Diabetologia (2009) 52:1197–1207
DOI 10.1007/s00125-009-1336-5

ARTICLE

Role of atypical protein kinase C in activation of sterol regulatory element binding protein-1c and nuclear factor kappa B (NFκB) in liver of rodents used as a model of diabetes, and relationships to hyperlipidaemia and insulin resistance

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Received: 22 January 2009 / Accepted: 25 February 2009 / Published online: 9 April 2009

Abstract
Aims/hypothesis Previous findings in rodents used as a model of diabetes suggest that insulin activation of atypical protein kinase C (aPKC) is impaired in muscle, but, unexpectedly, conserved in liver, despite impaired hepatic protein kinase B (PKB/Akt) activation. Moreover, aPKC at least partly regulates two major transactivators: (1) hepatic sterol receptor binding protein-1c (SREBP-1c), which controls lipid synthesis; and (2) nuclear factor kappa B (NFκB), which promotes inflammation and systemic insulin resistance.
Methods In Goto–Kakizaki rats used as a model of type 2 diabetes, we examined: (1) whether differences in hepatic aPKC and PKB activation reflect differences in activation of IRS-1- and IRS-2-dependent phosphatidylinositol 3-kinase (PI3K); (2) whether hepatic SREBP-1c and NFκB are excessively activated by aPKC; and (3) metabolic consequences of excessive activation of hepatic aPKC, SREBP-1c and NFκB.
Results In liver, as well as in muscle, IRS-2/PI3K activation by insulin was intact, whereas IRS-1/PI3K activation by insulin was impaired. Moreover, hepatic IRS-2 is known to control hepatic aPKC during insulin activation. Against this background, selective inhibition of hepatic aPKC by adenoviral-mediated expression of mRNA encoding kinase-inactive aPKC or short hairpin RNA targeting *Irs2* mRNA and partially depleting hepatic IRS-2 diminished hepatic SREBP-1c production and NFκB activities, concomitantly improving serum lipids and insulin signalling in muscle and liver. Similar improvements in SREBP-1c, NFκB and insulin signalling were seen in *ob/ob* mice following inhibition of hepatic aPKC.
Conclusions/interpretation In diabetic rodent liver, diminished PKB activation may largely reflect impaired IRS-1/PI3K activation, while conserved aPKC activation reflects retained IRS-2/PI3K activity. Hepatic aPKC may also contribute importantly to excessive SREPB-1c and NFκB activities. Excessive hepatic aPKC-dependent activation of SREBP-1c and NFκB may contribute importantly to hyperlipidaemia and systemic insulin resistance.

Keywords Atypical protein kinase C · Diabetes · Insulin · IRS-1 · IRS-2 · Liver · Muscle · Protein kinase B

Abbreviations

aPKC	Atypical protein kinase C
EMSA	Electrophoretic mobility shift assay

Electronic supplementary material The online version of this article (doi:10.1007/s00125-009-1336-5) contains supplementary material, which is available to authorised users.

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GK	Goto–Kakizaki
KI	Kinase-inactive
NFκB	Nuclear factor kappa B
IKK	Inhibitor of κB (IκB) kinase
PI3K	Phosphatidylinositol 3-kinase
PIP_3	Phosphatidylinositol-3,4,5-triphosphate
PKB/Akt	Protein kinase B
PKC	Protein kinase C
shRNA	Short hairpin RNA
SREBP-1c	Sterol receptor binding protein-1c

Introduction

Atypical protein kinase C (aPKC) and protein kinase B (PKB/Akt), operating downstream of phosphatidylinositol 3-kinase (PI3K), mediate specific insulin effects. In muscle and adipocytes, aPKC and PKB co-activate glucose transport and PKB increases glycogenesis. In liver, PKB diminishes glucose production and release; aPKC and PKB together increase lipid synthesis.

Normally, insulin increases glucose uptake and storage in muscle and adipocytes, diminishes hepatic glucose output and increases hepatic lipid synthesis. In type 2 diabetes, as expected, glucose clearance by muscle and adipocytes is diminished and hepatic glucose output is increased, yet inexplicably and paradoxically, lipid synthesis is increased [1].

With regard to the divergent insulin regulation of hepatic glucose and lipid metabolism in two models of type 2 diabetes, Goto–Kakizaki (GK) rats and *ob/ob* mice, PKB activation is impaired in muscle and liver [2, 3], whereas aPKC activation is impaired in muscle [2, 4], but conserved in liver [2].

Conserved aPKC activation in diabetic liver is noteworthy, as aPKC mediates insulin and feeding effects on levels and activation of hepatic sterol receptor binding protein-1c (SREBP-1c) [5, 6], which regulates levels of multiple enzymes engaged in lipid synthesis. Thus, conserved hepatic aPKC activation may contribute to paradoxical increases in lipid synthesis in diabetic liver. In this regard, although PKB co-regulates hepatic SREBP-1c production [7, 8], PKB activation is markedly impaired in diabetic liver [2] and is therefore unlikely to mediate increases in hepatic SREBP-1c levels.

Presently, there is limited information both on SREBP-1c production and activity, and on whether conserved aPKC activity may underlie paradoxical activation of SREBP-1c and SREBP-1c-dependent lipid abnormalities in diabetic liver. Hepatic expression of SREBP-1c is increased in *ob/ob* and lipodystrophic diabetic mice [9], but information in GK rats is lacking. In streptozotocin-induced hypoinsulinaemic models of diabetes, hepatic SREBP-1c expression is depressed, but promptly responds to insulin [10] by an uncertain signalling mechanism.

As with SREBP-1c, conserved hepatic aPKC activation in hyperinsulinaemic diabetic states may excessively activate hepatic nuclear factor kappa B (NFκB), since aPKC phosphorylates and activates IκB kinase (IKK)-β [11, 12], which phosphorylates IKKα/β, thereby negating its restraining and inhibitory effects on NFκB, and allowing nuclear transfer and subsequent action of NFκB to increase expression of cytokines that promote inflammation, atherosclerotic processes and systemic insulin resistance [13, 14]. Along with IKKβ activation, aPKC directly phosphorylates and activates NFκB [12]. It is not known whether insulin activates IKKβ/NFκB in liver.

The reason for conserved aPKC activation and impaired PKB activation in diabetic liver is uncertain, as information on activities of upstream activators, IRS-1- and IRS-2-dependent PI3K, is limited. In GK rats, insulin activation of IRS-1/PI3K is diminished in muscle [4], but there is no information on muscle IRS-2/PI3K or on hepatic IRS-1/PI3K or IRS-2/PI3K. In *ob/ob* mice, activation of IRS-1/PI3K and to a lesser extent IRS-2/PI3K during 1 min insulin treatment was impaired in liver and muscle [15], but longer studies are lacking.

With regard to divergent activation of aPKC and PKB in diabetic liver, insulin activation of aPKC [16] and PKB [16, 17] in IRS-1 knockout mice was impaired in muscle, whereas in liver, PKB activation was impaired [17], but aPKC activation intact [16]. In IRS-2-deficient hepatocytes, activation of aPKC and PKB was impaired [18]. Thus, in liver, aPKC is largely controlled by IRS-2 and PKB is controlled by IRS-1 and IRS-2.

Here, we tested the hypothesis that conserved hepatic aPKC activation by insulin and feeding causes excessive expression and activation of SREBP-1c, and activation of IKKβ/NFκB in GK rats and *ob/ob* mice. We also examined whether conserved aPKC activation and impaired PKB activation in diabetic liver may reflect differences in insulin-induced activation of IRS-2/PI3K and IRS-1/PI3K. Most importantly, since excessive SREBP-1c activity can increase serum lipids and since SREBP-1c (via lipids) and NFκB (via cytokines) can impair insulin action in muscle and liver, we questioned whether limiting the activity of hepatic aPKC or its upstream regulator IRS-2/PI3K (by administration of adenoviruses encoding factors that selectively alter hepatic signalling) would have salutary effects on serum lipids and insulin signalling mechanisms in these tissues.

Methods

Experimental rodents

Male Wistar non-diabetic rats (8–12 weeks of age, weight approx 250 g) were obtained from Harlan Industries

(Indianapolis, IN, USA). Male GK diabetic rats were obtained from our colony at the James A. Haley Veterans Hospital Vivarium in Tampa, FL, USA [2, 4]. Male *ob/ob* mice and lean counterparts (8–12 weeks of age, weight approx 35 g) were from Jackson Industries (Bar Harbor, MN, USA). Rodents were maintained in conditions of constant temperature and humidity, with 12 h light–dark cycle and access to standard rat chow. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of South Florida College of Medicine and the James A. Haley Veterans Administration Medical Center Research and Development Committee, and were in accordance with the Declaration of Helsinki.

Treatments

Rodents were injected intramuscularly or i.p. with physiological saline (0.9% NaCl, wt/vol.) or saline containing insulin (1 U/kg body weight) 5 or 15 min before killing to elicit maximal effects. Where indicated, rats were fasted overnight or fed their usual diet to observe effects of physiological, feeding-dependent increases in serum insulin or treated overnight with saline or long-acting glargine insulin (40 U/kg body weight), thereby increasing serum insulin levels in GK diabetic rats from approximately 480 to 960 pmol/l (fed serum insulin levels in non-diabetic Wistar rats approximately 160 pmol/l [4]) and decreasing serum glucose levels from approximately 13.9 to 8.9 mmol/l (fed serum glucose levels in Wistar rats approximately 8.3 mmol/l [4]).

Muscle and liver homogenates

As previously described [2, 4], quadriceps muscles and liver were homogenised (Polytron; Brinkman Instruments, Westbury, NY, USA) in buffer containing 0.25 mol/l sucrose, 20 mmol/l Tris/HCl (pH 7.5), 2 mmol/l EGTA, 2 mmol/l EDTA, 1 mmol/l phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 10 μg/ml aprotinin, 2 mmol/l $Na_4P_2O_7$, 2 mmol/l Na_3VO_4, 2 mmol/l NaF and 1 μmol/l microcystin. The homogenate was then centrifuged for 10 min at 700 *g* to remove debris, nuclei and fat, and the resulting lysates were supplemented with 1% (vol./vol.) Triton X-100, 0.6% (vol./vol.) Nonidet and 150 mmol/l NaCl, and centrifuged to remove insoluble materials.

Atypical PKC activation

As described [2, 4], aPKCs were immunoprecipitated from cell lysates with a rabbit polyclonal antiserum (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) that recognises the C-termini of PKC-ζ and PKC-λ, collected on Sepharose-AG beads and incubated for 8 min; ^{32}P-labelled substrates trapped on P-81 filter papers were counted.

PI3K activation

Lysate IRS-1/PI3K and IRS-2/PI3K activities were determined as described [2, 4]. In brief, IRS-1 and IRS-2 immunoprecipitates (rabbit polyclonal antibodies; Upstate Cell Signalling) were examined for incorporation of $^{32}PO_4$ into PI-3-PO_4, which was purified by thin-layer chromatography and quantified with a Phosphor-Imager (BioRad, Hercules, CA, USA).

PKB activation

Lysate PKB enzyme activity was measured using a kit (Upstate Cell Signalling, now Millipore, Bedford, MA, USA) as described [2, 4]. In brief, PKB was immunoprecipitated with rabbit polyclonal antibodies, collected on Sepharose-AG beads and incubated as directed. PKB activation was also assessed by immunoblotting for phosphorylation of serine-473.

Immunoblot studies

Western blot analyses were conducted as described [2, 4], using: rabbit polyclonal anti-PKC-ζ/λ antiserum (Santa Cruz Biotechnologies); rabbit polyclonal anti-PKB antiserum (Upstate Cell Signalling); rabbit polyclonal anti-phospho-serine-473-PKB antiserum (Cell Signalling Technology, Danvers, MA, USA); rabbit polyclonal anti-IRS-1 and anti-IRS-2 antisera (Upstate Cell Signalling); rabbit polyclonal anti-IRS-2 antiserum (Upstate Cell Signalling); rabbit polyclonal anti-p85/PI3K antiserum (Upstate Cell Signalling); rabbit polyclonal anti-SREBP-1c antiserum (Santa Cruz Biotechnologies); and rabbit polyclonal anti-phospho-serine-307-IRS-1 (Upstate Cell Signalling).

Adenoviral studies

Adenoviruses encoding kinase-inactive (KI)-*Prkcz* mRNA [19] and short hairpin RNA (shRNA) targeting *Irs2* mRNA [20] have been described previously. Adenovirus vector or adenovirus encoding KI-*Prkcz* mRNA, or shRNA targeting *Irs2* mRNA (2.5×10^{12} plaque forming units [PFU] per kg body weight) were administered intravenously through the tail vein. Where indicated, some rats, on the fourth day, were treated overnight with saline or long-acting glargine insulin (40 U/kg body weight); in other cases, on day 5, the rats were treated for 15 min with saline or regular insulin (1 U/kg body weight) in saline. Note that, relative to saline-treated controls, adenoviral treatments did not alter serum levels of aspartate aminotransferase or alanine aminotransferase, or food intake, and provoked changes in levels or activities of targeted signalling factors only in liver (inhibition of hepatic aPKC was accompanied by improved

insulin signalling to PKB in liver and to IRS-1/PI3K, aPKB and PKB in muscle).

Measurement of *Srebp-1c* and *Fas* mRNA

Hepatic *Srebp-1c* (also known as *Srebf1*) and *Fas* mRNAs were measured as described [21].

Nuclear preparations

Liver nuclei were prepared as described [21] and used: (1) to measure levels of active nuclear SREBP-1c fragment and p65/RelA subunit of NFκB; and (2) to perform NFκB electrophoretic mobility shift assays (EMSAs).

Electrophoretic mobility shift assays

NFκB-dependent shifts in mobility of specific $^{32}PO_4$-labelled DNA sequences were measured with a kit (Invitrogen, Carlsbad, CA, USA). The sequence of the consensus nucleotide NFκB probe provided in the kit used for detection of the NFκB/DNA complex was 5′-AGTTGAGGG GACTTTCCCAGGC-3′ and 3′-TCAACTCCCCTG AAAGGGTCCG-5′.

Serum triacylglycerol, cholesterol and glucose levels

Serum triacylglycerol and glucose levels were measured as described by Farese et al. [21].

Statistical evaluations

Data are expressed as mean±SE. We determined p values by a two-way ANOVA and the least-significant multiple comparison method.

Results

Studies in GK diabetic rats

IRS-1/PI3K and IRS-2/PI3K activation in liver Regarding the question of divergent activation of PKB and aPKC during insulin action in diabetic liver, we found: (1) insulin increased IRS-1/PI3K activity in Wistar non-diabetic liver, but not in GK diabetic liver (Fig. 1a); and in contrast (2) activation of IRS-2/PI3K by insulin was greater in GK diabetic liver (Fig. 1b). Levels of IRS-1, IRS-2 and the p85 subunit of PI3K (Fig. 1e) were comparable in liver of Wistar non-diabetic and GK diabetic rats. We did not observe increases in phosphorylation of Ser-307-IRS-1 in GK diabetic liver, relative to non-diabetic liver (not shown).

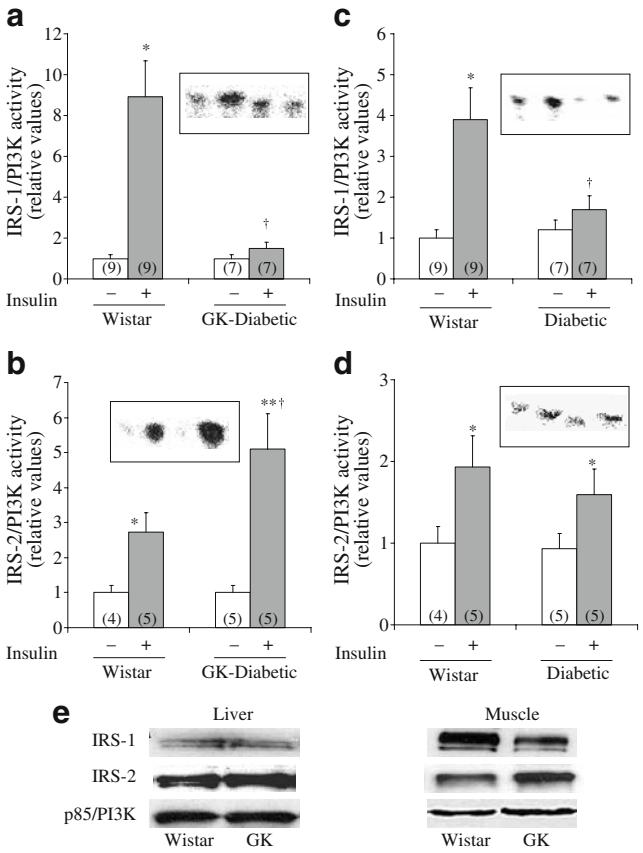

Fig. 1 Activation of IRS-1 (**a**, **c**) and IRS-2 (**b**, **d**) -dependent PI3K by insulin in liver (**a**, **b**) and muscle (**c**, **d**) of Wistar non-diabetic and GK diabetic rats. Insulin (1 U/kg body weight in saline; grey bars) or vehicle (white bars) was administered intramuscularly 15 min before killing in muscle studies and 5 min before killing in liver studies, i.e. at the optimal times for these tissues. Values are mean±SE of (n) rats. *p<0.05, **p<0.01, ***p<0.001 for insulin-stimulated samples vs adjacent basal resting (i.e. unstimulated) samples of the indicated rat group. †p<0.01 for insulin-stimulated samples of GK diabetic rats vs insulin-stimulated samples of Wistar non-diabetic rats. All p values were determined by ANOVA. The corresponding representative autoradiograms of PI3–$^{32}PO_4$, the lipid product of the PI3K assay, are also shown. **e** Representative blots showing levels of IRS-1, IRS-2 and the p85 subunit of PI3K in liver and muscle of GK diabetic and Wistar non-diabetic rats

IRS-1/PI3K and IRS-2/PI3K activation in muscle As previously observed [4], insulin effects on IRS-1/PI3K were diminished in GK diabetic muscle relative to Wistar non-diabetic muscle (Fig. 1c). In contrast, IRS-2/PI3K activation was not significantly diminished in GK diabetic muscle (Fig. 1d). Note that IRS-1 levels showed a downward and IRS-2 levels an upward trend in GK diabetic muscle (Fig. 1e), but changes were not significant. Levels of the PI3K p85 subunit levels were comparable in non-diabetic and GK diabetic muscle (Fig. 1e). As shown in the Electronic supplementary material (ESM) Fig. 1, phosphorylation of Ser-307-IRS-1 was increased in GK diabetic

muscle relative to non-diabetic muscle; in contrast, this phosphorylation was not altered in GK diabetic liver.

Activation of hepatic aPKC and PKB by feeding In Wistar non-diabetic rats, similarly to findings in insulin-treated rats [2], aPKC (Fig. 2a) and PKB (Fig. 2b) activities were increased in fed, relative to fasted rats. These feeding-dependent increases in hepatic aPKC and PKB activities probably reflect physiological increases in insulin secretion elicited by feeding, but other factors may also have contributed. Note that increases seen with insulin treatment in fed non-diabetic rats [2] (Fig. 3) imply that the effects of feeding on hepatic aPKC and PKB were sub-maximal.

Whereas in liver of GK diabetic rats, feeding-stimulated aPKC activity was greater than in Wistar non-diabetic controls (Fig. 2a), PKB activity failed to increase with feeding (Fig. 2b). Thus, the pattern of intact aPKC activation and impaired PKB activation in response to exogenous insulin treatment that had been observed previously in livers of fed GK diabetic rats [2] was reproduced in livers of fed vs fasted GK diabetic rats.

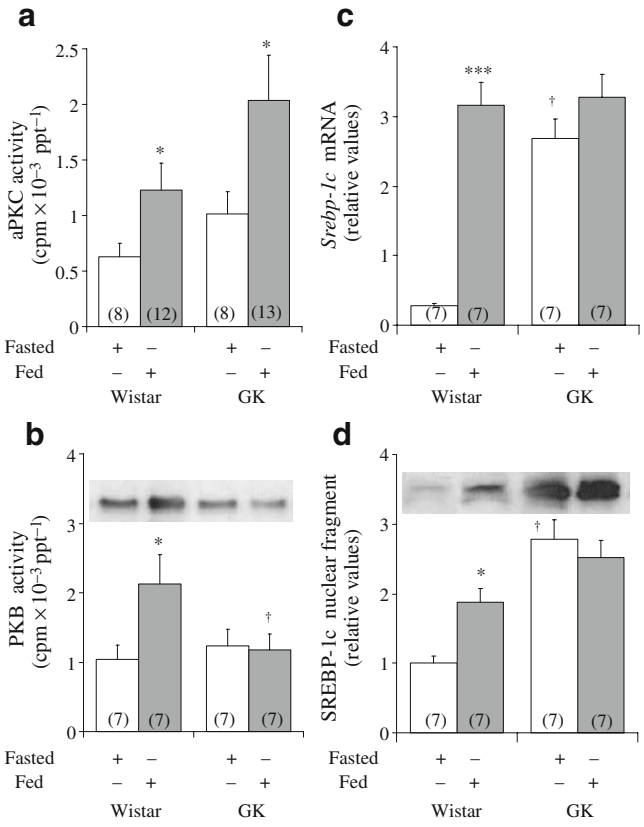

Fig. 2 Effects of feeding on hepatic activities of aPKC (**a**) and PKB (**b**), and on mRNA expression (**c**) and activation (**d**) of hepatic SREBP-1c in Wistar non-diabetic and GK diabetic rats. Rats were either fasted overnight or allowed access to their usual diets. Values are mean±SE of (*n*) rats. **p*<0.05, ****p*<0.001 for feeding-stimulated samples vs adjacent basal resting (i.e. fasting) samples of the indicated rat group. †*p*<0.001 for fasting or feeding-stimulated samples of GK diabetic rats vs fasting or feeding-stimulated samples of Wistar non-diabetic rats. All *p* values were determined by ANOVA. Representative western blots for phospho-Ser-473-PKB (**c**) and SREBP-1c nuclear fragment (**d**) are shown. ppt, immunoprecipitate

Hepatic Srebp-1c expression Relative to fasted Wistar non-diabetic rats, hepatic *Srebpc-1c* mRNA (Fig. 2c) and SREBP-1c active nuclear fragment levels (Fig. 2d) were increased in fed Wistar non-diabetic rats. However, in GK diabetic rats, fasting levels of hepatic *Srebp-1c* mRNA (Fig. 2c) and SREBP-1c nuclear protein levels (Fig. 2d) were increased relative to fasted Wistar non-diabetic rats, but did not increase further with feeding. Note that: (1) serum glucose levels were approximately 10 mmol/l and 5 mmol/l in fed and fasted Wistar rats, vs 18 mmol/l and 10 mmol/l in fed and fasted GK diabetic rats; and (2) treatment of GK diabetic rats with long-acting glargine insulin over 16 h did not elicit further increases in *Srebp-1c* mRNA or nuclear levels (not shown) above those seen with feeding alone. This indicates that activation of SREBP-1c in hyperinsulinaemic GK diabetic rats was persistently maximal, regardless of feeding status.

Effects of adenovirally mediated production of KI-Prkcz on hepatic aPKC and PKB To determine whether aPKC was required for maintaining hepatic SREBP-1c levels in fed GK diabetic rats, these rats were treated with adenovirus encoding KI-*Prkcz* mRNA or adenovirus vector alone. As seen in Fig. 3a, insulin provoked a 2.4-fold increase in hepatic aPKC activity in liver of GK diabetic rats treated with adenovirus vector, similar to previously reported increases observed in fed GK diabetic rats not treated with adenovirus [2]. However, following administration of adenovirus encoding KI-*Prkcz* mRNA, which increased total hepatic aPKC levels (Fig. 3a), insulin-induced increases in hepatic aPKC activity were virtually abolished in GK diabetic rats (Fig. 3a).

In contrast to hepatic aPKC, insulin-induced activation and Ser-473 phosphorylation of hepatic PKB was, as expected [2], markedly impaired in GK diabetic rats treated with adenovirus alone; however, insulin-stimulated PKB activity (Fig. 3b) and Ser-473 phosphorylation (Fig. 3b) improved substantially following treatment with adenovirus encoding KI-*Prkcz* mRNA.

Effects of adenovirally mediated production of kinase-inactive PKC-ζ on hepatic SREBP-1c and FAS Compared with results in GK diabetic rats treated with adenovirus vector alone, the administration of adenovirus encoding KI-*Prkcz* mRNA diminished hepatic levels of *Srebp-1c* mRNA (Fig. 4a) and the active nuclear SREBP-1c fragment (Fig. 4b). Similarly, hepatic *Fas* mRNA, which is regulated by SREBP-1c, was diminished by adenovirally mediated

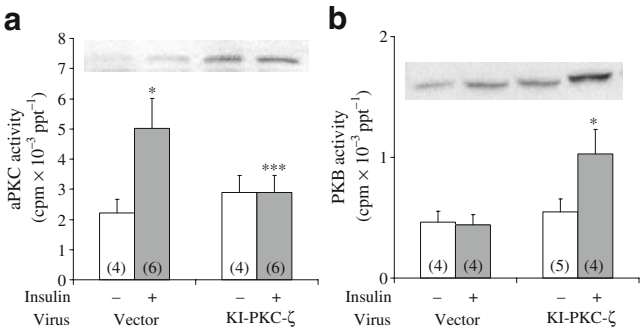

Fig. 3 Effects of adenovirally mediated production of KI-PKC-ζ on basal and insulin-stimulated activities of aPKC (**a**) and PKB (**b**) in liver of GK diabetic rats. Blot (**a**) shows KI-PKC-ζ-induced increases in levels of hepatic total aPKC; blot (**b**) shows immunoreactive phospho-Ser-473-PKB (representative blot). Insulin treatment in these studies was for 15 min, i.e. optimal for the depicted signalling factors. Values are mean±SE of (*n*) rats. **p*<0.05 for insulin-stimulated samples vs adjacent basal resting (i.e. unstimulated) samples of the indicated group. ****p*<0.001 for insulin-stimulated samples of vector-treated and KI-ζ-treated rats. All *p* values were determined by ANOVA. ppt, immunoprecipitate

production of KI-PKC-ζ (Fig. 4c). These findings suggest that the observed increases in SREBP-1c levels in GK diabetic liver were at least partly dependent on continued activation of hepatic aPKC.

Effects of adenovirally mediated production of KI-PKC-ζ on serum triacylglycerol and glucose As seen in Fig. 4d, serum triacylglycerol was increased in GK diabetic rats, relative to Wistar non-diabetic rats. Interestingly, in association with decreases in hepatic aPKC activity and mRNA and nuclear fragment levels of SREBP-1c following administration of adenovirus encoding KI-*Prkcz* mRNA, serum triacylglycerol diminished in GK diabetic rats (Fig. 4d). Decreases in serum glucose in response to insulin treatment were slightly but not significantly greater following administration of adenovirus encoding KI-*Prkcz* mRNA (Fig. 4e).

Effects of adenovirally mediated production of KI-PKC-ζ on hepatic IKKβ and NFκB As discussed, IKKβ and NFκB are regulated by aPKC in some cell types [11, 12] and chronic IKKβ and NFκB activation in mice fed a high-fat diet contributes importantly to systemic insulin resistance [13, 14]; however, effects of insulin and diabetes on these activities are unknown. Regarding these questions, we found that: (1) 16 h overnight treatment with long-acting glargine insulin provoked increases in hepatic IKKβ activity (Fig. 5a), NFκB electophoretic mobility shift activity (Fig. 5c) and active p65/RelA/NFκB nuclear levels (Fig. 5d) in fed Wistar non-diabetic rats (in contrast, acute 15 min insulin treatment was without effect, data not shown); and (2) activity of IKKβ (Fig. 5a) and NFκB (EMSA) (Fig. 5b,c), as well as active p65/RelA/NFκB nuclear levels (Fig. 5d) were increased in fed GK diabetic rats, although not further increased by overnight glargine insulin treatment. Moreover, after treatment of GK diabetic rats with adenovirus encoding KI-*Prkcz* mRNA, the activities of hepatic IKKβ (Fig. 5a) and NFκB (Fig. 5b–d) diminished to or below the levels seen in fed Wistar non-diabetic rats.

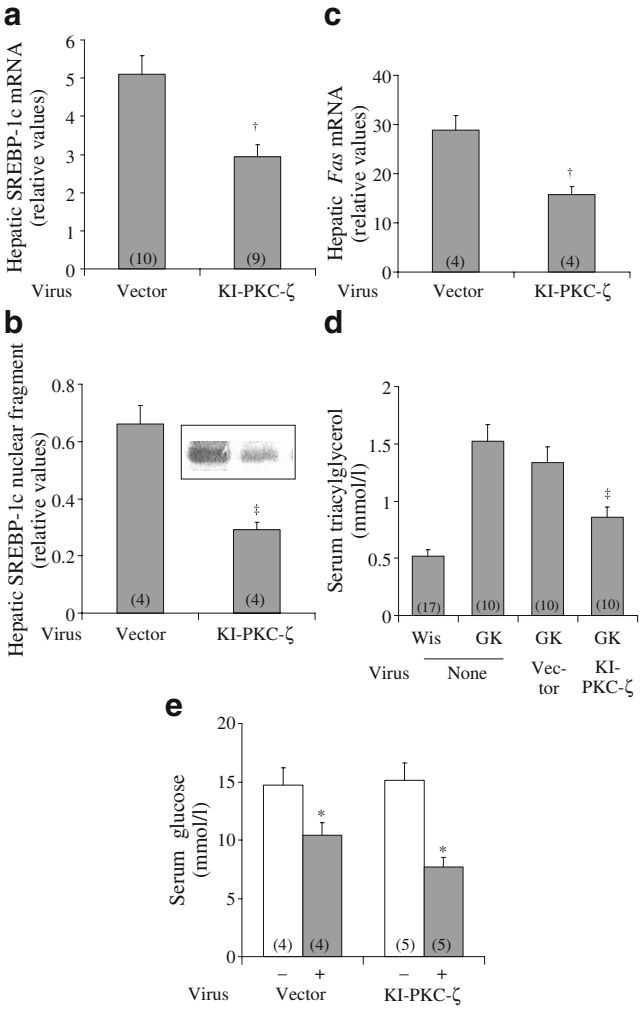

Fig. 4 Effects, in fed GK rats, of adenovirally mediated production of KI-PKC-ζ on **a** mRNA expression and **b** numerical values (with corresponding representative blot) active nuclear fragment levels of *Srebp-1c*, as well as on expression of *Fas* (**c**) in liver. **d** Effects of above on fasting serum triacylglycerol levels and **e** unstimulated and insulin-stimulated serum glucose levels. Rats were injected intravenously with adenovirus vector or adenovirus encoding KI-PKC-ζ; 5 days later (to allow time for expression), rats were treated where indicated with insulin for 15 min. Values are mean±SE of (*n*) rats. *p* values were determined by ANOVA. **p*<0.05, insulin-stimulated vs non-insulin-stimulated; †*p*<0.05 and ‡*p*<0.005, KI-PKC-ζ vs Vector

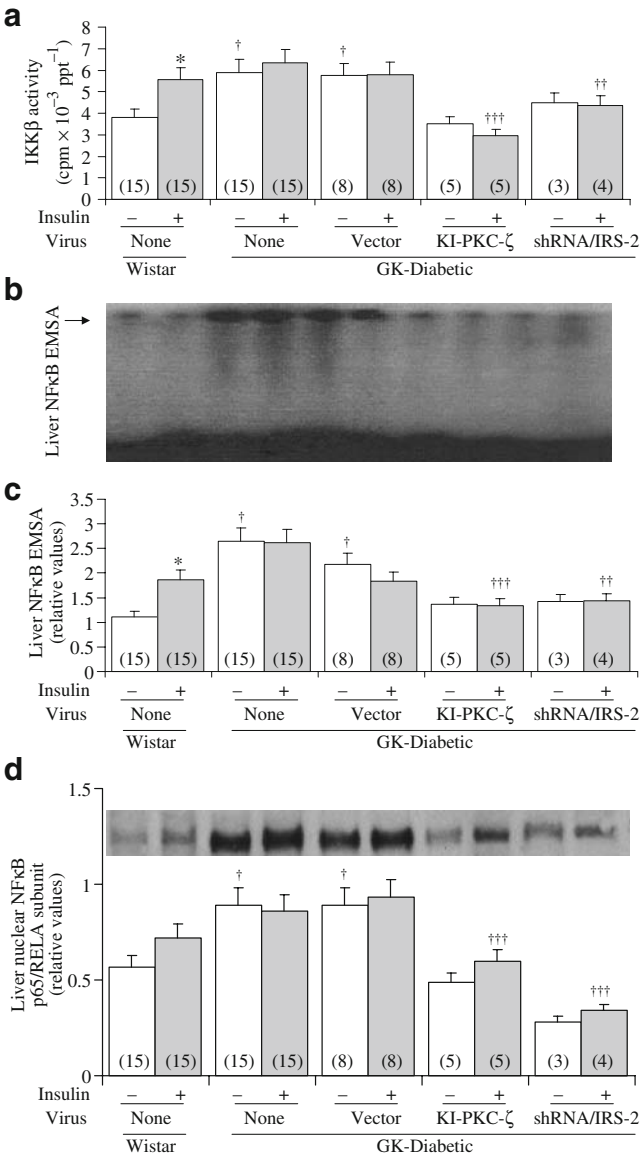

Fig. 5 Effects of adenovirally mediated production of KI-PKC-ζ or shRNA targeting *Irs2* mRNA on hepatic IKKβ activity (**a**), hepatic NFκB-dependent EMSA mobility shifts (**b**, **c**) and hepatic nuclear levels of the p65RelA subunit of NFκB (**d**) in fed GK diabetic rats. **b** Representative autoradiogram of NFκB EMSA mobility shifts of the indicated groups; the level of migration of the NFκB/DNA complex was determined with a standard provided with the assay kit and is shown by the arrow; the electrophoretic front is at the bottom of the autoradiogram. The representative immunoblot (**d**) is of nuclear levels of the p65/RelA subunit of NFκB. Where indicated, rats were injected intravenously with adenovirus vector alone or with adenovirus encoding KI-*Prkcz* mRNA or shRNA targeting *Irs2* mRNA. Five days later (to allow time for expression and knockdown), rats were treated intramuscularly overnight with saline or glargine insulin (40 U/kg body weight), as indicated. For comparisons in assays, basal and insulin-treated Wistar non-diabetic rats were examined in a similar manner. Values are mean ± SE of (*n*) rats. *p* values are by ANOVA. **p*<0.05, insulin-stimulated vs basal unstimulated Wistar; †*p*<0.05, unstimulated GK vs unstimulated Wistar; ††*p*<0.01 and †††*p*<0.001, GK KI-PKC-ζ-treated or GK shRNA/IRS-2-treated vs GK Vector-treated. Note that groups in all panels (**a**–**d**), including lanes in the representative autoradiogram (**b**) and immunoblots (**d**) are vertically aligned and identified at the bottom (**d**)

Effects of adenovirally mediated production of KI-PKC-ζ on muscle IRS-1/PI3K, aPKC and PKB activity Similar to previous findings in virus-untreated GK diabetic rats [2, 4] (Fig. 1c), insulin activation of aPKC (Fig. 6a) and IRS-1/PI3K (Fig. 6d), but not of PKB (Fig. 6c), was markedly impaired in muscles of adenoviral vector-treated GK diabetic rats. Remarkably, and completely opposite to findings in liver, insulin activation of aPKC after administration of adenovirus encoding KI-*Prkcz* mRNA was substantially increased in muscles of GK diabetic rats (Fig. 6a). This improvement in muscle aPKC activation in GK diabetic rats following treatment with adenovirus encoding KI-*Prkcz* mRNA was accompanied by and presumably at least partly due to improved activation of IRS-1/PI3K (Fig. 6d). Moreover, improvement in muscle

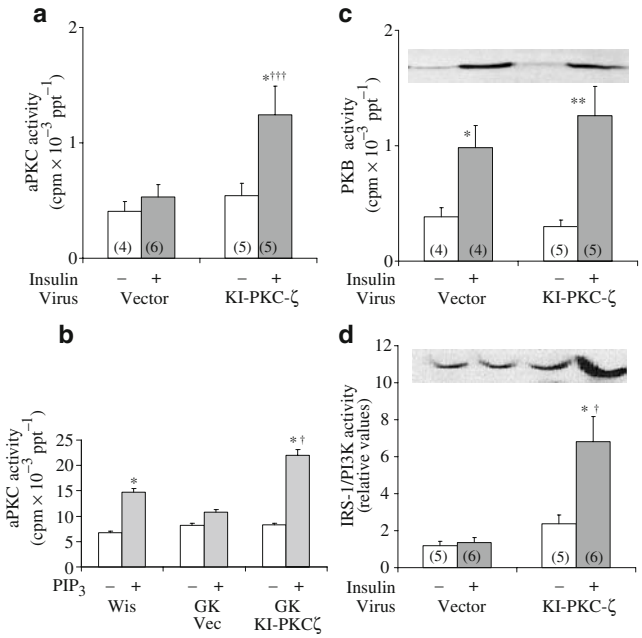

Fig. 6 Effects of adenovirally mediated production of KI-PKC-ζ on basal and insulin-stimulated activities of aPKC (**a**), PKB (**c**) and IRS-1-dependent PI3K (**d**) in vastus lateralis muscle of GK diabetic rats, with (**c**) representative immunoblot of phospho-Ser-473-PKB and (**d**) representative autoradiogram of PI-3-$^{32}PO_4$, the lipid product of the PI3K assay. (**b**) Bar graph of 10 μmol/l PIP_3-dependent activation of aPKC immunoprecipitated from muscles of indicated rats. Insulin treatment in these studies was for 15 min, i.e. optimal for the depicted signalling factors. Values are mean ± SE of (*n*) rats. All *p* values were determined by ANOVA. **p*<0.05, ***p*<0.01 for insulin-stimulated samples vs adjacent basal (i.e. unstimulated) samples of the indicated rat group. †*p*<0.05, †††*p*<0.001 for KI-PKC-ζ-treated samples of GK diabetic rats vs vector-treated samples of GK diabetic rats. ppt, immunoprecipitate

IRS-1/PI3K activation may have been due in part to diminished phosphorylation of serine-307-IRS-1, which was inordinately increased in muscles of GK diabetic rats (ESM Fig. 1).

In addition to improved activation of aPKC in muscles of GK diabetic rats in response to insulin treatment in vivo, responsiveness of immunoprecipitated muscle aPKC to phosphatidylinositol-3,4,5-triphosphate (PIP_3) was markedly improved following treatment with adenovirus encoding KI-*Prkcz* mRNA (Fig. 6b) (note increases in vector-treated GK rats, and normal increases in PIP_3-stimulated activity of immunoprecipitated aPKC obtained from Wistar non-diabetic rats). Presumably, this increase in responsiveness to PIP_3, in addition to increases in muscle IRS-1/PI3K activation, contributed to improved effects of insulin on muscle aPKC activity in GK diabetic rats treated with adenovirus encoding KI-*Prkcz* mRNA.

Note that, since PKB activation is diminished only slightly, if at all, in muscle of fed GK diabetic rats treated with comparably high doses of insulin [2, 4], it was not surprising that muscle PKB activation showed only a modest upward trend following administration of adenovirus encoding KI-*Prkcz* mRNA (Fig. 6c).

Effects of shRNA-mediated knockdown of hepatic IRS-2 on: hepatic aPKC, PKB, SREBP-1c, FAS and NFκB; muscle aPKC and IRS-1/PI3K; and serum triacylglycerol As discussed above, previous findings suggest that, unlike muscle, hepatic aPKC activation by insulin is dependent on IRS-2 [18], rather than on IRS-1 [16], whereas PKB activation is dependent on IRS-1 or IRS-2 or both [16–18]. Accordingly, administration of adenovirus encoding shRNA targeting *Irs2* mRNA, diminished IRS-2 protein levels within 5 days, as reported [20] (Fig. 7a), but note that IRS-2 levels

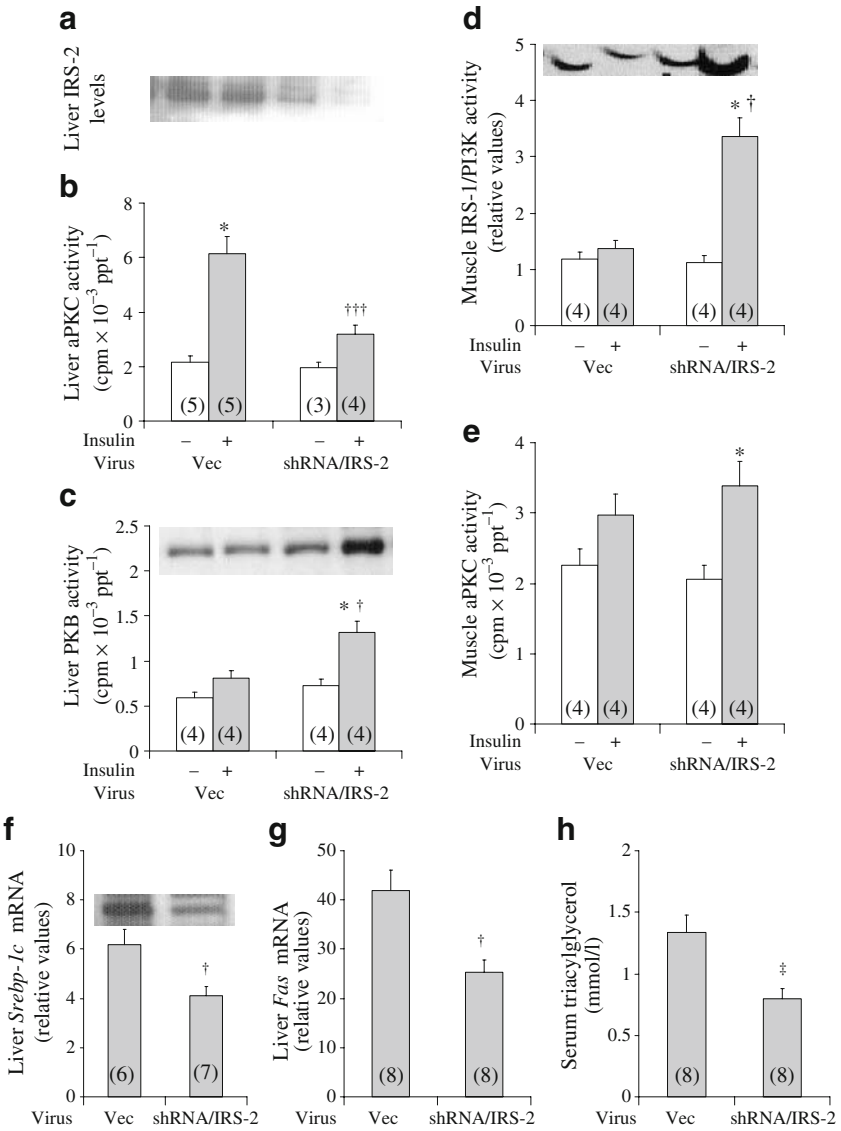

Fig. 7 Effects of adenovirally mediated expression of shRNA targeting *Irs2* mRNA on basal and insulin-stimulated levels of immunoreactive IRS-2 (**a**), aPKC (**b**) and PKB (**c**) activities in liver, and on basal and insulin-stimulated muscle activities of IRS-1-dependent PI3K (**d**) and aPKC (**e**) in vastus lateralis muscle. Effects as above on mRNA expression of *Srebp-1c* (**f**) and *Fas* (**g**) in liver, and on serum triacylglycerol (**h**) of fed GK diabetic rats. **c** Immunoreactive blot of phospho-ser-473-PKB; (**d**) autoradiogram of PI3-$^{32}PO_4$, the lipid product of the PI3K assay; and (**f**) immunoreactive blot of hepatic nuclear SREBP-1c fragment. Rats were injected intravenously with adenovirus vector (Vec) or adenovirus encoding shRNA targeting *Irs2* mRNA. Five days later, mice were treated intramuscularly for 15 min with saline (white bars) or insulin in saline (grey bars) before killing and removal of muscle and liver for analyses. Values are mean ± SE of (*n*) rats. All *p* values were determined by ANOVA. *$p<0.05$ for insulin-stimulated vs adjacent basal samples from rats treated with comparable virus. †$p<0.05$, †††$p<0.001$, ‡$p<0.005$ for indicated groups, i.e. insulin-stimulated adenovirus encoding shRNA targeting *Irs2* mRNA vs insulin-stimulated adenovirus vector. ppt, immunoprecipitate

in muscle were not altered (data not shown). Moreover, this decrease in hepatic IRS-2 markedly reduced insulin-stimulated aPKC activation (Fig. 7b) in GK diabetic liver. In marked contrast, hepatic PKB activation, which was markedly impaired in GK diabetic rats treated with adenovirus alone, significantly improved following hepatic IRS-2 depletion (Fig. 7c). This improvement in hepatic PKB activation in the face of sh-RNA-mediated hepatic IRS-2 depletion presumably reflected improved activation of IRS-1 and/or other forms of IRS by insulin.

In association with shRNA-mediated IRS-2 depletion and diminished insulin activation of aPKC in liver, insulin activation of IRS-1/PI3K (Fig. 7d) and aPKC (Fig. 7e) improved in muscle in GK diabetic rats. The improvement in muscle IRS-1/PI3K activation in muscle may have been due in part to diminished phosphorylation of serine-307-IRS-1, which was inordinately elevated in GK diabetic rats (ESM Fig. 1). Additionally, inordinate increases in hepatic *Srebp-1c* mRNA (Fig. 7f), SREBP-1c nuclear fragment levels (Fig. 7f), *Fas* mRNA (Fig. 7g), IKKβ activity (Fig. 5a), active nuclear p65RelA/NFκB levels (Fig. 5d) and NFκB EMSA activity (Fig. 5b,c) diminished following administration of adenovirus encoding shRNA targeting *IRS-2* mRNA in GK diabetic rats. Moreover, in conjunction with improved hepatic SREBP-1c, serum triacylglycerol levels (Fig. 7h) diminished in GK diabetic rats after administration of adenovirus encoding shRNA targeting *Irs2* mRNA.

As these improvements induced by IRS-2 knockdown were similar to those seen with inhibition of hepatic aPKC and since, in addition, IRS-2 activates hepatic aPKC, it follows that effects of IRS-2 depletion were at least partly due to decreased hepatic aPKC activation.

Studies in *ob/ob* mice

Effects of adenovirally mediated production of KI-PKC-ζ on hepatic aPKC, SREBP-1c and NFκB Despite poor activation of aPKC and PKB in muscle and poor activation of PKB in liver, aPKC activation in liver is intact [2] and, moreover, accompanied by excessive activation of hepatic SREBP-1c [9] in *ob/ob* obese diabetic mice. Comparable findings were seen in the *ob/ob* mice studied here (Fig. 8). We also found that, as in GK diabetic rats, administration of adenovirus encoding KI-*Prkcz* mRNA diminished hepatic aPKC activity (Fig. 8a) as expected and that this was accompanied by decreases in hepatic *Srebp-1c* mRNA and active nuclear fragment levels (Fig. 8e), SREBP-1c-dependent *Fas* expression (Fig. 8f), nuclear levels of hepatic p65/RelA/NFκB (Fig. 8g) and NFκB-dependent EMSA gel-shift activity (Fig. 8h). Furthermore, unlike hepatic aPKC activity, muscle aPKC activity increased (Fig. 8b) and was accompanied by increases in activities of hepatic PKB (Fig. 8c) and muscle PKB (Fig. 8d) following treatment with adenovirus encoding

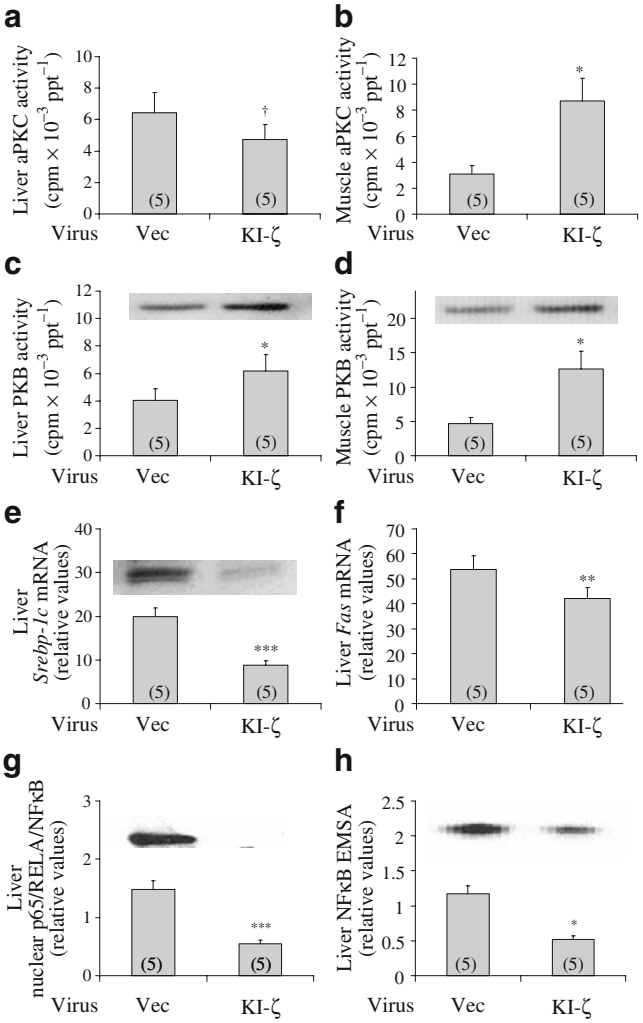

Fig. 8 Effects of adenovirally mediated production of KI-PKC-ζ on activities of aPKC (**a**) and PKB (**c**) in liver, and on aPKC (**b**) and PKB (**d**) in vastus lateralis muscle. **e** Effects as above on mRNA expression and nuclear levels (blot) of SREBP-1c, and **f** on *Fas* mRNA, **g** on nuclear levels and representative blot of p65/RelA subunit of NFκB and **h** on NFκB-dependent mobility shift values and representative autoradiogram in EMSAs in livers of fed *ob/ob* mice. Fed obese/diabetic *ob/ob* mice (presumably well-stimulated by marked hyperinsulinaemia) were injected intravenously with adenovirus vector (Vec) or adenovirus encoding *KI-PKC-ζ* mRNA (KI-ζ). Five days later (to allow time for expression), mice were killed, and muscle and liver removed and processed for analyses. Values are mean±SEM of (*n*) mice. **p*<0.05, ***p*<0.01, ****p*<0.001 and †*p*<0.02 for indicated groups, i.e. virus encoding KI-*Prkcz* vs Vector ppt, immunoprecipitate

KI-*Prkcz* mRNA. Like PKB activity, phosphorylation (pSer-473) of hepatic PKB and muscle PKB respectively increased from 1.00±0.29 to 3.16±0.76 ($p<0.05$) and from 1.00±0.35 to 2.85±0.58 ($p<0.05$).

It should be noted that IRS-2/PI3K activation, as measured 1 min after insulin administration, is impaired in *ob/ob* mice [15]. However, we observed a significant ($p<0.05$) increase in insulin-stimulated hepatic IRS-2/PI3K activity, i.e. from

1.00±0.20 (mean ± SE; n=7) to 1.94±0.23 (mean±SE; n=9), in *ob/ob* mice treated for 15 min with saline or insulin, respectively; this increase was only 28% less than that seen in lean controls.

Discussion

Findings in GK diabetic rats and *ob/ob* mice were consistent with the idea that conserved hepatic aPKC activation was explained by intact or retained IRS-2/PI3K activation, whereas diminished PKB activation was largely explained by impaired IRS-1/PI3K activation. This is also consistent with findings discussed above in IRS-1 knockout mice [16, 17] and IRS-2-deficient hepatocytes [18], and is further supported by our finding of diminished hepatic aPKC, but increased PKB activation in GK diabetic liver in which IRS-2 had been partially depleted by adenoviral/shRNA treatment. Also worthy of note is the fact that, in streptozotocin-induced diabetic rats, insulin activation of PKB and IRS-1/PI3K were markedly impaired, whereas activation of aPKC and IRS-2/PI3K were fully conserved (M. P. Sajan, M. L. Standaert, R. V. Farese, unpublished observations).

The diminished activation of IRS-1/PI3K, but not IRS-2/PI3K seen in GK diabetic muscle and liver raised the possibility of increased phosphorylation of inhibitory residues specific to IRS-1. In this regard, phosphorylation of serine-307-IRS-1 was increased in GK diabetic muscle and was also, moreover, reduced by inhibition of hepatic aPKC owing to production of KI-PKC-ζ or partial depletion of IRS-2. However, no such changes in serine-307-IRS-1 phosphorylation were observed in GK diabetic liver. Also in this regard, phosphorylation of serine-307-IRS-1 can be increased by multiple signalling pathways, and further studies are needed to identify the relevant pathway(s) in GK diabetic muscle.

Conserved aPKC activation in liver of GK diabetic rats and *ob/ob* mice is noteworthy for several reasons. First, aPKC participates in mediating insulin effects on hepatic SREBP-1c levels [5, 6], which transactivate an array of enzymes promoting lipid synthesis, and which are increased in these and other insulin-resistant hyperinsulinaemic states, including lipodystrophic mice [9] and muscle-specific PKC-λ knockout mice [21]. Accordingly, it was important to find that inhibition of hepatic aPKC was accompanied, not only by sizable decreases in expression and activation of hepatic SREPB-1c and FAS in both diabetes models, but also by remarkable improvement in serum triacylglycerol levels in GK diabetic rats. It may therefore be surmised that hepatic aPKC plays a pivotal role in the pathogenesis of excessive hepatic lipid synthesis and hyperlipidaemia in hyperinsulinaemic forms of diabetes.

Second, the conserved ability of insulin to activate aPKC appeared to be largely responsible for the excessive IKKβ and NFκB activation observed by us in diabetic liver. This is particularly important, as NFκB promotes the development of systemic insulin resistance [13, 14] and inflammatory responses that may abet thrombotic tendencies in atherosclerotic processes.

Improvements in insulin signalling to IRS-1/PI3K, aPKC and PKB in muscle, and to PKB in liver, as observed with inhibition of hepatic aPKC in GK diabetic rats and *ob/ob* mice, are most likely attributable to improvements in SREBP-1c-dependent hepatic lipid synthesis or NFκB-dependent cytokine production or both, as both have been linked to systemic insulin resistance [5, 13, 14, 22]. Although further studies are needed to determine relative contributions of SREBP-1c and NFκB to alterations in insulin signalling in diabetic muscle and liver, the fact that both factors are largely controlled by hepatic aPKC may facilitate development of a therapeutic agent that simultaneously limits excessive and deleterious activities of both factors.

The excessive increases in activities of SREBP-1c and NFκB observed by us in diabetic liver were apparently at least partly due to hyperinsulinaemia acting upon hepatic IRS-2 and aPKC. These increases in insulin action in diabetic liver contrast sharply with decreases in actions mediated through PKB. Stated differently, insulin actions in type 2 diabetic liver may be simultaneously excessive and deficient. Thus whereas metabolic processes in diabetic liver which are primarily controlled by signalling through IRS-1/2/PI3K and PKB, such as expression of genes diminishing hepatic glucose production and release, are likely to be impaired; those processes largely controlled by signalling through IRS-2, aPKC, SREBP-1c and NFκB, such as hepatic lipid synthesis and cytokine production, may be hyperactive, normoactive or hypoactive, depending upon circulating insulin levels. This bifurcation of insulin signalling to IRS-2/aPKC (conserved) and IRS-1/PKB (impaired) in liver provides a reasonable explanation for the paradoxical retention of lipid effects and simultaneous loss of carbohydrate effects of insulin and feeding in liver of type 2 diabetic animals [1].

It should be emphasised that, although excessive activation of hepatic aPKC in hyperinsulinaemic diabetic states is in part deleterious and hepatic aPKC is therefore a reasonable therapeutic target, activation of aPKC is critically needed, both for insulin-stimulated glucose transport in muscle [21] and adipocytes [19], and for glucose-dependent insulin secretion in pancreatic islet beta cells [23]. Thus, therapeutically effective aPKC inhibitors must be tailored to selectively inhibit hepatic aPKC. Development of such inhibitors promises to be a daunting, but, if successful, rewarding challenge.

Acknowledgements This study was supported by funds from the Department of Veterans Affairs Merit Review Program (to R. V. Farese) and from the National Institutes of Health Research grants DK-38079 (to R. V. Farese) and DK-30136 (C. R. Kahn).

Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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