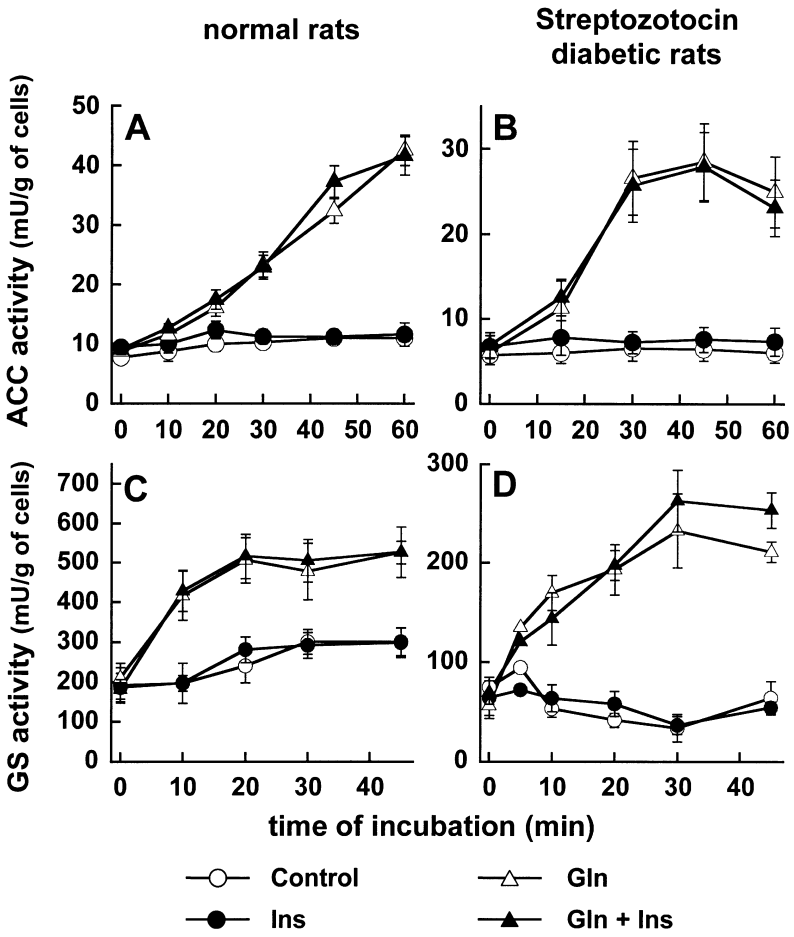

Fig. 1. Time-course of activation of acetyl-CoA carboxylase (ACC) and glycogen synthase (GS) in hepatocytes incubated with glutamine and insulin. Hepatocytes were isolated from normal (A,C) and streptozotocin-diabetic (B,D) rats. After a 15 min preincubation, they were further incubated for the indicated periods of time under control conditions (○), in the presence of insulin (●), glutamine (△), or both (▲). The values are the means ± SEM for five cell preparations.

performed in hepatocytes from streptozotocin-diabetic rats (Fig. 1B,D). The results obtained in these hepatocytes were qualitatively the same as those observed in nondiabetic animals. Although the relative activation (threefold to fourfold) was the same, the maximal activity of GS and ACC reached after amino acid treatment was lower in diabetic than in normal rats. Moreover, as for the nondiabetic controls, insulin alone had no effect and did not affect the stimulation of ACC and GS by glutamine.

Glutamine and leucine activate ACC and interfere differently with insulin

Like glutamine, leucine activated ACC but with a different time-course (compare Figs 1A and Fig. 2A). Interestingly, the activation by leucine, in contrast with that of glutamine, was enhanced in the presence of insulin (Fig. 2A). Maximal activation of ACC was achieved when hepatocytes were incubated with both glutamine and leucine (Fig. 2B).

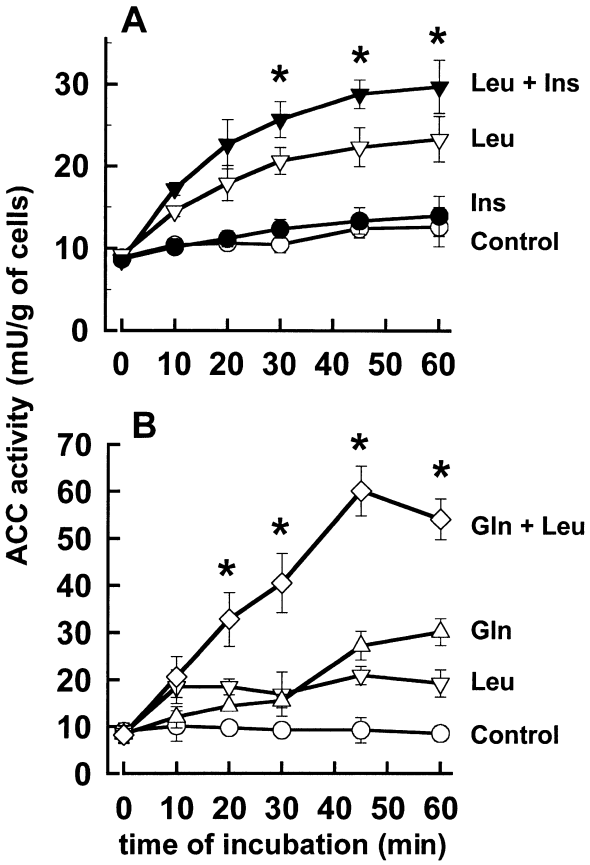

Fig. 2. Time-course of activation of ACC in hepatocytes incubated with insulin and/or amino acids. After a 15 min preincubation, hepatocytes were incubated for the indicated periods of time. (A) Interaction between leucine and insulin; (B) synergistic effect of leucine and glutamine. Hepatocytes were incubated under control conditions (○), in the presence of insulin (●), leucine (▽), glutamine (△), insulin plus leucine (▼) or glutamine plus leucine (◇). The values are the means ± SEM for four cell preparations. *, Values statistically different ($P < 0.05$) from the corresponding values obtained in hepatocytes incubated with leucine (A) or with glutamine (B).

Incubation of hepatocytes with glutamine leads to an increase in cell volume and an accumulation of glutamate [7,36]. The volume (Fig. 3A) and the intracellular concentration of glutamate (Fig. 3B) of hepatocytes incubated with both leucine and glutamine were greater than in the presence of glutamine alone. Incubation with leucine or insulin alone did not increase cell volume (leucine, 96 ± 1%; insulin, 98 ± 1%, means ± SEM, $n = 4$). It had also no effect on glutamate concentration (leucine, Fig. 3B; insulin, 2.0 ± 0.2 μmol·g cells^{-1}, means ± SEM, $n = 4$). Similarly, combination of leucine and insulin had no effect on cell volume (98 ± 2%, mean ± SEM, $n = 4$) and on glutamate concentration (2.3 ± 0.1 μmol·g cells^{-1}, means ± SEM, $n = 4$).

Leucine does not activate GS

In contrast with its effect on ACC, leucine did not activate GS (controls, 128 ± 5; leucine, 132 ± 4 mU·g cells^{-1}, means ± SEM, $n = 4$; 45 min incubation). In addition, leucine slightly inhibited GS activation by glutamine (glutamine, 246 ± 6; leucine plus glutamine, 188 ± 11 mU·g cells^{-1}, $P < 0.05$, means ± SEM, $n = 4$, 45 min incubation). Finally, combination of insulin and leucine did not result in GS activation (insulin, 142 ± 4; leucine plus insulin, 139 ± 6 mU·g cells^{-1}, means ± SEM, $n = 4$).

Insulin, but not amino acids, induces IR and IRS-1 phosphorylation as well as PtdIns3K and PKB activation

Because GS and ACC were not activated in insulin-treated hepatocytes, it was important to test the integrity of the insulin-signalling pathway. Insulin stimulation for 2 min increased the phosphorylation state of IR and IRS-1 (Fig. 4A) and caused a maximal 25-fold increase in IRS-1-associated PtdIns3K activity before progressively returning towards basal values (Fig. 4B). Insulin also stimulated the association between IRS-1 and the p85 subunit of PtdIns3K (Fig. 4C). Finally, the time-course of PKB activation by insulin (Fig. 5) followed that observed for PtdIns3K. A maximal fivefold to sevenfold activation within 2 min of insulin stimulation was followed by a progressive decrease towards basal values. No significant change in these parameters, namely, phosphorylation state of IR and IRS-1, association between IRS-1 and PtdIns3K, and activity of PtdIns3K and PKB could be detected in hepatocytes incubated with glutamine or leucine (Figs 4 and 5). As for the other elements of the insulin signalling pathway, there was no interaction between glutamine or leucine and insulin on PKB activity. Our findings in hepatocytes differ slightly from those observed in L6 muscle cells [37] or Fao hepatoma cells [8], where amino acids were reported to negatively interfere with the insulin signalling cascade. In any case, the synergistic effect of leucine and insulin on ACC activation (Fig. 2A) cannot be explained by an enhancement of insulin signalling.

Glutamine and leucine, but not insulin, activate p70S6K in hepatocytes

In several tissues or isolated cells, p70S6K is activated by both insulin and amino acids [15]. We compared the effects of glutamine, leucine and insulin on p70S6K

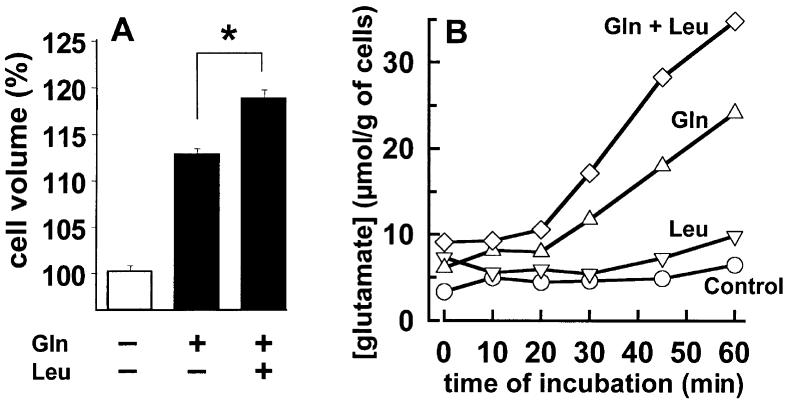

Fig. 3. Changes in cell volume (A) and glutamate concentration (B) induced by amino acids. Cells were incubated under control conditions or in the presence of amino acids for 45 min (A) or for the indicated period of time (B). Changes in cell volume (A) were determinated by weighing the centrifuged (10 000 ***g***, 3 s) hepatocytes at the end of the incubation period [16]. Control conditions (○), glutamine (△), leucine (▽), leucine plus glutamine (◇). Values are the means [in (A) ± SEM, $n = 3$] for two to three cell preparations. *, Values statistically different ($P < 0.05$) from the corresponding values obtained in hepatocytes incubated with glutamine.

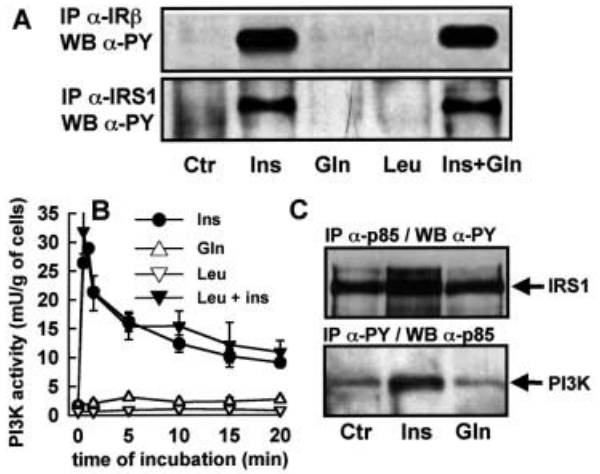

Fig. 4. Effect of amino acids on the insulin signalling cascade in hepatocytes. (A) Phosphorylation state of IR (upper blot) and IRS-1 (lower blot). Hepatocytes were incubated in control conditions (Ctr), for 2 min in the presence of insulin (Ins), 45 min with glutamine (Gln), 30 min with leucine (Leu), or 2 min with insulin following 45 min stimulation with glutamine (Ins + Gln). The β subunit of the IR or IRS-1 were immunoprecipitated (IP) with anti-IR (α-IRβ) or anti-IRS1 (α-IRS1) Ig, respectively, and the phosphorylation state of both was immunodetected (WB α-PY) with an anti-phosphotyrosine Ig (PY20). (B) IRS-1-associated PtdIns3K activity in hepatocytes after stimulation with insulin (●), glutamine (△), leucine (▽) or insulin plus leucine (▼). PtdIns3K was coimmunoprecipitated using an anti-(IRS-1) Ig, and its activity was measured. The values are the means ± SEM for three to four preparations. (C) Association of the p85 regulatory subunit of PtdIns3K with tyrosine-phosphorylated proteins. Hepatocytes were incubated with insulin (2 min) or glutamine (20 min). Upper blot: the p85 subunit of PtdIns3K was immunoprecipitated (IP α-p85) and associated IRS-1 was immunodetected (WB α-PY) with an anti-phosphotyrosine Ig (PY20). Lower blot: tyrosine-phosphorylated proteins were immunoprecipitated with the 4G10 antibody (IP α-PY) and association with the regulatory subunit of PtdIns3K was immunodetected (WB α-p85) using an anti-p85 Ig. PI3K, PtdIns3K.

activation in hepatocytes. Both glutamine (Fig. 6A) and leucine (Fig. 6B) activated p70S6K, however, the effect of leucine was slightly more rapid and transient but less pronounced than with glutamine. The glutamine-induced activation of p70S6K correlated with ACC activation (compare Fig. 6A and Fig. 1A), suggesting an upstream point of common control in their signalling pathways. As observed with ACC (Fig. 2B), maximal activation of p70S6K in hepatocytes was obtained with a combination of both amino acids (Fig. 6C), resulting in an activity as high as 1 $U \cdot g^{-1}$ of tissue. Insulin alone was without effect but it did enhance the leucine-induced activation of p70S6K (Fig. 6B).

To exclude the possibility that the lack of insulin effect could be an artefact resulting from the preparation of isolated hepatocytes by the collagenase method, the effect of insulin on p70S6K and PKB was tested in perfused rat livers. In this model, like in hepatocytes in suspension, insulin did not activate p70S6K (control, 9.0 ± 1.5; and insulin, 12.3 ± 1.3 $mU \cdot g$ $tissue^{-1}$, means ± SEM, $n = 5$, $P = 0.14$) but did activate PKB (control, 62 ± 14; and insulin, 271 ± 23 $mU \cdot g$ $tissue^{-1}$, means ± SEM, $n = 5$, $P < 0.01$). In contrast, injection (5 min) of insulin to intact rats was able to activate liver p70S6K fourfold (control, 24 ± 6; and insulin, 111 ± 23 $mU \cdot g$ $liver^{-1}$, means ± SEM, $n = 4$, $P < 0.01$).

In hepatocytes from diabetic rats, the activating effect of leucine was also observed, although the total p70S6K activity was significantly lower than in hepatocytes from control rats, and insulin was able to increase the stimulation of p70S6K by leucine (Fig. 6D).

Rapamycin inhibits p70S6K activation but has little effect on ACC activation

Rapamycin, a well-known inhibitor of p70S6K activation, has been shown to inhibit the effects of leucine in several cell types. In isolated hepatocytes, 300 nM rapamycin was found to completely inhibit p70S6K activation by leucine or

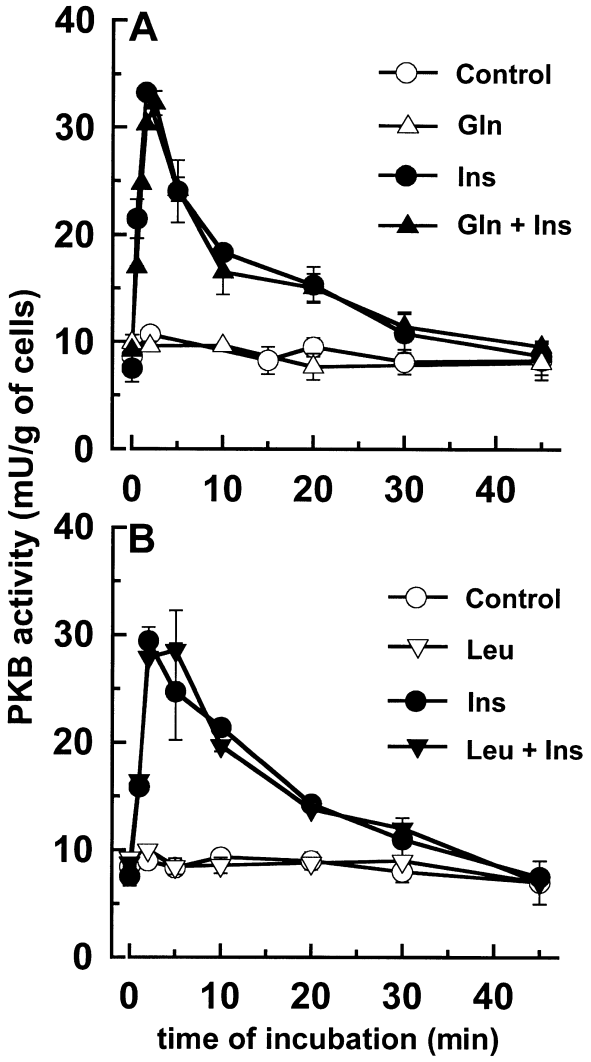

Fig. 5. Time-course of activation of PKB in hepatocytes incubated with insulin and amino acids. Hepatocytes were preincubated for 15 min and then further incubated with insulin and glutamine (A), or with insulin and leucine (B) for the indicated periods of time. Symbols are: control conditions (○), insulin (●) glutamine (△), leucine (▽), insulin plus glutamine (▲), insulin plus leucine (▼). Activation of PKB was blocked by 300 nM wortmannin (data not shown). The values are the means ± SEM for at least three, or the means for two cell preparations.

glutamine alone, or leucine together with glutamine or insulin (data not shown). In contrast, rapamycin had little, if any, effect on ACC activation. When present, the inhibition was smaller than 25% (data not shown) and was not specific for a leucine, glutamine or insulin.

The phosphorylation state of p70S6K is increased by both glutamine and leucine

p70S6K is activated by multiple phosphorylations on Ser/Thr residues. Isoforms corresponding to different phosphorylation states can be separated by SDS/PAGE in a low bisacrylamide gel. This method was used to investigate the effects of glutamine, leucine and insulin on the phosphorylation state of p70S6K (Fig. 7). In control hepatocytes, p70S6K seemed almost completely dephosphorylated, and this state was not changed by insulin. Leucine alone seemed to slightly increase phosphorylation. Incubation with glutamine or with glutamine together with leucine induced phosphorylation as reflected by the retardation of bands on SDS/PAGE (Fig. 7). Incubation with both amino acids induced a higher degree of retardation, suggesting the formation of hyper-phosphorylated forms. The same applied for incubation with insulin together with leucine (Fig. 7). In all cases, the phosphorylation state correlated with the extent of p70S6K activation. The highest activation was observed with glutamine plus leucine and corresponded to the highest mobility, whereas the lowest activation was found with leucine alone and corresponded to a slight modification of mobility.

DISCUSSION

Certain amino acids induce an anabolic response in hepatocytes independently of insulin

Amino acids like glutamine, alanine or proline, that enter the cell by cotransport with Na^+, induce cell swelling, which acts as an anabolic signal (reviewed in [38–41]) leading to the activation of GS, ACC and p70S6K [7]. Incubation with these amino acids leads to the accumulation of glutamate, which in turn causes swelling and stimulates the protein phosphatase GAPP [18]. There is a positive correlation between the degree of cell swelling and the extent of the anabolic response induced by amino acids [16]. On the other hand, leucine, which does not induce cell swelling and is poorly metabolized by the liver, exerts an anti-proteolytic effect (reviewed in [1]) and activates ACC and p70S6K. In hepatocytes, the effects of glutamine and leucine do not require insulin and persist in streptozotocin-diabetic rats. This may have some bearing on diabetes treatment. The liver response to these amino acids, which includes anti-ketotic and anti-proteolytic effects as well as a permissive effect on insulin action (see below), could improve the metabolic state of the diabetic patients and possibly help to decrease the required therapeutical doses of insulin. It should however, be remembered that large doses of amino acids can stimulate glucagon secretion.

The anabolic effect of insulin in liver requires the presence of leucine

The anabolic role of insulin in the liver is a matter of controversy. A 2.5-fold increase in ACC activity has been reported for liver *in vivo* [42], and increase in glycogen or activation of GS by insulin was found in hepatocytes in culture [35,43]. However, using freshly isolated hepatocytes in suspension, we show that insulin alone was able to initiate signalling pathway down to PKB. It was however, unable to elicit an anabolic response, but it enhanced the hepatocyte response to leucine. This is in agreement with the findings in Chinese hamster ovary cells where p70S6K was activated by insulin only in the presence of leucine [44] or of a mixture of amino acids [12], but in contrast with the results obtained in hepatoma cells, showing that the effect of insulin on p70S6K activation was also observed in the absence of amino acids [8]. Therefore we think that the

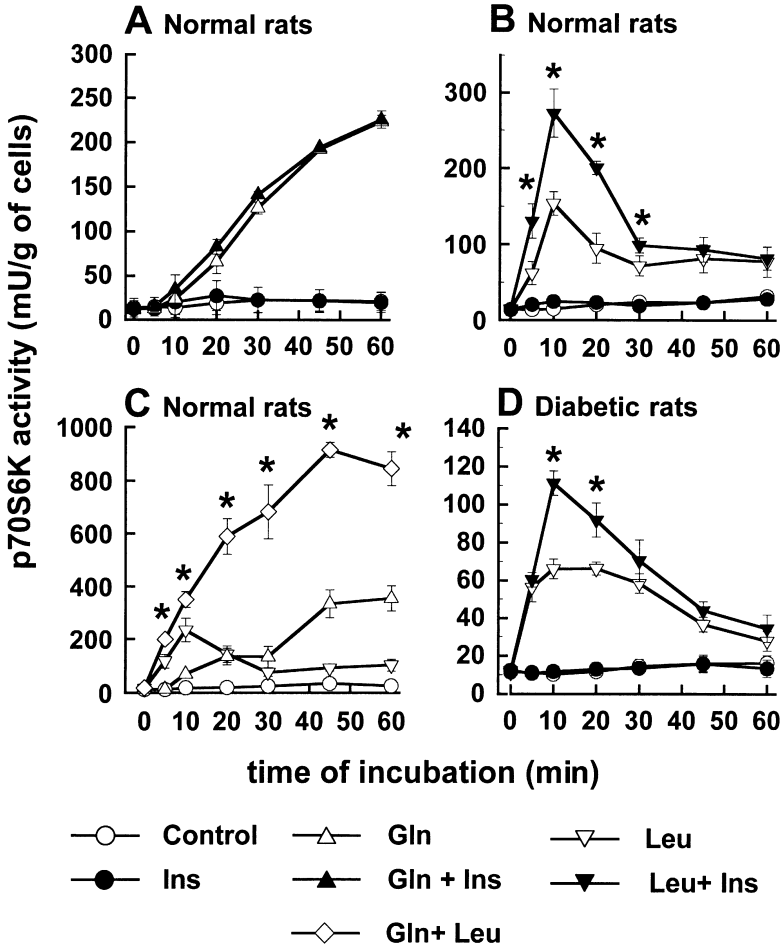

Fig. 6. Time-course of p70S6K activation in hepatocytes from normal and diabetic rats incubated with insulin and amino acids. Hepatocytes were isolated from normal (A–C) and streptozotocin-diabetic (D) rats. After a 15 min preincubation, cells were further incubated for the indicated periods of time. Interactions between glutamine and insulin (A), leucine and insulin (B,D) and glutamine and leucine (C) were investigated. Symbols are: control conditions (○), insulin (●), glutamine (△), leucine (▽), glutamine plus insulin (▲), leucine plus insulin (▼), leucine plus glutamine (◇). The values are the means ± SEM for at least four cell preparations. *Values statistically different ($P < 0.05$) from the corresponding values obtained in hepatocytes incubated with leucine (B,D) or glutamine (C).

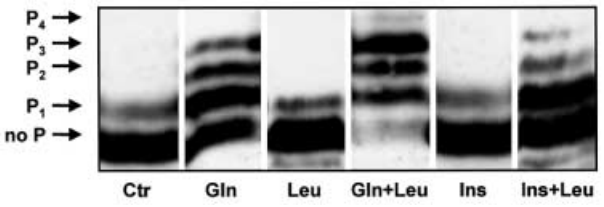

Fig. 7. Multiple phosphorylation states of p70S6K visualized by gel mobility shift assay. Hepatocytes were incubated under control conditions (Ctr), 45 min with glutamine (Gln) or glutamine plus leucine (Gln + Leu), 2 min with insulin (Ins), or 10 min with leucine (Leu) or with leucine plus insulin (Ins + Leu). Aliquots of extracts (150 μg of total protein) were separated on 7.5% SDS/PAGE using a low bisacrylamide concentration. p70S6K was immunodetected with anti-p70S6K Ig. No P, unphosphorylated enzyme; P1–P4, retarded bands of p70S6K corresponding to increased degrees in the phosphorylation state.

short-term effects of insulin alone in hepatocytes seem to be restricted to its well-known capacity to decrease cyclic AMP concentration and so to antagonize the effects of glucagon or β-adrenergic agonists [45,46]. In contrast, we demonstrate an activation of p70S6K by insulin in intact liver. By analogy to the situation in isolated hepatocytes, we interpret our observation obtained in intact liver as the presence of amino acids being the prerequisite for this anabolic insulin action *in vivo*.

Amino acids and insulin use different signalling pathways

The data reported here demonstrate that the signalling pathway mediating the response of hepatocytes to glutamine and leucine differed from the insulin signalling pathway. The first steps of the insulin signalling cascade including IR, IRS-1, IRS-1 associated PtdIns3K and PKB were not affected by the amino acids, whereas insulin readily increased their phosphorylation state and/or activity. Moreover it has been shown that, in hepatoma cells, amino acids inhibit insulin-stimulated PtdIns3K activity by decreasing insulin-stimulated IRS-1 phosphorylation and thereby inhibiting its association with PtdIns3K [8]. In L6 muscle cells, the deactivation by amino acids of insulin-stimulated PtdIns3K activity was explained by mTOR-mediated Ser/Thr phosphorylation of IRS-1 [37]. Accordingly, the increase in total PtdIns3K activity by glutamine (60%) and leucine (500%), which we [7] and others [19] have reported, did not correspond to an increased PtdIns3K activity associated with IRS-1. In addition, we failed to detect an increased PtdIns3K activity associated with tyrosine-phosphorylated proteins, including $p125^{FAK}$, which was found to be activated (twofold) by glutamine and was

tyrosine-phosphorylated (data not shown). Besides PtdIns3K, other wortmannin-sensitive lipid kinases could be involved. PtdIns3K could indeed work in concert with the lipid kinase 3-phosphatidylinositol 5-kinase to produce phosphatidylinositol-3,5-bisphosphate [PtdIns(3,5)P_2]. PtdIns(3,5)P_2 has been reported to be increased in cultured cells submitted to an hypo-osmotic shock [47]. According to this hypothesis, glutamine and hypo-osmotic media could cause an activation of 3-phosphatidylinositol 5-kinase and hence an increase in the concentration of PtdIns(3,5)P_2 in hepatocytes.

Our study of several signalling elements questions the central role of PtdIns3K/PKB in anabolic signalling in the liver. The activation of PtdIns3K and PKB by insulin was not sufficient to induce an activation of ACC and GS in liver in contrast with skeletal muscle or adipose tissue. In cultured hepatocytes, where GS is indeed activated by insulin, PKB activation might be necessary but is again not sufficient for GS activation [43]. In addition, we did not find a relationship between activation of PKB and of p70S6K. Indeed, insulin activated PKB but not p70S6K, whereas glutamine or leucine had the opposite effect. In other systems [13,19,48], p70S6K and PKB activation was correlated after stimulation of the cells with insulin rather than with amino acids.

The role of leucine

Although the mechanism of action of leucine has not been elucidated, some facts are worth discussing. Leucine stimulates ACC activation in synergism with glutamine. It also stimulates p70S6K activation in synergism with both glutamine and insulin. Unlike glutamine, leucine did not, however, activate GS. Leucine transport into the hepatocyte is independent of sodium ions and does not cause swelling. In contrast with skeletal muscle and pancreatic beta cells, the liver is unable to perform the first step of branched amino acid metabolism, namely their transamination to the corresponding keto acid. This may explain tissue differences in signalling and effects of branched amino acids [49,50]. However in liver, leucine is able to stimulate glutaminase and, hence, increases the intracellular concentration of glutamate [36]. The resulting stimulation of the type 2A protein phosphatase GAPP by glutamate [18] could explain the synergism with glutamine in ACC activation. In contrast, p70S6K activation did not result from dephosphorylation, because hyperphosphorylated forms were detected in hepatocytes incubated with both amino acids. In addition, a stimulating effect of insulin on p70S6K activation was observed with leucine, but not with glutamine. This confirms that control by leucine differs from that by glutamine. It also suggests that leucine and insulin signalling pathways converge on a common target, p70S6K, and that a hierarchical control is exerted by leucine on p70S6K phosphorylation. Finally, the synergism between insulin and leucine on p70S6K activation may explain the difference between insulin action on p70S6K in the intact animal and in isolated hepatocytes. This suggests that, to be observed, p70S6K activation by insulin requires the permissive action of leucine. This is in agreement with the permissive effect of amino acids on insulin action that has already been described for the inhibition of proteolysis in hepatocytes and for phosphorylation of S6 [3,4].

CONCLUSIONS

The main conclusions of this work are the following. Firstly, the effects of glutamine, a sodium-cotransported amino acid, leucine, a branched chain amino acid, and insulin on liver are mediated by different signalling pathways. Secondly, insulin alone is unable to trigger an anabolic response in hepatocytes. However, it does so in the presence of leucine, which exerts a permissive effect on p70S6K and ACC activation by this hormone. Finally, amino acids that are cotransported with sodium ions increase the volume of the liver. In so doing, they induce an anabolic response, which differs from, but is synergistic with, the anabolic effect of leucine, and which persists in diabetic animals.

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