Silencing *Jnk1* and *Jnk2* accelerates basal lipolysis and promotes fatty acid re-esterification in mouse adipocytes

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Abstract

Aims/hypothesis Elevated plasma levels of NEFA impair insulin action. Given the positive linear correlation between NEFA released by adipocytes and plasma NEFA levels, identification of mechanisms controlling adipocyte lipolysis and NEFA release could provide a guide to new therapies for insulin resistance and type 2 diabetes.

Methods Short hairpin RNA-mediated gene ablation was used to determine the functions of c-Jun N-terminal kinase (JNK)1 and JNK2 in adipocytes.

Results Combined JNK1/JNK2 deficiency drastically increased basal glycerol release, whereas individual JNK1- or JNK2-deficiency had no effect, indicating that JNK1/JNK2-deficiency enhances basal lipolysis, whereas the alternate subtype compensates for a single JNK subtype deficiency in the regulation of basal lipolysis. The profoundly increased glycerol release associated with JNK1/JNK2-deficiency was not accompanied by a concomitant increase in NEFA release over time. In addition, JNK1-deficiency, but not JNK2-deficiency, drastically decreased NEFA release as compared with that in JNK-intact cells, a result of increased NEFA re-esterification. In microarray, quantitative RT-PCR and western blotting, JNK1-, JNK2- and JNK1/JNK2-deficiencies selectively upregulated many genes involved in NEFA management,

without affecting the expression of genes involved in insulin signalling. Assays using reporter genes driven by peroxisome proliferator-activated receptor γ (PPAR- γ)-responsive promoters indicate distinct roles for JNK1 and JNK2 in regulating the transcriptional effects of PPAR- γ .

Conclusions/interpretation While JNK1 and JNK2 have shared roles in the regulation of basal lipolysis, JNK1 has a more profound role in supporting baseline NEFA release. Inhibition of JNK1 activity in adipocytes has potential therapeutic uses for management of elevated circulating NEFA levels at the onset of insulin resistance.

Keywords Adipocytes · Fatty acid · Fatty acid re-esterification · Gene expression · Gene silencing · Glycerol release · JNK1 · JNK2 · Lipolysis · PPAR- γ

Abbreviations

JNK	c-Jun N-terminal kinase
LC-CoA	long-chain fatty acyl-CoA
PEPCK	phosphoenolpyruvate carboxykinase 1, cytosolic
PPAR- γ	peroxisome proliferator-activated receptor γ
PPRE	PPAR- γ -response elements
qRT-PCR	quantitative RT-PCR
shRNA	short hairpin RNA

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Introduction

Insulin resistance is a fundamental defect in obesity-linked type 2 diabetes [1–4]. Despite extensive evidence implicating excess circulating NEFA in the induction and progression of systemic insulin resistance [4–6], the mechanisms by which plasma NEFA levels are elevated

are less clear. Adipose tissue is the largest NEFA depot. The amount of NEFA released from adipocytes, determined mainly by the balance between lipolysis and intra-adipocyte NEFA re-esterification, is linearly related to plasma NEFA levels [7, 8]. Moreover, insulin-mediated inhibition of NEFA release from adipocytes reduces plasma NEFA levels [9–12]. Thus, identification of the regulatory mechanisms controlling adipocyte lipolysis and NEFA release should provide a guide to new therapies for insulin resistance, a root cause of obesity-linked type 2 diabetes.

The c-Jun N-terminal kinases (JNKs), key regulators of both cell proliferation and growth, and apoptosis and metabolism [13–17], are activated by NEFA [18, 19] and pro-inflammatory cytokines [15]. Distinct genes encode three JNK subtypes. JNK3 is produced mainly in brain, heart and testis, whereas JNK1 and JNK2 are produced ubiquitously [14]. JNK activates several transcription factors including c-JUN [14], activating transcription factor 2 (ATF-2) [20] and ELK1, member of ETS oncogene family (ELK1) [21], and modulates the activities of IRS-1 [17, 22], IRS-2 [19] and peroxisome proliferator-activated receptor γ (PPAR- γ) [23]. The JNK pathway is highly relevant in this context, because JNK activity is elevated in insulin target tissues during obesity [24] and mice lacking JNK1, but not JNK2, are resistant to obesity induced by high-fat diet and have improved insulin sensitivity [24]. However, a genetic dissection of the involvement of JNK2 in overall insulin sensitivity may be masked by in vivo factors including JNK1 redundancy and the cross-talk between JNK subtypes. Indeed, the role of JNK2 in the regulation of body weight and insulin sensitivity is unmasked only in mice with heterozygous disruption of *Jnk1* (also known as *Mapk8*), but not in mice with full JNK1 activity [25]. Additionally, macrophage-specific ablation of *Jnk2* (also known as *Mapk9*), but not *Jnk1*, reduces atherosclerosis [26], while germ-line deletion of *Jnk2* reduces destructive insulinitis and protects against type 1 diabetes in mice [27], indicating differential physiological functions for JNK subtypes, depending on cell context. In light of these findings, it is important to identify the intrinsic roles of JNK1 and JNK2 in the regulation of adipocyte function, in the context of lipolysis and NEFA release, which is essential to systemic insulin sensitivity [6, 28].

Here, short hairpin RNA (shRNA)-mediated gene silencing of *Jnk1* and/or *Jnk2* in a model fat cell line, 3T3-L1 adipocytes, was used to directly and definitively test lipolysis and NEFA release in the absence of JNK1 and/or JNK2. Both shared and distinct roles for JNK1 and JNK2 in the regulation of adipocyte gene expression and function are reported and analysed in the context of obesity-linked diabetes.

Methods

Plasmids Short hairpin RNA constructs targeting *Jnk1* (5'-AGCCAGTCAGGCAAGAGAT-3') and *Jnk2* (5'-GCCTTGC GCCACCCGTATA-3') mRNAs were subcloned into pRetro-Super retroviral vector (Netherlands Cancer Institute, Amsterdam, the Netherlands). After transfection into the packaging cells, the supernatant fraction containing the retroviruses was collected and used to infect 3T3-L1 pre-adipocytes. The luciferase reporter genes driven by the *Pck1* and PPAR- γ -response elements (PPRE) promoters, and the cDNA encoding PPARG2 have been described previously [29].

Cell culture 3T3-L1 cells were purchased from American Type Culture Collection (Rockville, MD, USA), maintained as fibroblasts and differentiated into adipocytes using standard protocol except that troglitazone (1 μ mol/l) was added during the first 3 days of differentiation to achieve more uniform adipocyte differentiation (> 99%). The adipocytes were used within 10 days of induction of differentiation. After ~30 passages, the differentiation rates decline to ~80 to 85%. For this reason, new batches of cells were revived from frozen stocks to maintain greater than 95% of adipocyte differentiation in all four cell types.

Semi-qRT-PCR Total RNAs were isolated from indicated sources. Each sample of first-strand cDNA was synthesised from 1 μ g of total RNA. PCR reactions were performed for 31 cycles, which falls within the previously identified linear range for PCR amplification of *Jnk* mRNAs. Primers for amplification of *Jnk1* were 5'-TTCCCAGCTGACTCAGAGCATAAC-3' (forward), and 5'-TAGTCATCTACAGCAGCCCAGAGG-3' (reverse); for *Jnk2* were: 5'-GGTCCTCCCTCCTGAGCCTAGAT-3' (forward), and 5'-GTGTTCTGAGGCCGCTCTCCTAC-3' (reverse).

Western blot analysis Equal amounts of protein were resolved by SDS-PAGE, electroblotted onto a nitrocellulose membrane, and immunoblotted with indicated antibodies. Bound antibodies were detected using the ECL reagents (GE Healthcare, Piscataway, NJ, USA). For a list of suppliers of antibodies used, see [Electronic supplementary material \(ESM\)](#).

Microarray analysis Total RNA was isolated from JNK-intact and JNK1-, JNK2- and JNK1/JNK2-deficient 3T3-L1 adipocytes. Replicate samples of each cell type were analysed using Affymetrix mouse MG430A 2.0 arrays. A detailed description of microarray data collection and analysis is provided in the [ESM](#).

Lipolysis assay After 16 h of serum starvation, various adipocytes were incubated with DMEM containing 300 to

450 $\mu\text{mol/l}$ BSA and 25 mmol/l HEPES. After 10, 30, 60 and 90 min of incubation, 50 μl of medium was removed and replenished with 50 μl of fresh medium. After 120 min, the medium was completely removed. NEFA and free glycerol concentrations were determined using colorimetric kits (Wako Chemicals, Richmond, VA, USA). Protein concentrations were used to normalise sample values. Lipid loading and the NEFA re-esterification assay are described in the [ESM](#).

Statistical analysis Comparisons were performed using ANOVA (two- or three-way). Duncan's multiple-range test was used post hoc to compare differences between and within the various treatments and groups. A value of $p < 0.05$ was used as the level of statistical significance. All values are expressed as mean $\pm$ SEM.

Results

JNK1- and/or JNK2-deficiency do not impair 3T3-L1 pre-adipocyte differentiation We used a retroviral vector to deliver shRNAs against endogenous *Jnk1* and/or *Jnk2* mRNA into proliferating 3T3-L1 pre-adipocytes and achieved stable and highly efficient ($\sim 100\%$) transduction of shRNAs into target cells. The recombinant retrovirus integrates a single copy of the viral genome into the host genome, ensuring stable expression of shRNAs in all offspring passages of the infected cells. Thus, retroviral-mediated gene delivery permits analysis of large pools of stably infected cells with minimal intrinsic clone-to-clone variation. Control cells were 3T3-L1 pre-adipocytes infected with the empty vector, ruling out the possibility of retroviral infection- and/or antibiotic selection-mediated toxicity as a source of the observed phenotypic variation.

According to semi-quantitative RT (qRT)-PCR, retroviral delivery of *Jnk1* shRNA drastically reduced *Jnk1* mRNA levels from levels seen in adipocytes expressing vector only or *Jnk2* shRNA (Fig. 1a). The *Jnk1* shRNA did not affect endogenous JNK2 (Fig. 1a). Similarly, retroviral delivery of *Jnk2* shRNA reduced *Jnk2* mRNA without affecting the *Jnk1* mRNA level (Fig. 1a). Finally, co-expression of *Jnk1* and *Jnk2* shRNAs greatly reduced *Jnk1* and *Jnk2* mRNA levels (Fig. 1a).

Microarray analysis revealed no detectable *Jnk1* mRNA in adipocytes expressing *Jnk1* shRNA or both *Jnk1* and *Jnk2* shRNAs (Table 1). Similarly, *Jnk2* mRNA levels were undetectable in adipocytes expressing *Jnk2* shRNA or both *Jnk1* and *Jnk2* shRNAs. *Jnk1* or *Jnk2* mRNA was unaltered in adipocytes expressing the shRNA against the alternate JNK subtype (Table 1). The effects of JNK subtype-specific shRNA on JNK1 and JNK2 protein mass paralleled the

mRNA data (Fig. 1b). *Jnk1* shRNA selectively reduced JNK1 protein production but not that of JNK2. *Jnk2* shRNA greatly reduced JNK2 levels, whereas there was little change in JNK1 levels. Tubulin levels were unaffected (Fig. 1b). Thus, the effects of *Jnk1* and *Jnk2* shRNAs are subtype-specific with respect to mRNA expression and protein production.

Control (JNK-intact), JNK1-, JNK2- and JNK1/JNK2-deficient 3T3-L1 pre-adipocytes were generated and their differentiation was monitored. Three days after induction, all four cell lines had accumulated fat droplets, and after 10 days $\sim 100\%$ of the cells were adipocytes (Fig. 1c). Thus, deficiency of JNK1, JNK2 or both together does not impair cellular lipid accumulation during adipocyte differentiation. This concurs with previous data indicating that neither JNK1 or JNK2 is indispensable for adipose development in mice [24, 25].

Combined JNK1/JNK2-deficiency accelerates baseline triacylglycerol hydrolysis without affecting NEFA release, whereas silencing JNK1, but not JNK2, decreases NEFA release We compared baseline lipolysis in JNK-intact, JNK1-, JNK2- and JNK1/JNK2-deficient adipocytes. In control JNK-intact adipocytes, basal triacylglycerol hydrolysis increased media glycerol concentration over a 120 min period (Fig. 2a). Glycerol release from JNK1/JNK2-, JNK1- and JNK2-deficient cells was higher than that from JNK-intact cells at 10 min ($p = 0.0003$). However, at later times differences were not significant except for the JNK1/JNK2-deficient adipocytes, in which glycerol release increased by 44 to 154% over 120 min ($p < 0.0003$ at all time-points) (Fig. 2a). Thus, in the regulation of basal glycerol production, JNK1/JNK2 deficiency enhances basal glycerol release, whereas the alternate subtype compensates for a single JNK subtype deficiency. These effects are the same in the presence and absence of insulin (Fig. 2b): only JNK1/JNK2-deficiency affected glycerol release as seen by a 57 to 146% increase in media glycerol concentration (10 min, $p < 0.01$; 30 min, $p < 0.004$; 60 – 120 min, $p < 0.0001$).

According to ANOVA, insulin did not affect time-dependent increases in media glycerol concentrations in JNK-intact and JNK1/JNK2-deficient cells (Fig. 2a,b). In contrast, insulin increased glycerol release in JNK1-deficient (64%, $p = 0.01$) and JNK2-deficient (34%, $p = 0.004$) cells at 120 min. These data indicate that JNK1/JNK2-deficiency is more important than insulin in the regulation of glycerol release.

The media NEFA concentrations increased 46% ($p < 0.006$) in JNK-intact cells only at 120 min, whereas media NEFA concentrations for JNK1-, JNK2- and JNK1/JNK2-deficient cells did not change with time (Fig. 2c). Insulin decreased net NEFA release in JNK-intact cells only at prolonged incubations (-30% at 120 min, $p < 0.03$), but did

Fig. 1 Inactivation of JNK1 and/or JNK2 does not impair 3T3-L1 pre-adipocyte differentiation. Semi-qRT-PCR (**a**) and western blotting (**b**) analyses of JNK1 and JNK2 levels in the four stable 3T3-L1 pre-adipocyte cell lines expressing the indicated shRNAs or the empty vector. Semi-qRT-PCR was performed as described in the Methods. Tubulin was used as an internal control. **c** Adipocyte differentiation of indicated cells in the presence (+) or the absence (–) of troglitazone (1

$\mu\text{mol/l}$). On day 9 after differentiation induction, cells were washed with PBS, fixed with 4% (vol./vol.) formaldehyde for 15 min at room temperature and washed extensively. Cellular neutral lipids were then stained using 0.3% (wt/vol.) Oil Red O (ORO) dissolved in 60% (vol./vol.) isopropanol for 65 min at room temperature. After extensive washes, cells were examined and photographed. LM, phase-contrast light microscopy

not further suppress NEFA release in JNK1- and/or JNK2-deficient cells (Fig. 2c,d). JNK-intact adipocytes exhibited parallel increases in NEFA and glycerol release (Fig. 2a,c). In contrast, the profoundly increased glycerol release from JNK1/JNK2-deficient adipocytes was not accompanied by a concomitant increase in NEFA release over time (Fig. 2c). This suggests that NEFA re-esterification is much higher in

JNK1/JNK2-deficiency. Intriguingly, JNK1-deficiency, but not JNK2-deficiency, decreased NEFA release from the levels seen in JNK-intact cells in the absence ($p < 0.02$ for all time-points except for 10 min) (Fig. 2c) and the presence of insulin ($p < 0.01$ for all time-points except for 10 min) (Fig. 2d). Collectively, these data suggest that JNK1 and JNK2 have overlapping roles in the regulation of baseline

Table 1 Microarray analysis indicated that JNK1- and/or JNK2-deficiency preferentially represses genes for immune and inflammatory response and upregulates genes for triacylglycerol synthesis, lipolysis and NEFA re-esterification

Symbol	Gene name	<i>Jnk1</i> shRNA	<i>Jnk2</i> shRNA	<i>Jnk1/Jnk2</i> shRNA
Common markers of adipocytes				
<i>Adipoq</i>	Adiponectin	1.33	1.2	1.08
<i>Cfd</i>	Complement factor D (adipsin)	1.17	1.14	0.95
Adipogenic and lipogenic transcription factors				
<i>Pparg</i>	Peroxisome proliferator-activated receptor- γ	2.05 ^a	2.06 ^a	1.67 ^a
<i>Cebpa</i>	CCAAT/enhancer binding protein- α	2.64 ^a	2.76 ^a	3.45 ^a
<i>Cebpb</i>	CCAAT/enhancer binding protein- β	0.8	0.92	0.91
<i>Cebpd</i>	CCAAT/enhancer binding protein- δ	0.47 ^b	0.45 ^b	0.46 ^b
<i>Cebpg</i>	CCAAT/enhancer binding protein- γ	1.3	1.23	1.24
<i>Srebf1</i>	Sterol regulatory element binding transcription factor 1	1.14	1.43	1.37
Triacylglycerol synthesis and NEFA re-esterification				
<i>Slc2a1</i>	Solute carrier family 2, member 1 (also known as Glut1)	0.53 ^b	0.57 ^b	0.66 ^b
<i>Slc2a4</i>	Solute carrier family 2, member 4 (also known as Glut4)	2.84 ^a	1.78 ^a	3.59 ^a
<i>Lpl</i>	Lipoprotein lipase	1.35	1.6 ^a	1.37
<i>Slc27a1</i>	Fatty acid transport protein-1	1.44	1.48	1.37
<i>Fabp4</i>	Adipocyte fatty acid binding protein-4	1.22	1.14	0.93
<i>Scd1</i>	Stearoyl-CoA desaturase 1	1.29	1.24	0.95
<i>Scd2</i>	Stearoyl-CoA desaturase 2	2.25 ^a	1.55 ^a	1.71 ^a
<i>Acs11</i>	Acyl-CoA synthetase long-chain family member 1	3.41 ^a	2.21 ^a	3.7 ^a
<i>Acs14</i>	Acyl-CoA synthetase long-chain family member 4	1.23	1.44	1.47
<i>Gpam</i>	Glycerol-3-phosphate acyltransferase, mitochondrial	1.74 ^a	1.48 ^a	1.52 ^a
<i>Agpat1</i>	1-Acylglycerol-3-phosphate <i>O</i> -acyltransferase 1 (lysophosphatidic acid acyltransferase, alpha)	1.04	1.14	1.32
<i>Agpat3</i>	1-Acylglycerol-3-phosphate <i>O</i> -acyltransferase 3	1.43	1.48	1.91 ^a
<i>Agpat6</i>	1-Acylglycerol-3-phosphate <i>O</i> -acyltransferase 6	1.35	1.36	1.7 ^a
<i>Lpin1</i>	Lipin 1	2.87 ^a	2.4 ^a	3.32 ^a
<i>Dgat1</i>	Diacylglycerol <i>O</i> -acyltransferase 1	1.88 ^a	2.05 ^a	2.12 ^a
<i>Dgat2</i>	Diacylglycerol <i>O</i> -acyltransferase 2	1.25	1.33	2.4 ^a
<i>Pck1</i>	Phosphoenolpyruvate carboxykinase 1, cytosolic	1.53 ^a	2.01 ^a	4.66 ^a
<i>Pcx</i>	Pyruvate carboxylase	1.72 ^a	1.8 ^a	2.2 ^a
<i>Gyk</i>	Glycerol kinase	2.42 ^a	2.1 ^a	3.15 ^a
<i>Fasn</i>	Fatty acid synthase	2.56 ^a	2.06 ^a	2.77 ^a
Lipolysis				
<i>Ces3</i>	Triacylglycerol hydrolase	1.33	2.65 ^a	1.60 ^a
<i>Pnpla2</i>	Patatin-like phospholipase domain containing 2 (adipose triacylglycerol lipase, Atgl)	1.63 ^a	2.08 ^a	1.85 ^a
<i>Abhd5</i>	Abhydrolase domain containing 5 (CGI-58)	1.96 ^a	1.65 ^a	2.09 ^a
<i>Lipe</i>	Hormone sensitive lipase	1.65 ^a	1.99 ^a	1.75 ^a
<i>Mgl1</i>	Monoglyceride lipase	2.37 ^a	1.03	3.13 ^a
Signalling proteins				
<i>Insr</i>	Insulin receptor	1.27	1.17	1.05
<i>Irs1</i>	Insulin receptor substrate-1	1.11	1.18	1.23
<i>Pik3r1</i>	PI3 kinase regulatory subunit, p85 α	0.66 ^b	0.58 ^b	0.86
<i>Pik3r2</i>	PI3 kinase regulatory subunit, p85 β	0.78	0.86	0.94
<i>Pik3ca</i>	PI3 kinase catalytic subunit, α polypeptide	0.99	0.91	0.95
<i>Akt2</i>	Thymoma viral proto-oncogene 2 (Akt2)	1.32	1.26	1.74 ^a
<i>Akt1</i>	Thymoma viral proto-oncogene 1 (Akt1)	0.64 ^b	0.69 ^b	0.72 ^b
<i>Foxo1</i>	Forkhead box O1	1.37	1.14	1.14
<i>Gsk3b</i>	Glycogen synthase kinase 3 beta	0.93	1.14	1.42
<i>Frap1</i>	FK506 binding protein 12-rapamycin associated protein 1	0.98	1.05	1.22
<i>Rps6ka2</i>	Ribosomal protein S6 kinase, 90 kDa, polypeptide 2	0.58 ^b	0.63 ^b	0.65 ^b
<i>Rps6ka4</i>	Ribosomal protein S6 kinase, 90 kDa, polypeptide 4	0.49 ^b	0.52 ^b	0.58 ^b
<i>Rps6 kb2</i>	Ribosomal protein S6 kinase, 70 kDa, polypeptide 2	1.02	1.03	1.31
<i>Mapk1</i>	Mitogen-activated protein kinase 1 (Erk2)	0.83	0.8	0.95

Table 1 (continued)

Symbol	Gene name	<i>Jnk1</i> shRNA	<i>Jnk2</i> shRNA	<i>Jnk1/Jnk2</i> shRNA
<i>Mapk3</i>	Mitogen-activated protein kinase 3 (Erk1)	0.52 ^b	0.74 ^b	0.77
<i>Mapk8</i>	Mitogen-activated protein kinase 8 (Jnk1)	0 ^b	0.93	0 ^b
<i>Mapk9</i>	Mitogen-activated protein kinase 9 (Jnk2)	1.07	0.05 ^b	0.05 ^b
<i>Jun</i>	Jun oncogene (c-Jun)	0.53 ^b	0.63 ^b	0.34 ^b
<i>Jund1</i>	Jun proto-oncogene related gene d1	0.77	0.93	0.68 ^b
<i>Junb</i>	Jun-B oncogene	0.52 ^b	0.55 ^b	0.61 ^b
<i>Jundm2</i>	Jun dimerisation protein 2	0.45 ^b	0.55 ^b	0.55 ^b
<i>Elk1</i>	Elk-1, member of ETS oncogene family	0.82	0.85	1.1
<i>Atf2</i>	Activating transcription factor 2	1.15	0.89	1.11
<i>Fosb</i>	FBJ osteosarcoma oncogene B	1.43	1.44	1.33
<i>Mapk14</i>	Mitogen-activated protein kinase 14 (p38- α)	1.1	1.03	0.94
<i>Ikkkb</i>	Inhibitor of κ B kinase β	0.85	0.84	1
<i>Rela</i>	v-rel reticuloendotheliosis viral oncogene homologue A (p65 NF- κ B)	0.94	1.15	0.9
<i>Prkaca</i>	cAMP dependent protein kinase, catalytic, α subunit	1.12	1.2	1.59 ^a
<i>Prkab1</i>	cAMP-activated protein kinase, β subunit	0.88	1.09	0.89
<i>Prkag3</i>	cAMP-activated protein kinase, γ subunit	1.04	1.45	1.16
<i>Prkcd</i>	Protein kinase C, delta	0.86	0.69	1.21
<i>Prkcl</i>	Protein kinase C, iota	1.02	0.86	0.88
Immune, inflammatory and acute phase response genes				
<i>Ccl2</i>	Chemokine (C-C motif) ligand 2	0.2 ^b	0.42 ^b	0.26 ^b
<i>Ccl5</i>	Chemokine (C-C motif) ligand 5	0.23 ^b	0.2 ^b	0.21 ^b
<i>Ccl7</i>	Chemokine (C-C motif) ligand 7	0.25 ^b	0.33 ^b	0.35 ^b
<i>Ccl8</i>	Chemokine (C-C motif) ligand 8	0.33 ^b	0.29 ^b	0.34 ^b
<i>Ccl9</i>	Chemokine (C-C motif) ligand 9	0.3 ^b	0.25 ^b	0.24 ^b
<i>Cxcl1</i>	Chemokine (C-X-C motif) ligand 1	0.26 ^b	0.44 ^b	0.38 ^b
<i>Cxcl5</i>	Chemokine (C-X-C motif) ligand 5	0.31 ^b	0.31 ^b	0.4 ^b
<i>Cxcl12</i>	Chemokine (C-X-C motif) ligand 12	0.34 ^b	0.41 ^b	0.22 ^b
<i>Csfl</i>	Colony stimulating factor 1 (macrophage)	0.24 ^b	0.27 ^b	0.3 ^b
<i>C1s</i>	Complement component 1, s subcomponent	0.29 ^b	0.25 ^b	0.26 ^b
<i>C4</i>	Complement component 4 (within H-2S)	1.19	1.59 ^a	0.5 ^b
<i>H2-bf</i>	Histocompatibility 2, complement component factor B	0.39 ^b	0.74 ^b	0.45 ^b
<i>Cfh</i>	Complement component factor h	0.17 ^b	0.1 ^b	0.34 ^b
<i>Fn1</i>	Fibronectin	0 ^b	0 ^b	0 ^b
<i>Ifi202b</i>	Interferon activated gene 202B	0.27 ^b	0.37 ^b	0.29 ^b
<i>Ifi203</i>	Interferon activated gene 203	0.09 ^b	0.08 ^b	0.1 ^b
<i>Ifi205</i>	Interferon activated gene 205	0.24 ^b	0.15 ^b	0.24 ^b
<i>Ifi47</i>	Interferon gamma inducible protein, 47 kDa	0.2 ^b	0.48 ^b	0.37 ^b
<i>Ifi30</i>	Interferon gamma inducible protein 30	0.24 ^b	0.33 ^b	0.26 ^b
<i>Ifi1</i>	Interferon inducible protein 1	0.46 ^b	0.72 ^b	0.51 ^b
<i>Il33</i>	Interleukin 33	0.44 ^b	0 ^b	0.24 ^b
<i>Lbp</i>	Lipopolysaccharide binding protein	0 ^b	0 ^b	0.1 ^b
<i>Tlr2</i>	Toll-like receptor 2	0.24 ^b	0.27 ^b	0.22 ^b
<i>Tlr3</i>	Toll-like receptor 3	0.35 ^b	0.65 ^b	0.7 ^b
<i>Cd14</i>	Cd14 antigen	0.51 ^b	0.57 ^b	0.37 ^b
<i>Saa3</i>	Serum amyloid A3	0.48 ^b	0.57 ^b	0.6 ^b
<i>Ptx3</i>	Pentaxin related gene 3	0.17 ^b	0.18 ^b	0.13 ^b
<i>Adam10</i>	a disintegrin and metalloprotease domain 10	0.35 ^b	0.66 ^b	0.38 ^b
<i>Adam17</i>	a disintegrin and metalloproteinase domain 17	0.48 ^b	0.65 ^b	0.46 ^b
<i>Adamts1</i>	a disintegrin-like and metalloprotease with thrombospondin type 1 motif, 1	0.38 ^b	0.28 ^b	0.31 ^b

Genes shown were chosen from the significantly up- or downregulated Gene Ontology Clusters

Results shown are average changes in gene expression in JNK1-, JNK2- and JNK1/JNK2-deficient adipocytes, relative to JNK-intact adipocytes

^a Induction ≥ 1.5 -fold; ^b repression ≤ 0.75 -fold

lipolysis, whereas JNK1 has a predominant role in promoting basal NEFA release in adipocytes.

JNK1- and JNK1/JNK2-deficiencies increase the rate of NEFA re-esterification The discrepancy between glycerol and NEFA release in JNK1/JNK2-deficient cells and the diminished NEFA release from JNK1-deficient cells could be due to increased intracellular NEFA re-esterification. To test this hypothesis, we compared the kinetics of NEFA release in the four cell types in the presence of triacsin C, which blocks NEFA incorporation into triacylglycerol by inhibiting acyl-CoA synthetase [30, 31]. Triacsin C accelerated the rates of NEFA release; the differences in media NEFA concentrations were significant ($p<0.0001$) as early as 30 min and lasted for at least 120 min in all four cell types. Intriguingly, triacsin C abolished the differences in NEFA release between JNK-intact and JNK1-deficient cells (Fig. 2e), indicating that enhanced NEFA re-esterification reduces the baseline NEFA release that is associated with JNK1-deficiency. Notably, JNK1/JNK2-deficient cells had the highest NEFA release at some time-points (60 and 90 min, compared with JNK-intact cells; $p<0.03$) (Fig. 2e), indicating increased intrinsic lipolytic activity in JNK1/JNK2-deficient cells at these time-points. These data

suggest that JNK1-deficiency is both necessary and sufficient to enhance NEFA re-esterification in adipocytes. While accelerated NEFA re-esterification reduces NEFA release in JNK1-deficient cells, this effect was masked by the increased lipolysis in JNK1/JNK2-deficient cells.

JNK1 and/or JNK2-deficiency selectively upregulates adipocyte genes involved in triacylglycerol turnover Genome-wide expression profiling, qRT-PCR and western blotting were used to determine whether differences in NEFA release associated with subtype-specific JNK-deficiency could be linked to specific genes, particularly those coding for proteins involved in triacylglycerol metabolism and storage. Despite a normal cellular lipid accumulation and adipocyte phenotype, DNA microarray clearly revealed that JNK1- and JNK2-deficiency altered adipocyte gene transcription (Table 1).

The baseline expression levels of 623 genes were different in control and JNK1-deficient adipocytes. Of these, 425 showed at least a twofold decrease, whereas 198 genes showed an at least twofold increase in steady-state mRNA levels. Similarly, the basal expression levels of 571 and 658 genes were affected by JNK2- and JNK1/JNK2-deficiency, respectively. Of these, 431 (JNK2-defi-

Fig. 2 Combined and subtype-specific silencing of *Jnk1* and *Jnk2* differentially affect basal NEFA and glycerol release. Supernatant fractions from JNK-intact and JNK1-, JNK2- and JNK1/JNK2-deficient adipocytes were assayed at various time-points to determine glycerol concentrations in the absence (a) and presence (b) of insulin, as well as NEFA concentrations in the absence (c) and presence (d) of insulin using colorimetric methods. e Adipocytes grown in 12-well plates were loaded with [3 H]oleic acid as described in the ESM. After extensive washes at 37°C, cells were incubated with DMEM containing 25 mmol/l HEPES, 300 μ mol/l BSA and 5 μ mol/l triacsin C. At various time-points, aliquots of media were collected and the amount of [3 H]oleic acid release was determined by scintillation

counting. In all experiments, results were normalised using protein concentrations. Similar results were obtained in four independent experiments, each done in triplicate. Data shown are means $\pm$ SEM ($n=12$) and are the average of four experiments for each experimental condition. The curve fitting was done using the 'trendline' function in Microsoft Excel. The folds are relative to values from JNK-intact cells at 10 min under each condition. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs JNK-intact cells. White circles (with continuous trendline), control JNK-intact cells; black circles (with dashed trendline), JNK1-deficient; white squares (with dotted trendline), JNK2-deficient; black squares (with mixed dashed and dotted trendline), JNK1/JNK2-deficient cells

cient) and 361 (JNK1/JNK2-deficient) genes were repressed, whereas 140 (JNK2-deficient) and 297 (JNK1/JNK2-deficient) genes were upregulated by at least twofold (Fig. 3). Interestingly, JNK1-, JNK2 and JNK1/JNK2 deficiency altered many of the same target genes in the same way (Table 1). Repressed Gene Ontology clusters in JNK1- and/or JNK2-deficiency include immune and inflammatory responses, cytoskeleton, cell adhesion molecules and both secreted and extracellular matrix proteins. By contrast, upregulated Gene Ontology clusters include those involved in lipid, carbohydrate and protein metabolism. We also identified genes that responded specifically to either JNK1- or JNK2-deficiencies (Fig. 3).

Significantly upregulated common target genes include the lipogenic genes *Pparg*, *Cebpa*, *Slc2a4*, *Scd2*, *Acs1l*, *Lpin1*, *Pck1*, *Pcx*, *Gk* and *Fasn*, and the lipolytic genes *Ces3*, *Pnpla2*, *Abhd5* and *Lipe* (Table 1). However, genes whose encoded proteins are involved in insulin signalling such as *Insr*, *Irs1* and *Gsk3b* were not affected by JNK1- and/or JNK2-deficiency, neither were the mRNA levels of common adipocyte markers *Adipoq* and *Cfd* or signalling proteins *Ikbkb* and *Mapk14* (Table 1). Thus, upregulation was selective for genes involved in triacylglycerol synthesis, hydrolysis and NEFA re-esterification, and closely related to adipocyte function.

Real-time qRT-PCR was performed to confirm upregulation of eight adipocyte-abundant genes (*Pparg*, *Cebpa*, *Dgat1*, *Pcx*, *Pepck*, *Pnpla2*, *Abhd5* and *Lipe*) in various JNK-deficient cells (Fig. 4). Of eight genes, seven showed similar expression patterns as revealed by both qRT-PCR and DNA microarray (Fig. 4a–g). Although microarrays show increased *Pparg* mRNA levels in JNK1- (105%), JNK2- (106%) and JNK1/JNK2-deficient cells (67%) (Table 1), qRT-PCR revealed similar *Pparg* mRNA levels

among the four cell types (Fig. 4h). Moreover, according to western blot analysis, PPAR- γ protein levels were similar in the four cell types (data not shown). The discrepant observations could derive from intrinsic differences in the underlying principles of qPCR and microarray. Nevertheless, any differences in *Pparg* mRNA levels among the four cell types did not result in changes in PPAR- γ protein mass. The coordinated upregulation of multiple PPAR- γ target genes including *Pepck*, *Cebpa* and *Gk* (Table 1) supports the notion that increased PPAR- γ activity is probably involved in the upregulation of PPAR- γ target genes in JNK-deficient cells.

By western blotting, further induction of PEPCK protein was confirmed in cells with combined JNK1/JNK2-deficiency (359%, by densitometry) and JNK2-deficiency (152%); JNK1-deficient cells, however, showed decreased PEPCK protein mass compared with JNK-intact cells (–79%) (Fig. 5). The molecular mechanism(s) underlying the reduction in PEPCK protein mass mediated by JNK1-deficiency remains unclear. However, the decreased PEPCK protein level did not lead to increased NEFA release in JNK1-deficient cells (Fig. 2b), indicating a possible functional compensation from other genes involved in NEFA re-esterification such as glycerol kinase (Table 1) and/or increased intracellular NEFA utilisation including NEFA incorporation into phospholipids and oxidation.

JNK1-, JNK2- and JNK1/JNK2-deficiency differentially affect PPAR- γ -dependent gene transcription Based on our microarray studies suggesting the concomitant suppression of clusters of genes involved in immune and inflammatory response and cell adhesion, as well as upregulation of genes for adipocyte energy management in JNK1- and/or JNK2-

Fig. 3 Microarray analysis revealed that JNK1 and JNK2 have both common and distinct target genes. The numbers of genes that were repressed (**a**) or induced (**b**) by various JNK-deficiencies are shown in Venn diagrams. The overlapping regions of the Venn diagram indicate the number of common genes; the non-overlapping regions denote the number of unique genes regulated by the indicated deficiency of JNK

subtype(s). Fold changes were calculated using the average changes in gene expression in JNK1-, JNK2- and JNK1/JNK2-deficient adipocytes, relative to JNK-intact adipocytes. Genes with an average of a twofold or greater change in expression levels were included. For the entire set of microarray data, see ESM Table 1

deficient adipocytes (Table 1), we hypothesised that the link from JNK1- and/or JNK2-deficiency to adipocyte gene expression might be altered production or function of a transcription factor with overlapping transactivation and transrepression activities. One attractive candidate is PPAR- γ , a master regulator of adipocyte differentiation and function [32–34] and a negative regulator of inflammatory cytokine production in macrophages [35–37].

Given that PPAR- γ is a member of the ligand-dependent nuclear receptor superfamily and a phosphorylation target of JNK [38], we tested the impact of various forms of JNK-deficiency on two PPAR- γ -responsive promoters: *Pepck* promoter (–2250/+69), which is upregulated by PPAR- γ and has well-characterised PPAR- γ responsive elements (PPRE); and a synthetic promoter containing multimerised PPRE. In JNK-intact 3T3-L1 pre-adipocytes, exogenous PPAR- γ activated *Pepck* promoter (81%, $p<0.0001$) (Fig. 6a) in the presence of troglitazone. Despite baseline

Fig. 4 Validation of some DNA microarray findings by real-time qRT-PCR analysis. The steady-state mRNA levels of the eight adipocyte-abundant genes *Pck1* (a), *Pcx* (b), *Lipe* (c), *Pnpla2* (d), *Abhd5* (e), *Cebpa* (f), *Dgat1* (g) and *Pparg* (h) were examined using Taqman chemistry-based real-time qRT-PCR. All primers and probes were purchased from Applied Biosystems (Foster City, CA, USA). Eukaryotic 18S rRNA was used to normalise RNA sample loading. JNK1, JNK-intact; J1D, JNK1-deficient; J2D, JNK2-deficient; J12D, JNK1/JNK2-deficient adipocytes

Fig. 5 Western blot analysis of PEPCK levels in JNK-intact, JNK1-, JNK2- and JNK1/JNK2-deficient adipocytes. Total cellular proteins were analysed by SDS-PAGE and immunoblotted with anti-PEPCK antibody, quantified using Image J densitometry software and normalised with signal transducer and activator of transcription 1 (STAT-1). Results shown represent the results from five independent experiments

activity similar to that of the JNK-intact cells, in a JNK1-deficient cell background exogenous PPAR- γ increased *Pepck* promoter expression 283% ($p<0.0001$) in the presence of troglitazone, representing a 120% increase ($p<0.0001$) relative to JNK-intact cells and the highest stimulation among the four cell types ($p<0.0001$) (Fig. 6a). By contrast, JNK2-deficiency reduced basal (–21.5%, $p=0.01$), but had no effect on troglitazone-stimulated *Pepck* promoter expression as compared with JNK-intact cells (Fig. 6a). Intriguingly, in the presence of troglitazone, combined JNK1/JNK2-deficiency stimulated PPAR- γ -dependent *Pepck* promoter expression by only 106% ($p<0.0001$), a 44% reduction relative to that seen in JNK1-deficient cells ($p<0.0001$). This suggests that endogenous JNK1 activity represses and JNK2 activity promotes the ligand-dependent transactivation function of PPAR- γ . The inhibitory activity of JNK1 is dominant, whereas the stimulatory action of JNK2 is revealed only when JNK1 is absent.

When the PPRE-driven luciferase reporter gene was used, JNK1-deficient cells exhibited 64% higher basal ($p<0.0001$) and 70% enhancement of troglitazone-stimulated ($p<0.0001$) luciferase activity over JNK-intact cells (Fig. 6b), again demonstrating that JNK1 represses the transcription activation function of PPAR- γ . Consistent with our findings with the *Pepck* promoter, the enhancement of liganded PPAR- γ activity associated with JNK1-deficiency was attenuated by the simultaneous loss of JNK2 (–27%, $p<0.0001$). JNK2-deficiency alone did not affect baseline or PPAR- γ -dependent PPRE promoter expression as compared with JNK-intact cells (Fig. 6b). Taken together, these data suggest that while JNK1 suppresses and JNK2 stimulates PPAR- γ activity, overall endogenous JNK activity inhibits both ligand-independent and ligand-dependent transcription regulatory activity of PPAR- γ .

While our reporter gene assays indicated the highest levels of PPAR- γ -dependent transcription activity in JNK1-deficient cells (Fig. 6), several PPAR- γ target genes,

Fig. 6 JNK1- and/or JNK2-deficiency differentially affects ligand-independent and ligand-dependent activation of PPRES by PPAR- γ . 3T3-L1 pre-adipocytes were transiently transfected with luciferase reporter genes driven by the *Pck1* promoter (a) or a synthetic promoter containing multimerised PPRES (b) and were incubated with (grey

bars) or without (white bars) troglitazone (1 μ mol/l) for 20 to 40 h. Results shown are luciferase (Luc) activity (mean $\pm$ SEM) for three independent experiments relative to the basal value from control cells. Comparisons were performed using ANOVA as described in the Methods. * p <0.05, *** p <0.0001

including *Pepck*, *Cebpa*, *Slc2a4*, and *Gk*, showed dose-responses to the reduction of JNK subtype protein, with the highest expression seen in JNK1/JNK2-deficient adipocytes (Table 1). This difference is consistent with the hypothesis that JNK-regulated factors specifically present in adipocytes might act in concert with PPAR- γ -dependent and PPAR- γ -independent factors downstream of JNK to enhance transcription of these genes.

Discussion

Our data establish distinct and shared contributions of JNK1 and JNK2 to the regulation of adipocyte lipolysis and NEFA release, and can be summarised in four major findings. First, JNK1 and JNK2 are dispensable for adipocyte differentiation and cellular lipid accumulation. Second, combined JNK1/JNK2-deficiency accelerates basal lipolysis, whereas the alternate JNK subtype compensates for a single subtype deficiency in the regulation of basal lipolysis. Third, JNK1-deficiency is both necessary and sufficient to enhance NEFA re-esterification in adipocytes. Fourth, JNK1 and JNK2 have opposing roles in PPAR- γ -dependent reporter gene transcription in a pre-adipocyte cell background.

The amount of NEFA released from adipocytes, determined mainly by the balance between lipolysis and NEFA re-esterification, is regulated by energy demand and modulated by physiological and pathophysiological factors. Here, JNK1/JNK2-deficiency drastically enhanced basal lipolysis, whereas silencing *Jnk1* or *Jnk2* had no effect, suggesting a compensatory interplay between the two kinases in the regulation of basal lipolysis. Notably, insulin did not affect the kinetics of glycerol release in JNK-intact cells over a 120 min period, but increased glycerol release in JNK1- and JNK2-deficient cells at 120 min. Although both the acute and chronic stimulatory effects of insulin on adipocyte glycerol release have been demonstrated [39, 40], this is the first report that JNK1- or JNK2-deficiency

increases the stimulatory effect of insulin on glycerol release and that JNK1/JNK2-deficiency is more important in the regulation of lipolysis than insulin.

Intriguingly, the dramatically increased glycerol release associated with JNK1/JNK2-deficiency was not accompanied by a simultaneous increase in NEFA release over time. In addition, JNK1-deficiency, but not JNK2-deficiency, greatly decreased NEFA release without affecting glycerol release. We surmised that the observed discrepancy between lipolysis and NEFA release could be at least partially due to increased NEFA re-esterification. Measurements of NEFA release in the presence of triacsin C, which blocks NEFA re-esterification by inhibiting acyl-CoA synthetase, confirmed our prediction. Another interesting result is that insulin repressed net NEFA release in JNK-intact cells only during prolonged incubations of at least 120 min. Together with glycerol data, these indicate that during short-term incubation, insulin represses NEFA release through stimulation of NEFA re-esterification and not through inhibition of lipolysis. Insulin did not acutely alter NEFA release in JNK1-, JNK2- and JNK1/JNK2-deficient cells; however, the question of whether various types of JNK-deficiency will affect insulin response after a longer term exposure cannot be answered by our current study. Collectively, our data establish shared and distinct roles of JNK1 and JNK2 in the regulation of baseline lipolysis and NEFA release, and provide evidence linking the JNK pathway to the control of energy metabolism in adipocytes.

Both ligand-dependent and ligand-independent trans-activation activities of PPAR- γ are inhibited by JNK-mediated phosphorylation on its serine residue [38]; therefore inactivation of JNK1 and/or JNK2 is expected to enhance PPAR- γ activity. Indeed, our genome-wide expression profiling showed that JNK1-, JNK2- and JNK1/JNK2-deficiencies are associated with the upregulation of a subset of genes for triacylglycerol synthesis, NEFA re-esterification and lipolysis, suggesting that both JNK1 and JNK2 are involved in the repression of a selective set of adipocyte-abundant genes. Interestingly, several PPAR- γ

target genes including *Pepck*, *Cebpa*, *Slc2a4* and *Gk* showed dose-responses to the reduction of JNK subtype protein, with the highest expression seen in JNK1/JNK2-deficient adipocytes (Table 1). By contrast, some genes responded specifically to either JNK1- or JNK2-deficiencies (Fig. 3), whereas a number of immune and inflammatory genes require both JNK1 and JNK2 for expression at levels similar to JNK-intact cells (Table 1). It would be intriguing to identify the mechanism(s) by which various JNK-deficiencies differentially regulate adipocyte gene expression. However, such an undertaking would go beyond the scope of this report to the extent that a separate paper of interest would ensue.

In a pre-adipocyte background, JNK1-deficiency enhanced both ligand-independent and ligand-dependent transactivation functions of PPAR- γ . Interestingly, a simultaneous loss of JNK2 attenuated the enhancement of liganded-PPAR- γ activity associated with JNK1-deficiency. This indicates that endogenous JNK1 represses and JNK2 promotes ligand-dependent PPAR- γ activity. The inhibitory activity of JNK1 is dominant, whereas the stimulatory action of JNK2 is revealed only when JNK1 is absent. In contrast to these reporter gene analyses, our microarray data demonstrated that JNK2-deficiency upregulated many PPAR- γ target genes in adipocytes, in many cases, to a similar extent to that seen in JNK1-deficiency (Table 1), suggesting possible involvement of JNK-regulated factors specifically expressed in adipocytes. Taken together, our results suggest the likelihood of PPAR- γ being a critical target of both JNK1 and JNK2 in adipocytes, possibly through the phosphorylation of PPAR- γ and cofactors essential for PPAR- γ function.

Our data also demonstrate that both JNK1 and JNK2 are specifically required for the expression of the Jun family (*Jun*, *Jund*, *Junb* and *Jundm2*), but not the other adaptor protein-1 family members including *Fosb*, *Atf2* and *Elk1* (Table 1). The reductions in JUN production could contribute to the decreased expression of genes for immune and inflammatory responses. In addition, since JUN has been implicated in the repression of a number of genes [41–43], the reduction in JUN protein can potentially activate transcription of a subset of genes normally repressed by JUN in adipocytes. Alternatively, the altered adipocyte gene expression could result from the interplay of a network of signalling proteins, whose expression and/or activities are affected by the various types JNK-deficiencies. Supporting this notion, our microarray data indicate that the mRNA levels of key proteins downstream of the insulin receptor such as phosphoinositide-3-kinase, thymoma viral proto-oncogene (AKT) 1, AKT2, ribosomal protein S6 kinase (p90) and extracellular regulated kinase-1 were all altered by JNK1- and/or JNK2-deficiencies (Table 1). The exact mechanisms underlying the upregulation of adipocyte-

abundant genes associated with various JNK-deficiencies remain to be determined.

The drastic upregulation of PEPCK levels in JNK1/JNK2-deficient adipocytes (Fig. 5) probably contributes to the enhanced rate of NEFA re-esterification by providing glycerol-3-phosphate. In parallel, the enhanced rate of NEFA re-esterification could reduce intracellular concentrations of long-chain fatty acyl-CoA (LC-CoA). As inferred from perfused rat adipocytes, intracellular LC-CoA suppresses lipolysis by inhibiting triacylglycerol lipase activities [39, 44]; therefore the induction of PEPCK protein by JNK1/JNK2-deficiency could also lead to increased lipolysis. On the other hand, JNK1-deficiency drastically decreased PEPCK protein levels while simultaneously increasing NEFA re-esterification. This indicates that PEPCK is not the only factor governing NEFA release; rather, multiple factors including the expression and activities of proteins involved in triacylglycerol turnover and insulin signalling are likely to be involved in the coordinate regulation of adipocyte NEFA release. Further studies are needed to identify the mechanisms underlying the distinct roles of JNK1 and JNK2 in the control of NEFA release.

Our data provide compelling evidence to define the shared and distinct roles of JNK1 and JNK2 in the regulation of adipocyte function in the context of gene expression, lipolysis and NEFA release. Studies of genetically altered mice suggest a possible differential function of each JNK subtype in different cell types [24–27]. Thus, systemic inhibition of JNK would have limited translational value. Our studies showing that JNK1-deficiency inhibits baseline NEFA release suggest that inhibition of JNK1 activity could be a potential therapeutic approach for management of elevated NEFA levels at the onset of insulin resistance.

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