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ARTICLE

Deletion of protein kinase Cδ in mice modulates stability of inflammatory genes and protects against cytokine-stimulated beta cell death in vitro and in vivo

J. Cantley · E. Boslem · D. R. Laybutt · D. V. Cordery · G. Pearson · L. Carpenter · M. Leitges · T. J. Biden

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Abstract
Aims/hypothesis Proinflammatory cytokines contribute to beta cell destruction in type 1 diabetes, but the mechanisms are incompletely understood. The aim of the current study was to address the role of the protein kinase C (PKC) isoform PKCδ, a diverse regulator of cell death, in cytokine-stimulated apoptosis in primary beta cells.
Methods Islets isolated from wild-type or *Prkcd*$^{-/-}$ mice were treated with IL-1β, TNF-α and IFNγ and assayed for apoptosis, nitric oxide (NO) generation and insulin secretion. Activation of signalling pathways, apoptosis and endoplasmic reticulum (ER) stress were determined by immunoblotting. Stabilisation of mRNA transcripts was measured by RT-PCR following transcriptional arrest. Mice were injected with multiple low doses of streptozotocin (MLD-STZ) and fasting blood glucose monitored.
Results Deletion of *Prkcd* inhibited apoptosis and NO generation in islets stimulated ex vivo with cytokines. It also delayed the onset of hyperglycaemia in MLD-STZ-treated mice. Activation of ERK, p38, JNK, AKT1, the ER stress markers DDIT3 and phospho-EIF2α and the intrinsic apoptotic markers BCL2 and MCL1 was not different between genotypes. However, deletion of *Prkcd* destabilised mRNA transcripts for *Nos2*, and for multiple components of the toll-like receptor 2 (TLR2) signalling complex, which resulted in disrupted TLR2 signalling.
Conclusions/interpretation Loss of PKCδ partially protects against hyperglycaemia in the MLD-STZ model in vivo, and against cytokine-mediated apoptosis in vitro. This is accompanied by reduced NO generation and destabilisation of *Nos2* and components of the TLR2 signalling pathway. The results highlight a mechanism for regulating proinflammatory gene expression in beta cells independently of transcription.

Keywords Beta cell · Cytokines · Islets of Langerhans · mRNA stabilisation · NOS2 · Protein kinase C · Streptozotocin · Toll-like receptor · Type 1 diabetes

Abbreviations

AKT1	Thymoma viral proto-oncogene 1
BCL2	B-cell leukemia/lymphoma 2
DDIT3	DNA-damage-inducible transcript 3

J. Cantley and E. Boslem contributed equally to this study.

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J. Cantley · E. Boslem · D. R. Laybutt · D. V. Cordery · G. Pearson · L. Carpenter · T. J. Biden (✉)
Garvan Institute of Medical Research, St Vincent's Hospital,
384 Victoria Street, Darlinghurst,
Sydney, NSW 2010, Australia
e-mail: t.biden@garvan.org.au

E. Boslem · D. R. Laybutt · T. J. Biden
St Vincent's Clinical School, Faculty of Medicine,
University of New South Wales,
Sydney, NSW, Australia

M. Leitges
Biotechnology Centre of Oslo, University of Oslo,
Oslo, Norway

M. Leitges
Division of Nephrology, Department of Medicine,
Hannover Medical School,
Hannover, Germany

Present Address:
L. Carpenter
Stem Cells and Immunotherapies, NHS Blood and Transplant,
John Radcliffe Hospital,
Oxford, UK

EIF2AK3	Eukaryotic translation initiation factor 2 alpha kinase 3
ER	Endoplasmic reticulum
ERK	Extracellular signal regulated kinase
GSIS	Glucose-stimulated insulin secretion
JNK	c-Jun N-terminal kinase
PKC	Protein kinase C
MAPK	Mitogen-activated protein kinase
MCL1	Myeloid cell leukemia sequence 1
MLD-STZ	Multiple low dose streptozotocin
NFκB	Nuclear factor kappa B
NO	Nitric oxide
Nos2	Inducible nitric oxide synthase
P38	p38 MAPK
STZ	Streptozotocin
TLR	Toll-like receptor

Introduction

Apoptotic destruction of pancreatic beta cells as part of an auto-inflammatory response is the hallmark of type 1 diabetes [1–4]. The release of proinflammatory cytokines, such as IL-1β, TNF-α and IFNγ from the monocytic infiltrate is a key feature of the progression of the disease [1–4]. IL-1β signalling in beta cells includes activation of various mitogen-activated protein kinase (MAPK) cascades. Another important apoptotic pathway is mediated by nuclear factor kappa B (NFκB), which leads to increased expression of inducible nitric oxide synthase (NOS2), thereby enhancing generation of the free radical nitric oxide (NO). The latter is a key, but not sole, mediator of beta cell apoptosis following exposure to proinflammatory cytokines, and in experimental models of type 1 diabetes [1–4].

Another signalling pathway initiated by IL-1β involves activation of protein kinase C (PKC) [5]. This comprises a family of serine/threonine protein kinases consisting of 11 isoforms with differing tissue distributions and functions, as well as varying co-factor requirements and substrates [6]. Work from our laboratory established that the PKCδ isoform is activated following stimulation of INS-1 insulinoma cells with IL-1β, and that this activation contributed to cytokine-mediated apoptosis [7, 8]. In this model, PKCδ regulated *Nos2* at post-transcriptional level, by actively stabilising *Nos2* mRNA, thereby increasing its half-life [7]. However, PKCδ is now widely recognised as a component of apoptotic signalling pathways triggered by diverse cytotoxic agents, including those such as ultraviolet radiation and DNA-damaging agents, in which NOS2 induction does not play a role. In these instances, PKCδ can impinge upon substrates in the distal steps of the apoptotic cascade [9–11]. Furthermore, a recent study has demonstrated a requirement for PKCδ in mediating beta cell apoptosis in vivo in response to high-fat feeding, a situation not thought to involve NO generation [12]. To date, however, a role for PKCδ in animal models of type 1 diabetes has not been tested.

Another potential role of PKCδ is in linking endoplasmic reticulum (ER) stress to c-Jun N-terminal kinase (JNK) signalling and thus apoptosis [13]. This stress response is activated in beta cells by cytotoxic cytokines [14–18], potentially as a result of depletion of Ca^{2+} from the lumen of the ER, such that the ability of the latter to fold and export secretory protein is compromised [19]. Signalling molecules in the ER membrane sense the accumulation of unfolded proteins and initiate an adaptive response. However, if this fails to rebalance the folding capacity of the ER, apoptosis is activated [20, 21]. In beta cells, the best characterised of the transmembrane ER stress sensors is the protein kinase, eukaryotic translation initiation factor 2 alpha kinase 3 (EIF2AK3 also known as PERK), which (among other effects) is mainly responsible for transcriptional upregulation of the pro-apoptotic transcription factor, DNA-damage-inducible transcript 3 (DDIT3, also known as CHOP).

The goal of the current study was to determine whether activation of PKCδ by cytokines contributes to cytokine-mediated apoptosis in primary beta cells and to investigate the underlying mechanisms, with particular reference to ER stress and mRNA stabilisation of inflammatory genes such as *Nos2*. We also sought to test the potential involvement of PKCδ in a mouse model of type 1 diabetes.

Methods

Mice Prkcd null mice were generated by insertion of a LacZ/neo cassette into the first transcribed exon of the gene, as previously described [22, 23]. Routine genotyping was carried out by PCR analysis of tail-tip DNA, using a forward primer corresponding to a 5′ untranslated region of the *Prkcd* locus and reverse primers corresponding to either exon 1 (wild type)—or the Lac-Neo insert (see Electronic supplementary material [ESM] Table 1). Ethics approval for mouse studies was granted by the Garvan Institute/St Vincent's Hospital Animal Ethics Committee. Mice were maintained on a hybrid 129/SV C57BL/6 background, using *Prkcd* heterozygous breeding pairs. They were fed a standard chow diet and had free access to drinking water. Age-matched wild-type and $Prkcd^{-/-}$ littermates (8–12 weeks old) were used for experiments.

MLD-STZ treatment A stock solution of 4 mg/ml streptozotocin (STZ) was prepared in 0.1 mol/l sodium citrate

(pH 4.5) immediately before usage. After fasting for 4–6 h, mice were injected i.p. with 40 mg/kg STZ once a day for 5 days. On days 2, 5, 9, 11, 12, 16 and 24, food was withdrawn in the morning and blood withdrawn from the tail vein 6 h later for analysis of blood glucose. In some instances, mice were killed at day 11, and pancreases were removed and fixed in 4% paraformaldehyde for immunohistochemistry.

Islet isolation, cytokine treatment and insulin secretion assays Islets were isolated by pancreatic digestion, and purified using a Ficoll-paque gradient (GE Healthcare, Chalfont St Giles, UK) before overnight culture in RPMI 1640 with 11 mmol/l glucose and 10% FCS (Invitrogen, Mulgrave, Vic, Australia). A cytokine mixture comprising IL-1β, TNF-α and IFNγ was added at concentrations and for times indicated in the text. In some studies the selective TLR2 agonist Pam2CSK4 (Invivogen, San Diego, CA, USA) was added at 1 μg/ml for 30 min. For insulin secretion assays, islets were preincubated for 1 h in HEPES-buffered KRB containing 0.1% BSA and 2 mmol/l glucose. Batches of five islets were incubated at 37°C for 1 h in 130 μl KRB containing 0.1% BSA and 2 mmol/l glucose (basal), supplemented with glucose (20 mmol/l), or KCl (25 mmol/l) as indicated in the text. Insulin release was determined by RIA (Linco/Millipore, Billerica, MA, USA).

Apoptosis and NO assays Apoptosis was measured using an ELISA kit (Roche Applied Science, Castle Hill, NSW, Australia) which quantifies the apoptotic mono- and oligonucleosomes in a sample. Islets (40–80) were lysed in 0.2 ml of the supplied lysis buffer, incubated for 30 min at room temperature, and the lysate was spun at 200×*g* for 10 min [14]. The assay was performed using 20 μl of the supernatant fraction in the ELISA according to the manufacturer's instructions. NO was assayed using the Greiss reaction as previously described [7] using a 0.1 ml aliquot of the islet culture medium.

Western blotting Islets were washed, lysed and protein content determined by bicinchoninic acid assay (Pierce/Thermo Scientific, Rockford, IL, USA). Fifteen micrograms of protein was resolved on a 7% SDS-PAGE gel (Invitrogen) before transfer to polyvinylidene fluoride membranes. Membranes were blocked with milk and probed with the following antibodies for 2 h at room temperature: anti-PKCα (610108) and anti-PKCε (610086; BD biosciences, San Jose, CA, USA); anti-PKCβ (SC209), anti-PKCδ (SC213), anti-PKCζ (SC216), anti-DDIT3/CHOP (SC575), anti-IκBβ (SC945), anti-Mcl1 (SC819), anti-NOS2 (SC651) and anti-14-3-3β (SC1657; Santa Cruz Biotechnology, Santa Cruz, CA, USA); Anti-BCL2 (2876), anti-phospho-JNK (Thr183/Tyr185, 9251), anti-phospho-p44/42 MAPK (Thr202/Tyr204, 4377), anti-phospho-AKT1 (S473, 9271), anti-phospho-p38 MAPK (Thr180/Tyr182, 9211) and anti-phospho-EIF2AK3/PERK (Thr980, 3179; Cell Signaling Technology, Danvers, MA, USA); anti-α-tubulin (T90267) and anti-β-actin (Sigma, St Louis, MI, USA) and anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Following chemiluminescent detection, densitometry was performed using ImageJ1.38q (NIH, Bethesda, MD, USA).

Quantitative PCR and mRNA stabilisation Islets were exposed to 400 U/ml IL-1β for 6 h, and then treated with 1 μmol/l actinomycin D for times as indicated. RNA was extracted and RT-PCR was carried out as previously described using primers as listed in ESM Table 1 for *Nos2*, β-actin (*Actb*), and *Ddit3*. Alternatively, cDNA derived from islets before and 3 h after actinomycin D was applied to a PCR array containing 84 inflammation genes (SABiosciences, Frederick, MD, USA), RT-PCR was performed using an ABI PRISM7900 HT instrument (Applied Biosystems, CA, USA) and data were analysed according to the manufacturer's instructions. Gene expression was normalised to that of the mean of four housekeeping genes: *Gusb*, *Hprt1*, *Hsp90ab1* and *Gapdh*.

Statistics Statistics were performed using GraphPad Prism5 (Graphpad Software, La Jolla, CA, USA) or Excel (Microsoft, Redmond, WA, USA) software. Paired and unpaired *t* tests and two-way ANOVA were performed as appropriate. A p value of <0.05 was regarded as significant.

Results

Pancreatic islets from $Prkcd^{-/-}$ mice are deleted in PKCδ without compensation from other PKC isoforms Mice genetically deleted in *Prkcd* have been widely used to determine the function of this PKC isoform in multiple cell types [22–24]. Islets isolated from these animals showed no detectable expression of PKCδ in contrast to wild-type islets (Fig. 1a). Importantly, expression of other PKC isoforms was not altered in the absence of PKCδ (Fig. 1b, c).

Deletion of PKCδ partially protects against apoptosis due to cytokines, but does not alter glucose-stimulated insulin secretion Because of the widely reported pro-apoptotic role of PKCδ, we first compared the sensitivity of wild-type and $Prkcd^{-/-}$ islets ex vivo to a cytokine mixture, or the beta cell toxin STZ. As shown in Fig. 2a, cytokines markedly stimulated apoptosis in wild-type islets, but this was

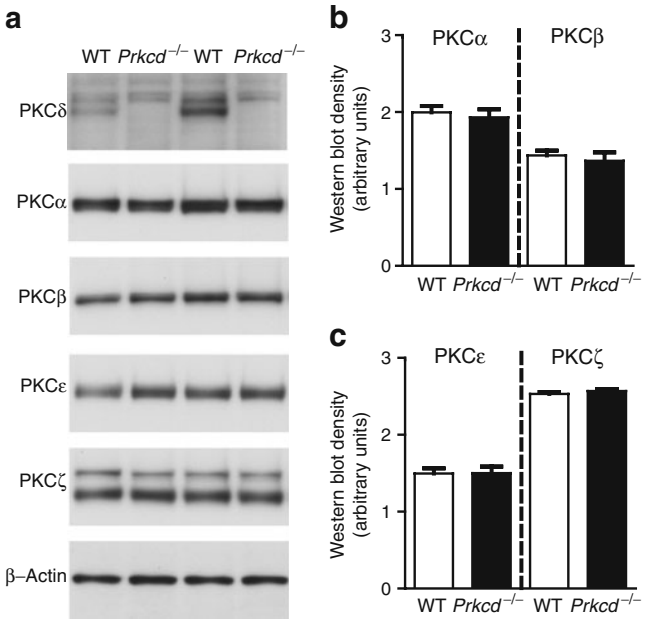

Fig. 1 PKC expression profile in islets isolated from *Prkcd*$^{-/-}$ mice. Islets were isolated from wild-type (WT) or *Prkcd*$^{-/-}$ mice. Cell lysates were separated by SDS-PAGE and immunoblotted for detection of PKC isoforms as indicated. Representative blots (**a**) and densitometric analyses (**b**, **c**) of PKC expression normalised for β-actin loading (n=3) are shown

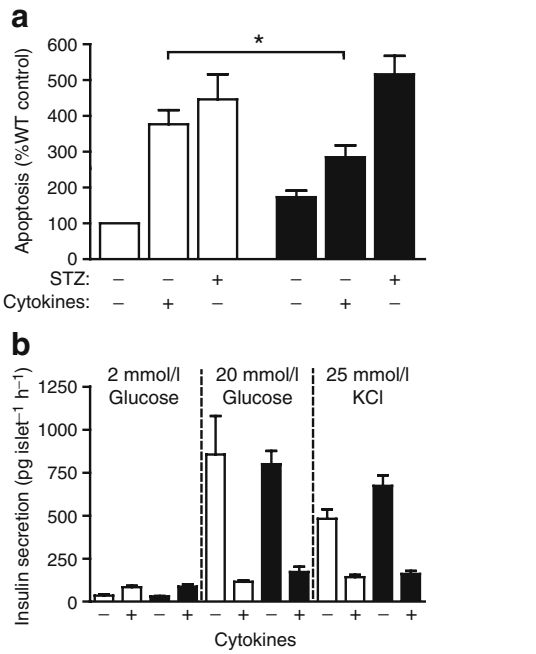

Fig. 2 Effect of *Prkcd* deletion on apoptosis and glucose-stimulated insulin secretion in isolated mouse islets. Islets were isolated from wild-type (WT) or *Prkcd*$^{-/-}$ mice. **a** Islets were treated for 24 h with either cytokine mixture comprising IL-1β (400 U/ml), TNF-α (400 U/ml) and IFNγ (400 U/ml, n=5) or STZ (10 mg/ml, n=3). Apoptosis was quantified in islet lysates using an ELISA for oligonucleosomal DNA. *p<0.05 vs corresponding WT value. **b** Islets were pretreated in culture for 24 h in the presence or absence of a mixture of cytokines comprising IL-1β (400 U/ml), TNF-α (400 U/ml) and IFNγ (400 U/ml). Batches of five islets were then incubated in KRB buffer containing 2 or 20 mmol/l glucose or 25 mmol/l KCl for 1 h (n=7–9). Insulin was quantified by radioimmunoassay. White bars, WT; black bars, *Prkcd*$^{-/-}$

inhibited >30% in the *Prkcd* null islets. By contrast, STZ-induced apoptosis was not affected by *Prkcd* deletion. Because cytokines impact on beta cell function as well as mass [25, 26], we then investigated the role of PKCδ in the inhibition of insulin secretion (Fig. 2b). Treatment of wild-type islets with cytokines enhanced basal secretion, and abolished the stimulation due to either 20 mmol/l glucose or 25 mmol/l KCl. Deletion of *Prkcd* did not alter glucose-stimulated insulin secretion (GSIS) in the absence of cytokines, nor did it modulate the effects of cytokines. Insulin contents were also unaltered between wild-type and *Prkcd*$^{-/-}$ islets under these conditions (results not shown).

Proximal cytokine signalling pathways in pancreatic islets are unaltered by deletion of Prkcd To address the mechanism underlying the protection against apoptosis due to deletion of *Prkcd*, we first investigated MAPK signalling. These pathways are important in cytokine-mediated apoptosis in beta cells [27, 28], and are influenced by PKCδ in other cell types [29, 30]. JNK phosphorylation was robustly stimulated by cytokines, without any apparent differences between wild-type and *Prkcd*$^{-/-}$ islets (Fig. 3a). As previously demonstrated, extracellular signal regulated kinase (ERK) phosphorylation tended to decrease with cytokine treatment [31], and there was a trend (albeit nonsignificant) toward further reduction in the absence of *Prkcd*. Basal phosphorylation of p38 MAPK (p38) was enhanced by *Prkcd* deletion (p=0.051), but the stimulated response was similar between the two groups (Fig. 3b). Likewise, the degradation of IκBα and IκBβ, which is integral to the activation of NFκB signalling, was similar following stimulation of islets from both wild-type and PKCδ null islets (Fig. 3b and results not shown). Thymoma viral proto-oncogene 1 (AKT1) is also a major regulator of beta cell survival [31, 32] but its activation, as measured by S-473 phosphorylation, was unaltered by either cytokines or *Prkcd* deletion.

Cytokine-stimulated ER stress is not modulated by Prkcd deletion DDIT3/CHOP is a transcription factor that serves as a major link between ER stress and apoptosis in beta cells [20, 33]. Although greatly induced over 24 h by cytokines in wild-type islets, this was not obviously affected by deletion of *Prkcd* at either the protein (Fig. 4) or mRNA level (results not shown). Phosphorylation of EIF2A3/PERK is an unequivocal marker of ER stress [20]. Its stimulation by approximately fourfold in response to cytokines was not significantly different between wild-type

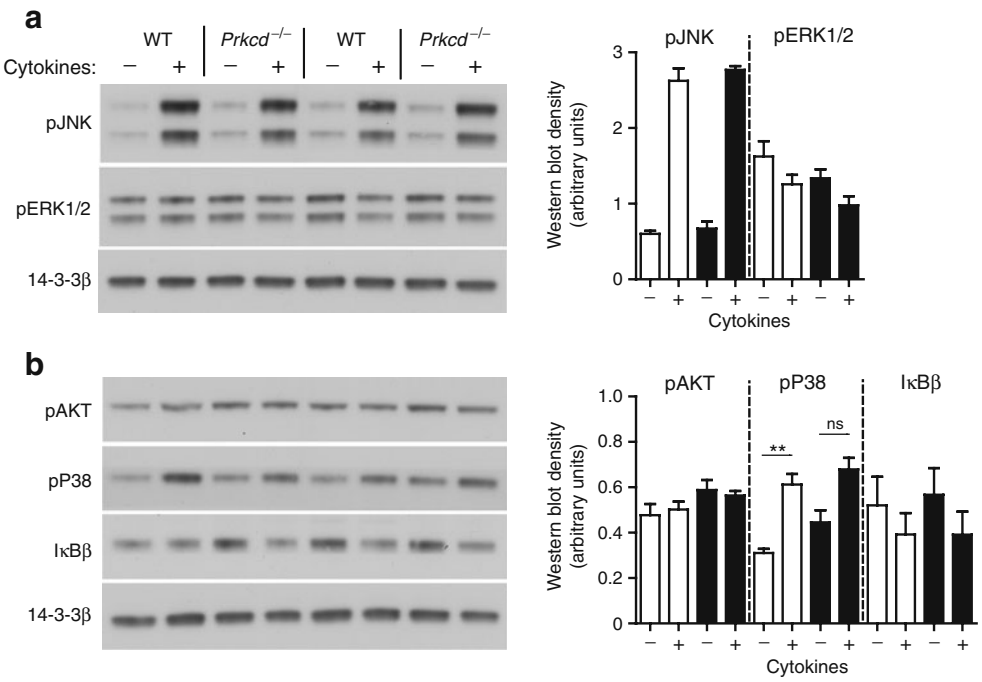

Fig. 3 Effect of *Prkcd* deletion on acute cytokine-stimulated signalling. Islets isolated from wild-type (WT) or *Prkcd*$^{-/-}$ mice were cultured overnight and then stimulated for 30 min with a cytokine mixture comprising IL-1β (400 U/ml), TNF-α (400 U/ml) and IFNγ (200 U/ml). Islets were lysed and proteins resolved by SDS-PAGE (**a**, **b**). Immunoblotting for phosphorylated forms of JNK, ERK, AKT1 and p38, and total IκBβ and 14-3-3β (loading control) was performed. The graphs show densitometric quantification of immunoblots normalised for loading (n=4); **p<0.01. White bars, WT; black bars, *Prkcd*$^{-/-}$

and *Prkcd*$^{-/-}$ islets (Fig. 4). In some cell types PKCδ has been reported to promote degradation of myeloid cell leukemia sequence 1 (MCL1) [34], a BH3-only protein that negatively regulates the intrinsic mitochondrial pathway. Although cytokine treatment tended to increase MCL1 protein in islets, this effect was similar in both wild-type and PKCδ$^{-/-}$ islets (Fig. 4). As expected, levels of another BH3-only protein, B-cell leukemia/lymphoma 2 (BCL2), tended to decrease with cytokine exposure, but in a manner independent of PKCδ expression [35].

PKCδ contributes to Nos2 mRNA stabilisation Generation of NO was increased more than fourfold in medium from wild-type islets stimulated with cytokines for 24 h. This was significantly reduced to threefold in *Prkcd*$^{-/-}$ islets (Fig. 5a). Correspondingly, we also observed a significant reduction in NOS2 protein in cytokine-stimulated *Prkcd*$^{-/-}$ islets vs wild-type islets (Fig. 5b). This is reminiscent of our previous findings using clonal beta cells in which the function of PKCδ was reciprocally modulated by overexpression of wild-type and kinase-dead PKCδ

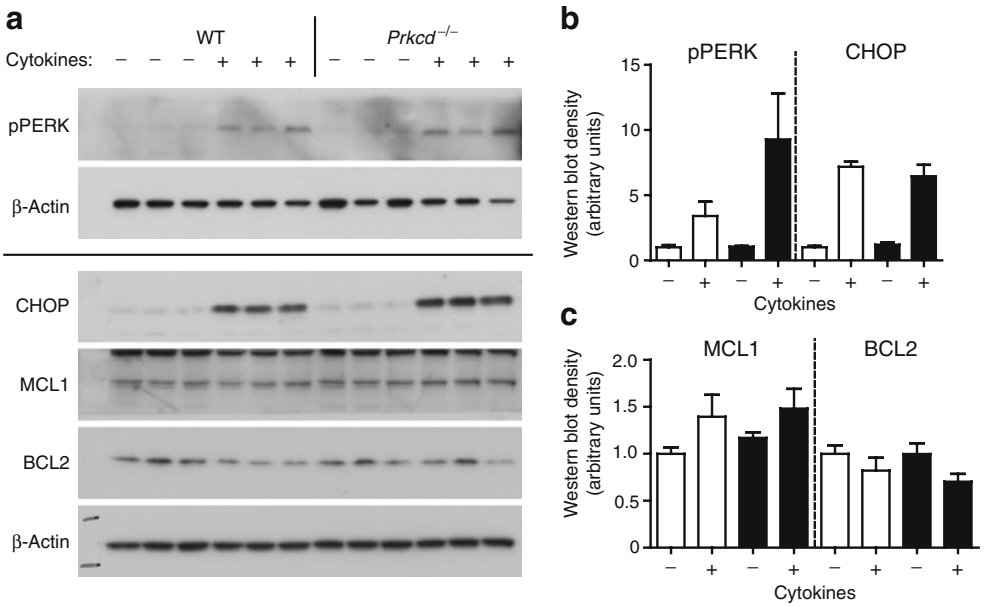

Fig. 4 Effect of PKCδ deletion on ER stress and apoptosis markers. Islets isolated from wild-type (WT) or *Prkcd*$^{-/-}$ mice were cultured for 24 h in presence or absence of a cytokine mixture comprising IL-1β (160 U/ml), TNF-α (40 U/ml) and IFNγ (400 U/ml). Islets were lysed and proteins resolved by SDS-PAGE. **a** Immunoblotting for DDIT3 (CHOP), MCL1, BCL2, phospho-EIF2A3 (PERK) and β-actin (loading control) was performed. **b**, **c** Densitometric quantification of immunoblots normalised for loading and expressed relative to wild-type control (n=4). White bars, WT; black bars, KO

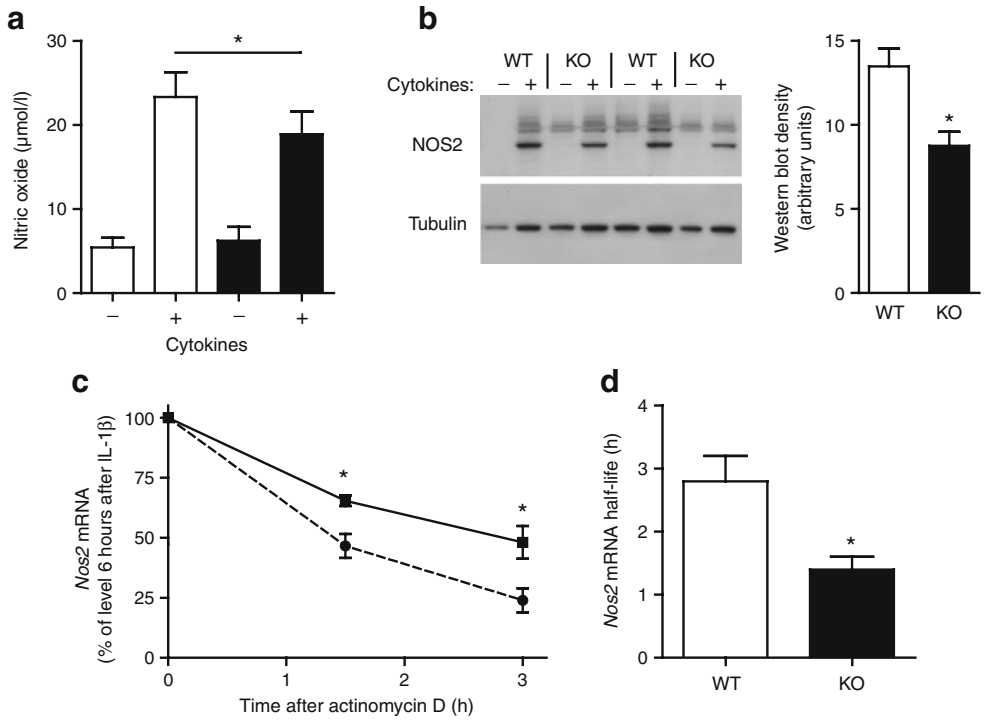

Fig. 5 *Prkcd* deletion reduces NO generation, NOS2 protein and destabilises *Nos2* mRNA. **a** Islets isolated from wild-type (WT) or *Prkcd*$^{-/-}$ (KO) mice were cultured for 24 h in the presence of a cytokine mixture comprising IL-1β (400 U/ml), TNF-α (400 U/ml) and IFNγ (400 U/ml). NO released into the medium was quantified using the Greiss reaction (n=4). *p<0.05 vs WT cytokine value. **b** Isolated islets were cultured for 24 h in the presence of IL-1β (400 U/ml). Islets were lysed and proteins resolved by SDS-PAGE. Representative immunoblot and densitometric analysis of stimulated NOS2 expression (n=4) *p<0.05. **c** Islets in tissue culture were stimulated for 6 h in the presence of (400 U/ml) IL-1β, at which time actinomycin D (1 μmol/l) was added. RNA was extracted at the indicated times and *Nos2* mRNA quantified by RT-PCR. **d** mRNA half-lives calculated from **b**. n=3; *p<0.05. black squares, WT; black circles, KO

adenoviruses [7]. In that instance we observed a partial requirement for PKCδ activation in the stabilisation of *Nos2* mRNA. Consistent with those findings we now demonstrate that *Nos2* mRNA decayed from its post-stimulation peak more rapidly in *Prkcd*$^{-/-}$ than wild-type mice (Fig. 5c). This corresponded to an approximate 50% decrease in the half-life from 2.8 to 1.4 h (Fig. 5d).

PKCδ post-transcriptionally regulates multiple mRNA transcripts in islets PKCδ is known to regulate transcripts in addition to *Nos2* in non beta cells, but to our knowledge this has only ever been assessed on a candidate-by-candidate basis [36, 37]. As a more comprehensive approach we used an RT-PCR array of 84 inflammatory genes for an unbiased screen of genes whose stability in beta cells might be influenced by loss of *Prkcd*. As expected, treatment for 3 h with actinomycin D tended to reduce expression of most genes, of which 33 were decreased significantly in either wild-type or *Prkcd*$^{-/-}$ islets (ESM Table 2). We next compared the change in gene expression following actinomycin D treatment between wild-type and *Prkcd*$^{-/-}$ islets and identified 13 gene transcripts that were significantly differentially regulated by genotype (ESM Table 2). Loss of *Prkcd* was associated with both increases and decreases in mRNA abundance (ESM Table 2). While this might point to an unexpected role for PKCδ in destabilising some transcripts, generally these transcripts did not decrease at all in the *Prkcd*$^{-/-}$ islets following addition of actinomycin D, and so these results should be viewed with caution. Therefore we chose to focus on the genes differentially regulated between the genotypes that also decreased in abundance following actinomycin D treatment in both genotypes, leaving us with six gene candidates for regulation by PKCδ (Fig. 6a). Although further experiments will be needed to validate these candidates, we propose that they are stabilised by PKCδ in wild-type islets. One of these genes is *Myd88*, a signalling partner of the IL-1 receptor, which itself appeared to be regulated by PKCδ. However, MYD88 also participates in signalling downstream of toll-like receptors (TLRs). Interestingly, there was also evidence of regulated expression of one of these receptors, *Tlr2*, along with *Tollip*, another component in this receptor/signalling complex. Several other genes were also decreased, albeit not significantly in the absence of *Prkcd*

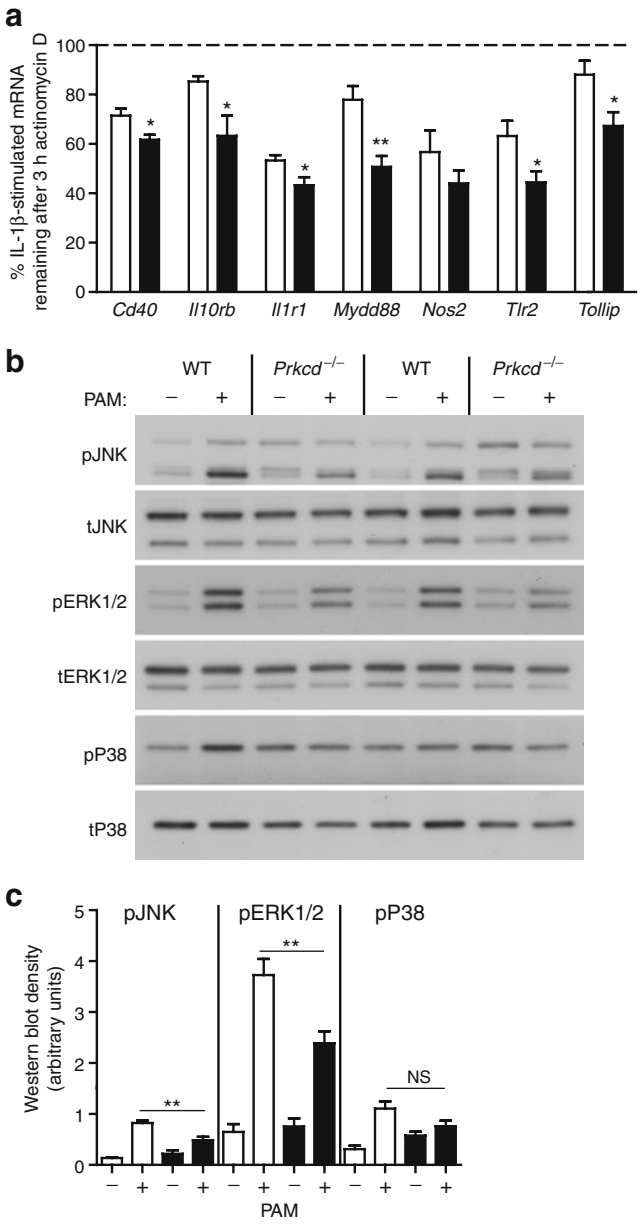

Fig. 6 PKCδ deletion regulates stability of multiple mRNA transcripts and inhibits TLR2 signalling. **a** Islets in tissue culture from wild-type (WT) or *Prkcd*$^{-/-}$ (KO) mice were stimulated for 6 h in the presence of IL-1β (400 U/ml). Actinomycin D (1 μmol/l) was then added and RNA extracted after a further 3 h. mRNA was quantified using a PCR array of 84 inflammatory genes. Results are normalised relative to four housekeeping genes and expressed as percentage of corresponding value at time of addition of actinomycin D (n=4). *p< 0.05 or **p<0.01 vs corresponding WT value. **b** Isolated islets were cultured overnight and then stimulated for 30 min with 1 μg/ml Pam2CSK4 (PAM). Islets were lysed and proteins resolved by SDS-PAGE. Immunoblotting for total (t) and phosphorylated (p) forms of JNK, ERK and p38 was performed. **c** The graph shows densitometric quantification of phosphorylation from immunoblots normalised for corresponding total protein control (n=4); **p<0.01. White bars, WT; black bars, *Prkcd*$^{-/-}$

(ESM Table 2). These might also prove interesting candidates for further investigation, given that they include *Nos2* (Fig. 6a), whose regulation by PKCδ has already been validated in more sensitive assays (Fig. 5c).

TLR2 signalling is inhibited by deletion of Prkcd A role for PKCδ in regulating expression of components of the TLR2 signalling complex might be expected to impact on signalling downstream of that receptor. We tested this directly using the specific TLR2 agonist Pam2CSK4. As shown in Fig. 6b, this compound resulted in large fold increases in the phosphorylation of JNK, ERK and p38 in wild-type islets. In all instances these responses were less pronounced in islets deleted in *Prkcd*, and this difference attained statistical significance in the case of JNK and ERK.

Mice deleted in Prkcd are partially protected in an autoimmune model of beta cell destruction Our current and previous data using primary islets and cell lines, respectively, suggested a role for PKCδ in beta cell death triggered by cytokines. We now sought to confirm the relevance of these findings in vivo. Mice were therefore injected with MLD-STZ, which leads to an immune-mediated destruction of pancreatic beta cells, and therefore represents a widely employed model of type 1 diabetes [38, 39]. We initially established that there was no difference in body weight in the mice used in these experiments (24.6± 1.2 g, n=11 for wild-type, and 25.5±0.6 g, n=14 for *Prkcd*$^{-/-}$) at day 24 after initial STZ injection. As shown in Fig. 7a, however, wild-type mice subjected to this protocol displayed a time-dependent increase in fasting blood glucose over the period of the study. This was significantly elevated by day 5 (p<0.01) compared with the blood glucose in mice before the STZ injections. By contrast, blood glucose was not significantly increased in the *Prkcd* null mice until day 11 (p< 0.005) of the treatment, and remained lower than the wild-type values from day 9 to day 24. Expression of these same data in terms of the percentage of mice displaying a fasting blood glucose >10 mmol/l (Fig. 7b) reveals that more than 25% of both wild-type and *Prkcd*$^{-/-}$ mice attained this threshold within 11–12 days. By 24 days, more than 80% of the wild-type animals were displaying a fasted blood glucose >10 mmol/l, compared with just over half of the *Prkcd*$^{-/-}$ mice. This might suggest that deletion of *Prkcd* slows the progression, but not the initiation, of beta cell destruction.

Discussion

PKCδ is a major mediator of diverse forms of apoptosis in many cell types [9–11]. Its role in beta cells, however, has

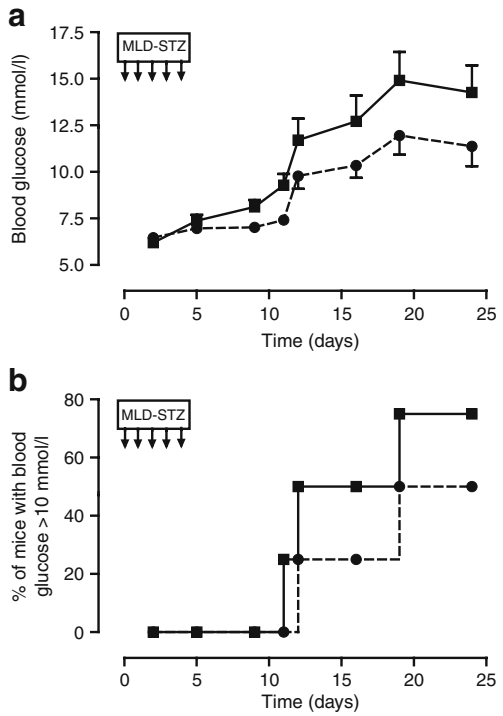

Fig. 7 PKCδ deletion delays the progression of hyperglycaemia in mice subjected to MLD-STZ injections. Wild-type (WT) or *Prkcd*$^{-/-}$ mice were injected i.p. with 40 mg/kg STZ once a day for 5 days. **a** Fasting blood glucose was measured at the days indicated. $p<0.0001$ for genotype by two-way ANOVA. **b** Data from **a**, showing numbers of mice developing fasting blood glucose >10 mmol/l with time. (n=11, WT; n=14, *Prkcd*$^{-/-}$). Black squares, WT; black circles, *Prkcd*$^{-/-}$

chiefly been addressed in the context of regulation of GSIS, which has proved controversial [40–42]. In our hands, functional inhibition of PKCδ by overexpression of a kinase-dead mutant of the enzyme in isolated rat islets using adenovirus was without effect on GSIS [40]. Moreover, overexpression of a similar construct specifically in beta cells of transgenic mice also failed to demonstrate a major role in regulating secretion [12]. By contrast, a partial requirement for PKCδ in GSIS was reported using islets from *Prkcd* knockout mice [43]. The results shown here, using a different knockout model, are in keeping with the results of the kinase-dead approach, suggesting that PKCδ plays little role in GSIS. We also demonstrate for the first time that activation of PKCδ does not appear to be involved in the inhibition of insulin secretion that occurs following exposure to cytokines.

As a member of the novel subgroup of PKC isoforms, PKCδ is traditionally activated in receptor signalling cascades by diacylglycerol, which is generated in turn from the breakdown of phosphoinositides by phospholipase C [6]. The best characterised function of PKCδ is as a positive regulator of apoptosis, but the underlying mechanisms are complex and diverse [9–11]. We now show that loss of PKCδ protects against beta cell death in response to cytokines but not STZ. These results point to a role for PKCδ specifically in cytokine-stimulated apoptosis, as STZ acts via chemical disruption of DNA and non-enzymatic generation of NO in vitro [44]. Note that this is different from its mode of action in vivo, where multiple low doses of STZ are thought to trigger an autoimmune attack on beta cells [38, 39]. Our current results are therefore consistent with previous data showing that cytokines, especially IL-1, activate the phospholipase C pathway in beta cells [5], and we previously provided evidence that PKCδ serves as a component of the downstream signalling cascade [7]. The present study, however, strongly supports the argument against a major involvement of PKCδ as an upstream component in the ERK, JNK and p38 pathways that are activated by cytokines and implicated in beta cell apoptosis.

Work from several laboratories has demonstrated that cytokines also induce ER stress in beta cells [14–18]. However, the extent to which this contributes to apoptosis under these conditions remains to be resolved. We favour the view that, at least in vitro, ER stress makes less of a contribution to beta cell apoptosis in response to cytokines than it does with other cytotoxic stimuli such as saturated fatty acids [14]. Here we demonstrate that induction of the ER stress markers phospho-EIF2A3 and DDIT3 by cytokines is not altered by deletion of *Prkcd*, even though there was a protection against apoptosis. This would suggest that PKCδ acts either independently of ER stress, or downstream of it. Because we observed modulation of NO generation, but not ER stress, our results would tend to support the argument against the view that NO serves as an upstream trigger of ER stress in cytokine-stimulated apoptosis [19, 45]. However, the 25% inhibition of NO demonstrated here may have been insufficient to affect ER stress.

Both our current results and previous studies [36, 37] suggest that a major protective role afforded by deletion of *Prkcd* might involve the destabilisation of multiple mRNA transcripts. Preeminent among these would be *Nos2* such that the corresponding mRNA would be degraded more rapidly in the absence of PKCδ, thereby limiting NO generation. In general, however, the role of PKCδ in *Nos2* expression and NO generation shown here for primary islets is more modest than that previously elaborated using INS-1 cells [7]. Despite this, we did observe a protective effect of *Prkcd* deletion using the MLD-STZ model. This suggests that mechanisms in addition to reduced NO generation might be active in vivo. In support of this, we provided data suggesting that PKCδ potentially regulates stabilisation of multiple beta cell gene transcripts, and to a greater extent than *Nos2*. Although these results will need to be confirmed and extended in future studies, it seems

more than coincidental that many of the candidate genes cluster to the IL-R1 and/or TLR2 signalling pathways. We believe the latter would provide the more productive avenue for further investigation. First, we were unable to provide direct evidence for a downregulation of IL-1R signalling (although we only examined this in conjunction with TNF-α and IFNγ, which may have confounded a modest effect). Second, TLR2 signalling to MAPK pathways was disrupted by deletion of *Prkcd*. This is noteworthy, as TLR2 has been implicated in beta cell dysfunction in both type 1 and type 2 diabetes [46–48]. Interestingly, the role of TLR2 in the setting of type 1 diabetes appears to involve recruitment of autoimmune cells that mediate secondary necrosis, rather than a beta cell autonomous effect on apoptosis [47]. Whether PKCδ also specifically modulates this process is a topic for future studies. However, $Prkcd^{-/-}$ mice subjected to the MLD-STZ protocol showed a significantly lower increase in fasting blood glucose compared with wild-type mice, although the time of onset of hyperglycaemia was similar between the two groups. This would be consistent with activation of PKCδ playing a modulatory role in progression of the disease. The MLD-STZ model, however, generates only a mild insulitis [38, 39], and so we were unable to quantify the extent to which loss of PKCδ protects via effects in the beta cell directly, versus effects within immune cells potentially regulating their recruitment during insulitis. Such analyses would be better undertaken in future by backcrossing *Prkcd* null mice onto the NOD background.

In conclusion, there is a growing appreciation that cytokines can regulate expression of (especially proinflammatory) genes via mRNA stabilisation, as well as via transcription [49, 50]. Various PKC isoforms, including PKCδ, are implicated in these stabilisation pathways [36, 37]. Although much effort has been devoted to elucidating the transcriptional networks regulated by cytokines in beta cells, our results suggest that mRNA stabilisation pathways might also be a topic of great relevance to type 1 diabetes, particularly in the context of a role for PKCδ.

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