Insulin stimulates the transcription of the sterol regulatory-element binding protein (SREBP) 1/ADD1 gene in liver. Hepatocytes in primary culture were used to delineate the insulin signalling pathway for induction of SREBP1 gene expression. The inhibitors of phosphoinositide 3-kinase (PI3-kinase), wortmannin and LY294002, abolished the insulin-dependent increase in SREBP1 mRNA, whereas the inhibitor of the mitogen-activated protein kinase cascade, PD98059, was without effect. To investigate the role of protein kinase B (PKB)/Akt downstream of PI3-kinase, hepatocytes were transduced with an adenovirus encoding a PKB-oestrogen receptor fusion protein. The PKB activity of this recombinant protein was rapidly activated in hepatocytes challenged with 4-hydroxytamoxifen (OHT), as was endogenous PKB in hepatocytes challenged with insulin. The addition of OHT to transduced hepatocytes resulted in accumulation of SREBP1 mRNA, with a time-course and magnitude similar to the effect of insulin in non-transduced cells. The level of SREBP1 mRNA was not increased by OHT in hepatocytes expressing a mutant form of the recombinant protein whose PKB activity was not activated by OHT. Thus acute activation of PKB is sufficient to induce SREBP1 mRNA accumulation in primary hepatocytes, and might be the major signalling event by which insulin induces SREBP1 gene expression in the liver.

Key words: hepatocytes, PKB/Akt, sterol regulatory-element binding protein (SREBP).

INTRODUCTION

The sterol regulatory-element binding proteins (SREBPs) are microsomal proteins which serve as precursors to transcriptional activators of the basic helix-loop-helix leucine zipper family. In mammals, two distinct genes termed SREBP1 (or adipocyte determination and differentiation factor 1 (ADD1)) and SREBP2 have been identified [1–3]. The SREBP1 gene is transcribed from optional promoters, giving rise to two mRNAs encoding precursors for SREBP1α and SREBP1c respectively [1]. The N-terminal moiety of each SREBP form, which harbours the transactivation and basic helix-loop-helix leucine zipper DNA-binding domains, is released from the full-length precursor by a two-step proteolytic cleavage, and is imported into the nucleus to activate the transcription of specific genes. The cleavage process is known to be activated when intracellular levels of sterols are low, and inhibited when levels are high [4].

The three forms of SREBP bind cis-acting elements of the SRE or E-box types [5], and are collectively involved in the transcriptional regulation of genes, encoding the low-density lipoprotein receptor and key enzymes of cholesterol and triacylglycerol biosynthesis [4]. Enzymes of fatty acid synthesis such as acetyl-CoA carboxylase and fatty acid synthase appear to be preferentially regulated by the products of the SREBP1 gene, in particular by SREBP1c in liver and adipose tissue [6,7]. This factor might also be a critical transcriptional activator of the gene for the regulatory enzyme of hepatic glucose metabolism, glucokinase [8].

Because glucokinase and the enzymes of triacylglycerol synthesis are inducible, the question arose whether SREBP1 gene expression might itself be hormonally regulated. Indeed, insulin was recently shown to stimulate the accumulation of SREBP1 mRNA in adipocytes and hepatocytes [7,9]. The present study addresses the mechanism of insulin signalling for induction of SREBP1 gene expression in hepatocytes. The effects of inhibitors of individual signal transduction pathways are reported. In addition, the specific role of protein kinase B (PKB)/Akt, a protein kinase critically involved in the metabolic actions of insulin [10,11], is analysed in cultured hepatocytes transduced with an adenovirus vector encoding a conditionally active form of PKB.

EXPERIMENTAL

Hepatocyte culture and transduction with adenovirus

Hepatocytes were isolated and cultured as described previously [12], except that the dexamethasone concentration in the medium was reduced to 10^{-8} M. Transduction with adenoviral vectors was performed 4 h after placing cells in culture. The hepatocytes, in 10-cm dishes, were exposed to recombinant viruses in 3 ml of culture medium for 1 h. The medium was removed and the hepatocytes were cultured in 10 ml of virus-free medium for 15 h.

The culture medium was replaced once more and the desired effectors were supplied to the cells 2 h after the last medium change. Inhibitors were supplied to cells 30 min before insulin, and in the case of wortmannin again at 2 h and 4 h after insulin. Cells were harvested at specified times after effector addition for isolation of total RNA as described [12].

Abbreviations used: SREBP, sterol regulatory-element binding protein; ADD1, adipocyte determination and differentiation factor 1; OHT, 4-hydroxytamoxifen; PI3-kinase, phosphoinositide 3-kinase; PKB, protein kinase B or cAkt; MER, myristoylated AktM4–129–oestrogen receptor or myrAktΔ4–129–ER; A2ER, A2myrAktΔ4–129–ER; HA, haemagglutinin.

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Adenovirus vectors

Recombinant DNA fragments encoding the fusion proteins myrAkt4-129-ER (MER) and A2myrAkt4-129-ER (A2ER) were isolated by BamHI/SalI digestion of pWZLneo retroviral vector plasmids, generously provided by Dr Richard A. Roth (Department of Molecular Pharmacology, Stanford University School of Medicine, Stanford, CA, U.S.A.) [13]. After blun- 
etting with the Klenow enzyme, the fragments were inserted into SwaI site of the cosmid pAdexCAG[14]. Adenoviral vectors 
were produced by in viva homologous recombination in 293 cells. 

Northern-blot assay of mRNAs

Blotting and hybridization with 32P-labelled cDNA probes was 
performed by described methods [16]. The amounts of probe 
hybridized to specific RNA bands were quantified by phos- 
phorimaging of the membranes. The rat SREBP1/ADD1 cDNA was 
kindly made available by Dr Bruce M. Spiegelman (Dana-
Darber Cancer Institute, Harvard Medical School, Boston, MA, 
U.S.A.) [2], and the rat glyceraldehyde 3-phosphate cDNA by Dr 
Philippe Fort (CRBM-CNRS, Montpellier, France) [17].

Immunoblotting and immunoprecipitation assays of PKB

For detection of MER and A2ER proteins expressed in virally 
transduced hepatocytes, total protein extracts were prepared 
as described previously [18]. Known amounts of protein were 
resolved by SDS/PAGE in 10% polyacrylamide separation gels 
and transferred to nitrocellulose membranes. Immunoblotting 
was performed using monoclonal antibodies (12CA5; Roche 
Molecular Biochemicals) against the haemagglutinin (HA) tag 
present in the middle of the recombinant proteins.

The protein kinase activity of authentic or recombinant PKB 
was assayed by immunoprecipitate enzyme assay. Hepatocytes in 
10-cm dishes were lysed in 1 ml of cell lysis buffer (New England 
Biolabs). Precipitation of endogenous PKB was with solid-phase 
antibodies to PKB (immobilized Akt1G1 monoclonal; New 
England Biolabs). Precipitation of endogenous PKB was with 
anti-HA immobilized on Protein A–Sepharose as described 
previously [18]. Known amounts of protein were 
resuspended in a kinase 
assay mixture containing a glycogen synthase kinase (GSK)-3 
with anti-HA immobilized on Protein A–Sepharose as described 
by Kohn et al. [10]. Precipitates were resuspended in a kinase 
reaction product was detected after 
SDS/PAGE by immunoblotting with Phospho-GSK-3 
(Ser21/9) antibodies (New England Biolabs). Secondary antibi-
odies conjugated with horseradish peroxidase were used for 
detection and revealed by enhanced chemiluminescence.

RESULTS

Insulin induction of SREBP1 mRNA is suppressed by inhibitors of phosphoinositide 3-kinase (PI 3-kinase)

The kinetics of effect of insulin on hepatic SREBP1 mRNA was 
studied using primary cultures of rat hepatocytes. As shown in 
Figure 1(A), the level of SREBP1 mRNA began to increase at 
4 h after hormone addition and plateaued after 24 h of treatment. 
Mean increases of SREBP1 mRNA were 6- and 20-fold of the 
starting level at 8 h and 24 h respectively in four separate 
hepatocyte experiments.

The level of SREBP1 mRNA in hepatocytes was investigated 
8 h after the addition of insulin to hepatocytes incubated with 
various inhibitors of insulin signalling pathways (Figure 1B). 
The inhibitor of PI 3-kinase, wortmannin, abolished the inductive 
effect of insulin. The insulin-dependent increase in SREBP1 
mRNA was also suppressed by LY 294002, a structurally 
distinct inhibitor of PI 3-kinase. In contrast, the response to 
inulin was essentially unaffected by PD 98059, an inhibitor of 
mitogen-activated protein kinase activation, and by rapamycin, 
an inhibitor of the protein kinase mTOR (mammalian target of 
rapamycin). With the latter inhibitor, the effect of insulin 
appeared to be slightly diminished, although the difference did 
not reach statistical significance. However, the absolute levels of 
SREBP1 mRNA in both the basal and insulin-stimulated states

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Protein kinase B/cAkt and sterol regulatory-element binding protein 1 gene expression

Figure 2  Expression and activation by OHT of PKB–oestrogen receptor fusion protein in adenovirus-transduced hepatocytes

(A) Hepatocytes were left uninfected or infected with adenovirus encoding LacZ or MER at the indicated multiplicity of infection (M.O.I.). Cell extracts were prepared 16 h after transduction and samples containing 30 μg protein were subjected to immunoblotting using antibodies to the HA tag. (B) Hepatocytes were not infected or infected at an M.O.I. of 4 with virus encoding MER or A2ER. Hepatocytes were left untreated or treated with insulin or OHT for 1 h. Cell extracts from equivalent number of cells were immunoprecipitated using antibodies to PKB (uninfected cells) or HA (cells infected with MER and A2ER viruses). Portions of the immunoprecipitates were assayed for PKB as described in the Experimental section. The phosphorylated GSK3 reaction product was revealed by immunoblot with phospho-specific antibodies. Portions of the immunoprecipitates were separately electrophoresed and blotted with antibodies to HA, to verify similar expression of MER and A2ER.

Figure 3  Induction of SREBP1 mRNA by OHT in hepatocytes expressing MER

Hepatocytes were infected with adenovirus encoding MER at a M.O.I. of 4 and were challenged with OHT 16 h after infection. (A) Time-course of change in SREBP1 mRNA in hepatocytes incubated with 0.2 μM OHT. (B) Dose-response of SREBP1 mRNA with the indicated concentrations of OHT 8 h after drug addition. The right-most lane shows the effect of 3 × 10⁻⁸ M insulin (ins) at 8 h. Lower panels in (A) and (B) depict the 28 S rRNA in the Acridine-Orange stained gel. (C) Uninfected hepatocytes or hepatocytes infected with adenovirus encoding MER or A2ER were left untreated, or exposed for 8 h to OHT at the doses indicated or to insulin (3 × 10⁻⁸ M). After Northern blotting, SREBP1 mRNA was quantified by phosphorimaging. In each group of cells, mRNA amounts were expressed as a percentage of the amount in cells incubated with insulin. Data are means ± S.D. of values in three independent culture experiments.

Expression of a conditionally activatable PKB fusion protein in cultured hepatocytes

The previous results suggested that insulin-dependent induction of SREBP1 required the activation of the PI 3-kinase. The role of PKB, a protein kinase downstream of PI 3-kinase, was investigated next. To this effect, hepatocytes were transduced with an adenoviral vector encoding a conditionally active form of PKB designed by Roth and colleagues [13]. This protein, termed MER (myristoylated AktΔ4–129–oestrogen receptor), is a chimaeric protein made up of a PKB moiety and an oestrogen receptor moiety, with an HA tag inserted to facilitate detection and immunoprecipitation. The expression of MER in transduced hepatocytes was quantified by immunoblotting with anti-HA antibodies (Figure 2A). Transduction with increasing numbers of viral particles resulted in dose-related expression of a recombinant protein with an apparent Mr of 89000, in agreement with the sequence of MER. This protein was absent both in non-transduced hepatocytes and in control hepatocytes transduced using a lacZ recombinant adenovirus. Staining the latter cells for β-galactosidase activity indicated that the transduction efficiency was 50–80% at a multiplicity of infection of 4 and 80% at a multiplicity of infection of 8 (results not shown).

The ability of the synthetic oestrogen 4-hydroxytamoxifen (OHT) to activate the chimaeric PKB was tested by immunoprecipitate kinase assay. The effect of OHT was compared with that of insulin on authentic PKB in non-transduced hepatocytes. As may be seen in Figure 2(B), hepatocytes transduced with the MER vector and incubated with OHT for 1 h exhibited strong activation of PKB activity of MER immunoprecipitated with anti-HA antibodies. The effect was comparable with the insulin stimulation of authentic PKB precipitated with anti-PKB antibodies in extracts of non-transduced hepatocytes.
For control purposes, hepatocytes were also transduced with a vector encoding a form of the PKB fusion protein termed A2ER, in which the N-terminal myristoylation signal of the fusion protein is inactivated by a Cys-2→Ala mutation [13]. As illustrated in Figure 2(B), the PKB activity of this protein assayed in anti-HA immunoprecipitates was not affected by incubation of the hepatocytes with OHT. Both the mutated and unaltered fusion proteins were expressed at similar levels, as verified by immunoblotting with anti-HA antibodies (bottom panel). The lack of OHT activation of the A2ER protein is consistent with the earlier results of Kohn et al. [13].

**Induction of SREBP1 gene expression by OHT in hepatocytes expressing MER**

The effect of OHT on the level of SREBP1 mRNA in hepatocytes transduced with the MER adenovirus was investigated. A time-dependent accumulation of SREBP1 mRNA was noted from 4–24 h after the addition of OHT (0.2 μM), as shown by Northern blotting (Figure 3A). The increase in specific mRNA was dose-related between 0.04 μM and 1 μM OHT (Figure 3B). Data from three separate experiments (Figure 3C) showed that OHT at 0.2 μM was as efficacious as SREBP1 induction as a maximal concentration of insulin (3 × 10−8 M). Importantly, induction of SREBP1 mRNA by OHT was restricted to hepatocytes expressing MER. A similar effect did not take place in non-transduced hepatocytes, or in hepatocytes transduced with the control A2ER adenovirus.

**DISCUSSION**

Using the run-on assay, Foretz et al. [7] have shown that insulin stimulates the transcription of the SREBP1 gene in rat liver cells. Therefore we infer that the regulation of SREBP1 mRNA in our experiments resulted mostly from regulation at a transcriptional level. The predominant transcript of the SREBP1 gene in liver is by far SREBP1c mRNA, and this form was the major insulin-regulated SREBP1 mRNA identified by RNase protection assay in hepatocytes [19]. On this basis, the effects reported herein most likely pertain to SREBP1c mRNA, although Northern blotting with SREBP cDNA probes actually does not discriminate between the two mRNAs.

The availability of the OHT-activated PKB fusion protein, and the use of adenovirus vectors to perform gene transfer in a very high percentage of primary hepatocytes, has allowed us to investigate the possible role of PKB in hepatic SREBP1 gene expression by a direct approach. The data conclusively establish that acute stimulation of PKB activity in isolated hepatocytes is sufficient to cause an increase in SREBP1 mRNA levels. Non-transduced hepatocytes did not induce SREBP1 mRNA in response to OHT. More importantly, SREBP1 mRNA was not increased in hepatocytes expressing a recombinant PKB refractory to activation by OHT. This provides stringent proof that the accumulation of SREBP1 mRNA after OHT addition to the hepatocytes expressing MER was specifically due to PKB activation. Furthermore, the effect elicited by OHT in cells expressing MER was similar in time-course and magnitude to the effect of insulin in non-transduced hepatocytes.

The insulin effect on SREBP1 gene expression was virtually unaffected by the MEK inhibitor, PD 98059, and thus does not appear to require activation of the mitogen-activated protein kinase cascade. By contrast, results with wortmannin and LY 294002 clearly implicated the PI 3-kinase signalling pathway. A major consequence of the activation of PI 3-kinase by insulin in many cell types, including hepatocytes, is a stimulation of PKB activity [20,20a]. The collective results of experiments using inhibitors of PI 3-kinase, on the one hand, and conditional activation of PKB, on the other hand, lead us to propose that induction of SREBP1 gene expression in the liver is most likely mediated via the PI 3-kinase/PKB branch of the insulin signalling pathways. Rapamycin, an inhibitor of mTOR, significantly reduced both induced and basal levels of SREBP1 mRNA, such that a strong insulin stimulation was conserved. However, a possible role of mTOR, which was recently shown to be a substrate for PKB [21], in maintaining basal SREBP1 gene expression would deserve further investigation.

The PI 3-kinase/PKB signalling pathway is thought to be involved in the insulin regulation of several genes, notably the gene for the insulin-like growth-factor binding protein (IGF-BP) 1 [22]. Recently, PKB was shown to phosphorylate several transcriptional activators of the forkhead family, including FKHR, AFX and FKHRL1, in vitro and in intact cells. The phosphorylation of specific residues in these factors inhibited their ability to activate transcription of target genes as a result of the sequestration of the phosphorylated factors in the cytoplasm [23–25]. Thus, the insulin-dependent repression of the IGF-BP1 gene, which harbours a cis-acting element capable of binding FKHR in its promoter, might depend, at least in part, on PKB-mediated phosphorylation and inactivation of this forkhead transactivator [22]. However, recent evidence has suggested that additional signals by-passing the PKB pathway might contribute to down-regulate the transactivating potential of FKHR [26]. Similarly, two distinct experimental systems using hepatoma cells have provided data either in favour of or against a critical role of PKB in the negative regulation of the phosphoenolpyruvate carboxykinase gene by insulin [27,28].

Evidence for a role of PKB in the positive regulation of specific gene expression by insulin is more limited. Recent publications suggest that GLUT1 and fatty acid synthase might be induced via PKB activation [29,30]. The transcription factors which might be affected directly or indirectly by PKB and transduce positive effects of insulin on gene expression are currently unknown. With respect to SREBP1 gene expression, the present data strongly suggest that the insulin effect might be mediated by PKB. A proof for this conclusion will require evidence for the abolition of the effect of insulin after complete and specific suppression of PKB activation. Moreover, insulin signalling via several, possibly redundant, pathways remains an intriguing possibility.

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