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Insulin induces Thr484 phosphorylation and stabilization of SIK2 in adipocytes

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ABSTRACT

Aims/hypothesis: Salt-inducible kinase 2 (SIK2) is downregulated in adipose tissue from obese or insulin-resistant individuals and inhibition of SIK isoforms results in reduced glucose uptake and insulin signalling in adipocytes. However, the regulation of SIK2 itself in response to insulin in adipocytes has not been studied in detail. The aim of our work was to investigate effects of insulin on various aspects of SIK2 function in adipocytes.
Methods: Primary adipocytes were isolated from human subcutaneous and rat epididymal adipose tissue. Insulin-induced phosphorylation of SIK2 and HDAC4 was analyzed using phosphospecific antibodies and changes in the catalytic activity of SIK2 with *in vitro* kinase assay. SIK2 protein levels were analyzed in primary adipocytes treated with the proteasome inhibitor MG132.
Results: We have identified a novel regulatory pathway of SIK2 in adipocytes, which involves insulin-induced phosphorylation at Thr484. This phosphorylation is impaired in individuals with a reduced insulin action. Insulin stimulation does not affect SIK2 catalytic activity or cellular activity towards HDAC4, but is associated with increased SIK2 protein levels in adipocytes.
Conclusion/interpretation: Our data suggest that downregulation of SIK2 in the adipose tissue of insulin-resistant individuals can partially be caused by impaired insulin signalling, which might result in defects in SIK2 expression and function.

1. Introduction

Salt-inducible kinase 2 (SIK2) is related to the metabolic regulator AMP-activated protein kinase (AMPK) [1] and displays abundant expression in white adipose tissue [2–5]. We have recently shown that SIK2 is downregulated in the adipose tissue of obese and insulin-resistant individuals [4]. Furthermore, pharmacological inhibition or genetic silencing/deletion of SIK2 is linked to reduced glucose uptake and insulin signalling in adipocytes [4,6,7].

SIK2, like AMPK, is catalytically activated through phosphorylation by the upstream master kinase liver kinase B1 (LKB1) on a T-loop residue (Thr175 in SIK2) [8]. LKB1 activity and Thr175 phosphorylation of SIK2 appears to be constitutively high, but SIK2 can be further regulated by additional phosphorylations in response to extracellular stimuli, of which cyclic AMP (cAMP)/protein kinase A (PKA)-inducing agents are the most studied [9–11]. These phosphorylations regulate various aspects of SIK2 function, such as protein-protein interactions, intracellular localization and protein stability, which might in turn control the cellular activity of SIK2 towards downstream substrates [6,9,12]. In white adipocytes, SIK2 is phosphorylated at several residues in response to cAMP/PKA-signalling (Ser343, Ser358, Thr484, Ser587), resulting in 14-3-3-binding and an intracellular re-localization that restricts its actions on downstream targets [6,9]. A similar regulation of SIK2 by PKA has been described in other cell types

Abbreviations: AMPK, AMP-activated protein kinase; BMI, body mass index; CaMK, calmodulin-dependent protein kinase; cAMP, cyclic AMP; CRTC, cAMP-responsive element-binding protein [CREB]-regulated transcription coactivator; HDAC, histone deacetylase; HSL, hormone sensitive lipase; IRS1, insulin receptor substrate 1; LKB1, liver kinase B1; PKA, protein kinase A; PKB/Akt, protein kinase B; SIK, salt-inducible kinase; TNF, tumor necrosis factor

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[10,11,13]. Reports addressing potential effects of insulin on SIK2 phosphorylation and function have however yielded contrasting and in some cases conflicting results, and detailed studies in adipocytes are lacking [9,10,14–16]. For example, one study reported that insulin increased SIK2 Ser358 phosphorylation and kinase activity in HEK-293 T cells and in hepatocytes [14], whereas in our own studies we found no effects of insulin on SIK2 in these cells [10] or in adipocytes [9]. Insulin stimulation of retinal glia was associated with increased SIK2 kinase activity but the underlying mechanism was not investigated [15]. On the other hand, in brown adipocytes it was shown that insulin stimulation results in phosphorylation of SIK2 at Ser587 with a concomitant decrease in the phosphorylation activity towards SIK substrate cAMP-responsive element-binding protein [CREB]-regulated transcription coactivator 2 (CRTC2) [16]. No studies have so far addressed the regulation of SIK2 in response to insulin in human adipocytes.

The aims of our study were thus to investigate effects of insulin on SIK2 in human adipocytes with regards to changes in phosphorylation, catalytic and cellular activity, and protein stability. Together, this will give a better understanding of whether SIK2 might mediate effects of insulin, and how SIK2 function in turn might be affected by insulin resistance.

2. Material and methods

2.1. Chemicals and reagents

The following reagents were used: complete protease inhibitor cocktail (Roche, Mannheim, Germany), HDAC5tide peptide (GL Biochem, Shanghai, China), pan-SIK inhibitor HG-9-91-01 (Cayman Chemical, Ann Arbor, MI, USA), insulin (Novo Nordisk, Bagsværd, Denmark), PKB/Akt-inhibitor MK-2206 (Active BioChem, Hong Kong), protein G-Sepharose (GE Healthcare, Little Chalfont, UK), bovine serum albumin (BSA), CL-316,243, Dulbecco's Modified Eagle's Medium (DMEM), gentamicin, isoprenaline, proteasome inhibitor MG132, PBS, phenylisopropyl adenosine (PIA) (all Sigma-Aldrich, St. Louis, MO, USA). All other standard chemicals were from Sigma-Aldrich.

2.2. Collection of adipose tissue and isolation of primary adipocytes

Abdominal subcutaneous adipose tissue was collected from patients who underwent laparoscopic cholecystectomy, gastric bypass surgery or reconstructive breast surgery (n = 28 individuals, body mass index (BMI) = 22–53 kg/m^2 [min-max], 30 ± 7 kg/m^2 [mean ± SD]). After excision, the adipose tissue was placed in PBS at room temperature and immediately transported to the laboratory for isolation of adipocytes. Patients with diagnosed type 2 diabetes were excluded from analyses. Epididymal adipose tissue was excised from 6-week-old male Sprague-Dawley rats (Charles River, Sulzfeld, Germany). Adipose tissue was minced and digested with collagenase (1 mg/ml) in a shaking incubator at 37 °C. Digests were filtered and washed in Krebs-Ringer buffer (KRB)-HEPES containing 25 mM HEPES (pH 7.4), 2 mM glucose, 1% (wt./vol.) BSA and 200 nM adenosine to isolate primary mature adipocytes.

2.3. Ethics statement

All subjects were given written and oral information about the study before providing their written informed consent. Human studies were approved by the Regional Ethical Review Board at Lund University. Animal experiments were approved by the Regional Ethical Committee on Animal Experiments in Malmö/Lund.

2.4. Cell cultures and treatments

Isolated primary human adipocytes were either directly stimulated, or incubated overnight in DMEM containing 1 mg/ml gentamicin, 200 nM PIA and 3.5% (wt./vol.) BSA at 37 °C, 5% CO_2. The following day, cells were washed in KRB-HEPES (with 1% wt./vol. BSA) and stimulated as indicated in figure legends. Isolated primary rat adipocytes were treated and stimulated as indicated in figure legends, without overnight recovery. After treatments, adipocytes were washed in KRB-HEPES (without BSA), and lysed in lysis buffer containing 50 mM TRIS-HCl (pH 7.5), 0.27 M sucrose, 1 mM EDTA, 1 mM EGTA, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 1 mM dithiothreitol (DTT), 1% (wt./vol.) NP-40 and complete protease inhibitor cocktail (one tablet/50 ml). Lysates were centrifuged at 13,000 *g* for 15 min (4 °C) and the supernatant was collected. Human mesenchymal stem cells were isolated from adipose tissue, cultured and differentiated *in vitro* as described previously [17]. Fully differentiated adipocytes were stimulated as indicated in figure legends. Protein concentration was determined by the Bradford assay. For activation of cAMP/PKA-signalling in adipocytes, we used either isoprenaline or CL-316,243 depending on species-specific distribution of β-adrenergic receptors. The β_3-adrenergic receptor agonist CL-316,243 was used in rat adipocytes which display high expression of β_3, whereas the pan-β-agonist isoprenaline was used in human adipocytes which instead express β_2 [18].

2.5. Western blotting and antibodies

Cell lysates (5–20 μg protein) were analyzed by SDS-PAGE and western blotting [19]. Detection was performed using horseradish peroxidase (HRP)-conjugated secondary antibodies and SuperSignal® West Pico and Femto Chemiluminescent Substrates (Thermo Fisher Scientific, Rockford, IL, USA). Chemiluminescence signals were visualized in a ChemiDoc XRS + (Bio-Rad, Hercules, C, USA) and quantified by densitometry using the software Image Lab™ 5.1 (Bio-Rad). The following primary antibodies were used for western blotting: mouse anti-β-actin (Sigma-Aldrich, dilution 1:5000), mouse anti-GAPDH (Sigma-Aldrich, dilution 1:2000), rabbit anti-p-HDAC4/5/7 Ser246/Ser259/Ser155 (Cell Signaling Technology (CST), Danvers, MA, USA, dilution 1:1000), rabbit anti-p-HSL Ser563 (CST, dilution 1:1000), mouse anti-HSP90 (BD Biosciences, San Jose, CA, USA, dilution 1:1000), rabbit anti-p-PKB/Akt Thr308 (CST, dilution 1:1000) and Ser473 (Thermo Fisher Scientific, Waltham, MA, USA, 1:5000). The following antibodies were raised in rabbit and affinity-purified by Innovagen (Lund, Sweden): SIK2 (dilution 0.5 μg/ml) and p-SIK2 Ser358 (dilution 1 μg/ml) [9], and p-SIK2 Thr484 (dilution 1 μg/ml) [4]. In house antibodies were validated using relevant recombinant wild type and mutant proteins (Supplementary Fig. 1). Antibodies towards p-SIK2 Ser343 (dilution 1 μg/ml) and p-SIK2 Thr484 (dilution 1 μg/ml) were generous gifts from K. Sakamoto (Diabetes & Circadian Rhythms, Nestlé Institute of Health Sciences, Lausanne, Switzerland) [10]. The following secondary antibodies were used: anti-mouse and anti-rabbit HRP-conjugated secondary antibodies were from Pierce Biotechnology (Thermo Fischer Scientific, Waltham, MA, USA) and GE Healthcare, respectively.

2.6. In vitro kinase assay

SIK2 *in vitro* kinase activity was measured as described previously [19] using a validated assay [6,10]. Briefly, lysates (10–25 μg protein) were incubated with SIK2 antibodies coupled to protein G-Sepharose. Immunoprecipitates were washed with lysis buffer (containing 0.5 M NaCl and 1 mM DTT), followed by kinase buffer (50 mM TRIS-HCl [pH 7.5], 0.1 mM EGTA and 1 mM DTT). Phosphotransferase activity towards the peptide substrate HDAC5tide (200 μM) was assayed and the incorporation of [^{32}P]-phosphate was determined by liquid scintillation counting. One unit of activity (U) was defined as that which catalyzed the transfer of 1 nmol ^{32}P/min to the substrate.

2.7. Statistical analysis

All values are presented as means (± SD). Statistical tests were

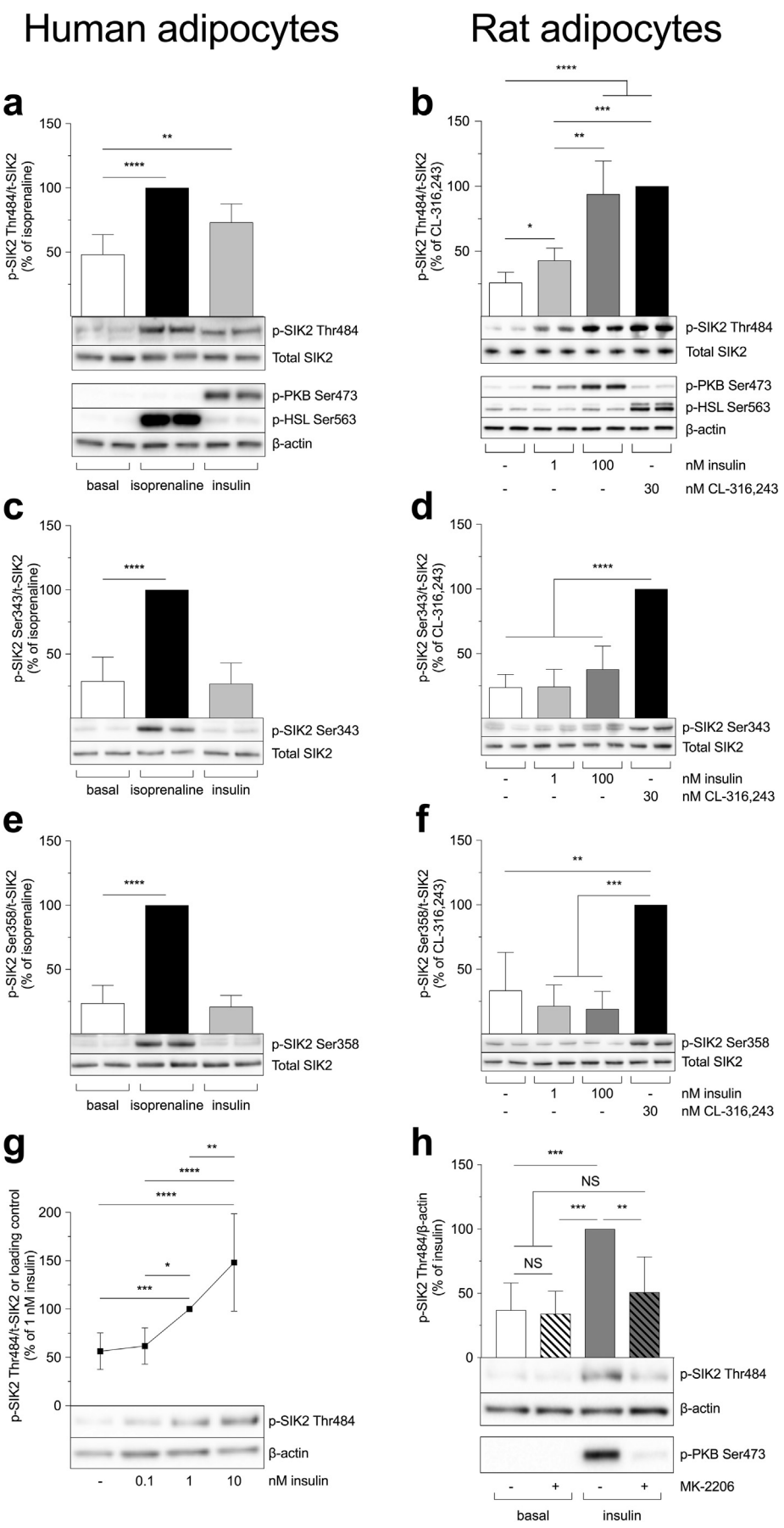

Fig. 1. SIK2 is phosphorylated at Thr484 in response to insulin in primary human and rat adipocytes.

(a-f) Phosphorylation of SIK2 at Thr484 **(a-b)**, Ser343 **(c-d)** and Ser358 **(e-f)** in **(a, c, e)** primary human adipocytes treated with insulin (1 nM, 10 min) or isoprenaline (100 nM, 30 min) (n = 3–9 individuals), and in **(b, d, f)** primary rat adipocytes treated with insulin (1 or 100 nM, 10 min) or the β_3-adrenergic receptor agonist CL-316,243 (30 nM, 30 min) (n = 4 independent experiments). Total SIK2 was used as loading control for normalization. **(g)** Phosphorylation of SIK2 at Thr484 in primary human adipocytes treated with insulin (0.1, 1 or 10 nM, 10–15 min) (n = 4–12 individuals). Total SIK2, β-actin or HSP90 were used as loading controls for normalization. **(h)** Phosphorylation of SIK2 at Thr484 in primary rat adipocytes treated with the PKB/Akt-inhibitor MK-2206 (10 μM, 1 h), followed by stimulation with insulin (100 nM, 10 min). B-actin was used as loading control for normalization (n = 5 independent experiments). The phosphorylation of PKB/Akt at Ser473 and HSL at Ser563 are shown as positive controls for insulin and isoprenaline/CL-316,243 stimulations, respectively. Statistical significance determined by one-way ANOVA followed by Dunnett's **(a, c, e)** or Tukey's **(b, d, f-g)** multiple comparisons post tests, or two-way ANOVA followed by Tukey's multiple comparisons post test **(h)**.

performed using GraphPad Prism 7 (La Jolla, CA, USA) as indicated in figure legends.

3. Results

3.1. SIK2 is phosphorylated at Thr484 in response to insulin in adipocytes

To address a potential regulation of SIK2 by insulin, we employed three different cell models; primary human adipocytes, primary rat adipocytes, and cultured *in vitro* differentiated human adipocytes. Cells were stimulated with insulin, and SIK2 phosphorylation was analyzed using phosphospecific antibodies towards the sites Ser343, Ser358 and Thr484. Stimulation with the β-adrenergic receptor agonists isoprenaline (human adipocytes) or CL-316,243 (rat adipocytes), to activate cAMP/PKA-signalling, was used for comparison since this is known to induce SIK2 phosphorylation [9]. As shown in Fig. 1, cAMP/PKA activation induced the phosphorylation of all three sites as expected, both in primary human (Fig. 1a, c, e) and rat (Fig. 1b, d, f) adipocytes. Interestingly, the phosphorylation at Thr484 was also induced in response to insulin (Fig. 1a-b), whereas the phosphorylations at Ser343 (Fig. 1c-d) or Ser358 (Fig. 1e-f) were unaffected by insulin stimulation. A similar phosphorylation pattern, including insulin-induced phosphorylation at Thr484, was observed in cultured human adipocytes (Supplementary Fig. 2a-c). Additionally, we titrated the induction in the phosphorylation at Thr484 in response to different doses of insulin and observed a dose-dependent increase (Fig. 1g). Together, our results suggest that phosphorylation of Thr484 might represent a novel regulatory pathway of SIK2 by insulin in adipocytes.

A key mediator downstream the insulin receptor is PKB/Akt [20]. To elucidate the role of PKB/Akt in mediating the insulin-induced phosphorylation of SIK2 at Thr484, primary rat adipocytes were treated with a highly selective PKB/Akt-inhibitor (MK-2206) in the absence or presence of insulin. As shown in Fig. 1h, the insulin-induced phosphorylation at Thr484 was reduced almost to the basal level in the inhibitor-treated cells suggesting that the major part of the phosphorylation is PKB/Akt-dependent.

3.2. Insulin-induced phosphorylation of SIK2 at Thr484 is impaired in humans with reduced insulin action

As insulin resistance is a common complication of obesity we investigated the association between the insulin-induced phosphorylation of SIK2 at Thr484 and that of PKB/Akt at Ser473 in primary adipocytes isolated from humans with varying body mass index (BMI). The insulin-induced phosphorylation of PKB/Akt at Ser473 was used as an indirect measure of insulin sensitivity, and displayed a negative correlation with BMI (Fig. 2a). Correspondingly, the insulin-induced phosphorylation of SIK2 at Thr484 displayed a positive correlation with that of PKB/Akt at Ser473 (Fig. 2b), indicating that a reduced insulin action results in impaired phosphorylation of SIK2 and further supporting that Thr484 phosphorylation is dependent on PKB/Akt activity.

3.3. Insulin stimulation does not alter the catalytic or cellular activity of SIK2 in adipocytes

To investigate the functional importance of Thr484 phosphorylation we first measured the *in vitro* kinase activity of SIK2, which had been immunoisolated from cells. The intrinsic catalytic activity was not altered in primary human (Fig. 3a) or rat (Fig. 3b) adipocytes in response to stimulation with cAMP/PKA-elevating agents or insulin. Similar results were obtained in cultured human adipocytes (Supplementary Fig. 2d). The phosphorylation status of the well-characterized SIK substrate histone deacetylase 4 (HDAC4) can be used as a readout for the cellular activity of SIKs [4,6]. Stimulation with insulin did not affect the phosphorylation of HDAC4 in primary human (Fig. 3c) or rat (Fig. 3d) adipocytes, whereas the phosphorylation of HDAC4 was increased in human adipocytes differentiated *in vitro* (Supplementary Fig. 2e). This suggests that insulin and Thr484 phosphorylation might regulate other aspects of SIK2 function than the intrinsic catalytic activity and the cellular activity towards HDAC4.

3.4. Insulin increases SIK2 protein levels in adipocytes possibly by reducing its proteasomal degradation

Another functional consequence of protein phosphorylation can be regulation of protein stability. Therefore, we hypothesized that SIK2 protein levels are regulated by proteasomal degradation in the basal state and that this degradation is inhibited/reduced after insulin stimulation, possibly *via* the insulin-induced phosphorylation at Thr484. Indeed, we observed an increase in SIK2 protein levels and Thr484 phosphorylation compared to basal after insulin stimulation in primary human (Fig. 4a [left bars]) and rat (Fig. 4c [top panels] and 4d [white squares]) adipocytes. In order to elucidate whether the increased SIK2 protein levels in the presence of insulin was a result of a reduced proteasomal degradation we employed the proteasome inhibitor MG132. We reasoned that in this context, we would observe a different pattern of SIK2 protein levels in the MG132-treated cells compared to control. Indeed, treatment with MG132 resulted in an accumulation of SIK2 protein levels in the basal state (Fig. 4b and e), similar to in cells treated with insulin alone (Fig. 4a [left bars] and 4d [white squares]), and there was no further increase in the presence of insulin (Fig. 4a [right bars], 4c [bottom panels] and 4d [black squares]), indicating that insulin stimulation might mediate stabilization of SIK2 protein levels by protecting it from proteasomal degradation.

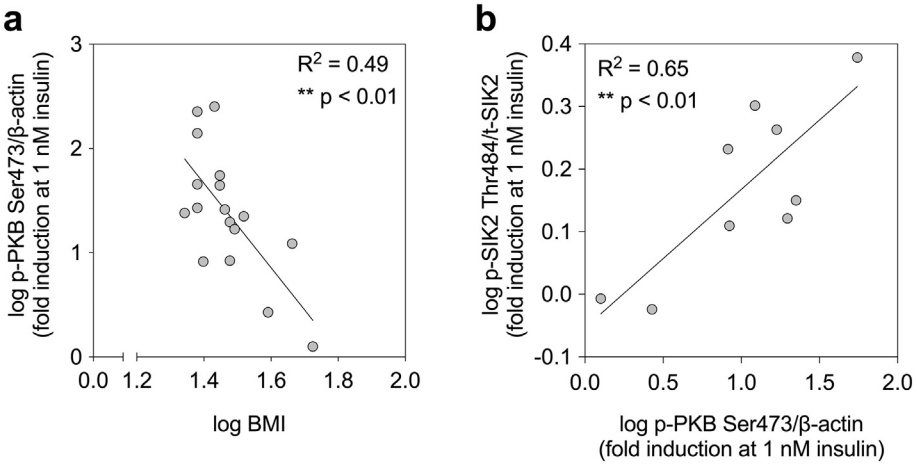

Fig. 2. Insulin-induced phosphorylation of SIK2 at Thr484 is impaired in humans with reduced insulin action.
(a-b) Primary human adipocytes were treated with insulin (1 nM, 10–15 min) and the phosphorylation of SIK2 at Thr484 and PKB/Akt at Ser473 were analyzed. Total SIK2 or β-actin were used as loading controls for normalization, respectively. **(a)** Fold induction of the insulin-induced phosphorylation of PKB/Akt at Ser473 plotted against BMI (n = 17 individuals). **(b)** Fold induction of the insulin-induced phosphorylation of SIK2 at Thr484 plotted against that of PKB/Akt at Ser473 (n = 9 individuals). Correlations made using Pearson correlation test.

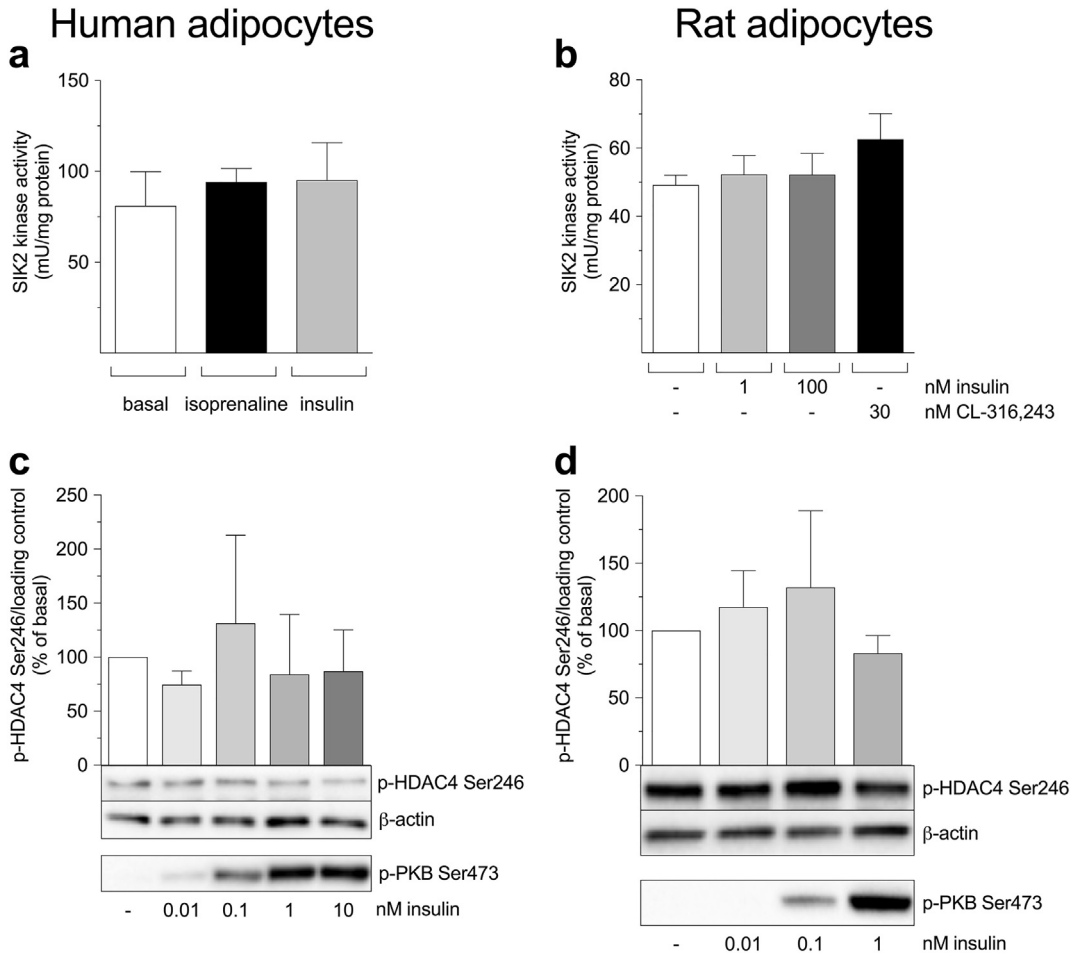

Fig. 3. Insulin stimulation does not alter SIK2 catalytic activity or cellular activity towards HDAC4 in primary human and rat adipocytes.
(a-b) SIK2 *in vitro* kinase activity against the peptide substrate HDAC5tide in **(a)** primary human adipocytes treated with insulin (1 nM, 10 min) or isoprenaline (100 nM, 30 min) (n = 3–6 individuals), and in **(b)** primary rat adipocytes treated with insulin (1 or 100 nM, 10 min) or CL-316,243 (30 nM, 30 min) (n = 3 independent experiments). Specific SIK2 activity ranged from 50.6–105.2 mU/mg protein for human adipocytes, and 45.3–69.9 mU/mg protein for rat adipocytes. Controls for stimulations are shown in Fig. 1a-b, respectively. **(c-d)** Phosphorylation of HDAC4 at Ser246 in **(c)** primary human adipocytes treated with insulin (0.01, 0.1, 1 or 10 nM, 10–20 min) (n = 3–12 individuals), and in **(d)** primary rat adipocytes treated with insulin (0.01, 0.1 or 1 nM, 10 min) (n = 5 independent experiments). B-actin, GAPDH or HSP90 were used as loading controls for normalization. The phosphorylation of PKB/Akt at Ser473 is shown as positive control for insulin stimulation. Statistical significance determined by one-way ANOVA followed by Dunnett's **(a)** or Tukey's **(b-d)** multiple comparisons post test.

4. Discussion

Our study identifies a novel regulatory pathway of SIK2 in response to insulin in adipocytes. Our findings demonstrate that SIK2 is phosphorylated at a specific residue (Thr484) by PKB/Akt, or a kinase downstream PKB/Akt, without altering the intrinsic catalytic activity. This insulin-induced phosphorylation occurs in several adipocyte models, including primary human adipocytes, strengthening the physiological relevance of this regulation. A number of previous studies have explored potential regulation of SIK2 by insulin, with contrasting results [10,14–16]. In our own previous work, we detected no changes in SIK2 Ser358 phosphorylation or intrinsic catalytic activity in response to insulin stimulation of primary rat or cultured 3 T3-L1 adipocytes [9]. However, in that study we did not specifically analyze the potential effect of insulin on other phosphorylation sites, such as Thr484 and Ser343. Interestingly, in our studies of SIK2 in hepatocytes we did not observe any clear changes in Thr484 phosphorylation [10], indicating that this effect of insulin is unique to adipocytes. Taken together, the regulation of SIK2 by insulin displays tissue-specific effects that likely enable specialized functions of SIK2 in different tissues. However, we cannot rule out that additional residues, apart from the ones included in our analysis (Ser343, Ser358 and Thr484), are phosphorylated in response to insulin in adipocytes.

SIK2 is known to relocalize within the cell upon cAMP/PKA activation, a process that likely permits or restricts access to downstream targets, rather than altering its intrinsic catalytic activity [9]. Notably, cAMP/PKA activation in adipocytes *reduces* the SIK2-dependent phosphorylation of its substrates CRTC2, CRTC3 and HDAC4 [6]. Using the phosphorylation status of HDAC4 as a readout for the cellular activity of SIK2 we did not observe any changes in HDAC4 phosphorylation in response to insulin in primary adipocytes. However, the phosphorylation was increased in human adipocytes differentiated *in vitro* indicating that cell-specific effects exist. Whether insulin stimulation involves an intracellular relocalization of SIK2 or changes in protein interactions need to be further studied. Also, we cannot rule out that other substrates are responsive to the insulin-dependent regulation of SIK2. It is interesting to note that Thr484 in SIK2 resides within a sequence that confers to both PKA and PKB/Akt consensus phosphorylation motifs. Indeed, SIK2 is also phosphorylated at Thr484 in response to cAMP-elevating agents (Fig. 1 and Supplementary Fig. 1, [9]), which typically have opposing effects on cellular functions compared to insulin. The specific role of this dual phosphorylation is not clear, but likely enables control of kinase function depending on the environmental context. It is also important to note that PKA in addition, and in

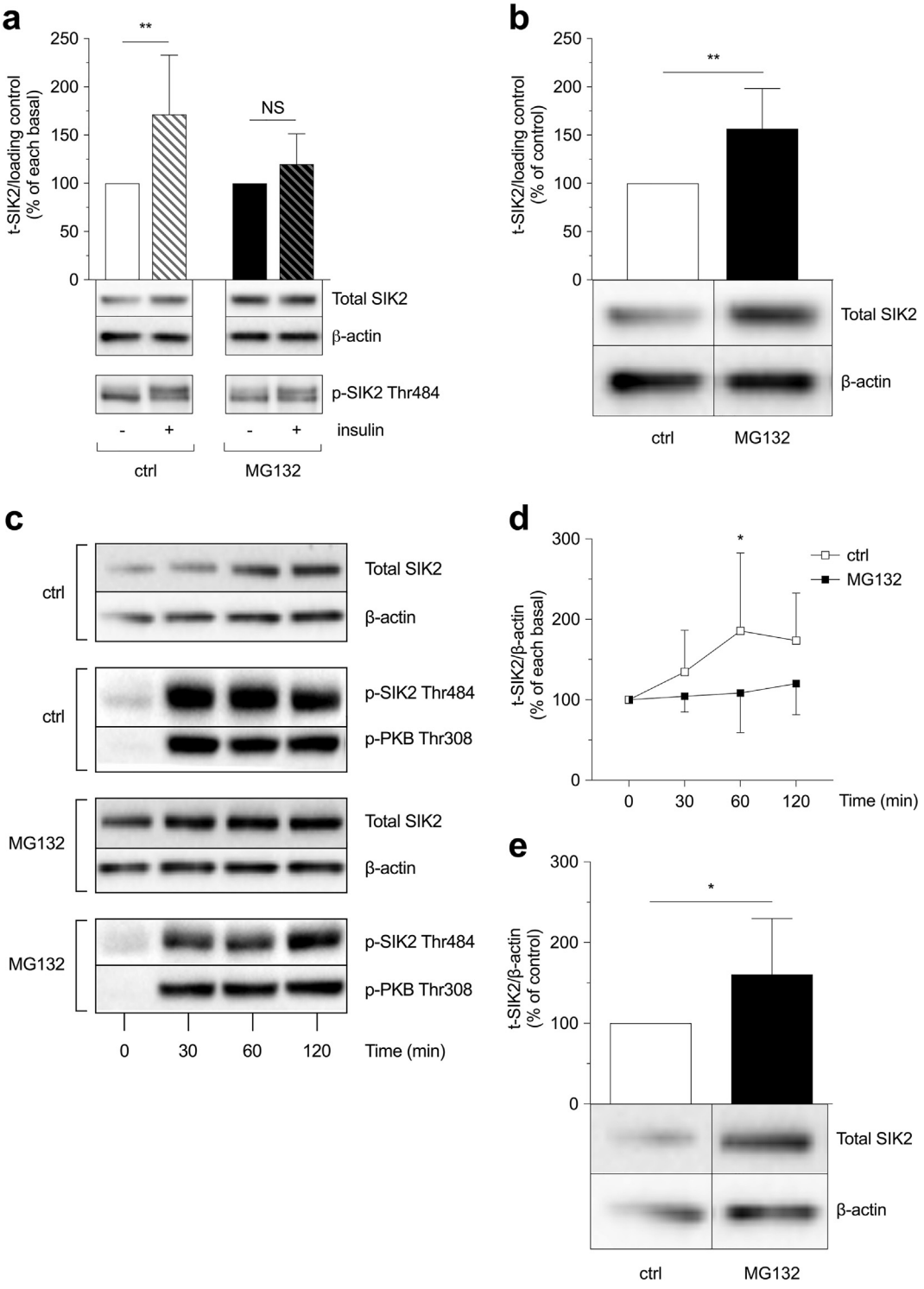

Fig. 4. Insulin increases SIK2 protein levels in adipocytes possibly by reducing its proteasomal degradation.
(a-b) Primary human adipocytes were stimulated with insulin (100 nM) overnight without or with pre-treatment with the proteasome inhibitor MG132 (10 μM, 30 min). Total SIK2 protein and phosphorylation at Thr484 was analyzed (n = 5 individuals). B-actin or HSP90 were used as loading controls for normalization. **(c-e)** Primary rat adipocytes were stimulated with insulin (100 nM) for 0–120 min without or with pre-treatment with the proteasome inhibitor MG132 (10 μM, 30 min). Total SIK2 protein and phosphorylation at Thr484 was analyzed (n = 4–5 independent experiments). B-actin was used as loading control for normalization. The phosphorylation of PKB/Akt at Thr308 is shown as positive control for insulin stimulation. A quantification of the SIK2 total blots in **(c)** is shown in **(d)**. **(b, e)** The effect of MG132 alone on total SIK2 protein in cells without insulin stimulation (basal). Blots shown below graphs originate from panels 4a and 4c, respectively. In all cases, control and MG132-treated samples were run on the same blotting membrane and developed using the same exposure. Statistical significance determined by two-way ANOVA with Sidak's multiple comparisons post test **(a)**, two-way ANOVA with Dunnett's multiple comparisons post test **(d)** or one-tailed unpaired Student's *t*-test **(b, e)**.

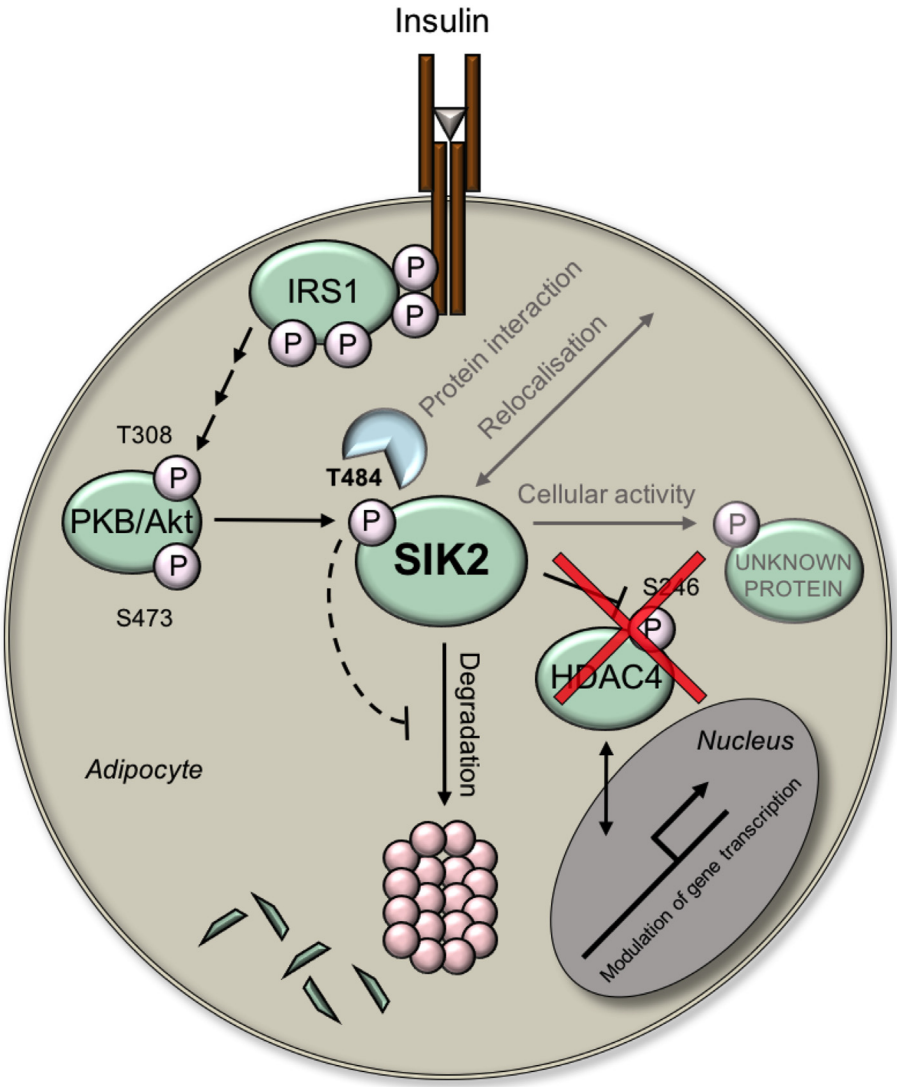

Fig. 5. Schematic summary.
Insulin stimulation of adipocytes results in phosphorylation of SIK2 at Thr484 (T484). Phosphorylation at Thr484 is dependent on PKB/Akt activity and is impaired in humans with reduced insulin action. Insulin stimulation does not regulate the intrinsic catalytic activity or the cellular activity of SIK2 towards the substrate HDAC4, but is associated with increased SIK2 protein levels. Insulin stimulation might mediate stabilization of SIK2 protein levels due to decreased proteasomal degradation, possibly *via* the insulin-induced phosphorylation at Thr484. In future studies, it will be important to determine if the insulin-mediated effects on SIK2 protein levels depend on Thr484 phosphorylation, as well as if Thr484 phosphorylation is involved in the regulation of SIK2 protein interactions, intracellular localization, or phosphorylation towards other, yet unknown, cellular substrates. Grey arrows and text illustrate unstudied processes, and dashed arrows illustrate that the primary mechanism is not known. IRS1, insulin receptor substrate 1; P, phosphorylated residue.

fact to a larger extent, phosphorylates other sites in SIK2, such as Ser358 [9].

Furthermore, we observed that long-term insulin stimulation was important for increasing SIK2 protein levels in adipocytes. Little is known about what controls SIK2 protein levels in adipocytes, but these findings demonstrate that SIK2 expression is not only regulated through changes in gene transcription but also at the post-translational level. In line with this, phosphorylation of SIK1 at the analogous site Thr475 in response to cAMP/PKA-signalling has been described to increase SIK1 protein stability in myoblasts [21]. Moreover, insulin stimulation of retinal glia was associated with increased SIK2 protein levels but the underlying mechanism was not elucidated [15]. On the other hand, Thr484 phosphorylation of SIK2 in response to activation of the Ca^{2+}/calmodulin-dependent protein kinase (CaMK) I/IV pathway resulted in destabilization and proteasomal degradation in neurons [12].

We have previously shown that SIK2 expression is downregulated in adipose tissue of obese or insulin-resistant humans, and that SIK2 mRNA and protein expression are negatively regulated by the pro-inflammatory cytokine TNF-α (tumor necrosis factor-α) in adipocytes [4]. The identified regulation of SIK2 protein stability in response to insulin provides additional insight into the mechanisms underlying the reduced SIK2 expression in human obesity. These findings also suggest that the inflammation and insulin resistance associated with obesity synergistically contribute to the decrease in SIK2 protein levels.

In summary, we have identified a novel regulatory pathway of SIK2 in adipocytes, through insulin-induced phosphorylation at Thr484. Moreover, this phosphorylation was impaired in individuals with a reduced insulin action. Furthermore, insulin stimulation might mediate stabilization of SIK2 protein levels by protecting it from proteasomal degradation, possibly *via* the insulin-induced phosphorylation at Thr484. Important future challenges include determining the requirement of Thr484 phosphorylation for the insulin-mediated regulation of SIK2 protein levels, and to further elucidate potential direct functionalities of Thr484 phosphorylation on the cellular activity of SIK2. A schematic summary of the findings in this study is illustrated in Fig. 5.

5. Conclusions

- SIK2 is phosphorylated at Thr484 in response to insulin in adipocytes.
- Phosphorylation at Thr484 is impaired in humans with reduced insulin action.
- Insulin stimulation does not alter SIK2 catalytic activity, or cellular activity towards HDAC4.
- SIK2 protein levels are increased in response to insulin in adipocytes.

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Duality of interest

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

Author contributions

J.S. contributed to experimental design, researched data and wrote the manuscript. W.L., F.N., F.K, B.H. and A.M.L.P. researched data and reviewed/edited the manuscript. M.E. collected human specimens and reviewed/edited the manuscript. J.L. and K.G.S. contributed to the experimental design and reviewed/edited the manuscript. E.D. assisted in the collection of human adipose tissue at Lund University, contributed to the design of the study and reviewed/edited the manuscript. O.G. contributed to the conception and design of the study and reviewed/edited the manuscript. O.G. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. All authors approved the final version of the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellsig.2018.12.011.

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