FEBS Letters 584 (2010) 4187–4192

FEBS Letters
journal homepage: www.FEBSLetters.org

Fas activation in adipocytes impairs insulin-stimulated glucose uptake by reducing Akt

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ARTICLE INFO

Article history:
Received 24 June 2010
Revised 18 August 2010
Accepted 30 August 2010
Available online 7 September 2010

Edited by Laszlo Nagy

Keywords:
Insulin resistance
Fas (CD95)
3T3-L1 adipocytes

ABSTRACT

Fas (CD95) belongs to the superfamily of the tumor necrosis factor (TNF) receptors. Besides its key role in apoptosis, Fas contributes to non-apoptotic pathways such as cell proliferation and inflammation. In 3T3-L1 adipocytes, activation of Fas by Fas ligand decreased insulin-stimulated glucose uptake, without affecting cell viability. This decrease in glucose uptake was accompanied by reduced protein expression and diminished phosphorylation of Akt. Similarly, insulin-stimulated glucose incorporation and protein levels of Akt were increased in isolated adipocytes from Fas deficient mice when compared to wild-type mice. In conclusion, Fas activation in adipocytes decreases Akt expression and thereby impairs insulin sensitivity.

1. Introduction

Fas (CD95) belongs to the super family of the tumor necrosis factor (TNF) receptors, and plays a key role in the induction of apoptosis [1,2]. However, recent studies identified a novel role for Fas in non-apoptotic pathways such as cell proliferation and inflammation in several cell lines and tissues, similar to the action of TNFα. Accordingly, Fas activation was shown to induce secretion of pro-inflammatory cytokines like IL-1α, IL-1β, IL-8 (KC) or MCP-1 [3,4]. Likewise, we previously reported that activation of the Fas signalling pathway in 3T3-L1 adipocytes increased the production of pro-inflammatory cytokines such as IL-6 and KC, without affecting cell viability [5]. Moreover, FasL-treated 3T3-L1 adipocytes became insulin resistant, as revealed by decreased insulin-stimulated glucose uptake and increased basal lipolysis [5]. However, the molecular mechanism involved in FasL-induced reduction in insulin-stimulated glucose uptake in 3T3-L1 adipocytes is not clear, and the present study seeks to unravel this mechanism. We demonstrate that Fas activation in 3T3-L1 adipocytes reduces Akt/PKB transcription and expression and diminishes phosphorylation of Akt (Thr308 and Ser473) as well as of its downstream target AS160 (Thr642). Moreover, FasL-induced insulin-resistance in 3T3-L1 adipocytes is reversible, and adipocytes isolated from Fas-deficient (Fas-def) mice show increased insulin-stimulated glucose incorporation compared to WT adipocytes paralleled by higher expression levels of Akt.

2. Materials and methods

2.1. Animals

C57BL/6J wild-type and total body Fas-deficient (Fas-def) mice backcrossed for >10 generations onto this same C57BL/6J inbred strain background (B6.MRL^{lpr}) were obtained from The Jackson Laboratory. Adipocyte-specific Fas knockout (AFasKO) mice were generated as described [5]. All mice were housed in a specific pathogen-free environment on a 12-hour light-dark cycle and fed a high fat diet (HFD) (D12331, Research Diets, New Brunswick, USA). All protocols conformed to the Swiss animal protection laws and were approved by the Cantonal Veterinary Office in Zurich, Switzerland.

2.2. Fat explants

White adipose tissue (retroperitoneal) was collected from wild-type and AFasKO mice [5]. Pads were rinsed in DMEM (4.5 g/L D-glucose, Invitrogen, Basel, Switzerland) containing 0.5% bovine serum albumin (Merck, Geneva, Switzerland) and minced into pieces of 2 cm^2 size and incubated for an hour at 37 °C and

Abbreviations: AFasKO, adipocyte-specific Fas knockout; Fas-def, Fas deficient

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doi:10.1016/j.febslet.2010.08.052

5% CO_2. After washing three times, fat pads were incubated over night with 5 ng/ml FasL at 37 °C. After incubation, pads were washed three times with DMEM, and then incubated with or without 100 nM insulin for 20 min. After washing in PBS, pads were snap frozen in liquid nitrogen and stored at −80° upon lysis.

2.3. Glucose incorporation into isolated adipocytes and glucose uptake by 3T3-L1 adipocytes

Adipocyte isolation and glucose incorporation were performed as described previously [6,7]. Glucose uptake by 3T3-L1 adipocyte was determined as described [8]. Mature 3T3-L1 adipocytes were incubated with 2 ng/ml membrane-bound FasL (Upstate, Lake Placid, NY, USA) for 12 h. For the last 3 h of incubation, cells were starved in FCS-free medium (DMEM, 5 mM glucose). Thirty minutes before glucose uptake, insulin (1 or 100 nM) was added to the medium as indicated.

2.4. Extraction of plasma membranes

Mature 3T3-L1 adipocytes were homogenised in a buffer containing 20 mM HEPES, 250 mM sucrose, 5 mM NaN_3, 1 mM EDTA, 0.2 mM PMSF and a 1:1000 dilution protease inhibitor cocktail (Sigma). Lysates were centrifuged at 1300×*g* for 10 min at 4 °C. Thereafter, supernatant was centrifuged at 229 000×g for 90 min at 4 °C (Kontron Instruments, Schlieren, Switzerland). Pellet was resuspended in 100 µl homogenization buffer and syringed 25 times (1 ml syringe, 25G5/8).

2.5. Western blotting

Cell lysates and tissue samples were homogenized and Western blotting was performed as described previously [5]. The following primary antibodies were used: Anti-phospho-Akt (Thr308), anti-phospho-Akt (Ser473), anti-Akt (Cell signalling, Beverly, MA, USA); anti-phospho-AS160, anti-IRS-1, anti-IR, anti-PPARγ_2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-GLUT4 and anti-GLUT1 (gift from Dr. A. Klip, The Hospital for Sick Children, Toronto, ON, Canada); anti-Fas, anti-AS160 and anti-phospho IRS-1 (Ser 307) (Upstate, Lake Placid, NY, USA). Membranes were exposed in an Image Reader and analyzed with Image Analyzer (FujiFilm, Dielsdorf, Switzerland).

2.6. RNA extraction and quantitative RT-PCR

Total RNA was extracted using the NucleoSpin Kit (Macherey-Nagel, Oensingen, Switzerland) and RT-PCR was performed as described [5]. Akt2 PCR primers Mm00545827_m1 and Mm02026778_g1 were purchased from Applied Biosystems.

2.7. Viability assay

Mature 3T3-L1 adipocytes were incubated for 12 h with 2 ng/ml FasL. Thereafter, cells were incubated with 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) for 1 h. Salt was extracted from the cells with DMSO. The amount of yellow MTT reduced to purple formazan was measured spectrophotometrically.

2.8. Data analysis

Data are presented as means ± SEM and were analyzed by Student's *t* test, one sample *t* test or by analysis of variance (ANOVA) with a Tukey correction for multiple group comparisons.

3. Results

3.1. Fas (CD95) activation in 3T3-L1 adipocytes reduces insulin-stimulated glucose uptake

Fas (CD95) receptor is expressed in 3T3-L1 adipocytes as well as in isolated adipocytes of WT mice, but not in adipocytes of adipocyte-specific Fas knockout (AFasKO) mice (Fig. 1A). Moreover, incubation of 3T3-L1 adipocytes with 2 ng/ml of membrane-bound FasL for 12 h decreased insulin-stimulated glucose uptake in 3T3-L1 adipocytes at different insulin concentrations (1 and 100 nM) without affecting cell viability, while a concentration of 1 ng/ml FasL had no effect on glucose uptake (Fig. 1B and C).

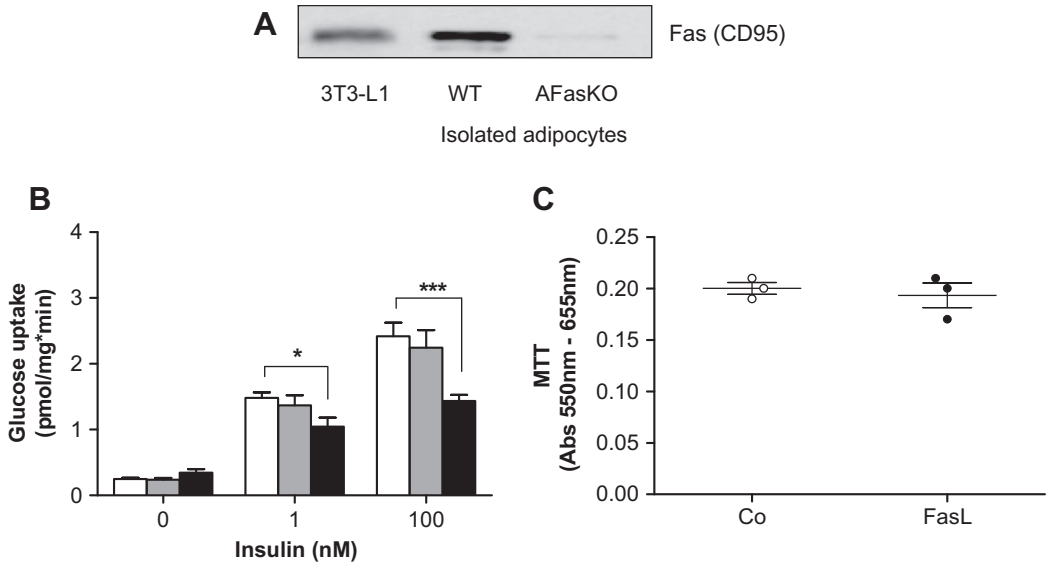

Fig. 1. Fas activation impairs insulin-stimulated glucose uptake. (A) Lysates were prepared from mature 3T3-L1 adipocytes and from isolated white adipocytes, resolved by LDS–PAGE and immunoblotted with anti-Fas antibody. (B) Mature 3T3-L1 adipocytes were incubated without (open bars), with 1 ng/ml (grey bars), or 2 ng/ml (black bars) FasL for 12 h and glucose uptake was determined as outlined in the Section 2. Results are the mean ± SEM of 4–9 independent experiments. $^{*}P < 0.05$; $^{***}P < 0.0005$ (ANOVA). (C) Mature 3T3-L1 adipocytes were incubated for 12 h with or without 2 ng/ml FasL. Thereafter, yellow MTT reduced to purple formazan was measured spectrophotometrically (550–655 nm). Results are the mean ± SEM of three independent experiments.

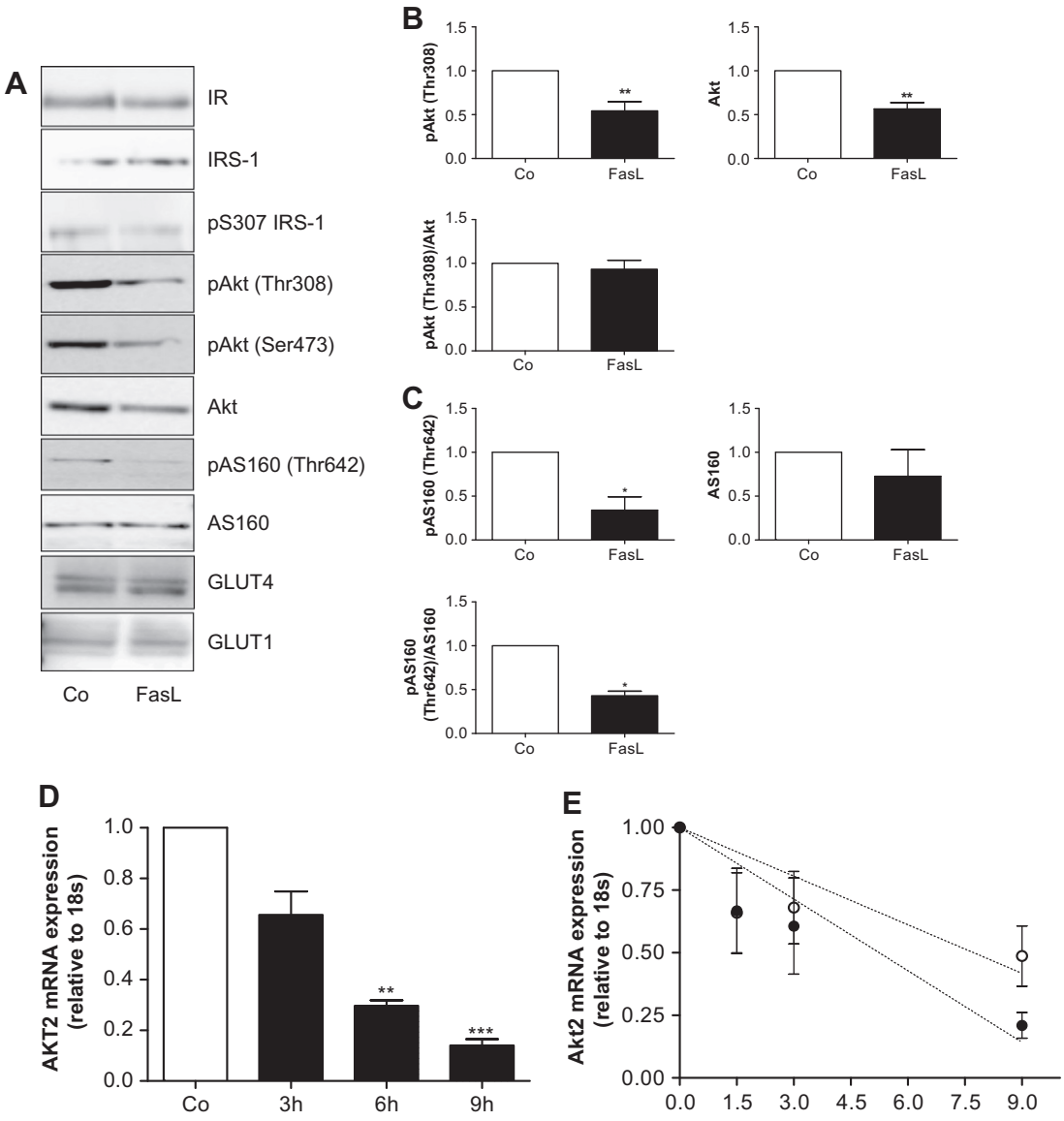

Fig. 2. Fas activation down regulates Akt expression. (A) Total lysates of untreated and FasL treated (2 ng/ml for 12 h) 3T3-L1 adipocytes were resolved by LDS–PAGE and immunoblotted with respective antibodies. Total membrane lysates were used for immunoblotting with GLUT4 and GLUT1 antibody. (B) Expression levels of phosphorylated, total and the ratio of phosphorylated to total Akt (B) and AS160 (C) was calculated in untreated and FasL treated (12 h) cells. Results are the mean ± SEM of 3–6 independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$ (one sample *t* test). (D) mRNA expression of Akt2 in 3T3-L1 adipocytes treated without (open bar) or with (black bars) FasL for 3, 6, and 9 h. Results are the means ± SEM of three independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$ vs untreated control (one sample *t* test). (E) Mature 3T3-L1 adipocytes treated with 5 μg/ml actinomycin D (ActD; Sigma) for various time periods (0, 1.5, 3 and 9 h) were incubated either with (filled circles) or without (open circles) FasL. mRNA expression of Akt2 was determined with two different set of primers (shown are results for one primer) and linear regression analysis (dotted line) was performed to calculate $t_{1/2}$. Results are the means ± SEM of three independent experiments.

Intracellular contents of proteins involved in the insulin signalling pathway were analyzed next. Incubation of 3T3-L1 adipocytes with 2 ng/ml FasL for 12 h had no effect on IR, IRS-1, pIRS-1 (Ser307), total AS160, GLUT4 and GLUT1 levels (Fig. 2A and Table 1). However, levels of phosphorylated Akt (Thr308 and Ser473) as well as of total Akt were significantly down regulated upon Fas activation (Fig. 2A and Table 1). Interestingly, the ratio of phosphorylated Akt to total Akt was not affected upon Fas activation, suggesting that Fas activation reduces primarily Akt expression and does not directly interfere with insulin signalling upstream of Akt (Fig. 2B). In order to investigate whether decreased pAkt levels lead to reduced propagation of the insulin signal, phosphorylation of one of Akt's downstream target, AS160, was determined. Indeed, incubation of 3T3-L1 adipocytes with 2 ng/ml FasL for 12 h attenuated phosphorylation of AS160 on Thr642 (Fig. 2A and Table 1) without affecting total AS160 levels (Fig. 2C), suggesting impaired insulin signalling downstream of Akt. To explore whether Fas activation affects transcript levels of Akt, mRNA levels of Akt were determined in untreated and FasL-treated 3T3-L1 adipocytes. Transcript levels of Akt2, the most abundant and for glucose metabolism most important Akt isoform in adipocytes [9], were significantly down regulated upon FasL incubation (Fig. 2D), demonstrating that Fas activation down regulates Akt expression on transcriptional level. To investigate whether Fas activation has an effect on mRNA stability of Akt2, half life time ($t_{1/2}$) of the mRNA of untreated and FasL-treated cells was determined. As shown in Fig. 2E, $t_{1/2}$ was diminished. However this difference did not reach statistical significance (8.4 ± 1.5 h in untreated vs 5.2 ± 0.6 h in treated cells; $P = 0.12$). Hence, although there is a trend to decreased mRNA stability upon FasL treatment, these data suggest

Table 1
Fas activation affects protein levels of insulin signalling cascade in 3T3-L1 adipocytes.

Protein	Control	FasL
IR	1.0	0.99 ± 0.19
IRS-1	1.0	0.82 ± 0.25
pS307 IRS-1	1.0	1.06 ± 0.16
pAkt (Thr308)	1.0	0.52 ± 0.09**
pAkt (Ser473)	1.0	0.63 ± 0.13*
Akt	1.0	0.56 ± 0.07**
pAS160 (Thr642)	1.0	0.34 ± 0.15*
AS160	1.0	0.73 ± 0.3
GLUT1	1.0	0.83 ± 0.07
GLUT4	1.0	0.97 ± 0.15

Values are means ± SEM of 3–10 independent experiments; *$P < 0.05$, **$P < 0.01$ (One sample t test).

that Fas activation in 3T3-L1 adipocytes may also decrease Akt2 mRNA transcription.

3.2. Impaired insulin-stimulated glucose uptake into 3T3-L1 adipocytes upon Fas activation is reversible

The similar viability of FasL-treated and untreated cells (Fig. 1C), as well as the fact that many proteins of the insulin signalling pathway were not affected by Fas activation (Fig. 2A and Table 1) suggested that incubation of 3T3-L1 adipocytes with 2 ng/ml FasL for 12 h activated mostly non-apoptotic processes. To further support this idea, experiments to determine the reversibility of FasL-induced alterations were performed. 3T3-L1 adipocytes were incubated with 2 ng/ml FasL for 12 h; thereafter cells were washed with PBS, and were recovered in media (DMEM, 5 mM glucose) without FasL for 9 h. Upon recovery, the reduction in insulin-stimulated glucose uptake was reverted (Fig. 3A), suggesting that 3T3-L1 adipocytes were not determined to apoptosis after incubation with 2 ng/ml FasL for 12 h. Similarly, phosphorylation of Akt (Thr308) recovered, accompanied by restoration of total Akt levels (Fig. 3B).

3.3. Membrane-bound FasL signals via Fas receptor

To check whether the observed effects of FasL treatment on 3T3-L1 adipocytes were specific to the activation of the Fas receptor and not to (unspecific) effects, e.g., via activation of the TNF receptor, experiments were performed in fat explants harvested from AFasKO mice. As expected, Fas expression was reduced in white adipose tissue of AFasKO mice compared to WT (Supplementary Fig. 1). However since Fas is still expressed in the stromal vascular fraction of adipose tissue, Fas is still present in adipose tissue of AFasKO mice [5], while it is almost completely absent in isolated adipocytes (Fig. 1A). Whereas incubation of fat explants with FasL from WT mice decreased Akt phosphorylation (Thr308) (Supplementary Fig. 1), similar to what was observed in 3T3-L1 adipocytes (Fig. 2A), FasL treatment had no effect on Akt phosphorylation in fat explants from AFasKO mice (Supplementary Fig. 1). Such data therefore suggest that the membrane-bound FasL used in this study signals specifically via the Fas receptor.

3.4. Akt expression and insulin-stimulated glucose incorporation are increased in Fas-def adipocytes

To investigate whether Fas activation in adipocytes has an effect on Akt expression in vivo, expression levels of Akt were analyzed in isolated adipocytes of WT and Fas-def mice fed a high fat diet (HFD) for 6 weeks. We previously showed that Fas is up regulated in adipocytes of WT mice upon 6 weeks of HFD [5]. As shown in Fig. 4A, deletion of Fas from adipocytes resulted in significantly increased Akt expression compared to WT adipocytes. In addition, insulin-stimulated (0.5 and 100 nM) glucose incorporation into adipocytes isolated from Fas-def mice was preserved (Fig. 4B), suggesting improved insulin sensitivity due to higher Akt levels.

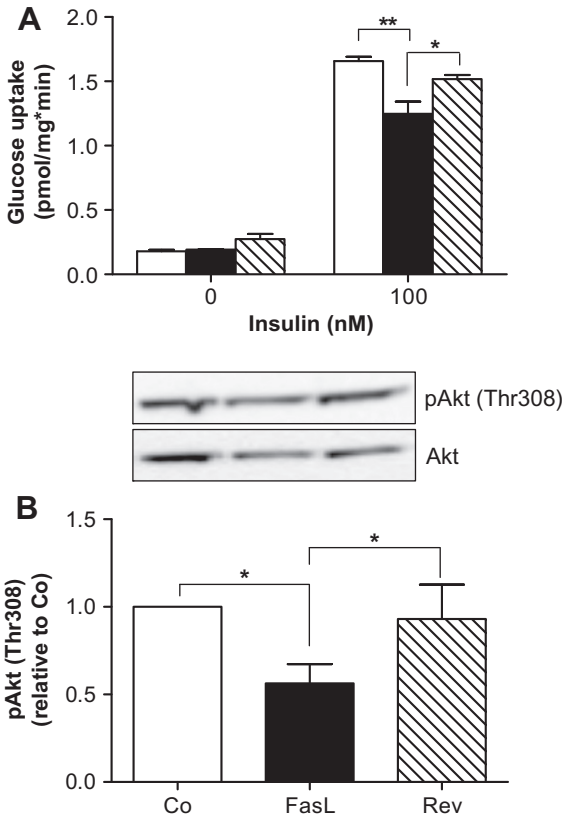

Fig. 3. Recovery of Akt restores insulin-stimulated glucose uptake. (A) Mature 3T3-L1 adipocytes were incubated with (black bars; FasL) or without (open bars; Co) 2 ng/ml FasL for 12 h. Cells were then incubated with normal glucose medium (DMEM, 5 nM, 2% FCS) without FasL for 9 h (hatched bars; Rev) and 2-deoxyglucose glucose uptake was determined as outlined in Fig. 1. Results are the mean ± SEM of three independent experiments. *$P < 0.05$, **$P < 0.01$ (ANOVA). (B) Total lysates of 3T3-L1 adipocytes were treated as outlined in Fig. 3A, stimulated with 100 nM human insulin for 30 min, resolved by LDS–PAGE and immunoblotted with anti-pAkt (Thr308) or anti-Akt antibody. Results are the mean ± SEM of three independent experiments. *$P < 0.05$ (one sample t test, Student's t test).

4. Discussion

Fas (CD95), a member of the TNF receptor super family, is a major contributor to apoptosis in many cells. Findings over the last years, however, revealed that activation of the Fas signalling pathway can evoke non-apoptotic responses in different cells, similar to the action of TNFα [3–5]. We recently provided evidence that activation of Fas may contribute to obesity-induced insulin resistance since mice lacking Fas in adipocytes were partly protected from developing insulin resistance. In particular, Fas activation led to increased release of pro-inflammatory cytokines, and reduced insulin-stimulated glucose uptake in 3T3-L1 adipocytes [5]. The aim of the present study was to further characterize the mechanism involved in the latter. Here we show that Fas activation in 3T3-L1 adipocytes decreased Akt mRNA levels by reducing its transcription (Fig. 2D and E) and, subsequently, protein content. Consequently, reduction in Akt protein levels led to reduced propagation of the insulin signal as manifested by decreased phosphorylation of AS160 albeit its protein levels were not significantly decreased. Similar reduction of to-

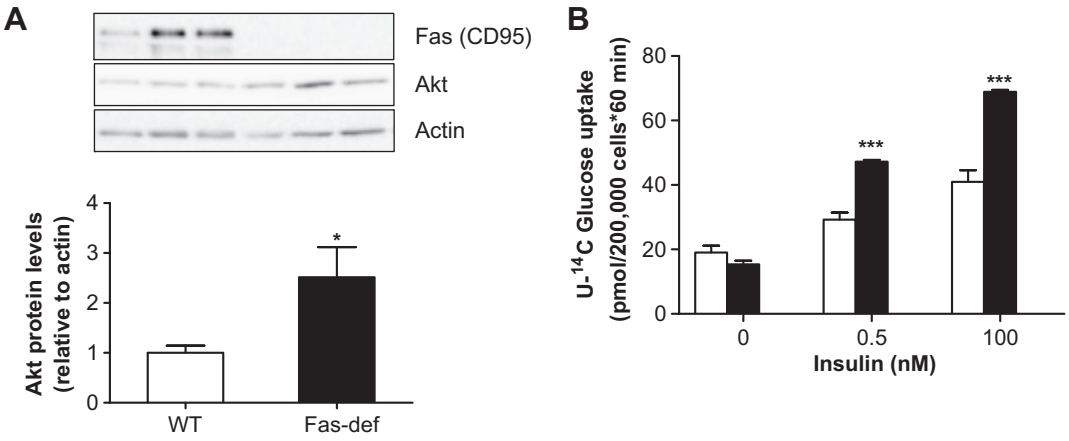

Fig. 4. Akt expression is increased in Fas-def adipocytes. (A) Total cell lysates were prepared from isolated adipocytes of wild-type and Fas-def mice. Lysates were resolved by LDS–PAGE and immunoblotted with anti-Fas, anti-Akt or anti-actin antibody. Results represent the mean ± SEM of 4–5 experiments. $^{*}P < 0.05$ (Student's t test). (B) ^{14}C-D-glucose incorporation into isolated adipocytes from HFD-fed WT (open bars) and Fas-def (black bars) mice was determined in the absence or presence of insulin as indicated. Shown are absolute values of glucose incorporation. Results represent the mean ± SEM of six experiments. $^{***}P < 0.001$ (Student's t test).

tal Akt and pAkt protein levels (Fig. 2A and B and Table 1) upon FasL stimulation suggests that insulin signalling is not altered upstream of Akt. Accordingly, levels of IR, IRS-1 and pS307 IRS-1 were not affected by the activation of the Fas signalling pathway in 3T3-L1 adipocytes (Fig. 2A and Table 1).

Similar findings of reduced Akt levels were reported for the activation of the TNFα pathway in 3T3-L1 adipocytes [10]. However, TNFα levels were not increased in the supernatant of 3T3-L1 adipocytes treated for 12 h with FasL (45.2 ± 1.0 pg/ml in untreated vs 47.4 ± 0.7 pg/ml in FasL-treated cells). In addition, we show herein that membrane-bound FasL signals specifically via the Fas receptor, as FasL incubation of fat pads harvested from AFasKO mice had no effect on the phosphorylation of Akt (Supplementary Fig. 1). Therefore, neither a paracrine TNFα effect nor signalling of FasL via the TNF receptor is responsible for the observed effects. Possibly, increased release of cytokines like IL-6 upon Fas activation contributes to reduced insulin-stimulated glucose uptake in 3T3-L1 adipocytes [5]. However, IL-6 was shown to impair glucose uptake via down regulation of GLUT4 [11], an effect that we did not observe in FasL-treated cells (Fig. 2A and Table 1). In addition, FasL-induced inhibition of glucose uptake could not be reverted using a neutralizing IL-6 antibody (data not shown). Moreover, Fas activation induced secretion of IL-6 into the supernatant was only observed after 6 h of incubation (Supplementary Fig. 2), whereas Akt mRNA was already decreased upon 3 h of FasL incubation (Fig. 2D). These data suggest that increased IL-6 secretion is not involved in decreased Akt mRNA transcription upon Fas activation. What could be the mechanism of reduced Akt transcription upon FasL incubation in 3T3-L1 adipocytes? Fas activation was previously shown to activate the NF-κB, JNK and ERK signalling pathways in several cells and therefore to affect transcription [12]. However, incubation of 3T3-L1 adipocytes with membrane-bound FasL did neither activate the NF-κB signalling pathway (Supplementary Fig. 3) nor the JNK pathway (data not shown). In contrast, incubation of 3T3-L1 adipocytes with 2 ng/ml FasL activated the ERK signalling pathway. Inhibition of ERK activation with the MEK inhibitor U0126 during FasL incubation, however, could not prevent Fas activation induced Akt down regulation (data not shown). Hence, three presently known transcription pathways induced by Fas activation could be excluded as potential contributors to the observed effects in 3T3-L1 adipocytes. Akt2 promotor analysis (www.genecards.org, August 2010) suggests that PPARγ binds to the promotor region of Akt2. Treatment of 3T3-L1 adipocytes with FasL significantly reduces PPARγ protein levels (Supplementary Fig. 4). Interestingly, increased activation of PPARγ by treatment with rosiglitazone prevented Fas-induced down regulation of Akt2 mRNA (Supplementary Fig. 4), suggesting that reduced PPARγ levels upon Fas activation might be involved in reduced Akt2 transcription. Moreover, besides reducing Akt2 transcription, Fas activation might also reduce mRNA stability of Akt2 (Fig. 2E).

Activation of the Fas signalling pathway may contribute to obesity-induced insulin-resistance of adipocytes [5]. Consistent with such notion, insulin-stimulated glucose incorporation into isolated adipocytes from Fas-def mice was increased and paralleled by enhanced expression of Akt, suggesting that the absence of Fas prevented adipocytes from Fas-induced down regulation of Akt and, therefore, decreased insulin sensitivity. In addition, reduced expression of pro-inflammatory cytokines in WAT of Fas-def mice may have contributed to increased insulin sensitivity of adipocytes. Further studies are needed to unravel this question.

In conclusion, our results suggest that Fas activation in adipocytes decreases Akt expression and, therefore, reduces insulin-stimulated glucose uptake/incorporation.

5. Conflict of interest

None.

Acknowledgments

We would like to thank Markus Niessen and Dora Schmid for advice and help regarding 3T3-L1 adipocyte culture. This work was supported by grants from the Swiss National Science Foundation (# 310000-112275 and # 310030-124729) and a "Forschungskredit" from the University of Zurich (both to D.K.).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.08.052.

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