Activation of protein kinase B/cAkt in hepatocytes is sufficient for the induction of expression of the gene encoding glucokinase

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Inhibitors of signalling pathways were used to dissect the mechanism of insulin action on expression of the gene encoding glucokinase in cultured rat hepatocytes. Wortmannin and LY 294002 completely prevented the insulin-induced increase in glucokinase mRNA seen in uninhibited cells, indicating that the phosphoinositide 3-kinase module has a key role. A ligand inducible protein kinase B (PKB, also termed cAkt) fusion protein was expressed by using adenoviral transduction of hepatocytes in primary culture. The PKB activity of this protein was shown to be activated in transduced hepatocytes within 30 min of the addition of 4-hydroxytamoxifen and to stay high for 8 h, as a result of serine phosphorylation at position 473 of PKB. The increase in PKB activity was reflected in the hyperphosphorylation of phosphorylated, heat and acid stable regulated by insulin protein (PHAS-I; also termed 4E-BP1, for eukaryotic initiation factor 4E-binding protein 1), a protein involved in the regulation of translation initiation. These effects were comparable to the insulin-induced activation of endogenous PKB and phosphorylation of PHAS-I in non-transduced hepa-

tocytes. The addition of tamoxifen to transduced hepatocytes resulted in an induction of glucokinase mRNA with kinetics and magnitude similar to those of insulin-induced mRNA accumulation. The effect of tamoxifen depended on stimulated PKB activity because it did not occur in hepatocytes that were transduced with a mutant PKB fusion protein that was refractory to activation with tamoxifen. These results establish that acute activation of PKB is sufficient to produce an insulin-like induction of glucokinase in isolated hepatocytes. Together with the inhibition by phosphoinositide 3-kinase inhibitors, they suggest that the activation of PKB might be critical in mediating the induction of glucokinase by insulin. In addition, experiments showed that PD98059 decreased by half the increase in glucokinase mRNA brought about by insulin, suggesting a contributory role of the mitogen-activated protein kinase cascade.

Key words: insulin, MAP kinase, phosphoenolpyruvate carboxykinase, phosphoinositide 3-kinase, signal transduction.

INTRODUCTION

Many genes are known to be transcriptionally regulated by insulin in target tissues. One particularly interesting example is the gene encoding glucokinase in the liver [1]. The level of hepatic glucokinase drops in insulin-deficient diabetes, owing to the cessation of specific gene transcription, and this change is reversed on insulin administration to diabetic animals [2,3]. The gene for glucokinase is not transcribed in hepatocytes cultured in insulin-free medium. Transcription of the gene resumes rapidly after the addition of insulin to the hepatocytes, leading to the reappearance of glucokinase mRNA and enzyme [4–7]. Thus the expression of glucokinase in the liver cell seems to be strictly dependent on the presence of insulin. In view of the key role of glucokinase in hepatic glucose metabolism, and specifically in glycogen synthesis [8], the stimulation of glucokinase gene transcription should be regarded as a central aspect of the metabolic response of the liver to insulin.

The mechanisms that underlie the induction of liver glucokinase by insulin have not been elucidated. Transcription of the gene for glucokinase in the liver initiates at a promoter known to be used only in this tissue. Cell-type-restricted transcription depends at least in part on a hepatocyte-specific enhancer localized 0.6–1 kbp upstream of the initiation site [9]. However, neither this enhancer nor the proximal promoter region confers insulin-inducibility on reporter gene constructs after transient transfection into hepatocytes or hepatoma cells [10]. At the gene

level, the discovery of *cis*-acting elements that might act as binding sites for putative insulin-regulated transcriptional factors remains an important objective.

Another unsettled issue is to identify the signalling events that are triggered by insulin and eventually lead to the stimulation of glucokinase gene transcription. In the present study, two complementary approaches were used to address this question. First, inhibitors of signal transduction pathways were tested for their effects on the insulin-induced accumulation of glucokinase mRNA in cultured rat hepatocytes. The second line of investigation focused on the hypothetical role of protein kinase B (PKB, also termed cAkt), a serine/threonine protein kinase that is known to be rapidly activated in response to insulin and is thought to be involved in many of the metabolic effects of insulin [11]. Primary rat hepatocytes were transduced with an adenovirus vector for expression of a form of PKB that could be activated at will by the addition of 4-hydroxytamoxifen (OHT) to the cell culture medium. The system allowed us to examine whether the acute activation of PKB with this artificial device would mimic the effect of insulin on expression of the gene for glucokinase.

EXPERIMENTAL

Hepatocyte culture and transduction with adenovirus

Hepatocytes were isolated by perfusion with collagenase from the livers of 2-day-fasted male Wistar rats and were maintained

Abbreviations used: GSK3, glycogen synthase kinase 3; HA, haemagglutinin; MAP, mitogen-activated protein; mTOR, mammalian target of rapamycin; OHT, 4-hydroxytamoxifen; PDEs, phosphodiesterases; PEPCK, phosphoenolpyruvate carboxykinase; PHAS-I, phosphorylated, heat and acid stable regulated by insulin protein (also termed 4E-BP1, for eukaryotic initiation factor 4E-binding protein-1); PI-3K, phosphoinositide 3-kinase; PKB, protein kinase B (also termed cAkt); SREBP, sterol-regulatory-element-binding protein.

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in culture under conditions described previously [6,12], except that the fetal bovine serum concentration in the maintenance medium was decreased to 4% (v/v) and the dexamethasone concentration to 10 nM. Transduction of the hepatocytes with adenoviral vectors was performed as described previously [13], usually with a multiplicity of infection of 4. The hepatocytes were fed with fresh culture medium 14 h after transduction and stimulated with insulin or OHT 2 h thereafter. Inhibitors were supplied to cells 30 min before insulin. Total RNA was isolated from the hepatocytes as described [6].

Adenovirus vectors

The subcloning of DNA fragments encoding the fusion proteins myrAktΔ4-129-ER (MER) and A2myrAktΔ4-129-ER (A2ER) [14] into the adenoviral shuttle vector pAdexCAG and the production of recombinant adenoviruses has been described previously [13].

Northern blot assay of mRNA species

Electrophoresis of RNA, blotting and hybridization with ³²P-labelled cDNA probes were done by published methods [7].

Immunoblotting and immunoprecipitation assays of PKB

The expression of MER and A2ER proteins in virally transduced hepatocytes was analysed by immunoblotting with monoclonal antibodies to the haemagglutinin (HA) tag (12CA5; Roche Molecular Biochemicals). Hepatocytes were lysed in a buffer containing 25 mM Tris/phosphate, pH 7.8, 2 mM *trans*-1,2-diaminocyclohexane-N,N,N',N'-tetra-acetic acid, 1% (w/v) Triton X-100, 10% (w/v) glycerol, 1 mM dithiothreitol and 250 μM PMSF. The lysates were sonicated briefly on ice, centrifuged at 4 °C and 12000 *g* for 30 s and the supernatants were collected for assay of protein concentration (Bradford method) and SDS/PAGE [10% (w/v) gel]. Western transfer and blotting with primary and secondary antibodies were performed essentially as described [15], except that 5% (w/v) non-fat dried milk and 0.1% (v/v) Tween 20 were used instead of gelatin and Triton X-405 respectively.

The degree of phosphorylation of PKB at position Ser-473, and of proteins MER or A2ER at the cognate residue, was assessed by immunoblotting with a phospho-specific antibody specific for the phosphorylated 466–479 polypeptide sequence of PKB. Hepatocytes were lysed in 1 ml of cell lysis buffer (New England Biolabs, Beverly, MA, U.S.A.) per 10 cm dish. Electrophoresis and Western blot analysis were done as described above, except that incubation of the blots with antibodies was performed in the presence of 5% (w/v) BSA.

The protein kinase activities of endogenous PKB or recombinant MER and A2ER proteins were assayed by immunoprecipitate enzyme assay as described previously [13]. In brief, immunoprecipitates obtained with antibodies against endogenous PKB or against the HA tag of the recombinant proteins were reacted in an assay mix containing recombinant myosin-GSK3 (GSK3 being glycogen synthase kinase 3) (New England Biolabs) and unlabelled ATP as substrates. The extent of specific GSK3 phosphorylation was estimated by immunoblotting with phospho-specific antibodies against the PKB target polypeptide sequence in the presence of 5% (w/v) BSA.

Immunoblotting of PHAS-I

The phosphorylation status of phosphorylated, heat and acid stable regulated by insulin protein (PHAS-I; also termed 4E-

BP1, for eukaryotic initiation factor 4E-binding protein 1), a protein involved in the regulation of translation initiation, was estimated from electrophoretic mobility shift on SDS/PAGE [15% (w/v) gel], with the same hepatocytes lysates as used for the PKB phosphorylation and activity assay. The PHAS-I polypeptide bands were detected by immunoblotting with affinity-purified polyclonal rabbit anti-(PHAS-I) antibodies (Zymed Laboratories, San Francisco, CA, U.S.A.) diluted in buffer containing 5% (w/v) non-fat dried milk.

RESULTS

Effects of inhibitors of insulin signalling on induction of glucokinase mRNA

The regulation of glucokinase mRNA by insulin was studied in primary rat hepatocytes 1 day after the cells had been placed in culture. In agreement with published data [6], levels of glucokinase mRNA were very low in cells maintained in basal medium and were induced by insulin approx. 20-fold in 8 h (Figure 1A). The effect of insulin was significantly blunted by PD98059, an inhibitor of the mitogen-activated protein (MAP) kinase cascade, and by rapamycin, an inhibitor of mammalian target of rapamycin (mTOR). Both inhibitors caused an approx. 50% decrease in the level of induced glucokinase mRNA. This degree of inhibition reflected the maximal effects of both inhibitors, as verified in preliminary dose-response experiments. In contrast with the partial inhibition caused by the above agents, complete suppression of the insulin effect was noted with two distinct inhibitors of phosphoinositide 3-kinase (PI-3K): wortmannin and LY 294002. The dose-dependent decrease of insulin-induced glucokinase mRNA with LY 294002 is shown in Figure 1(B).

The effects of inhibitors on another insulin response, the de-induction of the gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK), were studied in the same hepatocytes. As expected, insulin caused a more than 20-fold decrease in the level of PEPCK mRNA. The negative effect of insulin was unaffected after blockades of the MAP kinase cascade by PD98059, or of mTOR by rapamycin. In contrast, wortmannin completely prevented the insulin-dependent de-induction of PEPCK mRNA (Figure 1C).

Time course of expression of MER in hepatocytes after transduction with adenovirus

Downstream of PI-3K, activated PKB is thought to be instrumental in signal transduction for at least some of the metabolic effects of insulin. To address the possible role of PKB in glucokinase mRNA regulation, a conditionally active form of PKB, termed MER, was expressed in primary hepatocytes by using adenovirus-mediated gene transfer. The time course of accumulation of this protein was analysed in virally infected hepatocytes by immunoblotting with antibodies against the HA tag. An immunoreactive protein was readily detectable at 16 h after infection and its amount increased approx. 3-fold in the next 24 h (Figure 2A). The apparent molecular mass of 89 kDa estimated from the electrophoretic migration agreed well with the calculated molecular mass of the MER protein. The immunoreactive band was previously shown to be absent from extracts of non-transduced hepatocytes or hepatocytes transduced with a control lacZ adenovirus [13]. Moreover, in hepatocytes transduced with the MER-containing adenovirus, the 89 kDa protein was recognized, together with endogenous PKB, by antibodies against PKB. Only authentic PKB was discernible in non-transduced hepatocytes (Figure 2B).

Figure 1 Effects of inhibitors of insulin signalling on the induction of glucokinase mRNA (A, B) and repression of PEPCK mRNA (C) in primary hepatocytes

Hepatocytes were isolated from rats fasted for 48 h. Cells were maintained in primary culture for 22 h before the addition of insulin to a final concentration of 30 nM. Inhibitors were added 30 min before insulin to the following concentrations: PD98059, 50 μM; rapamycin (rapa), 200 nM; wortmannin (wort), 150 nM. Wortmannin additions were repeated at 2 and 4 h after insulin. Concentrations of LY 294002 are shown on the abscissa in (B). Hepatocytes were harvested for RNA isolation 8 h after the addition of insulin. Total RNA was subjected to Northern blotting with glucokinase cDNA (A, B) or PEPCK cDNA probes (C). Quantification of the specific mRNA bands was done by phosphorimaging. Results are means $\pm$ S.E.M. for four distinct culture experiments in (A) and (C), and five experiments in (B).

Time-controlled activation of MER protein by OHT in transduced hepatocytes

The effects of insulin and OHT on the phosphorylation status of PKB and MER protein respectively were investigated (Figure 3A). This analysis was performed by immunoblotting with anti-phosphopeptide antibodies that recognized the polypeptide se-

Figure 2 Time course of MER expression in adenovirally transduced hepatocytes

Primary hepatocytes were left to attach to culture dishes for 4 h after cell isolation. The culture medium with unattached cells was aspirated and the hepatocytes were supplied with maintenance culture medium containing the adenovirus vector encoding myrAkt Δ 4-129-ER (MER). They were incubated with virus for 1 h, after which the medium was replaced by virus-free medium. The medium was changed once more after 14 h and cells were harvested for the preparation of total protein extracts 2 h after the last change of medium (16 h after transduction) or at later times as indicated. (A) Samples containing 15 μg of protein were loaded in each lane and immunoblotting (ib) was performed with anti-HA antibodies. (B) Protein samples (30 μg) were obtained from non-infected control hepatocytes or hepatocytes at 16 h after infection with the MER virus and blotted with an antiserum against the C-terminus of PKB α [32]. The positions of molecular mass markers are indicated (in kDa) between the panels.

quence surrounding Ser-473 of PKB when this residue was phosphorylated. Phosphorylation of Ser-473 was not discernible in PKB of non-stimulated hepatocytes. Insulin elicited a rapid and durable phosphorylation of Ser-473, the effect being maximal within 30 min and prolonged throughout the 8 h of the experiment. In hepatocytes transduced for the expression of MER, this protein was not detectably phosphorylated in the basal state. The addition of OHT to the cells resulted in phosphorylation of the critical serine residue within 30 min. Hyperphosphorylation was sustained for 8 h. Endogenous PKB was not phosphorylated in response to OHT in transduced cells. The effect of OHT was also investigated in hepatocytes transduced with an adenovirus vector coding for A2ER, a G2A mutant of MER protein. This protein cannot be myristylated and therefore cannot be anchored to the plasma membrane after the binding of ligand. Exposure of A2ER-transduced hepatocytes to OHT did not result in appreciable Ser-473 phosphorylation of the PKB moiety of A2ER at 30 min (Figure 3A) or at any later time in the experiments. Expression levels of MER and A2ER proteins in hepatocytes transduced with the respective vectors were previously shown to be similar ([13]; see also below).

Figure 3 Phosphorylation and activation of PKB by insulin and of MER by OHT in hepatocytes

Hepatocytes in primary culture were transduced with adenoviruses encoding myrAkt Δ 4-129-ER (MER) or A2myrAkt Δ 4-129-ER (A2ER), or left uninfected (no virus). Insulin (ins) or OHT was added to the cultures 16 h after infection and cells were lysed at the time points indicated. **(A)** Time course of phosphorylation of PKB or MER detected by immunoblotting with anti-phospho-PKB (Ser-473). Samples contained 44 μ g of protein from non-transduced hepatocytes and 11 μ g from MER-transduced or A2ER-transduced cells. The positions of molecular mass markers are indicated (in kDa) at the right. **(B)** Time course of increase in PKB activity detected by immunoprecipitate (ipt) kinase assay. Immunoprecipitates from non-transduced hepatocytes were obtained with an antibody against PKB and immunoprecipitates from MER-transduced or A2ER-transduced cells were obtained with an antibody against the HA epitope. The precipitate kinase assay was performed with a myosin-GSK3 fusion protein as substrate. The phosphorylated product detected by immunoblotting (ibt) with phospho-GSK3-specific antibodies is shown in the upper panels of **(B)**. The products obtained from cell lysate volumes equivalent to 200 μ g of protein from non-transduced hepatocytes and 120 μ g of protein from transduced hepatocytes are shown. Recoveries of PKB, MER or A2ER in the immunoprecipitates were assessed by blotting with antibodies against PKB α or against the HA epitope as appropriate, and are shown in the lower panels of **(B)**. **(C)** Effects of insulin and OHT on the phosphorylation of PHAS-I in hepatocytes. Non-transduced hepatocytes were treated with insulin, and MER-expressing hepatocytes with OHT, for 30 min. Protein samples (54 μ g) were subjected to SDS/PAGE [15% (w/v) gel] and to immunoblotting with affinity-purified antibodies against PHAS-I. All results are representative of experiments that were performed at least twice.

The effects of insulin and OHT on the state of activation of PKB and MER or A2ER proteins were assessed by kinase assays of immunoprecipitates formed with anti-PKB or anti-HA antibodies respectively. The substrate was a recombinant myosin-GSK3 protein; its degree of phosphorylation was estimated by immunoblotting with an anti-phosphopeptide antibody specific for a PKB phosphorylation site in GSK3. In non-transduced hepatocytes, PKB activity was negligible in the basal state and was strongly activated in response to insulin. The time course of the insulin effect was characterized by a sharp increase in PKB activity within 30 min, a peak at 4 h and a clear-cut stimulation persisting at 8 h. In hepatocytes expressing MER, the addition of OHT resulted in activation of the PKB activity of MER with a time-course comparable to the effect of insulin in non-transduced hepatocytes. It is interesting to note that MER exhibited low, but measurable, PKB activity in the basal state, before the addition of OHT. In hepatocytes transduced with the A2ER vector there was no activation of PKB activity of the A2ER protein after the addition of OHT.

One cellular substrate for PKB is the protein kinase mTOR, which is activated as a result of its phosphorylation by PKB [16]. In turn, mTOR catalyses the phosphorylation of PHAS-I [17]. As an indirect marker of PKB activation in hepatocytes, the phosphorylation status of PHAS-I was analysed with a simple migration shift assay on SDS/PAGE. Immunoblotting of hepatocyte proteins with anti-(PHAS-I) antibodies revealed a triplet of closely spaced bands, termed α , β and γ , with apparent molecular masses of 17, 18 and 19 kDa respectively. The retarded migration of bands β and γ is known to reflect various degrees of phosphorylation of multiple serine and threonine residues [18]. On stimulation of non-transduced hepatocytes with insulin, the bands α and β seen in the basal state were shifted to the position of band γ . A similar shift was induced by OHT in MER-expressing hepatocytes. Interestingly, in these cells, band γ was already discernible before stimulation with OHT, accounting for approx. 25–35% of total immunoreactive PHAS-I. Phosphorylation of PHAS-I was not induced by OHT in A2ER-expressing hepatocytes (results not shown).

Stimulation of PKB activity in hepatocytes is sufficient for the induction of glucokinase mRNA and the de-induction of PEPCK mRNA

Having established in the previous experiments that OHT produces insulin-like effects on PKB activity and downstream protein phosphorylation events in hepatocytes expressing MER, we investigated the possibility of mimicking insulin action on specific gene expression with OHT. A robust induction of glucokinase mRNA occurred in response to stimulation with OHT. In the experiment shown in Figure 4(A), glucokinase mRNA was increased 4-fold over the starting level at 2 h after the addition of OHT and 13-fold at 8 h. Concomitantly, the level of PEPCK mRNA was markedly decreased.

The induction of glucokinase mRNA by OHT in hepatocytes expressing MER protein was dose-dependent, as summarized in Figure 4(B). The level of glucokinase mRNA in cells induced with 1 μ M OHT was slightly lower than, but statistically not different from, the level reached after stimulation with 30 nM insulin, a concentration known to exert maximal effect [6]. In important control experiments, glucokinase mRNA was shown to be unaffected by OHT in hepatocytes that had been transduced with the adenovirus vector encoding A2ER (Figure 4B), the non-activatable PKB construct. OHT was also without effect on glucokinase mRNA in non-transduced hepatocytes.

Figure 4 Induction of glucokinase mRNA and repression of PEPCK mRNA by OHT in hepatocytes expressing MER

(A) Time course of changes in glucokinase mRNA and PEPCK mRNA. Hepatocytes were transduced with the adenoviral vector encoding myrAkt Δ 4-129-ER (MER) as in Figure 3 and challenged with OHT 16 h later (time 0). Total RNA was isolated from the hepatocytes at the times indicated and samples (20 μ g) were subjected to Northern blotting. Autoradiographs of a membrane hybridized with glucokinase (top panel) and PEPCK (bottom panel) cDNA probes are shown. (B) Dose-dependence of the effect of OHT on glucokinase mRNA. Hepatocytes were left uninfected ($\square$) or infected with the MER-encoding ($\circ$) or A2myrAkt Δ 4-129-ER (A2ER)-encoding ($\triangle$) viruses. The three groups of cells were treated with OHT at the indicated doses or with insulin (Ins). Total RNA was extracted 8 h after the additions and glucokinase mRNA was quantified by Northern blotting and phosphorimaging. (C) Dose-dependence of OHT effect on PEPCK mRNA quantified in the same groups of hepatocytes as in (B). Results in (B) and (C) are means $\pm$ S.E.M. for three independent culture experiments.

The ability of OHT to mimic the effect of insulin on PEPCK mRNA was also investigated in these hepatocytes. The level of PEPCK mRNA was found to be substantially decreased in MER-expressing cells before any addition of effector. The addition of OHT to these cells resulted in a further dose-related decline in the mRNA. At 1 μ M OHT, PEPCK mRNA became undetectable, as also occurred in hepatocytes challenged with a maximal concentration of insulin. Complete suppression of PEPCK mRNA by OHT was specific to hepatocytes expressing MER protein. Unexpectedly, however, a biphasic effect of OHT on PEPCK mRNA was noted in non-transduced hepatocytes or hepatocytes expressing A2ER. In both group of cells, PEPCK mRNA increased at low doses and decreased at high doses of OHT.

DISCUSSION

The tamoxifen-activated PKB system provides an attractive tool for studies of insulin signalling. Most earlier studies with the tamoxifen-activated PKB system relied on stable integration of the gene encoding MER into established cell lines [14,19–21]. The present study illustrates the usefulness of the system as applied to primary cultures of rat hepatocytes. Primary hepatocytes have a limited life span in culture and cannot be readily subjected to methods for stable gene transfer. However, they can be very efficiently transduced with adenovirus vectors. The expression of the gene for MER in a large fraction of the cell population circumvented the need for the use of reporter genes, and instead allowed the responses of the endogenous genes for glucokinase and PEPCK to be monitored.

Ideally, a conditional activation system such as MER protein would require stable expression of the ligand-dependent protein at a fixed level during the period of study. This requirement is difficult, if not impossible, to achieve with a short-lived cell culture system such as primary hepatocytes. The protocol selected here, with regard to multiplicity of infection and time allowed for the accumulation of MER before challenge with OHT, was a compromise between the need for the adequate expression of MER in a majority of cells at the time of addition of OHT, and the risk of promiscuous activation of the protein in case of exaggerated overexpression. Although the level of MER protein continued to increase during the experimental period in the present study, this did not lead to confounding effects.

The ability to trigger an increase in PKB activity by the addition of OHT to hepatocytes expressing MER was examined in detail. The time course of activation by OHT was comparable to the time course of activation of endogenous PKB by insulin in normal hepatocytes, both effectors causing a strong activation within 30 min and activation being sustained over the entire 8 h period of the study. However, an interesting difference was noted in that PKB immunoprecipitates from control cells before stimulation with insulin were entirely devoid of activity, whereas low but measurable PKB activity was detected in immunoprecipitated MER protein before activation by OHT. Thus the conditional activation system seemed somewhat 'leaky' in the hepatocytes. This might have been due to the overexpression and adventitious activation of MER in some cells. In agreement with earlier results of Kohn et al. [14], the OHT-dependent activation of MER was accompanied by phosphorylation of the Ser residue corresponding to Ser-473 of PKB. This was attributed to the anchoring of OHT-liganded MER, via its myristylation signal, to the plasma membrane, and to its phosphorylation, presumably by membrane-bound phosphoinositide-dependent kinase 1 active in the resting state. Consistent with this mechanism was the observation that the myristylation-defective A2ER did not

undergo Ser-473 phosphorylation and activation in OHT-treated hepatocytes.

A distal effect of insulin mediated by the PI-3K/PKB/mTOR cascade in muscle or adipose cells is the phosphorylation of PHAS-I [21]. Phosphorylated PHAS-I dissociates from eIF4E, which is thereby released in active form for the translational initiation of capped mRNA species. This effect occurred in insulin-stimulated hepatocytes and was mimicked by OHT in MER-expressing hepatocytes. Probably related to some leakiness of the conditional system was the observation that PHAS-I seemed to be partly hyperphosphorylated in MER-expressing hepatocytes before the addition of OHT.

The activation of MER by OHT was sufficient to cause an increase in glucokinase mRNA similar to the effect of insulin. The induction of glucokinase mRNA in response to OHT can be traced to an acute increase in cellular PKB activity, because it occurred only when MER was expressed in hepatocytes. Glucokinase mRNA was not induced in non-transduced hepatocytes or in cells expressing A2ER, excluding an effect of OHT itself or an unexpected consequence of adenoviral infection. Interestingly, the glucokinase mRNA level was slightly increased in the resting state in MER-expressing hepatocytes, compared with non-transduced or A2ER-expressing hepatocytes, probably reflecting minor activation of MER independently of OHT stimulation.

Insulin has reciprocal effects on hepatic glucokinase and PEPCK mRNA species. In an earlier study with H42E hepatoma cells and stably integrated MER protein, Liao et al. [20] showed that acute stimulation of cellular PKB activity by OHT was sufficient to de-induce PEPCK gene expression in an insulin-like manner. However, a role for PKB in PEPCK repression has been questioned by other workers, because insulin was still active in cells expressing 'dominant-negative' mutants of PKB [22]. The present experiments with primary hepatocytes confirm that PEPCK mRNA levels decrease rapidly after activation of a ligand-inducible form of PKB. However, the interpretation of these experiments is somewhat blurred by non-specific effects of OHT that became apparent in control hepatocytes. In non-transduced as well as in A2ER-transduced hepatocytes, OHT brought about an increase in PEPCK mRNA at low doses and a decrease at higher doses. The mechanism of these effects is unknown. In spite of these side effects, a decrease in PEPCK mRNA levels, specifically related to PKB activation, can be clearly discerned from the comparison of MER-expressing and A2ER-expressing hepatocytes respectively, at each of the OHT concentrations used.

From the two premises (1) that the activation of PKB is an integral part of the response of hepatocytes to insulin and (2) that increased cellular PKB activity is sufficient to induce an increase in glucokinase mRNA in hepatocytes, it follows that the induction of hepatic glucokinase by insulin can be mediated, at least in part, via PKB. Consistent with this view is the observation that blockade of insulin signalling at the PI-3K step, which precedes PKB activation, completely prevented an increase in glucokinase mRNA. However, these results do not exclude the possibility that unidentified effector pathways downstream of PI-3K, in parallel with PKB, could contribute to induction of the gene for glucokinase following stimulation with insulin. The partial inhibition of the insulin effect by rapamycin suggests that cellular effects resulting from the activation of mTOR and p70 S6-kinase, perhaps translational effects, might contribute to the full-blown increase of glucokinase mRNA. In an earlier study we showed that inhibitors of cAMP phosphodiesterases (PDEs) abolished the induction of glucokinase mRNA by insulin in cultured hepatocytes [23]. This finding suggested that the insulin-dependent stimulation of PDE activity might have a role in the

induction of the gene encoding glucokinase. It is interesting to note that PDE3B has recently been reported to be phosphorylated and activated, in the test tube and in cells, by PKB [24–26] or another protein kinase under the control of PI-3K [27].

Although hepatocytes treated with the MEK inhibitor PD98059 were able to induce glucokinase mRNA in response to insulin, the hormone response was blunted compared with that of untreated hepatocytes. In the same experiments, PD98059 did not significantly affect the level of PEPCK mRNA in hepatocytes cultured without insulin, or the extent of insulin repression of PEPCK mRNA, in agreement with the results of earlier experiments with hepatoma cells [28]. These results suggest that the MAP kinase cascade is not involved in the insulin regulation of the PEPCK gene but might have a contributory role in signal transduction for induction of the gene encoding glucokinase.

Recently, Foretz et al. [29] have shown evidence for a role of the sterol-regulatory-element-binding protein (SREBP)1c transcriptional activator in the regulation of glucokinase gene expression in hepatocytes. In addition, SREBP2 and SREBP1a were found by others to be phosphorylated and activated by MAP kinase [30]. If SREBP1c is similarly activated after stimulation of the MAP kinase cascade, the results of Foretz et al. [29] and those obtained here, which implicate the MAP kinase pathway, would suggest a hypothetical transduction mechanism that could account for part of the effect of insulin on glucokinase gene expression. However, it should be stressed that a role for the SREBPs in the transcriptional regulation of hepatic glucokinase remains an unsettled issue because the overexpression of any of the three isoforms of SREBP in the liver of transgenic mice was not accompanied by an increase in the steady-state level of glucokinase mRNA [31].

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