Analysis of the role of protein kinase B (cAKT) in insulin-dependent induction of glucokinase and sterol regulatory element-binding protein 1 (SREBP1) mRNAs in hepatocytes

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Previous work showed that acute stimulation of a conditionally active protein kinase B (PKB or cAKT) was sufficient to elicit insulin-like induction of GCK (glucokinase) and SREBP1 (sterol regulatory element-binding protein 1) in hepatocytes [Iynedjian, Roth, Fleischmann and Gjinovci (2000) *Biochem. J.* **351**, 621–627; Fleischmann and Iynedjian (2000) *Biochem. J.* **349**, 13–17]. The objective of the present study was to determine whether activation of PKB during insulin stimulation of hepatocytes was a necessary condition for the induction of the two genes. Activation of PKB by insulin was inhibited by pretreatment of the hepatocytes with C2 ceramide. This resulted in the inhibition of insulin-dependent increases in GCK and SREBP1 mRNAs. A triple mutant of PKB failed to interfere with insulin activation of PKB in hepatocytes even at high overexpression levels achieved after adenovirus transduction. A PKB–CaaX

fusion protein, which can act as a dominant-negative inhibitor of PKB activation in other cells, was shown to be constitutively activated in hepatocytes and to trigger insulin-like induction of GCK and SREBP1. In addition, constitutive PKB–CaaX activity caused refractoriness of the hepatocytes to insulin signalling at an upstream step resulting in the inhibition of both extracellular-signal-regulated kinase 1/2 and endogenous PKB activation. The stimulation of gene expression by constitutively active PKB–CaaX and inhibition of the insulin effect by ceramide are compatible with a role for PKB in the insulin-dependent induction of GCK and SREBP1.

Key words: ceramide, glucokinase, insulin, liver, protein kinase B, signalling.

INTRODUCTION

Insulin action in the liver is crucial for the maintenance of metabolic homeostasis [1]. Among the effects triggered by insulin are the induction or repression of the transcription of enzyme genes. A key liver enzyme that is transcriptionally induced by insulin in hepatocytes is hexokinase IV, commonly called GCK (glucokinase). The insulin-dependent maintenance of adequate GCK activity is essential for the liver to take up glucose and convert it into glycogen and triacylglycerols at times of plentiful supply of dietary carbohydrates [2].

The induction of liver GCK by insulin is not well understood at the molecular level. Regulatory DNA elements in the gene that might confer insulin responsiveness to the liver GCK promoter have not been identified. Therefore candidate transcription regulatory proteins that might be the target of insulin signalling remain to be defined. Some evidence suggests that the induction of the liver GCK gene could be a secondary effect resulting from the accumulation of nuclear SREBP1c (sterol regulatory element-binding protein 1c), a transacting factor which is itself induced at the transcriptional level by insulin in the liver [3]. This hypothesis arose from a report by Foretz et al. [4] that the level of GCK mRNA was increased in cultured hepatocytes overexpressing the nuclear form of SREBP1c. However, a more recent study failed to confirm this finding [5], which should therefore be re-examined independently. Moreover, the stimulation of GCK gene transcription after exposure of hepatocytes to insulin reaches a maximum 1 h after adding insulin to hepatocytes [6], whereas the increase in

SREBP1 mRNA and by inference *de novo* SREBP1c protein accumulation has barely begun at the 1 h time point. This kinetic discrepancy strongly argues against an indirect mechanism for GCK induction depending on prior transcriptional induction of an intermediary factor.

Regardless of the identity of putative target transcriptional regulators that could undergo post-translational modifications triggered by insulin, previous evidence from our laboratory suggested that insulin signalling to the GCK gene might be mediated by the PI3K (phosphoinositide 3-kinase)–protein kinase B (PKB or cAKT) pathway. By overexpressing an oestrogen receptor–PKB fusion protein in cultured hepatocytes, we could show that acute stimulation of PKB activity by tamoxifen was sufficient to elicit an insulin-like effect on GCK mRNA [7]. Stimulation of the conditionally active form of PKB in the hepatocytes was also sufficient to mimic the inductive effect of insulin on SREBP1 mRNA [8], suggesting a common signalling path for the two effects. In the present study, we examined whether the activation of endogenous PKB in hepatocytes during an insulin challenge was necessary for the induction of these mRNAs. Two approaches were used in an attempt to interfere with the insulin activation of PKB. The first was to pretreat the hepatocytes with the ceramide analogue C2 ceramide, a sphingolipid which was shown in other insulin-sensitive cell types to inhibit PKB activation at a step after the production of 3'-phosphoinositides [9,10]. The second approach tested was to overexpress protein variants, which are widely used as dominant-negative forms of PKB in transfected cells.

Abbreviations used: CIPh-cAMP, chlorophenylthio-cAMP; 4E-BP1, eukaryotic initiation factor 4E-binding protein-1; ERK, extracellular-signal-regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GCK, glucokinase; GSK, glycogen synthase kinase; HA, haemagglutinin; IRS-1, insulin receptor substrate 1; PEPCK, phosphoenolpyruvate carboxykinase; PI3K, phosphoinositide 3-kinase; PKB, protein kinase B; SREBP1, sterol regulatory element-binding protein 1.

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EXPERIMENTAL

Materials

Human insulin (Actrapid HM) was purchased from Novo-Nordisk (Bagsvaerd, Denmark), and CIPh-cAMP (chlorophenylthio-cAMP) was from Sigma (Buchs, Switzerland). C2 ceramide was obtained from Tocris (Avonmouth, U.K.) and C2 dihydroceramide from Calbiochem (San Diego, CA, U.S.A.). Rabbit antibodies to the p85 subunit of PI3K, to IRS-1 (insulin receptor substrate 1) and to the pleckstrin homology domain of PKB- α were from Upstate (Lake Placid, NY, U.S.A.). The rabbit antibody to total PKB (α , β and γ) was from Cell Signaling Technology (Beverly, MA, U.S.A.). Antibodies to PKB phosphorylated at Ser⁴⁷³, to ERK1 (extracellular-signal-regulated kinase 1) phosphorylated at Thr²⁰²/Tyr²⁰⁴ and to GSK3- α (glycogen synthase kinase 3- α) and GSK3- β phosphorylated at residues Ser²¹ and Ser⁹ respectively were from Cell Signaling Technology. The monoclonal antibody to the HA (haemagglutinin) tag (12CA5) was obtained from Roche Molecular Biochemicals (Rotkreuz, Switzerland), that to GSK3 was from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.) and the rabbit antibody to 4E-BP1 (eukaryotic initiation factor 4E-binding protein-1) was from Zymed Laboratories (San Francisco, CA, U.S.A.). Secondary antibodies were affinity-purified goat antibodies against rabbit or mouse IgG conjugated with peroxidase (Bio-Rad Laboratories, Hercules, CA, U.S.A.). The solid-phase monoclonal antibody against PKB was from Cell Signaling Technology. Protein A-Sepharose (EzviewTM Red Protein A affinity gel) was obtained from Sigma. The peptide RPRAATF (Akt/SGK-specific peptide) was obtained from Upstate. Phosphocellulose filter units (SpinZymeTM) were bought from Pierce Chemical (Rockford, IL, U.S.A.). Reagents for enhanced chemiluminescence (ECL[®]; SuperSignal West Pico) were from Pierce. Plasmids pShuttle-CMV and pAdEasy-1 vector were bought from Stratagene (La Jolla, CA, U.S.A.).

Adenovirus vectors

A recombinant DNA fragment encoding the protein HA-PKB-AAA was isolated from plasmid pcDNA3 HA-PKB-AAA, which was generously provided by Dr J. R. Woodgett (Ontario Cancer Institute, Toronto, Canada). A fragment encoding the protein HA-PKB-CaaX was isolated from plasmid pSG5 HA-PKB-CaaX, which was given by Dr B. M. Burgering (Department of Physiological Chemistry and Center for Biomedical Genetics, University Medical Center, Utrecht, The Netherlands). The fragments were subcloned in the plasmid pShuttle-CMV by standard cloning techniques to generate plasmids pShuttle-CMV HA-PKB-AAA and pShuttle-CMV HA-PKB-CaaX. A recombinant pShuttle-CMV HA-PKB-K179A-CaaX plasmid was produced by inserting an *ScaI*-*XhoI* 1 kb fragment from pShuttle-CMV HA-PKB-CaaX into the 8 kb *XhoI*-*ScaI* fragment of pShuttle-CMV HA-PKB-AAA. The PKB sequence of the above plasmids is that of bovine PKB- α . Relevant regions of the plasmids were sequenced to verify the presence of the mutations and the in-frame fusion of the CaaX sequence.

All adenovirus recombinant vectors were constructed by homologous recombination of the pShuttle-CMV plasmids containing appropriate inserts and the pAdEasy-1 vector, using the *Escherichia coli* strain BJ5183. The cloned recombinant pAdEasy-1 plasmids were then transfected into HEK-293 (human embryonic kidney 293) cells to produce recombinant viruses. The titre of stocks of recombinant viruses was assessed by a cytopathic effect test in HEK-293 cells. Proteins encoded by the viruses are designated throughout this study as PKB-AAA, PKB-CaaX and

PKB-K179A-CaaX without the mention of the N-terminal HA tag.

Hepatocyte culture and viral transduction

Experiments were approved by the State Commissioner on Animal Care. Hepatocytes were isolated from male Wistar rats, fasted for 2 days before the experiments. Cell isolation and culture were performed as described previously [7], except that the period of cell attachment was reduced to 1 h. When specified, hepatocytes were transduced with adenovirus as described previously [8], except that transduction was started 5 h after placing cells in the culture. When hepatocytes were treated with C2 ceramide or C2 dihydroceramide, these compounds (dissolved in DMSO) were added to the culture 90 min before insulin or CIPh-cAMP.

Protein extraction and immunoblotting

Total protein lysates were obtained from hepatocyte monolayers by extraction in cell lysis buffer as described previously [11]. The amounts of protein specified in the Figure legends were resolved by SDS/PAGE and transferred on to nitrocellulose membranes. Immunoblotting was performed in a buffer [10 mM Tris/HCl, (pH 7.5)/500 mM NaCl/0.08 % Tween 20], containing non-fat dried milk or BSA and individual antibodies at concentrations as recommended by each supplier. Blotting with the anti-IRS-1 antibody was done in the above buffer containing 3 % (w/v) BSA plus 2 % (w/v) non-fat dried milk. The bands on the immunoblots were revealed by ECL[®]. Quantification was accomplished by densitometer analysis of the X-ray films, using calibration curves obtained with graded inputs of protein.

Immunoprecipitation of IRS-1

Approx. 120 μ l of cell lysates containing 450 μ g of protein were added to 250 μ l of PBS and incubated overnight at 4 °C with 1.8 μ g of antibody to IRS-1. Prewashed Protein A-Sepharose beads (20 μ l) were added to the reactions, and tubes were incubated for a further 4 h at 4 °C with rocking. After centrifugation, supernatants were removed and beads were washed three times in ice-cold PBS. After the last wash, 35 μ l of twice-concentrated Laemmli sample buffer was added to the beads and the samples were heated in a heat block at 105 °C for 8 min. Samples of 25 μ l of supernatant were loaded on to SDS/polyacrylamide gels and subjected to immunoblotting with antibodies to IRS-1 or the p85 subunit of PI3K.

Immunoprecipitate kinase assay

Cell lysates (450 μ l) containing approx. 1.2 mg of protein were incubated with 15 μ l of packed, prewashed solid-phase antibody against PKB at 4 °C for 3 h with rocking. The beads were washed twice in ice-cold cell-lysis buffer and twice in kinase buffer containing 25 mM Tris/HCl (pH 7.5), 10 mM MgCl₂, 5 mM β -glycerophosphate, 0.1 mM sodium orthovanadate and 2 mM dithiothreitol. To initiate the protein kinase activity assay, 40 μ l of kinase buffer supplemented with 160 μ M peptide substrate for PKB (RPRAATF), 75 μ M ATP and 6 μ Ci of [γ -³²P]ATP was added to the pelleted beads. The samples were incubated at 30 °C with occasional shaking for 30 min. After pelleting the beads, samples of 25 μ l of supernatant were loaded on to phosphocellulose filter units. The units were washed twice with 500 μ l of 75 mM phosphoric acid and were placed in 10 ml of liquid-scintillation fluid for the measurement of radioactive phosphate incorporated into the PKB substrate. Replicate protein kinase

Figure 1 Inhibition of insulin-dependent activation of PKB in hepatocytes pretreated with C2 ceramide

Hepatocytes were cultured for 20 h before addition of the indicated concentrations of C2 ceramide, C2 dihydroceramide or the solvent DMSO. Insulin was added 90 min later. Hepatocytes were lysed for the extraction of total cell protein, 60 min after adding insulin. (A) Top to bottom: immunoblot analyses of PKB phosphorylated at Ser⁴⁷³, total PKB (antibody to total PKB from Cell Signaling), GSK3- α and GSK3- β phosphorylated at Ser²¹ or Ser⁹ respectively and total GSK3. Samples of 30–50 μ g of protein were loaded on to gels. Position of molecular-mass markers is indicated on the left. (B) Effect of insulin on PKB activity measured by immunoprecipitate kinase assay (filled bars) and on phosphorylation of PKB at Ser⁴⁷³ (open bars). Results, expressed as percentages of values in cells stimulated with insulin without ceramide pretreatment, are given as means $\pm$ S.D. from three separate experiments. (C) Effect of insulin on association of p85 subunit of PI3K with IRS-1.

assays without peptide substrate were performed to measure background radioactivity, which was subtracted from the radioactivity measured in the presence of substrate.

RNA extraction and Northern-blot analysis

Total RNA from cell monolayers was extracted and subjected to Northern-blot analysis as described in [12,13]. The ³²P-labelled cDNA probes used were a rat liver GCK cDNA [14], a rat SREBP1 cDNA [15] and a rat PEPCK (phosphoenolpyruvate carboxykinase) cDNA [13]. Equal loading of all lanes was verified by Acridine Orange staining of the gel and by hybridization of the blots with a GAPDH (glyceraldehyde-3-phosphate dehydrogenase) cDNA [16]. The quantification of specific mRNAs was performed by phosphorimaging of the membranes using a Cyclone Phosphorimager (Packard Instrument, Meriden, CT, U.S.A.).

RESULTS

Ceramide prevents insulin-dependent activation of PKB in hepatocytes

We tested the effect of pretreatment with C2 ceramide on the ability of hepatocytes in primary culture to activate PKB in response to insulin. The hepatocytes were cultured in the presence of C2 ceramide for 90 min before the addition of insulin at a concentration of 30 nM, which was known to elicit maximal effects in the present hepatocyte culture system [12]. Insulin was allowed to act for 60 min, after which total protein was harvested from the cells for immunoblot analysis of Ser⁴⁷³ phosphorylation of PKB, an event reflecting the activation of this protein kinase.

Pretreatment of the hepatocytes with concentrations of C2 ceramide between 50 and 150 μ M decreased the extent of insulin-dependent phosphorylation of PKB at Ser⁴⁷³, with virtually complete suppression of the insulin effect at the highest dose (Figure 1A). No inhibition of insulin-stimulated phosphorylation of PKB occurred in hepatocytes treated with the inactive ceramide analogue C2 dihydroceramide used as control.

In parallel, the phosphorylation status of GSK3- α and GSK3- β on Ser²¹ and Ser⁹ respectively was analysed by immunoblotting with phosphospecific antibodies. The above serine residues in the GSK3 isoenzymes are targets for phosphorylation by PKB [17], and their phosphorylation state can serve as an estimate of 'in-cell' PKB activity. As seen in Figure 1(A), the insulin-induced overphosphorylation of GSK3- α was abrogated and that of GSK3- β markedly blunted in hepatocytes exposed to the higher doses of C2 ceramide.

The relationship between insulin-stimulated phosphorylation of Ser⁴⁷³ of PKB and PKB activity was investigated by analysis of protein extracts from hepatocytes using both phosphospecific immunoblotting and immunoprecipitate kinase assay. The results depicted in Figure 1(B) show that insulin caused an approx. 10-fold stimulation of PKB activity in cells not pretreated with C2 ceramide, and that both insulin stimulation of PKB activity and extent of Ser⁴⁷³ phosphorylation were progressively inhibited with identical dose-responses after exposure of the cells to increasing concentrations of C2 ceramide.

The question of the level in the PI3K/PKB cascade that was inhibited by treatment with C2 ceramide was addressed by analysing the effect of insulin on the association of the regulatory p85 subunit of PI3K with IRS-1. The binding of this regulatory subunit to tyrosine-phosphorylated IRS-1 is recognized as a key mechanistic step for the activation of PI3K [18]. Cell

lysates from hepatocytes treated with various concentrations of C2 ceramide and subsequently challenged with insulin were subjected to immunoprecipitation using anti-IRS-1 antibodies, followed by analysis of the immunoprecipitated complexes by immunoblotting with anti-p85 antibodies. As expected, insulin was found to promote the association of p85 with IRS-1. This physical interaction was not impaired at any of the C2 ceramide doses (Figure 1C). The foregoing results are consistent with intact activation of PI3K by insulin in C2 ceramide-treated hepatocytes.

Ceramide prevents insulin-dependent induction of GCK and SREBP1 mRNAs

The ability of insulin to induce GCK and SREBP1 gene expression in hepatocytes cultured in a medium with C2 ceramide at the concentrations shown to inhibit insulin-stimulated PKB activity was investigated next. Treatment with C2 ceramide, but not C2 dihydroceramide, inhibited the increase in hepatic GCK mRNA dose dependently, as shown in the autoradiogram of a Northern-blot analysis in Figure 2(A). The effect of insulin on SREBP1 mRNA was also inhibited by C2 ceramide. However, the dose-response curve for SREBP1 mRNA appeared to be biphasic, with a slight overshoot of SREBP1 mRNA noted in insulin-induced hepatocytes treated with the lowest concentration of C2 ceramide, and with complete inhibition of the inductive effect of insulin at the highest concentration of the sphingolipid. A control Northern-blot analysis hybridized with a GAPDH cDNA showed little effect of either insulin or C2 ceramide on GAPDH mRNA.

We attempted to establish a correlation between inhibition of insulin activation of PKB by C2 ceramide on the one hand and of GCK mRNA induction on the other. The results for two separate hepatocyte culture experiments in which Ser⁴⁷³ phosphorylation of PKB was quantified by immunoblotting with phosphospecific antibodies and GCK mRNA was quantified by Northern-blot analysis are summarized as a regression analysis in Figure 2(B). The results clearly illustrate that the degree of inhibition of GCK mRNA induction was proportional to the degree of inhibition of PKB phosphorylation on Ser⁴⁷³, and by inference from Figure 1(B) to the degree of inhibition of PKB activity.

Since ceramides can elicit pleiotropic responses in cells, including apoptosis [19], it was important to verify that hepatocytes pretreated with C2 ceramide were not incapacitated non-specifically for the induction of specific genes, e.g. as a result of a general inhibition of transcription. To address this issue, hepatocytes exposed to C2 ceramide as in previous experiments were tested for their ability to induce the gluconeogenic enzyme PEPCK in response to its archetypal inducer, cAMP [20]. As shown in Figure 2(C), the analogue CIPh-cAMP (100 μ M) elicited a robust increase in PEPCK mRNA, and this response was completely preserved in C2 ceramide-treated hepatocytes.

Triple alanine mutant of PKB does not interfere with PKB signalling in hepatocytes

A non-natural mutant of PKB with alanine substitutions for both the Thr³⁰⁸ and Ser⁴⁷³ regulatory phosphorylation sites, as well as the Lys¹⁷⁹ residue at the active site of the kinase, was shown by Wang et al. [21] to interfere effectively with effector-mediated PKB activation in transfected cells. An adenovirus vector encoding this mutant, termed PKB-AAA, was produced to allow efficient gene transfer and protein overexpression in primary hepatocytes. Control hepatocytes were transduced with an equivalent multiplicity of infection of a control adenovirus coding for β -galactosidase. The hepatocytes were then exposed to insulin and harvested at appropriate times for monitoring signal

Figure 2 Inhibition of insulin-dependent induction of GCK and SREBP1 mRNAs in hepatocytes pretreated with C2 ceramide

Hepatocyte culture, pretreatment with ceramide analogues and stimulation with insulin were done as in Figure 1. Total RNA was extracted from cells 7 h after adding insulin. (A) Top to bottom: Northern-blot analyses of GCK mRNA, SREBP1 mRNA and GAPDH mRNA. Samples of 15 μ g of RNA were loaded on to gels. (B) Relative level of GCK mRNA as a function of inhibition of insulin-stimulated PKB phosphorylation by doses of C2 ceramide of 50 μ M ($\times$), 100 μ M (Δ) and 150 μ M ($\bullet$). The amount of GCK mRNA is given as a percentage of insulin-induced level in the absence of C2 ceramide ($\circ$). Values are from two separate experiments. The straight line and 95% confidence limits were calculated by linear regression analysis. (C) Pretreatment with C2 ceramide does not inhibit the effect of CIPh-cAMP (cAMP) on PEPCK mRNA in hepatocytes.

transduction along the PKB pathway and measuring the levels of GCK and SREBP1 mRNAs. An immunoblot depicting the amounts of total PKB (endogenous and PKB-AAA) (Figure 3A, top panel) shows that the hepatocytes overexpressed PKB-AAA approx. 12-fold above the endogenous PKB level. At this level of overexpression, the extent of insulin-stimulated phosphorylation of Ser⁴⁷³ of endogenous PKB was essentially unaffected when compared with that in non-transduced hepatocytes or hepatocytes transduced with the β -galactosidase virus (Figure 3A, middle panel). In line with unimpaired PKB activation, the insulin-dependent phosphorylation of GSK3- α and GSK3- β was also

Figure 3 Lack of effect of PKB-AAA on insulin activation of PKB and induction of GSK and SREBP1 mRNAs

Hepatocytes were placed in primary culture and transduced 5 h after seeding with adenoviruses encoding PKB-AAA or lacZ at the specified multiplicities of infection (MOI) for 75 min. Culture was continued and the hepatocytes were challenged with insulin 23 h after the start of culture. Protein lysates were prepared 1 h after adding insulin. Total RNA was extracted from hepatocytes 7 h after adding insulin. (A) Immunoblot analyses of total PKB (antibody to pleckstrin homology domain of PKB from Upstate; top panel), PKB phosphorylated at Ser⁴⁷³ (middle panel) and phosphorylated GSK3-α and GSK3-β (bottom panel). Samples of 50–90 μg of protein were loaded on to gels. (B) Northern-blot analyses of GSK3 mRNA (top panel) and SREBP1 mRNA (middle panel). Samples of 20 μg of RNA were loaded on to gels. The photograph at the bottom depicts 18 S rRNA detected by UV light transillumination after Acridine Orange staining of the agarose gel. Identical results were obtained from two separate experiments.

unaltered (Figure 3A, bottom panel). Finally, the insulin induction of GSK and SREBP1 mRNAs was virtually identical in the hepatocytes overexpressing PKB-AAA and in the controls, as shown in Figure 3(B).

Variant PKB-CaaX has constitutive PKB activity and induces GSK and SREBP1 mRNAs

Since PKB-AAA failed to inhibit insulin-stimulated PKB activity in primary hepatocytes, a version of engineered PKB bearing a CaaX sequence at its C-terminal end was tested. This variant, termed PKB-CaaX, was shown to interfere with PKB activation when expressed in A14 cells [22]. Hepatocytes were transduced with an adenovirus encoding PKB-CaaX and analysed for the level of expression of the non-natural PKB protein and for the ability to respond to insulin by an increase in Ser⁴⁷³ phosphorylation

Figure 4 Constitutive PKB activity of PKB-CaaX in hepatocytes

The culture of hepatocytes, transduction with adenovirus vectors encoding PKB-CaaX or PKB-K179A-CaaX, stimulation with insulin and lysis for protein or RNA extractions were as described in Figure 3. (A) Immunoblot analyses of total PKB (antibody to pleckstrin homology of PKB from Upstate; upper panel) and PKB phosphorylated on Ser⁴⁷³ (lower panel). Samples containing 50 μg of protein were electrophoresed. (B) Immunoblot analyses of phosphorylated GSK3-α and GSK3-β (upper panel) and total GSK3 (lower panel), using samples of 90 μg of protein. (C) Top to bottom: immunoblot analyses of levels of expression of PKB-CaaX or PKB-K179A-CaaX using antibody to the HA tag (* designates non-specific band), and analyses of insulin-stimulated phosphorylation of PKB (Ser⁴⁷³), of GSK3-α and GSK3-β (Ser²¹ and Ser⁹) and 4E-BP1 (mobility shift). The α-form of 4E-BP1 was discernible in the 1st and 5th lanes on the original X-ray film. Similar results were obtained from three separate experiments.

of endogenous PKB. Increasing multiplicities of infection produced stepwise increases in levels of PKB-CaaX, and overexpression of PKB-CaaX progressively interfered with the effect of insulin on PKB phosphorylation at Ser⁴⁷³ (Figure 4A). This result was consistent with a dominant-negative effect of PKB-CaaX.

Quite unexpectedly, however, we found that GSK3-α and GSK3-β were increasingly hyperphosphorylated in hepatocytes

Figure 5 Induction of GSK3 mRNA and SREBP1 mRNA in hepatocytes expressing constitutively active PKB-CaaX

Hepatocytes were transduced with adenovirus vectors encoding PKB-CaaX or PKB-K179A-CaaX and stimulated with insulin as in Figure 4(C). Total RNA was extracted from the cells 7 h after insulin and subjected to gel electrophoresis using samples of 20 μ g of RNA. Northern-blot analyses of GSK3 mRNA (top panel) and SREBP1 mRNA (middle panel); 18 S rRNA was imaged by fluorescence staining of the gel (bottom panel). Identical results were obtained from two separate experiments.

in the basal state (non-stimulated by insulin) in function of PKB-CaaX overexpression (Figure 4B). At the highest level of PKB-CaaX expression, the phosphorylation state of the GSK3 isoenzymes in the absence of insulin approached the insulin-stimulated level seen in non-transduced hepatocytes, and displayed little further increase in the presence of insulin. This observation suggested that, contrary to our expectation, PKB-CaaX could perform as a constitutively activated PKB in primary hepatocytes.

To verify this point, a mutant sequence for PKB-CaaX with an alanine substitution for Lys¹⁷⁹, designated PKB-K179A-CaaX, was cloned into an adenovirus vector. Hepatocytes transduced with the original PKB-CaaX and the PKB-K179A-CaaX viruses, at multiplicities yielding equal levels of overexpression, were analysed for insulin-stimulated Ser⁴⁷³ phosphorylation of endogenous PKB as well as downstream signalling steps. As seen above, the insulin stimulation of PKB phosphorylation at Ser⁴⁷³ was severely depressed in hepatocytes expressing PKB-CaaX, and strong phosphorylation of GSK3 isoenzymes was noted in the basal state. Moreover, a shift in electrophoretic migration of the translation regulatory protein 4E-BP1 [also called PHAS-1 (phosphorylated, heat and acid stable regulated by insulin protein)] was noted in the absence of insulin in hepatocytes expressing PKB-CaaX, similar to the effect of insulin in non-transduced cells. The mobility shift is indicative of phosphorylation by the protein kinase mammalian target of rapamycin [23], which is itself phosphorylated and activated by PKB [24]. In contrast, hepatocytes expressing PKB-K179A-CaaX exhibited none of these effects and their behaviour was identical with non-transduced hepatocytes (Figure 4C). These experiments demonstrate that the effects of PKB-CaaX, both to inhibit the insulin-dependent phosphorylation of endogenous PKB and to elicit downstream signalling events mimicking insulin, are due to the intrinsic kinase activity of PKB-CaaX, which is constitutively activated in hepatocytes.

It was then of interest to assay GSK3 and SREBP1 mRNAs in hepatocytes transduced with the above viruses (Figure 5). As predicted, both mRNAs were low in the absence of insulin in non-transduced cells and were markedly induced by insulin. Low

Figure 6 Inhibition of insulin signalling in hepatocytes expressing constitutively active PKB-CaaX

Hepatocytes were cultured, transduced with adenovirus vectors and stimulated with insulin as described in Figure 4(C). (A) Protein samples (90 μ g) for analysis of phosphorylation of ERK1/ERK2 were obtained 5 min after adding insulin. (B) Protein samples for immunoprecipitation of IRS-1 followed by immunoblotting with the same antibody to IRS-1 were obtained 60 min after adding insulin. Similar results were obtained from two separate experiments.

basal amounts of the mRNAs and strong insulin induction also prevailed in hepatocytes overexpressing the 'kinase-dead' PKB-K179A-CaaX. In contrast, hepatocytes expressing PKB-CaaX exhibited high levels of GSK3 and SREBP1 mRNAs in the basal state, comparable with the insulin-induced levels in the control groups. Insulin addition did not further increase the mRNAs in the PKB-CaaX hepatocytes (Figure 5).

Constitutive activity of PKB in hepatocytes interferes with insulin signalling at an upstream step

As shown previously (Figure 4C), the expression of PKB-CaaX, but not the 'kinase-dead' PKB-K179A-CaaX version of this protein, resulted in the inhibition of the insulin-stimulated phosphorylation of endogenous PKB. This finding suggested that the protein kinase function of PKB-CaaX was instrumental in eliciting this inhibitory effect. To address the issue of whether the inhibitory effect specifically targeted endogenous PKB or reflected a more general suppression of insulin signal transduction, the acute effect of insulin on the ERK1/ERK2 branch of the insulin signalling cascades was investigated. Immunoblotting of hepatocyte protein with antibodies specific to phosphorylated threonine and tyrosine residues in ERK1 and ERK2 revealed a strong stimulation by insulin in non-transduced hepatocytes as well as in hepatocytes transduced with the viral vector encoding PKB-K179A-CaaX (Figure 6A). However, in hepatocytes expressing PKB-CaaX at a similar level, the response was nearly abolished.

This finding suggested that activated PKB could exert negative feedback at an early stage of insulin signalling in hepatocytes. Recent evidence has suggested that the activation of PKB in cells can directly or indirectly result in hyperphosphorylation of serine or threonine residues in IRS-1 [25,26], leading in some cases to increased turnover of IRS-1 [27]. In the present experiments, IRS-1 was analysed by immunoprecipitation of total protein extracts from hepatocytes with anti-IRS-1 antibodies, followed by immunoblotting of the precipitated protein complexes. As seen in Figure 6(B), an electrophoretic mobility shift of IRS-1 was discernible after stimulation of non-transduced hepatocytes by insulin, compatible with an hyperphosphorylated state of IRS-1. The same insulin-dependent shift occurred in hepatocytes

expressing PKB-K179A-CaaX. In contrast, hepatocytes expressing PKB-CaaX exhibited the retarded form of IRS-1 in the absence of insulin, and little further change occurred after exposure to insulin. Neither insulin nor constitutively active PKB-CaaX appeared to cause any obvious decrease in the amount of immunoreactive IRS-1.

DISCUSSION

Previous work in our laboratory has shown that acute activation of an oestrogen receptor-PKB chimaeric protein by tamoxifen in primary hepatocytes resulted in rapid induction of GCK and SREBP1 mRNAs, similar to the effect of insulin on the expression of these target genes [7,8]. The primary purpose of the present study was to determine whether the activation of PKB is a necessary step in the action of insulin on the expression of these genes. Insulin-dependent activation of PKB was inhibited, apparently at a step downstream of PI3K, by a short pretreatment of primary hepatocytes with the sphingolipid C2 ceramide. This was accompanied by a weakening of the insulin effect on GCK and SREBP1 mRNAs in C2 ceramide-treated hepatocytes. More importantly, the degree of inhibition of mRNA induction was correlated with the degree of inhibition of insulin activation of PKB, consistent with the notion that insulin signalling to the target genes was mediated via PKB. In a comparable study with L6 myoblasts stimulated with insulin, Hajdich et al. [9] showed that the translocation of PKB to the plasma membrane and its activation, as well as downstream effects on glucose transport and glycogen synthesis, were all markedly inhibited in cells pretreated with C2 ceramide, in the face of a fully preserved increase in PI3K activity. Similarly, platelet-derived growth factor stimulated the production of 3'-phosphoinositides normally in ceramide-treated 3T3 fibroblasts, but the translocation to the plasma membrane of selected pleckstrin homology domains, including that of PKB, was severely impaired [28]. These studies suggested that C2 ceramide might exert a rather specific inhibition on the mechanism of activation of PKB, at a step between the generation of 3'-phosphoinositides and the phosphorylation of PKB. However, multiple effects of C2 ceramide besides the inhibition of PKB activation, including effects on several protein kinases and phosphatases, have also been noted in various cell types [29]. Therefore caution is required in ascribing ceramide effects to the inhibition of PKB activation.

An alternative approach to block hormone-dependent activation of PKB in cells is via forced expression of 'dominant-negative' mutants of PKB, although criticism has been raised on this type of experiments [30]. On the basis of experimental evidence and theoretical considerations, Coffey et al. [31] argued that the most effective dominant interfering form of PKB was PKB-AAA. Surprisingly, in primary hepatocytes, PKB-AAA failed to prevent the activation of endogenous PKB by insulin, as estimated by Ser⁴⁷³ phosphorylation of PKB and immunoprecipitate kinase activity (results not shown), as well as by phosphorylation of GSK3- α and GSK3- β on Ser²¹ and Ser⁹ respectively. There was no significant reduction of the insulin effects on GCK and SREBP1 mRNAs in the hepatocytes expressing PKB-AAA. The ineffectiveness of PKB-AAA was noted in hepatocytes which overexpressed this protein by as much as 12 times the amount of endogenous PKB. This degree of overexpression should have been sufficient to interfere strongly with the activation of endogenous PKB, according to the model proposed by Coffey et al. [31] in which PKB functions in cells as a trimer. Whether even higher levels would result in a dominant-negative effect is not known, but unspecific effects would be difficult to exclude consequent to such a massive overexpression.

Recently, Matsumoto et al. [32] used an adenoviral vector encoding a double T308A/S473A mutant of PKB for gene transfer in cultured hepatocytes and showed that the insulin inductions of GCK and SREBP1 mRNAs were normal in transduced hepatocytes. However, they reported that the insulin activation of PKB- β was inhibited. It is difficult to compare this finding with our own results because the level of expression of the mutant protein relative to endogenous PKB was not reported by Matsumoto et al. [32], and because the activation status of total PKB, represented mostly by PKB- α in liver tissue [33], was not ascertained. Another important result in [32] was a strong inhibition of insulin-dependent increases in GCK and SREBP1 mRNAs in hepatocytes overexpressing a truncated variant of IRS-1. This was accompanied by a nearly complete suppression of PI3K activity associated with IRS-1, whereas insulin-stimulated phosphorylation of bulk PKB on Ser⁴⁷³ was minimally affected. A plausible interpretation that would reconcile these and our results would be that insulin signalling to the GCK and SREBP1 genes is transduced via IRS-1, PI3K and a restricted subfraction of PKB that is inhibited in ceramide-treated hepatocytes, but refractory to inhibition by expression of 'dominant-negative' variants of PKB.

The attachment of consensus amino acid sequences for fatty acylation to signalling proteins can result in membrane anchoring and stimulus-independent signalling activity of such proteins. A fusion protein with a CaaX farnesylation motif added to the C-terminus of PKB, termed PKB-CaaX, was generated by van Weeren et al. [22]. Quite unexpectedly, van Weeren et al. [22] found that PKB-CaaX was devoid of kinase activity in the basal as well as in insulin-stimulated state, after its transfection into A14 cells. Moreover, PKB-CaaX acted as a 'dominant-negative', in that insulin activation of PKB and the ensuing inactivation of GSK3- β due to Ser⁹ phosphorylation were inhibited in A14 cells [22]. In the current experiments, forced expression of PKB-CaaX resulted in a markedly different outcome in primary hepatocytes. Hepatocytes expressing PKB-CaaX displayed increased phosphorylation of GSK3 and phosphorylation-dependent electrophoretic mobility shift of 4E-BP1 in the basal state, similar to the effects of insulin in non-transduced cells. Additionally, GCK and SREBP1 mRNAs were strongly increased in the absence of insulin to levels similar to those induced by insulin in non-transduced cells. These changes appeared to be a consequence of constitutive activity of PKB-CaaX, because they were absent from hepatocytes expressing a 'kinase-dead' version of PKB-CaaX. The reason for the difference between hepatocytes and A14 cells in the ability to activate PKB-CaaX is unclear, but may reflect differences in the amount and localization of upstream kinases, such as phosphoinositide-dependent kinases, capable of activating PKB. In any case, the sustained induction of GCK and SREBP1 mRNAs in hepatocytes expressing a chronically active form of PKB extends the previous findings in this laboratory of the rapid induction of these messages on acute stimulation by tamoxifen of a conditional form of PKB [7,8].

Another interesting consequence of the expression of PKB-CaaX in hepatocytes was the refractoriness of the cells to insulin signalling. This was manifested by a weakening of the effects of insulin both on Ser⁴⁷³ phosphorylation of endogenous PKB and on threonine/tyrosine phosphorylation of ERK1/ERK2. Because PKB and ERK1/ERK2 belong to distinct branches of the insulin-signalling cascades, the inhibition of both branches suggested an interference at an early step after receptor activation. Hepatocytes expressing PKB-CaaX exhibited a shift in the electrophoretic mobility of IRS-1 in SDS/polyacrylamide gels, a change occurring after the phosphorylation of serine residues in IRS-1 [27,34]. Phosphorylation of IRS-1 on serine residues by a number of serine/threonine protein kinases can adversely affect insulin signal

transduction [35]. One specific phosphorylation site implicated in negative regulation of insulin signalling is Ser³⁰⁷, which has been shown to be hyperphosphorylated in response to the activation of the mammalian target of rapamycin [27], itself under the dependence of the PI3K/PKB signalling pathway. Our preliminary experiments using a phosphospecific antibody to phosphorylated Ser³⁰⁷ did not reveal any marked effect of PKB–CaaX or insulin on the phosphorylation of this residue in hepatocytes. Also, the amount of the p85 subunit of PI3K co-precipitating with IRS-1, either in the basal state or after insulin stimulation, was not altered in hepatocytes expressing constitutively active PKB–CaaX compared with untransduced cells or cells transduced with the ‘kinase-dead’ PKB-K179A–CaaX. Interestingly, inhibition of insulin-stimulated PI3K activity, without decrease in insulin-stimulated association of p85 with IRS-1, has been noted as a consequence of serine phosphorylation of IRS-1 in cells overexpressing atypical PKC- ζ [36]. Further investigations will be necessary to identify specific phosphorylation sites that might be targeted directly or indirectly by PKB or other insulin-activated protein kinases in liver tissue, and to analyse the functional consequences of such phosphorylation in more details.

In summary, four conclusions emerge from the present study. First, exposure of hepatocytes to the ceramide analogue C2 ceramide caused an inhibition of the effect of insulin on the activation of PKB and a proportional inhibition of the induction of GCK and SREBP1 gene expression, consistent with a necessary role of PKB activation for the insulin induction of these genes. Secondly, variants of PKB known to act as dominant-negative forms of PKB in other cell systems failed to inhibit insulin-dependent PKB activation in hepatocytes, even when abundantly overexpressed, raising an important caveat to the use of these variants at least in some cell systems. Thirdly, a constitutively activated form of PKB triggered insulin-like induction of GCK and SREBP1 mRNAs in hepatocytes, again compatible with a mediator role of PKB in the effects of insulin. Finally, chronic activation of PKB induced a state of refractoriness to insulin along both the PKB and ERK1/ERK2 signalling pathways in liver cells, similar to cases of insulin resistance in other cell types.

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REFERENCES

- Michael, M. D., Kulkarni, R. N., Postic, C., Previs, S. F., Shulman, G. I., Magnuson, M. A. and Kahn, C. R. (2000) Loss of insulin signaling in hepatocytes leads to severe insulin resistance and progressive hepatic dysfunction. *Mol. Cell* **6**, 87–97
- Iynedjian, P. B. (1993) Mammalian glucokinase and its gene. *Biochem. J.* **293**, 1–13
- Foretz, M., Pacot, C., Dugail, I., Lemarchand, P., Guichard, C., Le Liepvre, X., Berthelot-Lubrano, C., Spiegelman, B., Kim, J. B., Ferre, P. et al. (1999) ADD1/SREBP-1c is required in the activation of hepatic lipogenic gene expression by glucose. *Mol. Cell. Biol.* **19**, 3760–3768
- Foretz, M., Guichard, C., Ferre, P. and Foulfelle, F. (1999) Sterol regulatory element binding protein-1c is a major mediator of insulin action on the hepatic expression of glucokinase and lipogenesis-related genes. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 12737–12742
- Stoeckman, A. K. and Towle, H. C. (2002) The role of SREBP-1c in nutritional regulation of lipogenic enzyme gene expression. *J. Biol. Chem.* **277**, 27029–27035
- Nouspikel, T. and Iynedjian, P. B. (1992) Insulin signalling and regulation of glucokinase gene expression in cultured hepatocytes. *Eur. J. Biochem.* **210**, 365–373
- Iynedjian, P. B., Roth, R. A., Fleischmann, M. and Gjinovci, A. (2000) Activation of protein kinase B/Akt in hepatocytes is sufficient for the induction of expression of the gene encoding glucokinase. *Biochem. J.* **351**, 621–627
- Fleischmann, M. and Iynedjian, P. B. (2000) Regulation of sterol regulatory-element binding protein 1 gene expression in liver: role of insulin and protein kinase B/Akt. *Biochem. J.* **349**, 13–17
- Hajdich, E., Balendran, A., Batty, I. H., Litherland, G. J., Blair, A. S., Downes, C. P. and Hundal, H. S. (2001) Ceramide impairs the insulin-dependent membrane recruitment of protein kinase B leading to a loss in downstream signalling in L6 skeletal muscle cells. *Diabetologia* **44**, 173–183
- Teruel, T., Hernandez, R. and Lorenzo, M. (2001) Ceramide mediates insulin resistance by tumor necrosis factor- α in brown adipocytes by maintaining Akt in an inactive dephosphorylated state. *Diabetes* **50**, 2563–2571
- Ribaux, P., Gjinovci, A., Sadowski, H. B. and Iynedjian, P. B. (2002) Discrimination between signaling pathways in regulation of specific gene expression by insulin and growth hormone in hepatocytes. *Endocrinology* **143**, 3766–3772
- Iynedjian, P. B., Jotterand, D., Nouspikel, T., Asfari, M. and Pilot, P. R. (1989) Transcriptional induction of glucokinase gene by insulin in cultured liver cells and its repression by the glucagon-cAMP system. *J. Biol. Chem.* **264**, 21824–21829
- Iynedjian, P. B., Marie, S., Gjinovci, A., Genin, B., Deng, S. P., Buhler, L., Morel, P. and Mentha, G. (1995) Glucokinase and cytosolic phosphoenolpyruvate carboxykinase (GTP) in the human liver. Regulation of gene expression in cultured hepatocytes. *J. Clin. Invest.* **95**, 1966–1973
- Hayzer, D. J. and Iynedjian, P. B. (1990) Alternative splicing of glucokinase mRNA in rat liver. *Biochem. J.* **270**, 261–263
- Tontonoz, P., Kim, J. B., Graves, R. A. and Spiegelman, B. M. (1993) ADD1: a novel helix–loop–helix transcription factor associated with adipocyte determination and differentiation. *Mol. Cell. Biol.* **13**, 4753–4759
- Fort, P., Marty, L., Piechaczyk, M., El Sabrouy, S., Dani, C., Jeanteur, P. and Blanchard, J. M. (1985) Various rat adult tissues express only one major mRNA species from the glyceraldehyde-3-phosphate-dehydrogenase multigenic family. *Nucleic Acids Res.* **13**, 1431–1442
- Cross, D. A., Alessi, D. R., Cohen, P., Andjelkovich, M. and Hemmings, B. A. (1995) Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature (London)* **378**, 785–789
- Shepherd, P. R., Withers, D. J. and Siddle, K. (1998) Phosphoinositide 3-kinase: the key switch mechanism in insulin signalling. *Biochem. J.* **333**, 471–490
- Kolesnick, R. (2002) The therapeutic potential of modulating the ceramide/sphingomyelin pathway. *J. Clin. Invest.* **110**, 3–8
- Iynedjian, P. B. and Hanson, R. W. (1977) Increase in level of functional messenger RNA coding for phosphoenolpyruvate carboxykinase (GTP) during induction by cyclic adenosine 3':5'-monophosphate. *J. Biol. Chem.* **252**, 655–662
- Wang, Q., Somwar, R., Bilan, P. J., Liu, Z., Jin, J., Woodgett, J. R. and Klip, A. (1999) Protein kinase B/Akt participates in GLUT4 translocation by insulin in L6 myoblasts. *Mol. Cell. Biol.* **19**, 4008–4018
- van Weeren, P. C., de Bruyn, K. M., de Vries-Smits, A. M., van Lint, J. and Burgering, B. M. (1998) Essential role for protein kinase B (PKB) in insulin-induced glycogen synthase kinase 3 inactivation. Characterization of dominant-negative mutant of PKB. *J. Biol. Chem.* **273**, 13150–13156
- Choi, K. M., McMahon, L. P. and Lawrence, Jr, J. C. (2003) Two motifs in the translational repressor PHAS-I required for efficient phosphorylation by mammalian target of rapamycin and for recognition by raptor. *J. Biol. Chem.* **278**, 19667–19673
- Scott, P. H., Brunn, G. J., Kohn, A. D., Roth, R. A. and Lawrence, Jr, J. C. (1998) Evidence of insulin-stimulated phosphorylation and activation of the mammalian target of rapamycin mediated by a protein kinase B signaling pathway. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 7772–7777
- Li, J., DeFea, K. and Roth, R. A. (1999) Modulation of insulin receptor substrate-1 tyrosine phosphorylation by an Akt/phosphatidylinositol 3-kinase pathway. *J. Biol. Chem.* **274**, 9351–9356
- Ozes, O. N., Akca, H., Mayo, L. D., Gustin, J. A., Maehama, T., Dixon, J. E. and Donner, D. B. (2001) A phosphatidylinositol 3-kinase/Akt/mTOR pathway mediates and PTEN antagonizes tumor necrosis factor inhibition of insulin signaling through insulin receptor substrate-1. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 4640–4645
- Greene, M. W., Sakaue, H., Wang, L., Alessi, D. R. and Roth, R. A. (2003) Modulation of insulin-stimulated degradation of human insulin receptor substrate-1 by serine 312 phosphorylation. *J. Biol. Chem.* **278**, 8199–8211
- Stratford, S., DeWald, D. B. and Summers, S. A. (2001) Ceramide dissociates 3'-phosphoinositide production from pleckstrin homology domain translocation. *Biochem. J.* **354**, 359–368
- van Blitterswijk, W. J., van der Luit, A. H., Veldman, R. J., Verheij, M. and Borst, J. (2003) Ceramide: second messenger or modulator of membrane structure and dynamics? *Biochem. J.* **369**, 199–211
- Vanhaesebroeck, B. and Alessi, D. R. (2000) The PI3K–PDK1 connection: more than just a road to PKB. *Biochem. J.* **346**, 561–576

-
- 31 Coffey, P. J., Jin, J. and Woodgett, J. R. (1998) Protein kinase B (c-Akt): a multifunctional mediator of phosphatidylinositol 3-kinase activation. *Biochem. J.* **335**, 1–13
- 32 Matsumoto, M., Ogawa, W., Teshigawara, K., Inoue, H., Miyake, K., Sakaue, H. and Kasuga, M. (2002) Role of the insulin receptor substrate 1 and phosphatidylinositol 3-kinase signaling pathway in insulin-induced expression of sterol regulatory element binding protein 1c and glucokinase genes in rat hepatocytes. *Diabetes* **51**, 1672–1680
- 33 Walker, K. S., Deak, M., Paterson, A., Hudson, K., Cohen, P. and Alessi, D. R. (1998) Activation of protein kinase B β and γ isoforms by insulin *in vivo* and by 3-phosphoinositide-dependent protein kinase-1 *in vitro*: comparison with protein kinase B α . *Biochem. J.* **331**, 299–308
- 34 Potashnik, R., Bloch-Damti, A., Bashan, N. and Rudich, A. (2003) IRS1 degradation and increased serine phosphorylation cannot predict the degree of metabolic insulin resistance induced by oxidative stress. *Diabetologia* **46**, 639–648
- 35 Johnston, A. M., Pirola, L. and Van Obberghen, E. (2003) Molecular mechanisms of insulin receptor substrate protein-mediated modulation of insulin signalling. *FEBS Lett.* **546**, 32–36
- 36 Ravichandran, L. V., Esposito, D. L., Chen, J. and Quon, M. J. (2001) Protein kinase C- ζ phosphorylates insulin receptor substrate-1 and impairs its ability to activate phosphatidylinositol 3-kinase in response to insulin. *J. Biol. Chem.* **276**, 3543–3549
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