Diabetologia (2010) 53:946–954
DOI 10.1007/s00125-010-1668-1

ARTICLE

Protein kinase C-δ is involved in the inflammatory effect of IL-6 in mouse adipose cells

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Received: 22 September 2009 / Accepted: 17 December 2009 / Published online: 12 February 2010

Abstract
Aims/hypothesis The aim of the study was to address the role of protein kinase C-δ (PKCδ) on phosphorylation of signal transducer and activator of transcription 3 (STAT3) and activation of inflammatory genes in response to IL-6 in adipose cells.
Methods Differentiated mouse 3T3-L1 adipocytes preincubated with the PKCδ inhibitor rottlerin and mouse embryonic fibroblasts (MEFs) lacking PKCδ were incubated with IL-6 and/or insulin. RNA was extracted and the gene expression was analysed by real-time PCR, while the proteins from total, nuclear and cytoplasmic lysates were analysed by immunoblotting.
Results Inhibition of PKCδ by rottlerin significantly reduced both Ser-727 and Tyr-705 phosphorylation of STAT3. Consequently, nuclear translocation of STAT3 and the IL-6-induced gene transcription and protein release of the inflammatory molecule serum amyloid A 3 (SAA3) were reduced. Similarly, the IL-6-regulated gene transcription of *Il-6* (also known as *Il6*) to *Hp* and the feedback inhibitor of IL-6, *Socs3*, were also attenuated by rottlerin. Furthermore, PKCδ was found to translocate to the nucleus following IL-6 treatment and this was also reduced by rottlerin. In agreement with the effect of rottlerin, *Pkcδ* (also known as *Prkcd*)$^{-/-}$ MEFs also displayed a markedly reduced ability of IL-6 to activate the transcription of *Saa3*, *Hp, Socs3* and *Il6* genes compared with wild-type MEFs. These results correlated with a reduced nuclear translocation and phosphorylation of STAT3.
Conclusions/interpretation These results show that PKCδ plays a key role in the inflammatory effect of IL-6 in adipose cells and may be a suitable target for novel anti-inflammatory agents.

Keywords 3T3-L1 · IL-6 · Inflammation · Insulin resistance · Obesity · PKC delta · STAT3

Abbreviations

ERK	Extracellular signal-regulated kinase
gp130	Glycoprotein 130
Hp	Haptoglobin
HRP	Horseradish peroxidase
IRS-1	Insulin receptor substrate-1
JAK2	Janus kinase 2
JNK	c-Jun N-terminal kinase
MAPK	Mitogen-activated protein kinase
MEF	Mouse embryonic fibroblast
mTOR	Mammalian target of rapamycin
NPM	Nucleophosmin
PKCδ	Protein kinase C-δ
PLCγ-1	Phospholipase C γ-1
RQ	Relative quantity
SAA3	Serum amyloid A 3
siRNA	Small interfering RNA
SOCS3	Suppressor of cytokine signalling 3
STAT	Signal transducer and activator of transcription
WT	Wild-type

Electronic supplementary material The online version of this article (doi:10.1007/s00125-010-1668-1) contains supplementary material, which is available to authorised users.

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Introduction

The adipose tissue is not only the main organ for lipid storage and release but it also has important paracrine and endocrine functions by secreting different adipokines, including cytokines, chemokines, growth factors and complement factors. The expression of inflammatory molecules is increased in obesity and associated with inflammation in the adipose tissue, which, in turn, negatively affects insulin sensitivity [1]. A major secreted inflammatory cytokine is IL-6 [2]. The interstitial level of IL-6 in the adipose tissue is considerably higher than in the peripheral circulation and correlates with the size of fat cells [3], a marker of local inflammation in adipose tissue. Furthermore, ~30% of the circulating levels of IL-6 originate from adipose tissue [4].

Chronically elevated IL-6 levels have been shown to negatively affect insulin signalling and action in liver [5], adipose cells [6–8] and skeletal muscles [9]. Important target molecules for IL-6 are peroxisome proliferator-activated receptor γ, adiponectin, GLUT4 and insulin receptor substrate-1 (IRS-1) [3, 8, 10]. IL-6 both impairs the transcriptional activation of these molecules and increases the degradation of IRS-1 [11, 12]. One mechanism for this is the IL-6-induced increase of the suppressor of cytokine signalling 3 (SOCS3), a feed-back inhibitor of the signal transducer and activator of transcription (STAT) 3 signalling, which also binds to IRS-1 targeting it for proteasomal degradation. The overall effect of IL-6 is the induction of insulin resistance through an impaired insulin signal transduction [11–13] and decreased insulin-stimulated glucose uptake in both adipocytes [8, 14] and skeletal muscle [9].

IL-6 signals through a complex of heterodimerised IL-6 and glycoprotein 130 (gp130) receptors. This complex activates tyrosine kinases from the Janus kinase (JAK) family allowing phosphorylation of gp130. STATs, mainly STAT3, bind via the SH2 domain to phosphotyrosine residues on the gp130 receptor, allowing STAT3 to become phosphorylated on Tyr-705. This phosphorylation is necessary for the homo- or heterodimerisation of STAT3 and STAT1, and a prerequisite for the nuclear translocation of the STAT molecules [15, 16].

The additional Ser-727 phosphorylation has been reported to increase the transcriptional activation of STAT3 [17–21]. Depending on cell origin, many different kinases have been shown to increase Ser-727 phosphorylation of STAT3, such as protein kinase C-δ (PKCδ), extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (MAPK) and the mammalian target of rapamycin (mTOR) [18–22].

We have previously reported that insulin exerts an anti-inflammatory effect on the IL-6 signalling pathway by reducing Tyr-705 phosphorylation and nuclear translocation of STAT3, leading to a reduction of IL-6-activated genes. We also showed that insulin increases Ser-727 phosphorylation of STAT3 through a MAPK-dependent pathway that was different from that of IL-6 [23].

In this study, we further characterised Ser-727 phosphorylation of STAT3 by IL-6 and its potential role in mouse 3T3-L1 adipocytes by using specific inhibitors for the kinase pathways presented above [18–22]. We concluded that PKCδ was not only important for Ser-727 phosphorylation, but also for the Tyr-705 phosphorylation, nuclear translocation and transcriptional properties of STAT3. Furthermore, IL-6-inducible inflammatory genes such as *Il-6* (also known as *Il6*), serum amyloid A 3 *(Saa3*) and *Hp*, as well as the feedback regulator *Socs3*, were all reduced by inhibition of PKCδ. This effect appears specific for IL-6-induced inflammatory genes, since other non-IL-6-inducible but STAT-regulated genes were not altered.

Methods

Cell cultures Mouse 3T3-L1 preadipocytes, wild-type (WT) mouse embryonic fibroblasts (MEFs) and *Pkcδ* (also known as *Prkcd)*$^{-/-}$ MEFs (a kind gift from M. E. Reyland, University of Colorado Health Sciences Center, Aurora, CO, USA) [24] were grown in DMEM supplemented with 10% (vol./vol.) FBS, 2 mmol/l glutamine and 1% penicillin/streptomycin (1% [vol./vol.]) [24]. MEFs were also supplemented with non-essential amino acids (Lonza, Rockland, ME, USA). The 3T3-L1 cells were differentiated 2 days after confluence, as described before [23]. On day 8, the differentiated 3T3-L1 adipocytes were starved in DMEM containing 1.5% (vol./vol.) FBS for 8 h, washed twice with PBS and then incubated with DMEM supplemented with 0.5% (wt/vol.) BSA for another 16 h before starting the experiment on day 9. Insulin (100 nmol/l) was added 10 min before IL-6 (20 ng/ml; Chemicon, Temecula, CA, USA) while rottlerin (6μmol/l; Sigma-Aldrich, St Louis, MO, USA) was added 30 min before addition of IL-6. MEFs were starved for 16 h in DMEM supplemented with 0.5% (wt/vol.) BSA before addition of 30 ng/ml IL-6 together with 30 ng/ml soluble IL-6 receptor (Sigma-Aldrich) as indicated. Transfection of PKCδ small interfering RNA (siRNA; Sigma-Aldrich) in 3T3-L1 cells was performed on day 3 in the differentiation cycle using Liposome 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA).

Cell lysates and immunoblotting Differentiated 3T3-L1 adipocytes were cultured in 25 cm^2 flasks, washed twice with ice-cold PBS and lysed in lysis buffer as previously described [23]. The cell lysate was centrifuged at 20,000×*g* for 10 min and the supernatant fraction was kept at −80°C.

The nuclear cell extract was prepared as suggested by the manufacturer of the STAT3 transcription factor assay kit (Active Motif, Carlsbad, CA, USA). Protein concentration was quantified by the bicinchonic acid method (Pierce, Rockford, IL) or a Bradford protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). The lysates were boiled in SDS loading buffer for 5 min and applied to SDS-PAGE (Lonza, Rockland, ME, USA). Following gel electrophoresis and gel transference, the nitrocellulose membranes were blocked for 1 h with 5% (wt/vol.) fat-free milk powder diluted in PBS–TWEEN 20 (0.05% vol./vol.). Membranes were probed with primary antibodies to the following proteins: Tyr-705-phosphorylated STAT3, Tyr-701-phosphorylated STAT1, nucleophosmin (NPM) and PKCδ (Cell Signaling Technologies, Beverly, MA, USA), Ser-727-phosphorylated STAT3 and gp130 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), phospholipase C (PLC) γ-1 (Upstate Biotechnology, Lake Placid, NY, USA), STAT3 (BD Biosciences, San Jose, CA, USA) and murine SAA3 (kind gift from P. Scherer, University of Texas Southwestern Medical Center, Dallas, TX, USA). Antibodies were diluted according to the recommendation of the manufacturers. The blots were probed with secondary antibody linked to horseradish peroxidase (HRP; Cell Signaling) diluted 1:1,000 in blocking solution. Detection was with an Immun-STAR HRP chemiluminescence kit using a ChemiDoc XRS detection system (Bio-Rad).

STAT3 transcription assay Nuclear extracts were prepared and analysed on a 96 well plate coated with oligonucleotides corresponding to the STAT3 consensus binding site. STAT3 primary antibody was added to the plate followed by HRP-conjugated secondary antibody. The absorbance was read on a spectrophotometer. Thus, this kit measures nuclear STAT3 binding to its consensus binding site. The protocol was essentially performed as suggested by the manufacturer of the STAT3 transcription factor assay (Active Motif).

RT-PCR and quantitative real-time PCR Cells were washed in RNAse-free PBS and mRNA was extracted using the RNeasy Mini Protocol (Qiagen, Valencia, CA, USA). cDNA was synthesised from 0.4 μg total RNA using the High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems, Foster City, CA, USA). The samples were quantified using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems) using gene-specific primers and probes. The sequences are available upon request. 18S ribosomal RNA was used as an endogenous control for each sample.

Statistical analyses Statistical analyses were performed in SPSS or Microsoft Excel with a paired Wilcoxon test, unpaired Mann–Whitney test or paired and unpaired Student's *t* tests as indicated in figure legends. $p<0.05$ was considered statistically significant. Results shown in the figures are means±SEM.

Results

Inhibition of PKCδ with rottlerin inhibits STAT3 signalling We have previously shown that incubation of differentiated 3T3-L1 cells with insulin or IL-6 increases Ser-727 phosphorylation of STAT3 through different protein kinases [23]. An extensive search for known potential kinases inducing Ser-727 phosphorylation of STAT3 in response to IL-6 was performed by using inhibitors to PKCδ, ERK, JNK, p38 MAPK and mTOR (data not shown), as they have previously been described to induce this phosphorylation [18–22]. Our study revealed PKCδ as the main target molecule. We then analysed the effect of the PKCδ inhibitor, rottlerin, on STAT3 activation. Pretreatment with rottlerin significantly reduced the IL-6-induced Ser-727 phosphorylation of STAT3, whereas no significant effect was seen on the insulin-induced phosphorylation (Fig. 1a, c). We also examined the effect of rottlerin on Tyr-705 phosphorylation of STAT3; pretreatment with rottlerin for 30 min significantly inhibited this phosphorylation (Fig. 1d, f), while total STAT3 was not altered in the experiments (Fig. 1b, e).

Rottlerin reduces the transcriptional effect of IL-6 on inflammatory genes We have previously reported that IL-6 activates the transcription of *Socs3*, *Saa3* and *Hp* genes in differentiated 3T3-L1 adipocytes [23]. Pretreatment with rottlerin significantly reduced the IL-6-induced activation of *Socs3* and the *Il-6* gene itself (1 h), as well as of *Hp* and *Saa3* (24 h; Fig. 2a). Another general PKC inhibitor, H7, also totally blocked the activation of these genes by IL-6 (Electronic supplementary material [ESM] Fig. 1). However, other non-IL-6-inducible STAT-regulated genes, such as *Pepck* (also known as *Pck1*) or *G6pc* [25], were not significantly altered (Fig. 2b), suggesting that PKCδ is particularly important for the activation of inflammatory genes by IL-6.

Secreted SAA3 protein to the culture medium was also analysed by immunoblotting. Culture media from differentiated 3T3-L1 cells incubated for 24 h with IL-6 alone displayed an increased secretion of SAA3, whereas this was inhibited by rottlerin (Fig. 2c, d).

Effect of rottlerin on the transcriptional properties and nuclear localisation of STAT3 As rottlerin markedly impaired IL-6-induced STAT3 phosphorylation and gene activation, we also investigated the effect of rottlerin on

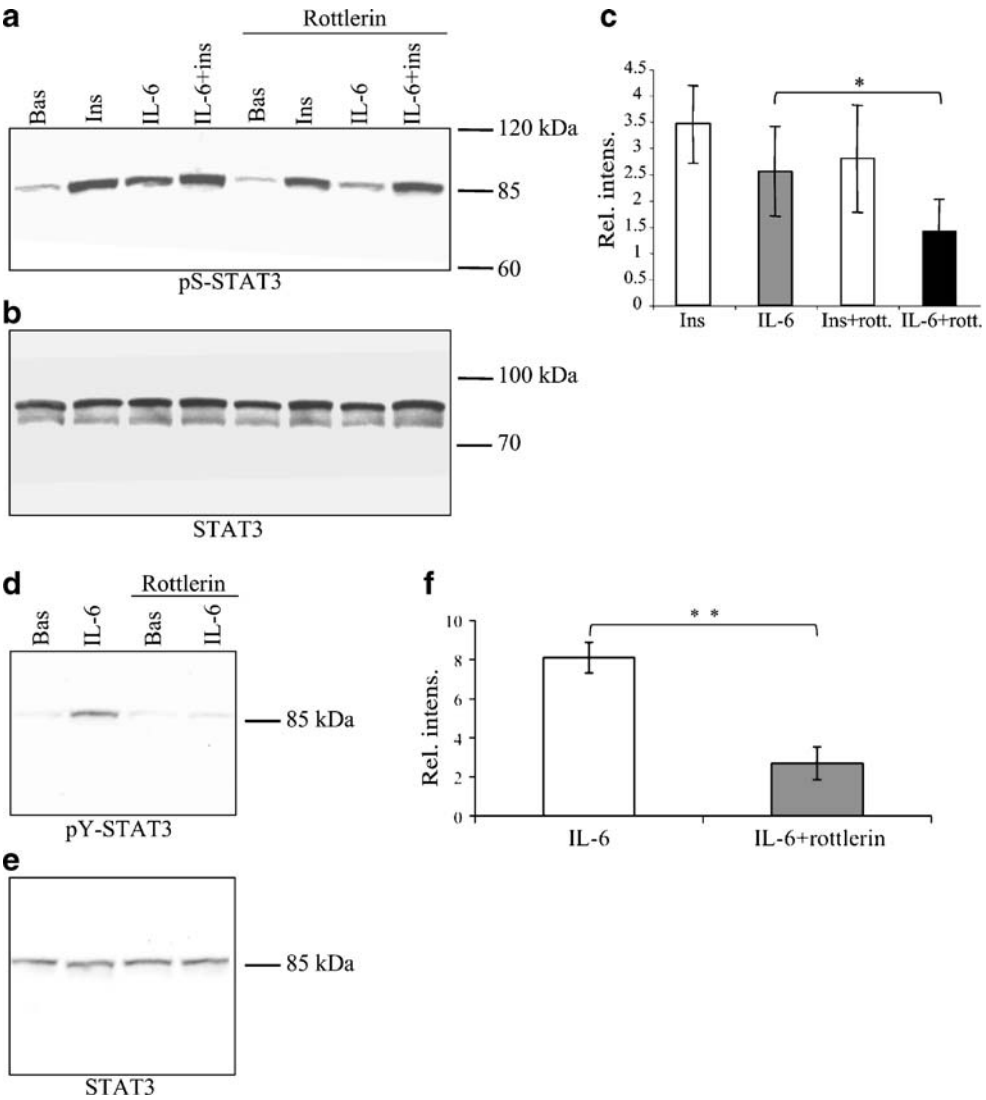

Fig. 1 Effect of rottlerin on STAT3 phosphorylation. Immunoblots of lysates from differentiated 3T3-L1 adipose cells pretreated with rottlerin before addition of insulin and/or IL-6 for 30 min. Immunoblots showing Ser-727-phosphorylated STAT3 (pS-STAT3) (**a**) and total STAT3 protein (**b**). **c** Bar chart of the corresponding relative band intensities (Rel. intens.) from five individual experiments (means±SEM, basal = 1, *$p<0.05$, paired Student's *t* test). Immunoblots showing Tyr-705-phosphorylated STAT3 (pY-STAT3) (**d**) and total STAT3 (**e**). **f** Bar chart of the corresponding band intensities from six individual experiments (means±SEM, basal = 1, **$p<0.01$, paired Student's *t* test). Bas, basal; ins, insulin; rott., rottlerin

STAT3 nuclear translocation and its transcriptional properties (STAT3 binding to consensus sequence). Differentiated 3T3-L1 cells, pretreated with rottlerin before IL-6 addition, showed 42% ($p<0.05$) reduced DNA binding of STAT3 compared with IL-6 alone (Fig. 3a). Furthermore, nuclear translocation of STAT3 was also markedly reduced (Fig. 3b, c), which is consistent with the inhibitory effect of rottlerin on STAT3 phosphorylation. In agreement with Fig. 1a, Ser-727-phosphorylated STAT3 in the nucleus also appeared to be reduced in the presence of rottlerin, although this difference did not reach statistical significance (ESM Fig. 2a).

Rottlerin reduces the nuclear translocation of PKCδ in 3T3-L1 cells To investigate the potential co-localisation of PKCδ with STAT3, we examined nuclear extracts from differentiated 3T3-L1 adipocytes and found that nuclear translocation of PKCδ was increased in response to IL-6 and that this could be inhibited by the addition of rottlerin (Fig. 3d, e). At this time point, STAT3 is also translocated to the nucleus following IL-6 (Fig. 3b).

Reduced effect of IL-6-induced gene expression in Pkcδ$^{-/-}$ MEFs We also investigated the role of PKCδ in the IL-6-induced inflammatory response in *Pkcδ*$^{-/-}$ MEF cells. IL-6 alone increased *Socs3* and *Il-6* mRNA levels after 1 h in both cell types (Fig. 4). However, the inductions of these genes were much lower (~80%) in *Pkcδ*$^{-/-}$ MEFs compared with WT MEFs (Fig. 4). Furthermore, in contrast to WT MEFs, *Pkcδ*$^{-/-}$ cells did not increase gene transcription of *Hp* or *Saa3* when stimulated with IL-6 for 24 h (Fig. 4). These findings further support a critical role for PKCδ in STAT3 activation of inflammatory genes in response to IL-6. In addition we attempted to transfect differentiated 3T3-L1 cells with PKCδ-siRNA. We were able to downregulate ~25% of the PKCδ expression when cells were transfected on the third day in the differentiation cycle (ESM Fig. 3). On day 8, cells were incubated with IL-6 for 24 h followed

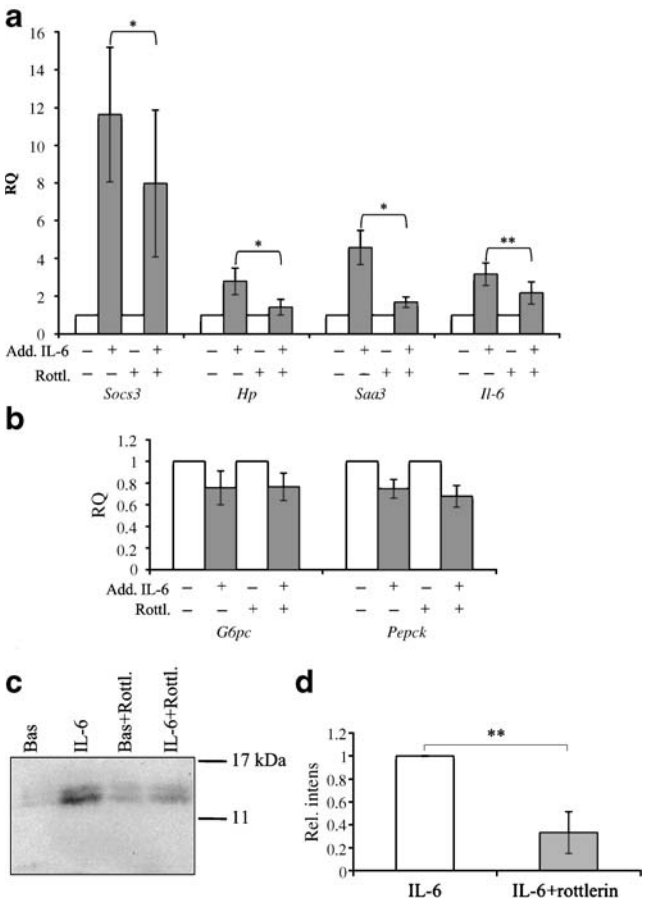

Fig. 2 The effect of rottlerin on IL-6-induced mRNA expression was measured by real-time PCR (white bars, basal samples; grey bars, IL-6 samples). **a** mRNA expression of IL-6-activated genes. Differentiated 3T3-L1 adipose cells were pretreated with rottlerin before incubation with IL-6. The mRNA levels of *Socs3* and *Il-6* were analysed 1 h after IL-6 addition (Add.) (n=6 experiments) and *Hp* and *Saa3* were analysed 24 h after IL-6 addition (n=6 experiments). Means±SEM, *p<0.05, paired Wilcoxon test. **b** Control experiment showing no alteration (paired Student's t test) of mRNA levels of the non-inflammatory STAT3-regulated genes *G6pc* and *Pepck* (means ± SEM, n=4 experiments) when pretreated with rottlerin and subjected to IL-6 for 1 h. **c** Immunoblot showing secreted SAA3 protein in culture media from differentiated 3T3-L1 cells incubated with IL-6 for 24 h with or without rottlerin. The bar chart (**d**) shows corresponding relative band intensities (Rel. Intens.) from four individual experiments, IL-6 samples are set to 1. Means±SEM, **p<0.01, paired Student's t test. Bas, basal; Rottl., rottlerin

by RNA preparation. Real-time PCR analysis of the IL-6-induced gene expression of *Saa3* and *Hp* also showed lower expression of these genes in PKCδ-siRNA-transfected cells compared with non-transfected cells (ESM Fig. 3). However, owing to large variation this reduction did not reach statistical significance.

Reduced nuclear localisation of STAT3 in Pkcδ$^{-/-}$ MEFs We also analysed the nuclear translocation of STAT3 in *Pkcδ*$^{-/-}$ MEFs. Similar to the findings in 3T3-L1 cells incubated with rottlerin (Fig. 3b), nuclear translocation of STAT3 in response to IL-6 was markedly reduced in *Pkcδ*$^{-/-}$ MEFs (Fig. 5a, b). The reduced nuclear localisation of STAT3 was also associated with a decreased Tyr-705

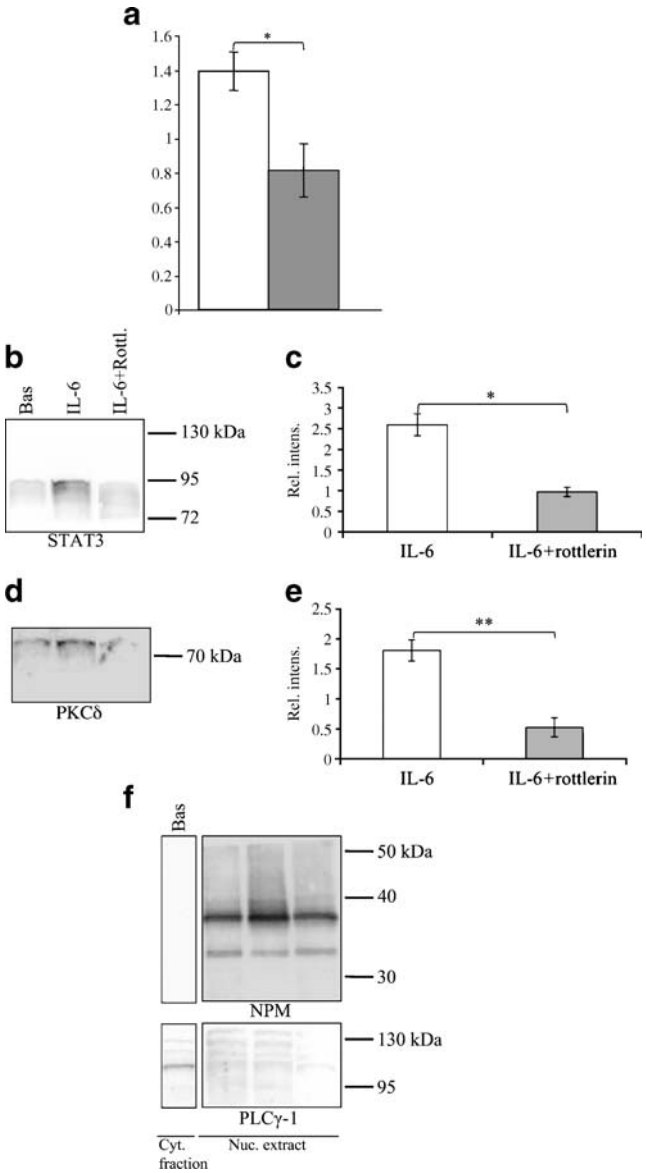

Fig. 3 Effect of rottlerin on transcriptional activation properties and nuclear localisation of STAT3 (white bars, basal samples; grey bars, IL-6 samples). **a** Transcriptional activity of STAT3. Bar chart showing the ability of nuclear STAT3 to bind to its consensus sequence in samples pretreated with rottlerin and incubated with IL-6 for 15 min (n=6 experiments). Means±SEM, *p<0.05, paired Wilcoxon test. **b–e** Nuclear localisation of STAT3 and PKCδ. Immunoblots showing STAT3 (**b**) and PKCδ (**d**) in nuclear lysates from differentiated 3T3-L1 adipose cells incubated with IL-6 or IL-6+rottlerin for 15 min and bar charts of the average corresponding relative band intensities (Rel. intens.) for STAT3 (**c**) (n=3) and PKCδ (**e**) (n=5). Means±SEM, *p< 0.05, **p<0.01, paired Student's t test. **f** Blots showing NPM and PLCγ-1 used as nuclear (Nuc.) and cytoplasmic (Cyt.) markers, respectively. Bas, basal; Rel. intens., relative intensity (basal sample set to 1); Rottl., rottlerin

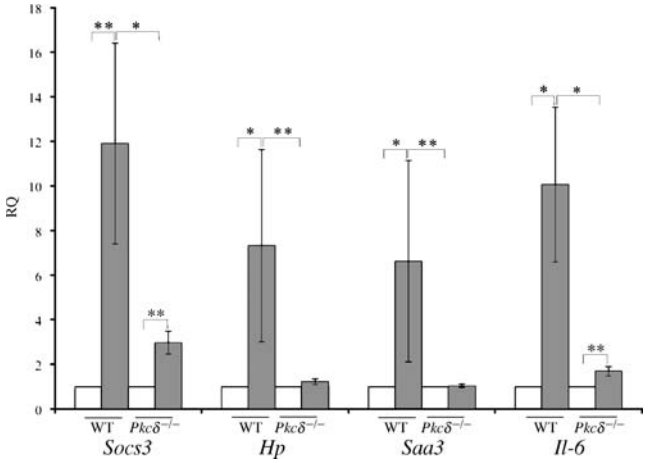

Fig. 4 Reduced effect of IL-6 in *Pkcδ*$^{-/-}$ MEFs. WT MEFs and *Pkcδ*$^{-/-}$ MEFs incubated with IL-6 (white bars, basal samples; grey bars, IL-6 samples). The mRNA levels of *Socs3* and *Il-6* after 1 h, and of *Hp* and *Saa3* after 24 h, were measured by real-time PCR. Results are means±SEM of 6–12 individual experiments. *$p<0.05$, **$p<0.01$; paired Wilcoxon test for basal–IL-6 samples and unpaired Mann–Whitney test for IL-6–IL-6 samples

phosphorylation in response to IL-6 in *Pkcδ*$^{-/-}$ cells (Fig. 5a, b). As for rottlerin-treated adipocytes, IL-6-induced Ser-727 phosphorylation of nuclear STAT3 also tended to be reduced, but the difference did not reach statistical significance (ESM Fig. 2b). Lysates from the cytoplasmic fraction were also analysed by immunoblotting using the same STAT3 antibody. In WT MEFs, cytoplasmic STAT3 protein levels in response to IL-6 were reduced, but this was not seen in *Pkcδ*$^{-/-}$ MEFs (Fig. 5c, d). This correlates with the STAT3 translocation seen in the nuclear extracts (Fig. 5a).

Discussion

As for humans, genetic variations between different mice strains are associated with a differential sensitivity to the complications of obesity. In a comparison between C57Bl/6 (B6) mice and 129S6/SvEvTac, the B6 mice were more sensitive to becoming obese, insulin resistant and glucose intolerant, independently of the fat content in the food. Interestingly, these mice also displayed an upregulation of PKCδ in skeletal muscle, liver and, especially, in epididymal fat [26]. Interestingly, it has recently been shown that PKCδ-deficient mice fed on high-fat diet displayed improved glucose tolerance because of increased insulin sensitivity. Same mice also showed reduced levels of triacylglycerol in the liver [27]. In another study, isolated adipocytes from B6 mice fed a high-fat diet showed impaired insulin-induced glucose uptake, oxidative stress and activation of PKCδ, which were suggested to be important for obesity-induced insulin resistance in the adipose tissue [28]. It has also been shown that PKCδ is involved in insulin-induced Ser-318 phosphorylation of IRS-1, thus impairing insulin signalling [29].

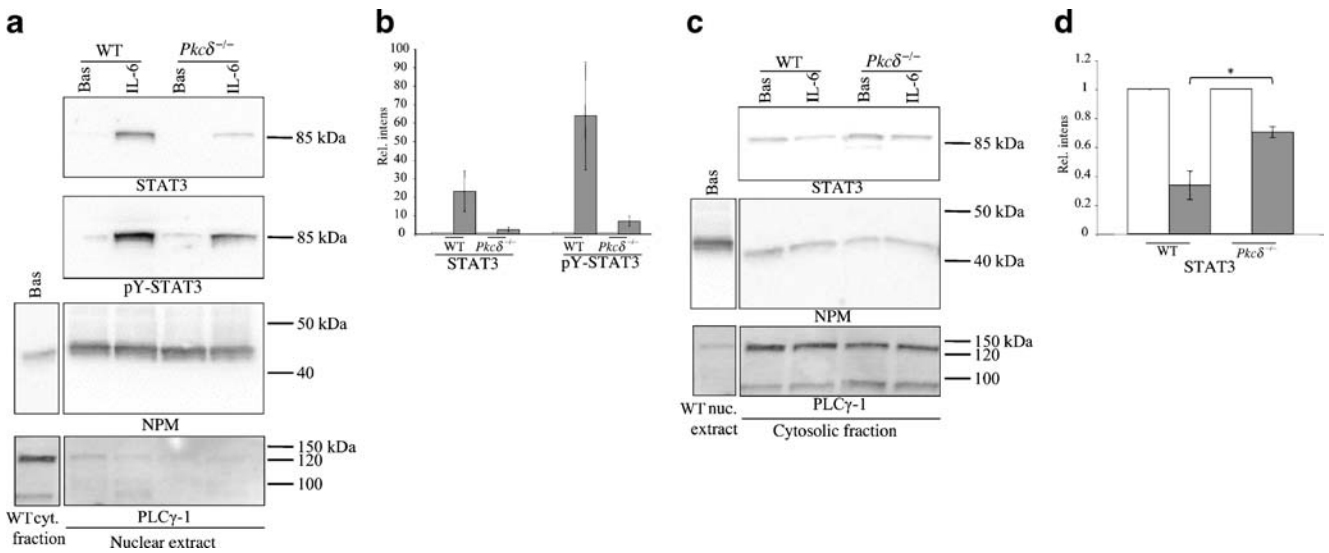

Fig. 5 Reduced nuclear translocation of STAT3 in *Pkcδ*$^{-/-}$ MEFs. Immunoblots showing STAT3 and Tyr-705-phosphorylated STAT3 (pY-STAT3) in nuclear lysates (**a**) and a bar chart (**b**, means±SEM) of the corresponding relative band intensities (Rel. intens.) for nuclear STAT3 and Tyr-705-phosphorylated STAT3 ($n=3$ experiments) for WT MEFs and *Pkcδ*$^{-/-}$ MEFs samples incubated with IL-6 for 15 min. Immunoblots showing STAT3 in the cytoplasmic fraction (**c**) and a bar chart (**d**) of corresponding relative band intensities (Rel. intens.) from WT MEFs and *Pkcδ*$^{-/-}$ MEFs (white bars, basal samples; grey bars, IL-6 samples) incubated with IL-6 for 15 min ($n=3$; basal is set to 1 for each cell type). Means±SEM, *$p<0.05$, unpaired Student's t test. Blots showing NPM and PLCγ-1 are used as nuclear (nuc.) and cytoplasmic (cyt.) markers, respectively (**a**, **c**)

In this study, the role of several kinases (e.g. PKCδ, ERK, JNK, p38 MAPK and mTOR) on IL-6 signalling and activation of inflammatory genes in 3T3-L1 adipose cells was examined. We found that PKCδ plays a critical role in STAT-3 signalling in these cells, since the addition of rottlerin inhibited: (1) IL-6-induced Tyr-705 and Ser-727 phosphorylation of STAT3; (2) nuclear translocation and transcriptional binding of STAT3; (3) IL-6-induced activation of inflammatory genes like *Il-6*, *Saa3* and *Hp*, as well as the feed-back inhibitor *Socs3*; and (4) SAA3 secretion to the culture medium. Although rottlerin has been claimed to be a specific inhibitor of PKCδ [30], recent studies have shown that it may have additional effects in the mitochondria and on 5′-AMP-activated protein kinase [31, 32]. Therefore, we further corroborated our findings in 3T3-L1 cells with rottlerin by using *Pkcδ*$^{-/-}$ MEFs. Essentially all results seen with rottlerin and IL-6 in 3T3-L1 adipocytes were confirmed in *Pkcδ*$^{-/-}$ MEFs. Additionally, we were able to attenuate the expression of PKCδ using siRNA, which also tended to decrease the IL-6-induced gene transcription of *Saa3* and *Hp,* further supporting a role for PKCδ in IL-6 signalling. Interestingly, we found in both *Pkcδ*$^{-/-}$ MEFs and 3T3-L1 adipose cells incubated with rottlerin that the effect of IL-6 on Tyr-705 phosphorylation and on Ser-727 phosphorylation of STAT3 was reduced. Together, these results show that PKCδ plays a key role in IL-6-induced STAT3 phosphorylation and activation. Tyr-705 phosphorylation is crucial for dimerisation and nuclear translocation of STAT3 [15]. Thus, the reduced nuclear translocation of STAT3 seen in the presence of rottlerin and in *Pkcδ*$^{-/-}$ MEFs is an expected consequence of the reduced tyrosine phosphorylation.

There are few reports focusing on the effect of rottlerin on the Tyr-705 phosphorylation of STAT3. In HepG2 cells, Schuringa et al. [18] found no inhibition of IL-6-induced STAT3 Tyr-705 phosphorylation while Xu et al. [33] found that pretreatment with rottlerin totally blocked IL-13-induced STAT3–DNA binding in monocytes. Another report showed that prolactin- or rat placental lactogen 1-induced Tyr-705 phosphorylation of STAT3 is reduced in response to rottlerin in granulosa cells and, thus, also STAT3–DNA binding [34].

PKCδ is obviously involved in (a) critical site(s) of STAT3 activation in response to IL-6. It may play a role in JAK2 activation by IL-6, and this suggestion is supported by a previous report showing that rottlerin reduced the activation of JAK2 in NCM460-NK-1R cells [35]. However, we did not find any difference in JAK2 phosphorylation in the presence or absence of rottlerin (data not shown). Another possibility is that PKCδ is important for stabilising STAT3 binding to gp130. Reports have shown that PKCδ associates with Tyr-705-phosphorylated STAT3 in the cytoplasm [22, 36] and that it can also bind and phosphorylate gp130 [37]. We have also detected an interaction between STAT3 and PKCδ by co-immunoprecipitation, but this interaction was not altered by either IL-6 or rottlerin (ESM Fig. 4a). Nor did rottlerin interfere with the formation of the STAT3–gp130 complex, but the IL-6-induced tyrosine phosphorylation of STAT3 in this complex was reduced by the presence of rottlerin (ESM Fig. 4b), suggesting that inhibition of PKCδ affects an upstream kinase. Furthermore, as both STAT1 and STAT3 are similarly activated [15], we analysed IL-6-induced activation of STAT1 and found a small (but detectable) phosphorylation of Tyr-701 (ESM Fig. 5). This phosphorylation was also reduced in the presence of rottlerin, which further supports a role for PKCδ on an upstream kinase(s).

In order to elucidate if IL-6 activates PKCδ by phosphorylation, the PKCδ phosphorylation sites Thr-505, Ser-643 and Tyr-311 were examined by immunoblotting. However, we could not detect an IL-6-dependent alteration in any of these sites (data not shown). There are very few reports describing an IL-6-induced phosphorylation of PKCδ. Schuringa et al. [18] showed in HepG2 cells that PKCδ Thr-505 is already phosphorylated in the nucleus 2 min after IL-6 addition. However, we were not able to confirm these results in differentiated 3T3-L1 cells.

Nuclear translocation of PKCδ has also been previously studied in HepG2 cells. Schuringa et al. [18] showed that PKCδ was present in the nucleus independently of IL-6 addition, whereas Jain et al. [22] detected PKCδ exclusively in the cytoplasm and not in the nucleus in the HepG2 cell line.

We found that PKCδ is translocated to the nucleus in an IL-6-dependent manner in differentiated 3T3-L1 cells, implying a role for PKCδ in the transcriptional activation of IL-6-inducible genes. Rottlerin reduced the nuclear co-localisation of PKCδ and STAT3 as well as the transcription and secretion of IL-6-regulated inflammatory genes. This further supports our concept that PKCδ is important for IL-6 signal transduction and activation of gene transcription. However, the mRNA levels of several STAT3-regulated but non-IL-6-inducible genes, like *Pepck* and *G6pc* were not changed, suggesting that PKCδ is particularly important for IL-6-inducible inflammatory genes.

In summary, we here demonstrate that PKCδ is important for IL-6-induced Tyr-705 and Ser-727 phosphorylation of STAT3, and nuclear translocation, as well as the activation of genes associated with inflammation in 3T3-L1 adipose cells. Our finding that PKCδ is translocated to the nucleus in response to IL-6, like STAT3, further supports the importance of PKCδ as a regulator of this pathway. These findings suggest that PKCδ is a potential target for the development of anti-inflammatory agents focusing on the effect of IL-6.

Acknowledgements This study was supported by grants from the European Commission (HEPADIP LSHM-CT-2005-018734), the Swedish Diabetes Association, the Swedish Research Council, the

Novo Nordisk Foundation, the Sonya Hedenbratt Memorial Fund, the Martina and Willhelm Lundgren Foundation, the IngaBritt and Arne Lundberg Foundation, the Torsten and Ragnar Söderberg's Foundation, the Gothenburg Royal Society of Arts, the Swedish Foundation for Strategic Research, the Lars Hierta Memorial Fund, the O.E. and Edla Johansson Foundation, the Swedish Insurance Society, the Thuring Foundation, the Magn Bergvall Foundation, the Åke Wiberg Foundation and the Gothenburg Medical Society.

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

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