**IκB kinase β (IKKβ) does not mediate feedback inhibition of the insulin signalling cascade**

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INTRODUCTION

Insulin resistance, which can be defined as a failure of insulin to regulate the metabolism of its target tissues [1], is a hallmark of obesity and is implicated in the development of Type 2 diabetes. The insulin signalling cascade consists of a complex and highly integrated network that regulates a diverse array of cellular responses such as glucose uptake, glucose synthesis, gluconeogenesis, protein synthesis, cell growth and differentiation [2]. Binding of insulin to the insulin receptor activates the intrinsic tyrosine kinase activity of the latter, resulting in receptor autophosphorylation as well as IRS (insulin receptor substrate) phosphorylation, which promotes the recruitment of PI3K (phosphoinositide 3-kinase) to the plasma membrane and, consequently, the phosphorylation of PKB (protein kinase B) and 2-deoxyglucose uptake in differentiated 3T3-L1 adipocytes. BMS-345541 did not prevent insulin-induced IRS1 serine phosphorylation on known IKKβ target sites. Secondly, adenovirus-mediated overexpression of wild-type IKKβ in differentiated 3T3-L1 adipocytes did not suppress insulin-stimulated 2-deoxyglucose uptake, IRS1 tyrosine phosphorylation, PI3K phosphorylation or 2-deoxyglucose uptake in differentiated 3T3-L1 adipocytes. BMS-345541 did not prevent insulin-induced IRS1 serine phosphorylation on known IKKβ target sites. Thirdly, insulin signalling was not potentiated in mouse embryonic fibroblasts lacking IKKβ. Finally, insulin treatment of 3T3-L1 adipocytes did not promote the recruitment of IKKβ to IRS1, supporting our findings that IKKβ, although activated by insulin, does not promote direct serine phosphorylation of IRS1 and does not contribute to the feedback inhibition of the insulin signalling cascade.

Key words: adipocyte, diabetes, inhibitor of nuclear factor κB kinase β (IKKβ), insulin, phosphoinositide 3-kinase (PI3K), protein kinase B (PKB).

In the present study, we have examined whether IKKβ [IκB (inhibitor of nuclear factor κB) kinase β] plays a role in feedback inhibition of the insulin signalling cascade. Insulin induces the phosphorylation of IKKβ, in vitro and in vivo, and this effect is dependent on intact signalling via PI3K (phosphoinositide 3-kinase), but not PKB (protein kinase B). To test the hypothesis that insulin activates IKKβ as a means of negative feedback, we employed a variety of experimental approaches. First, pharmacological inhibition of IKKβ via BMS-345541 did not potentiate insulin-induced IRS1 (insulin receptor substrate 1) tyrosine phosphorylation, PKB phosphorylation or 2-deoxyglucose uptake in differentiated 3T3-L1 adipocytes. BMS-345541 did not prevent insulin-induced IRS1 serine phosphorylation on known IKKβ target sites. Secondly, adenovirus-mediated overexpression of wild-type IKKβ in differentiated 3T3-L1 adipocytes did not suppress insulin-stimulated 2-deoxyglucose uptake, IRS1 tyrosine phosphorylation, PI3K phosphorylation or 2-deoxyglucose uptake in differentiated 3T3-L1 adipocytes. BMS-345541 did not prevent insulin-induced IRS1 serine phosphorylation on known IKKβ target sites. Thirdly, insulin signalling was not potentiated in mouse embryonic fibroblasts lacking IKKβ. Finally, insulin treatment of 3T3-L1 adipocytes did not promote the recruitment of IKKβ to IRS1, supporting our findings that IKKβ, although activated by insulin, does not promote direct serine phosphorylation of IRS1 and does not contribute to the feedback inhibition of the insulin signalling cascade.

INTRODUCTION

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The serine phosphorylation of IRS1 also serves as an important negative regulatory mechanism during insulin signalling, interfering with tyrosine phosphorylation and thus preventing the propagation of insulin signal transduction. More than 100 potential serine phosphorylation sites are present in IRS1, and IRS1 serine phosphorylation plays a particularly important role in the development of obesity-associated insulin resistance [3], with numerous serine kinases, e.g. JNK (c-Jun N-terminal kinase), IKKβ [IκB (inhibitor of nuclear factor-κB) kinase β], mTOR, S6K (ribosomal S6 kinase), ERK (extracellular-signal-regulated kinase) 1/2, mPLK (mouse pelle-like kinase), PKCθ (protein kinase Cθ) and AMPK (AMP-activated protein kinase), implicated in the development of insulin resistance in response to a wide range of stimuli [4].

However, it is important to note that IRS1 serine phosphorylation does not exclusively mediate cellular insulin resistance in the context of obesity. Insulin rapidly promotes IRS1 serine phosphorylation [5] as a means of feedback inhibition of the insulin signalling cascade. Although the role of JNK as a mediator...
of inflammation-induced insulin resistance is well established [6–8], it has also been demonstrated that insulin rapidly activates JNK, and this acute insulin-induced JNK activation negatively regulates the insulin signalling cascade via transient IRS1 Ser10 phosphorylation [5]. The identity of other insulin-responsive serine kinases that contribute to the acute feedback inhibition of the insulin signalling cascade is not known. In an analogous manner to JNK, IKKβ is also implicated in inflammation-induced insulin resistance [9,10]. Thus IKKβ has been shown to interact physically with IRS1 and promote serine phosphorylation [11]. Additionally, TNFα (tumour necrosis factor α)-induced IKKβ activation promotes IRS1 serine phosphorylation and, consequently, insulin resistance [12,13].

Insulin treatment has been demonstrated to activate the NF-κB (nuclear factor κB) family of transcription factors [14,15], an effect that is likely to be due to the activation of catalytic α and β subunits of the IKK complex. Collectively, these findings led us to hypothesize that insulin rapidly activates IKKβ and that insulin-activated IKKβ plays a role in the feedback inhibition of insulin signalling via the serine phosphorylation of IRS1. We have shown that insulin does, indeed, rapidly activate IKKβ in adipocytes, both in vitro and in vivo, in a PI3K-dependent manner. However, in contrast with our expectations, insulin-induced IKKβ activation does not regulate IRS1 serine phosphorylation and therefore does not contribute to the feedback inhibition of insulin signalling.

EXPERIMENTAL

Animal experiments

Male C57/B16 mice were fed either standard mouse chow (8% of calories from fat, 21% from protein, 71% from carbohydrate) or on high-fat diets [42% of calories from fat (lard), 20% from protein and 35% from carbohydrate] starting at 8 weeks of age, for a period of 12 weeks. Following completion of the 12-week dietary period, animals were anaesthetized and the peritoneal cavity was ‘opened’. Samples of liver, epididymal fat and skeletal muscle (quadriceps) were collected and snap-frozen in liquid nitrogen. From these samples, additional samples of liver, epididymal fat and skeletal muscle were collected. These samples were subsequently homogenized in protein lysis buffer and the phosphorylation status of IKKβ was assessed by Western blotting, as described below. All procedures were approved by the Alfred Medical Research and Education Precinct animal ethics committee and were in accordance with the National Health and Medical Research Council of Australia guidelines for animal experimentation.

Cell culture, treatments, lysis, immunoprecipitations, Western blotting and real-time PCR

3T3-L1 fibroblasts were grown and passaged in DMEM (Dulbecco’s modified Eagle’s medium) supplemented with 10% (v/v) FBS (fetal bovine serum), 100 units/ml penicillin and 0.1 mg/ml streptomycin. Cells were used for differentiation at 2 days post-confluence by treatment with 250 nM dexamethasone, 0.5 mM IBMX (3-isobutyl-1-methylxanthine) and 350 nM insulin in DMEM containing 5% (v/v) FBS. After 3 days, the differentiation medium was replaced with DMEM supplemented with 5% (v/v) FBS and 350 nM insulin. After 2 days, insulin medium was replaced with maintenance medium [DMEM with 5% (v/v) FBS] for a further 2–3 days until cells displayed fat droplets, before overnight serum-starvation and subsequent treatment. MEFs (mouse embryonic fibroblasts) derived from E16 WT (wild-type) and Ikkβ−/− mouse embryos were cultured in DMEM supplemented with 10% (v/v) FBS, 100 units/ml penicillin and 0.1 mg/ml streptomycin. RAW264.7 murine macrophages were grown in DMEM containing 5.5 mM glucose and supplemented with 10% (v/v) FBS, 100 units/ml penicillin and 0.1 mg/ml streptomycin.

3T3-L1 adipocytes and WT and Ikkβ−/− MEFs were grown in six-well plates and serum-starved overnight in DMEM supplemented with 0.5–1% (v/v) FBS before stimulation with insulin. For inhibitor studies, cells were pre-treated for 1 h with 0.1, 0.5 or 1 μM wortmannin, 10, 25 or 50 μM LY294002, 1 or 5 μM Akti-1/2 or 5 μM BMS-345541 before insulin stimulation. Antioxidant pre-treatment was carried out on WT and Ikkβ−/− MEFs for 2 h with 100 μM BHA (butylated hydroxyanisole) before and during insulin stimulation. RAW264.7 murine macrophages were grown in six-well plates in DMEM containing 10% (v/v) FBS and 1% penicillin and 0.1 mg/ml streptomycin. During treatment, RAW264.7 cells were maintained in DMEM containing 5% (v/v) FBS.

Following treatments, cells were washed twice with ice-cold PBS and scraped in ice-cold lysis buffer consisting of 50 mM Tris/HCl (pH 7.4), 130 mM NaCl, 5 mM EDTA, 1% Nonidet P40, 1 mM PMSF, 1 mM NaF, phosphatase inhibitor cocktail (containing sodium vanadate, sodium molybdate, sodium tartrate and imidazole; Sigma–Aldrich) and protease inhibitor cocktail (containing AEBSF [4-(2-aminoethyl)benzenesulfonyl fluoride], pepstatin A, E-64 [trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane], bestatin, leupeptin and aprotenin; Sigma–Aldrich). Protein concentration was determined using the BCA (bicinchoninic acid) method (Pierce) and samples were solubilized in Laemmli’s buffer and heated at 95°C for ∼5 min. Within each experiment, equal protein amounts (20–30 μg of total protein) were resolved by SDS/PAGE (10% gels) and transferred onto nitrocellulose membranes (0.2 μm pore size; Bio-Rad Laboratories) for Western blotting. Membranes were developed using an ECL (enhanced chemiluminescence) Advance Western Blotting Detection Kit (GE Healthcare). Band intensity was quantified using the Molecular Imager ChemiDoc System and Quantity One 1-D Analysis Software (Bio-Rad Laboratories). For immunoprecipitation, 300 μg of total protein was incubated with 2 μg of anti-IRS1 antibody for 4 h at 4°C. Protein–Sepharose beads were added to the protein–anti-IRS1 antibody complex and incubated overnight at 4°C. Immunocomplexes were washed three times with ice-cold PBS, treated with 1× Laemmli’s buffer, heated at 95°C, resolved by SDS/PAGE (10% gels) and immunoblotted with specific antibodies. With the exception of antibodies against phospho-IRS1 (Tyr112) (Sigma–Aldrich, catalogue number I2658), PI3K p85 (Millipore, catalogue number 9101), total ERK (catalogue number 9272), β-catenin (catalogue number I2658), PI3K p85 (Millipore, catalogue number 9331), total PKB (catalogue number 9272), β-actin (catalogue number 4812), phospho-IKKβ (catalogue number 2678), Ibo (catalogue number 4812), phospho-IRS1 Ser636/Ser639 (catalogue number 2388), phospho-IRS1 Ser473 (catalogue number 2381), phospho-JNK (catalogue number 9251), phospho-S6K (catalogue number 9234) and total S6K (catalogue number 9202).

Total RNA was extracted from cells in 800 μl of TRIzol® reagent according to the manufacturer’s instructions (Invitrogen).
Total RNA quantity and quality was measured using the ND-1000 NanoDrop Spectrophotometer (Thermo Scientific). For each sample, RNA was reverse-transcribed and converted into cDNA using TaqMan® Reverse Transcription Reagents (Applied Biosystems). Gene expression analysis was performed using TaqMan® Gene Expression Assays (Applied Biosystems), including 18S probe and primers for housekeeping gene measurement. A total of 30 ng of total cDNA per real-time PCR was used. The comparative Ct method was used to quantify results from real-time PCR.

Adenoviral vectors and infection
The adenoviral vector encoding WT IKKβ and GFP was kindly provided by Professor Yehiel Zick (Department of Molecular Cell Biology, Weizmann Institute of Science, Hebrew University of Jerusalem, Jerusalem, Israel). The control adenovirus pAdTrack-CMV (AdGo) encodes GFP only. Differentiated 3T3-L1 adipocytes and WT and Ikkβ−/− MEFs were infected with 10–40 PFU (plaque-forming units)/cell in DMEM for 2 h. Medium was subsequently supplemented with 10% (v/v) FBS. Subsequent experiments were conducted 24–48 h after infection, following overnight serum starvation.

Measurement of 2-deoxyglucose uptake
Acute [3H]2-deoxyglucose uptake was determined as described previously [16]. In brief, serum-starved 3T3-L1 adipocytes and WT and Ikkβ−/− MEFs were incubated in glucose-free DMEM for 1 h before 10 min of stimulation with 100 nM insulin. The medium was subsequently removed and 1 μCi/ml 2-deoxy-D-[2,6-3H]glucose was added. The assay was stopped after 10 min by washing the cells three times with ice-cold PBS. Cells were lysed with 0.3 M NaOH and an aliquot was taken for protein determination using the BCA method. Radioactivity incorporated into the cells was measured by liquid-scintillation counting.

Statistical analysis
Results are expressed as means ± S.E.M. Statistical significance was calculated using Student’s t test and one- or two-way ANOVA as appropriate for the dataset using SigmaStat Statistical Software Version 3.5. Statistical significance was set at P < 0.05.

RESULTS
Insulin promotes rapid phosphorylation of IKKβ in vivo and in vitro
To determine whether insulin activates IKKβ in vivo, we injected standard chow-fed C57/B16 mice with 1 unit of insulin/kg of lean body mass. Insulin treatment (3 min) increased IKKβ phosphorylation at Ser181, a site required for IKKβ activation, in WAT (white adipose tissue) (Figure 1A), but not skeletal muscle or liver (results not shown). Insulin-stimulated PKB phosphorylation was increased in WAT, skeletal muscle and liver, compared with control (Figure 1A, and results not shown), confirming the efficacy of the insulin treatment. Using differentiated 3T3-L1 adipocytes, we found that insulin rapidly, and transiently, activates IKKβ (Figure 1B); the phosphorylation of PKB was determined throughout the present study to confirm the efficacy of the various treatments. Of note, insulin-stimulated IKKβ phosphorylation is relatively transient, whereas insulin-stimulated PKB phosphorylation is more sustained (Figure 1B). The dose–response relationships of insulin-stimulated IKKβ and PKB phosphorylation were very similar, with insulin dose-dependently increasing the level of phosphorylation of both IKKβ and PKB (Figure 1C). The concentration of insulin required to elicit a significant increase in PKB phosphorylation was lower than that required to elicit a significant increase in IKKβ phosphorylation (Figure 1C).

Comparison of the effects of insulin and LPS (lipopolysaccharide) on IKKβ phosphorylation and NF-κB target gene expression in 3T3-L1 adipocytes and RAW264.7 macrophages
As insulin stimulated robust activation of IKKβ signalling, we next examined whether this resulted in an increase in the expression of canonical NF-κB target genes. Furthermore, we compared signalling and gene expression responses in insulin-treated 3T3-L1 adipocytes with LPS, a prototypical activator of IKKβ/NF-κB signalling. Finally, we compared these responses with insulin- and LPS-stimulated IKKβ signalling and NF-κB target gene expression in RAW264.7 murine macrophages. As expected, in RAW264.7 macrophages, LPS induced a pronounced
increase in IKKβ phosphorylation (Figure 2A; compare lane 8 with lane 14) and this was associated with significant increases in the expression of numerous NF-κB target genes (Figures 2B–2E). Insulin stimulation did not promote IKKβ phosphorylation and did not affect the expression of the NF-κB target genes assessed in RAW264.7 macrophages (Figure 2). In 3T3-L1 adipocytes, both insulin and LPS induced transient IKKβ phosphorylation (Figure 2A). Of note, this response was more rapid than that observed in RAW264.7 macrophages, but did not appear as quantitatively large, as inferred from the maximal observed IKKβ phosphorylation (Figure 2A). Strikingly, and despite robust IKKβ phosphorylation, insulin stimulation did not increase NF-κB target gene expression in 3T3-L1 adipocytes (Figures 2B–2E); indeed, significant decreases were observed for both *tnf* (TNFα gene) and *Il1b* (interleukin 1B gene) (Figures 2B and 2C). Similarly, whereas LPS did stimulate increases in *Il6* (interleukin 6 gene) and *Ccl2* (chemokine CC ligand 2 gene) in 3T3-L1 adipocytes (Figures 2D and 2E), these increases were markedly lower than that observed in RAW264.7 macrophages (2.7-fold compared with 38.7-fold for *Ccl2*; 6.9-fold compared with 233.3-fold for *Il6*). The finding of robust activation of IKKβ by insulin in the absence of an increase in the expression of canonical NF-κB target gene expression was surprising. To address this discrepancy further, we examined the protein level of IκBα. The degradation of IκBα is required for the induction of NF-κB-dependent target genes and is mediated by IKKβ-dependent phosphorylation. Consistent with increased IKKβ phosphorylation and NF-κB target gene expression, LPS treatment in RAW264.7 macrophages caused a marked reduction in total IκBα levels (Figure 2A; compare lanes 8 and 14). However, in 3T3-L1 adipocytes, whereas insulin stimulated IKKβ phosphorylation, no IκBα degradation was observed, consistent with the lack of change in NF-κB target genes (Figure 2A; compare lane 1 with lanes 2, 3 and 4). The differences in signalling and gene expression we observed in macrophages and adipocytes are consistent with their primary functions, i.e. responding to pathogens and growth factors. Intuitively, it would not be expected that insulin would stimulate a strong pro-inflammatory response. These findings thus raise the intriguing question of the biological function of insulin-induced IKKβ activation.
Insulin-induced IKKβ phosphorylation is PI3K-dependent, but PKB-independent

It was reported previously that IGF (insulin-like growth factor) I and II induce the expression of NF-κB-dependent genes in a PI3K-dependent manner [17,18]. Therefore we examined whether activation of PI3K was required for insulin-induced phosphorylation of IKKβ. As shown in Figure 3(A), wortmannin and LY294002, non-isoform-specific PI3K inhibitors, dose-dependently prevented insulin-stimulated IKKβ phosphorylation in differentiated 3T3-L1 adipocytes. The bottom panels in Figure 3(A) show the efficacy of the various PI3K inhibitors in preventing insulin-stimulated PKB phosphorylation. It has been shown previously that PDGF (platelet-derived growth factor) is able to activate IKKα and IKKβ, and that this effect is dependent on the ‘upstream’ activation of PKB [19]. Indeed, IKKα has been shown to be a direct substrate of PKB [20], although other work has challenged this notion [21]. To examine the potential requirement for PKB activation in insulin-stimulated IKKβ phosphorylation, we treated MEFs or 3T3-L1 adipocytes with Akti-1/2, a PKB-specific inhibitor [22]. Our results demonstrate that, whereas Akti-1/2 completely prevented insulin-stimulated PKB and GSK3β phosphorylation (Figure 3B and Figure 3C, bottom panels), insulin-stimulated IKKβ phosphorylation was not impaired (Figure 3B and Figure 3C, top panel). These results demonstrate that the phosphorylation of IKKβ in response to insulin is dependent on intact signalling via PI3K, but not via PKB.

Neither acute pharmacological inhibition of IKKβ nor adenovirus-mediated overexpression of IKKβ inhibits insulin-stimulated 2-deoxyglucose uptake or insulin signalling in differentiated 3T3-L1 adipocytes

On the basis of insulin’s ability to rapidly promote IKKβ phosphorylation and given that IKKβ is able to serine-phosphorylate IRS1 [11], we hypothesized that the activation of IKKβ by insulin contributes to the negative regulation of the insulin signalling cascade by promoting the recruitment of active IKKβ to IRS1, subsequently inducing its serine phosphorylation. An analogous scenario has been reported for JNK [5]. Therefore inhibiting the activation of IKKβ should potentiate insulin’s actions. To test this hypothesis, we pre-treated 3T3-L1 adipocytes with BMS-345541, a highly selective inhibitor of IKKβ [23], before examining insulin signalling and glucose uptake. BMS-345541 has been used in many experimental contexts, both in vivo and in vitro, to inhibit IKKβ; nonetheless, we confirmed that BMS-345541 potently inhibited the up-regulation of canonical NF-κβ target genes in our hands (see Supplementary Figure S1 at http://www.BiochemJ.org/bj/442/bj4420723add.htm). In contrast with our hypothesis, BMS-345541 had no effect on insulin-stimulated PKB phosphorylation (Figure 4A), tyrosine phosphorylation of IRS1 (Figure 4B), recruitment of the p85 catalytic subunit of PI3K to IRS1 (Figure 4B) or 2-deoxyglucose uptake (Figure 4C). BMS-345541 was also without effect when we used lower concentrations of insulin (see Supplementary Figure S2 at http://www.BiochemJ.org/bj/442/bj4420723add.htm).
Importantly, when we examined the phosphorylation status of serine sites within IRS1 linked to impaired insulin action, BMS-345541 treatment did not affect either Ser307 or Ser636/Ser639 phosphorylation (Figure 4B).

To test further the potential of IKKβ to negatively regulate insulin signalling, we transduced differentiated 3T3-L1 adipocytes with an adenovirus expressing IKKβ [24]. We hypothesized that if IKKβ does indeed negatively regulate insulin signalling, then its overexpression would suppress insulin’s actions. However, IKKβ overexpression did not affect insulin-stimulated 2-deoxyglucose uptake (Figure 4D).

To examine whether IKKβ overexpression affected insulin signalling, we examined insulin-stimulated PKB phosphorylation and immunoprecipitated IRS1 and examined its tyrosine-phosphorylation status and association with the p85 regulatory subunit of PI3K. Consistent with the 2-deoxyglucose-uptake results, IKKβ overexpression did not affect insulin’s ability to promote PKB phosphorylation (Figure 4E), IRS1 tyrosine phosphorylation at Tyr612 or its association with the p85 regulatory subunit of PI3K (Figure 4F). It has been demonstrated previously that IKKβ is capable of interacting physically with IRS1 [11]. Finally, we examined whether the insulin-induced activation of IKKβ promoted its association with IRS1. Fully differentiated 3T3-L1 adipocytes were treated in the presence or absence of insulin for 10 min and IRS1 was immunoprecipitated. Whereas insulin induced IKKβ phosphorylation and promoted the recruitment of the p85 subunit of PI3K to IRS1 (Figure 4G), we were unable to detect any recruitment of IKKβ to IRS1 (Figure 4G). Collectively, these findings argue that the insulin-induced activation of IKKβ does not promote IRS1 serine phosphorylation and, importantly, does not mediate negative feedback of the insulin signalling cascade.

Ablation of IKKβ in MEFs does not potentiate insulin signalling

The results presented so far demonstrate that, whereas insulin stimulates the phosphorylation of IKKβ, IKKβ does not mediate feedback inhibition of insulin signalling. We sought to confirm these results in an experimental model in which IKKβ expression is ablated (Figure 5A). Accordingly, we treated WT and IKKβ−/− MEFs with insulin and examined activation of the insulin signalling pathway. If IKKβ was indeed able to mediate the feedback inhibition of the insulin signalling cascade, then deletion of IKKβ should potentiate insulin

Figure 4 Neither acute pharmacological inhibition of IKKβ nor adenovirus-mediated overexpression of WT IKKβ inhibits insulin-stimulated 2-deoxyglucose uptake or insulin signalling in 3T3-L1 adipocytes

(A) Overnight serum-starved differentiated 3T3-L1 adipocytes were pre-treated with 5 μM BMS-345541 for 1 h, before 10 nM insulin treatment for the indicated periods of time. Results are from one experiment performed in triplicate and are representative of three independent experiments. (B) Overnight serum-starved differentiated 3T3-L1 adipocytes were pre-treated with 5 μM BMS-345541 for 1 h, before 100 nM insulin treatment for 10 min. Results are representative of two independent experiments performed in triplicate. (C) Overnight serum-starved differentiated 3T3-L1 adipocytes were pre-treated with 5 μM BMS-345541 for 1 h, before 100 nM insulin treatment. Results are means ± S.E.M. from six replicates and are representative of two independent experiments. (D-G) Serum-starved differentiated 3T3-L1 adipocytes were infected with adenoviral constructs expressing either Adeno-FLAG–WT-IKKβ (IKKβ WT) or control pAdTrack-CMV (AdGo). At 48 h after infection, cells were serum-starved overnight and stimulated with 10 nM insulin for the indicated periods. (D) Insulin-stimulated glucose uptake. End, endogenous IKKβ; exo, adenovirally delivered IKKβ WT. (E) WT IKKβ and AdGo-infected 3T3-L1 adipocytes were serum-starved overnight and stimulated with 10 nM insulin for 30 min. Blots are representative of six replicates from two independent experiments. (F) Samples from the experiment described in (B) were used. Blots are representative of six replicates from two independent experiments. (G) Samples from the experiment described in (B) were used. Blots are representative of two independent experiments performed in triplicate. IP, immunoprecipitation; P-, phospho; T-, total.
signalling. Surprisingly, deletion of IKKβ suppressed insulin-stimulated PKB phosphorylation (Figure 5B), IRS1 tyrosine phosphorylation at Tyr612 and its association with the p85 regulatory subunit of PI3K (Figure 5C), and S6K phosphorylation (Figure 5D). To examine whether these decreases may, paradoxically, be mediated via an enhanced IRS1 serine phosphorylation, we immunoprecipitated IRS1 and examined its serine phosphorylation status. Consistent with the general decrease in insulin sensitivity observed in Ikkβ−/− MEFs, IRS1 serine phosphorylation was reduced relative to WT MEFs (Figure 5F). Interestingly, the insulin-stimulated phosphorylation of ERK1/2 was slightly, although statistically significantly, increased in Ikkβ−/− MEFs compared with WT MEFs (Figure 5E), suggesting that the deletion of IKKβ does not cause a generalized reduction in insulin signalling, but, instead, exerts more specific effects. Adenovirus-mediated overexpression of WT IKKβ in Ikkβ−/− MEFs restored insulin-stimulated PKB phosphorylation (Figure 5G). Whereas the level of IKKβ in the reconstituted Ikkβ−/− MEFs was greater than the endogenous IKKβ levels of the WT MEFs, a similar level of IKKβ

Figure 5 Ablation of IKKβ in MEFs impairs insulin signalling

(A) Total IKKβ and β-actin expression in WT and Ikkβ−/− MEFs. (B, D and E) Serum-starved WT and Ikkβ−/− MEFs were stimulated with 10 nM insulin for the indicated time periods. Blots are representative of at least six blots from two independent experiments. Results in the histograms are means ± S.E.M. * P < 0.05, WT compared with Ikkβ−/− MEFs. (C) WT and Ikkβ−/− MEFs were serum-starved overnight and stimulated with 10 nM insulin for 10 min. Blots are representative of six replicates from two independent experiments. * P < 0.05, WT MEFs treated with insulin compared with WT MEFs not treated with insulin. (F) WT and Ikkβ−/− MEFs were serum-starved overnight and stimulated with 10 nM insulin for 10 min. Blots shown are representative of triplicates. (G) WT and Ikkβ−/− MEFs were infected with adenoviral constructs expressing either WT IKKβ or control AdGo. At 24 h after infection, cells were serum-starved overnight and stimulated with 10 nM insulin for 10 min. Results are from one experiment performed in triplicate, representative of two independent experiments. * P < 0.05, insulin-treated AdGo Ikkβ−/− MEFs compared with insulin-treated WT IKKβ virus-infected Ikkβ−/− MEFs. End, endogenous IKKβ; exo, adenovirally delivered IKKβ; IP, immunoprecipitation; P-, phospho; T-, total. All results in histograms are means ± S.E.M.
overexpression in the WT MEFs did not affect insulin-stimulated PKB phosphorylation (Figure 5G). Consistent with the lack of effect of WT IKKβ overexpression in 3T3-L1 adipocytes on insulin-stimulated PKB phosphorylation, overexpression of WT IKKβ in WT MEFs similarly did not suppress insulin-stimulated PKB phosphorylation. It is clear from Figure 5(B) that the basal levels of PKB phosphorylation are lower in Ikkβ−/− MEFs. We re-expressed the data in Figure 5(B) relative to the zero time point for each genotype, as opposed to expressing the data relative to the zero time point within the WT MEFs as shown in Figure 5(B). These results show that, despite lower basal levels of phosphorylated PKB in the Ikkβ−/− MEFs, the fold induction in response to insulin is still significantly lower compared with the WT MEFs, providing further evidence that Ikkβ−/− MEFs do, indeed, have an impaired capacity to respond to insulin (see Supplementary Figure S3 at http://www.BiochemJ.org/bj/442/bj4420723add.htm). Additionally, we repeated some of these experiments at both 5 and 10 nM insulin and showed that, at both concentrations, Ikkβ−/− MEFs have an impaired response to insulin (see Supplementary Figure S4 at http://www.BiochemJ.org/bj/442/bj4420723add.htm).

It is interesting to note that the phenotype of impaired insulin-stimulated PKB phosphorylation seen in the Ikkβ−/− MEFs is not seen with the pharmacological inhibition of IKKβ via BMS-345541 in 3T3-L1 adipocytes. Consistent with its lack of effect in 3T3-L1 adipocytes (Figure 4), BMS-345541 was also without effect on insulin-stimulated PKB phosphorylation in WT MEFs (see Supplementary Figure S5 at http://www.BiochemJ.org/bj/442/bj4420723add.htm), suggesting that cell specificity does not account for the differences observed between Ikkβ−/− MEFs and BMS-345541-treated cells. We hypothesize that this difference is due to the nature of the IKKβ manipulation in the two different experimental systems. In the inhibitor studies, WT MEFs and 3T3-L1 adipocytes were pre-treated for 1 h before insulin stimulation; however, in experiments using the Ikkβ−/− MEFs, IKKβ has been deleted. Given that NF-κB controls many hundreds of genes, in Ikkβ−/− MEFs the expression of many proteins is likely to be affected as a result of IKKβ deletion. However, in the BMS-345541 inhibitor studies, because of the very acute nature of the IKKβ manipulation, it is unlikely that proteins other than IKKβ are affected.

Finally, Ikkβ−/− MEFs do not have an intrinsic defect in their ability to activate PKB as PKB phosphorylation following treatment with H2O2 was not impaired in Ikkβ−/− MEFs, compared with WT MEFs (see Supplementary Figure S6 at http://www.BiochemJ.org/bj/442/bj4420723add.htm). The exacerbated PKB phosphorylation observed in H2O2-treated Ikkβ−/− MEFs is likely to be due to the reduced expression of NF-κB-dependent antioxidant genes in Ikkβ−/− MEFs compared with WT MEFs, resulting in a heightened sensitivity to ROS (reactive oxygen species) [25,26]. These findings argue that the deletion of IKKβ does not mediate feedback inhibition of the insulin signalling cascade.

Excess ROS generation or aberrant JNK activation plays no significant role in impaired insulin responsiveness in Ikkβ−/− MEFs

A feature of Ikkβ−/− MEFs is excessive accumulation of ROS and prolonged JNK activation due to the reduced expression of NF-κB-dependent antioxidant genes [25,26]. As both excess ROS accumulation and JNK activation are associated with impaired insulin signalling [5,27], we examined the potential role of ROS and JNK as mediators of impaired insulin signalling in Ikkβ−/− MEFs. Treatment of Ikkβ−/− MEFs with the antioxidant BHA did not result in a significant restoration of insulin-stimulated PKB phosphorylation (Figure 6A). We obtained similar results using a second antioxidant, N-acetylcysteine (not shown). Similarly, JNK phosphorylation was not exacerbated in Ikkβ−/− MEFs, compared with WT MEFs, under either basal or insulin-stimulated conditions (Figure 6B). Collectively, these findings argue against a significant role for either excess ROS generation or aberrant JNK activation as key mediators of the impaired insulin signalling of Ikkβ−/− MEFs.

DISCUSSION

The role of IKKβ in obesity-induced insulin resistance is well established. Thus, in the context of TNFα or saturated fatty acid treatment in vitro or obesity in vivo, the inhibition or genetic deletion of IKKβ prevents the development of insulin resistance.
function of insulin-induced IKK cascade, this raises an important question: what is the biological importance of insulin-induced IKKβ activation following insulin treatment, we were unable to detect a physical association between IKKβ and IRS1, suggesting that, in response to insulin, IKKβ does not serine-phosphorylate IRS1. Indeed, IKKβ inhibition via BMS-345541 did not affect IRS1 serine phosphorylation.

Given that insulin is a potent activator of IKKβ both in vitro and in vivo, but that the insulin-induced activation of IKKβ is not required for the feedback inhibition of the insulin signalling cascade, this raises an important question: what is the biological function of insulin-induced IKKβ activation? Although we did not assess the activation of NF-κB itself in the present study, the activation of NF-κB following stimulation with growth factors, including insulin, has been reported [14,15,19]. It is likely that a key function of growth-factor-induced NF-κB activation is the up-regulation of anti-apoptotic gene expression. For example, inhibition of NF-κB activation by a non-degradable form of IκBβ augments apoptosis induced by growth factor depletion and prevents the anti-apoptotic effects of insulin [19,30]. Accordingly, we hypothesize that perhaps one key function of insulin-induced IKKβ/NF-κB activation may be the up-regulation of genes required for cell survival. Intriguingly, increased adipocyte cell death is postulated to be a potential initiator of adipose tissue macrophage recruitment, adipose tissue inflammation and insulin resistance [31]. Of note, in the present study, we found in high-fat-fed insulin-resistant mice that the ability of insulin to activate IKKβ was markedly suppressed (see Supplementary Figure S7 at http://www.BiochemJ.org/bj/442/bj4420723add.htm; compare with data in chow-fed mice presented in Figure 1A). It is therefore possible that in insulin-resistant adipocytes the inability of insulin, and probably other growth factors, to activate the IKKβ/NF-κB pathway, and to consequently promote the expression of anti-apoptotic genes, may provide a mechanistic basis for the enhanced adipocyte cell death observed in the adipose tissue of obese animals.

We originally chose to use IKKβ-deficient MEFs as a model to test the hypothesis that the deletion of IKKβ would potentiate insulin signalling. As discussed above, deletion of IKKβ did not augment insulin signalling, but, and much to our surprise, caused a marked suppression in the ability of insulin to stimulate growth factor signalling. In support of our findings, it has previously been shown, using knockout MEFs, that both IKKβ and IKKα are required for the activation of S6K in response to insulin [32]. Our initial experiments focused on using the phosphorylation status of PKB and S6K as indicators of insulin signalling; however, to more clearly define the mechanistic basis of the impaired insulin signalling in IKKβ−/− MEFs, we determined IRS1 tyrosine phosphorylation and IRS1 association with the p85 regulatory subunit of PI3K. Consistent with our PKB and S6K phosphorylation findings, the ability of insulin to promote IRS1 tyrosine phosphorylation and binding to p85 was markedly suppressed in IKKβ−/− MEFs. Although one previous study has observed a reduced activation of S6K following insulin-stimulation in IKKα and IKKβ MEFs [32], our results extend these findings and argue that IKKβ-deficient MEFs have a compromised ability to respond to insulin. The mechanism by which IKKβ deletion in MEFs resulted in impaired growth factor signalling is not clear. We consider it extremely unlikely that IKKβ is an obligate mediator in cellular growth factor signalling pathways. More likely, given the large number of genes that the IKKβ/NF-κB axis regulates [33], is that perturbation to IKKβ/NF-κB signalling affects key molecules that act as inhibitors or mediators in the insulin signalling pathway.

In conclusion, we have found that insulin is a potent inducer of IKKβ phosphorylation both in vitro and in vivo. Importantly, and in contrast with our hypothesis, we show, in several different experimental models, that, despite rapid activation of IKKβ by insulin, IKKβ does not play a role in the feedback inhibition of the insulin signalling cascade. The function and physiological importance of insulin-induced IKKβ activation is, at present, not known, but may relate to the promotion of adipocyte cell survival. Characterization of the precise loci of the defect(s) to insulin signalling in IKKβ-deficient MEFs requires future work.

AUTHOR CONTRIBUTION

Graeme Lancaster designed and performed experiments, analysed the data and wrote the first draft of the paper. Beata Skiba designed and performed experiments and analysed data. Christine Yang, Hayley Nicholls, Katherine Langley, Stanley Chan and Clinton Bruce performed experiments. Gordon Rewcastle, Peter Shepherd and Michael Karin provided critical reagents. Mark Febbraio designed experiments. All authors contributed to the drafting of the paper.

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SUPPLEMENTARY ONLINE DATA

*IκB kinase β (IKKβ) does not mediate feedback inhibition of the insulin signalling cascade*

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Figure S1  BMS-345541 inhibits pro-inflammatory gene expression

RAW264.7 murine macrophages were pre-treated for 1 h with either BMS-345541 or vehicle control before subsequent treatment with either 100 ng/ml LPS or its vehicle control for a further 6 h. Results are means ± S.E.M. from one experiment performed in triplicate, but are representative of data from several independent experiments.

Figure S2  BMS-345541 does not inhibit insulin-stimulated PKB phosphorylation

WT MEFs were treated in reduced serum medium overnight before being pre-treated for 1 h with either BMS-345541 or vehicle before stimulation with the indicated concentrations of insulin for 10 min. Blots are from one experiment performed in triplicate. P-, phospho; T-, total.

Figure S3 Insulin-stimulated PKB phosphorylation in WT and IKKβ−/− MEFs

Re-expression of the data shown in Figure 5(B) of the main text. Results are expressed as the fold change from untreated for each genotype. *P < 0.05, WT MEFs compared with IKKβ−/− MEFs. Results are means ± S.E.M.

Figure S4 Effect of insulin dose on PKB phosphorylation in WT and IKKβ−/− MEFs

WT and IKKβ−/− MEFs were treated in reduced serum medium overnight before stimulation with the indicated concentrations of insulin for 10 min. Blots are representative of triplicate samples from two independent experiments. Results in the histogram are means ± S.E.M. *P < 0.05, WT MEFs compared with IKKβ−/− MEFs. P-, phospho; T-, total.

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Figure S5  BMS-345541 does not affect insulin-stimulated PKB phosphorylation in WT MEFs
WT MEFs were treated in reduced serum medium overnight before being pre-treated with either BMS-345541 or vehicle before stimulation with 10 nM insulin for the indicated durations. Blots are from one experiment performed in triplicate. Results in the histogram are means ± S.E.M. P-, phospho; T-, total.

Figure S6  Effect of H2O2 on PKB phosphorylation in WT and IKKβ−/− MEFs
WT and Ikβ−/− MEFs were serum-starved overnight and treated with or without 1 mM H2O2 for 1 h. Blots are representative of triplicate samples from two independent experiments. Results in the histogram are means ± S.E.M. P-, phospho; V, vehicle.

Figure S7  Effect of a high-fat diet on insulin-stimulated IKKβ phosphorylation
C57/Bl6 mice were fed on a high-fat diet for 12 weeks before 3 min of stimulation with 1 unit of insulin/kg of lean body mass. A portion of the epididymal fat pad was removed immediately before and then following insulin administration. Extracts were prepared and analysed for levels of phosphorylated IKKβ (Ser181) by Western blotting. Results are from n = 8 in each group and are means ± S.E.M. P-, phospho.

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