The role of ATF2 in insulin action

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Chapter 5

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Identification of insulin-regulated ATF2-target genes in 3T3L1 adipocytes and A14 fibroblasts

Bart Baan[1], Terrence A.T. Wanga[1], Gerard C.M. van der Zon[1],
J. Antonie Maassen[2] and D. Margriet Ouwens[1]

From the [1]Department of Molecular Cell Biology, Leiden University Medical Centre, Leiden, [2]Department of Endocrinology/Diabetes Centre, VU University Medical Centre, Amsterdam, the Netherlands

Activating transcription factor 2 (ATF2) is strongly associated with the response to inducers of cellular stress, like viral infection and DNA damage. However, ATF2 has also been identified as a component of insulin signalling, both *in vitro and in vivo*. Studies in mouse and Drosophila have linked ATF2 to the regulation of glucose and lipid metabolism via induction of PPARγ coactivator 1α (PGC1α) and phosphoenolpyruvatecarboxykinase (PEPCK) expression. This study aimed at detailing the role of ATF2 in insulin action and metabolic control through the characterization of ATF2 target genes in insulin-sensitive cell lines. Therefore, we analyzed the expression of 16 putative ATF2-target genes by real-time PCR in 3T3L1 adipocytes and A14 cells. Using the JNK-inhibitor SP600125, which abrogates the induction of ATF2 phosphorylation by insulin, and shRNA-mediated silencing of the ATF2 gene, we identified ATF3, *c-jun*, Egr1, MKP1, and SREBP1c as insulin-induced ATF2-dependent genes. Furthermore, we found that the induction of adiponectin by insulin is JNK-dependent.

Activating transcription factor 2 (ATF2), a ubiquitously expressed member of the cAMP-responsive element binding protein family of basic region leucine-zipper transcription factors, has been implicated in multiple responses in mammalian cells by regulating the expression of a broad spectrum of target genes (1-3). Among the ATF2 target genes are cell cycle regulators (cyclin A, cyclin D1), proteins related with invasion (MMP2, UPA), cell adhesion molecules (E-selectin, P-selectin, and VCAM), anti-apoptotic factors (Bcl-2), transcription factors (*c-jun*, ATF3, CHOP, *c-fos*, junB), growth factor receptors and cytokines (PDGFRα, IL8, Fas ligand, TNFα, IL1β, and IL6), and proteins engaged in the response to endoplasmic reticulum (ER) stress and DNA damage (Grp78,ATM, XPA) (1-4). The nature of these target genes indicates a key role for ATF2 in oncogenesis and protection against cellular stress and DNA damage.

Activation of ATF2, however, is not confined to inducers of cellular stress. Also insulin has been shown to activate ATF2 *in vitro* and *in vivo* (5-8). Moreover, ATF2 has been implicated in the regulation of proteins involved in metabolic control, including expression of PPARγ coactivator 1α (PGC1α) in brown adipose tissue (9) and phosphoenolpyruvate carboxykinase (PEPCK) in the liver and white adipose tissue (10-12). Finally, selective ablation of ATF2 in the Drosophila fat body, the fly equivalent of mammalian liver and adipose tissue, not only confirms ATF2 regulation of PEPCK expression, but also identifies ATF2 as critical regulator of lipid metabolism (13).

Collectively, these findings strongly suggest that ATF2 also has an important function in the regulation of metabolic control by insulin.

To detail the role of ATF2 in insulin action, the present study aimed to characterize insulin-regulated ATF2 target genes in 3T3L1 adipocytes and A14 fibroblasts. We used real-time PCR to examine the effects of insulin on the expression of 16 putative ATF2 targets identified in previous studies (1;3;4). To validate these genes as bona fide ATF2 targets, the expression of the insulin-regulated genes was subsequently studied following treatment with the JNK-inhibitor SP600125, which abrogates insulin-mediated ATF2 phosphorylation (5), and after shRNA-mediated silencing of the ATF2 gene.

Results

Regulation of gene expression by insulin. Using real-time PCR, we examined the effect of insulin on the expression 16 putative ATF2-target genes in 3T3L1 adipocytes. The candidate genes, which are listed in Table 1, were selected on the basis of previous studies and have regulatory functions in insulin action and lipid metabolism. Adiponectin was included as potential ATF2 target gene based on a promoter analysis, which identified two potential cyclic AMP response elements (CRE), the binding site for ATF2, at position -3177 (TGACGTAA) and -335 (TGACGTCC), respectively.

Table 1. Potential ATF2 target genes

Target	References	Role in insulin action & metabolism
Transcriptional regulators		
ATF3	(1;3;20)	apoptosis
FOXO3a	(4)	metabolic gene expression
c-jun	(1;3;4)	proliferation
CREB1	(1;3)	metabolic gene expression
Egr1	(4)	proliferation
HIF1α	(4)	Angiogenesis
PGC1α	(9)	metabolic gene expression
PPARα	(4)	metabolic gene expression
SREBP1c	(4)	lipid metabolism
Hormones		
adiponectin		positive regulation insulin action
IGF2	(4)	adipocyte function
IGFBP6	(4)	
IL1β	(1;21)	β-cell destruction, inflammation
TNFα	(21)	inhibition of insulin signalling
Metabolic enzymes		
MKP1	(4;49)	inhibition of insulin signalling
PEPCK	(10;11;13)	gluconeogenesis, glyceroneogenesis

Figure 1 shows that a 30 min incubation with insulin increased the expression of 7 genes (adiponectin, ATF3, *c-jun*, Egr1, IGFBP6, MKP1, and PEPCK), lowered the levels of 3 genes (FOXO3a, IGF2, PPARα), and did not affect the abundance of 4 genes (CREB1, HIF1α, PGC1α, and SREBP1c) in 3T3L1 adipocytes. No reproducible data were obtained for IL1β and TNFα. Except for ATF3, all insulin-mediated alterations in gene expression,

both positive and negative, were transient. Compared to 30 min of insulin incubation, the levels of *c-jun* and Egr1 were lower after 60 min exposure to insulin, or had returned to basal level in the case of adiponectin, IGFBP6, MKP1, and PEPCK (Figure 1).

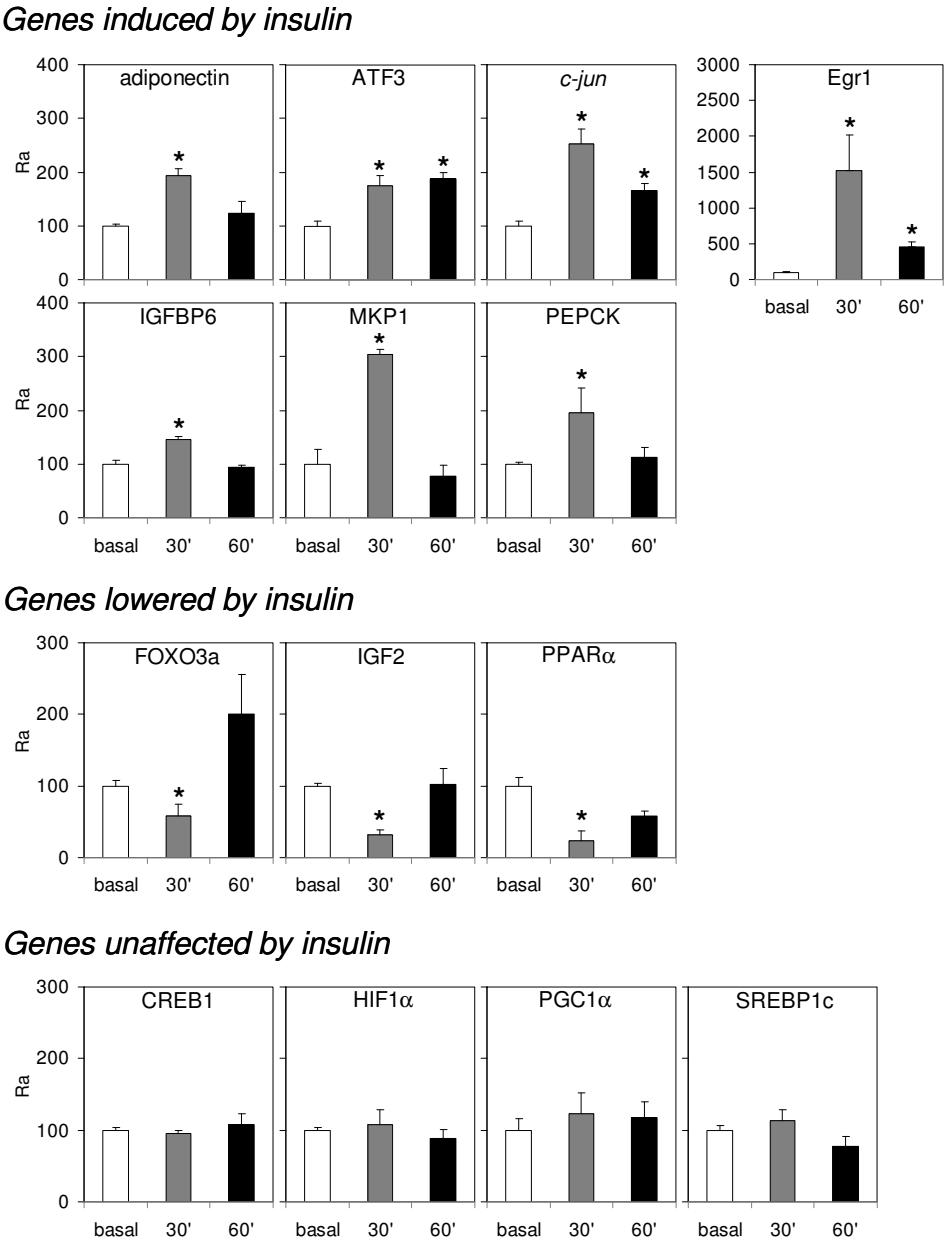

Figure 1. Regulation of gene expression by insulin in 3T3L1 adipocytes. Serum-starved 3T3L1 adipocytes were kept untreated (basal; open bars), or stimulated for 30 min (30'; grey bars) or 60 min (60'; black bars) with 10 nM insulin. Gene expression levels were determined by real-time PCR. The relative mRNA abundance (Ra) in untreated cells was set at 100. Data are mean ± SEM of 3 independent experiments performed in duplicate. *, $P<0.05$ versus basal.

We used A14 cells to substantiate the insulin-mediated induction of putative ATF2-target genes. Compared to 3T3L1 adipocytes, insulin increased the mRNA levels of ATF3, *c-jun*, Egr1 and MKP1 in A14 cells (Figure 2). Only in A14 cells, insulin also induced HIF1α and SREBP1c mRNA (Figure 2). The levels of CREB1, IGFBP6, and PGC1α were either lowered or unaffected by insulin (Figure 2). We did not determine the abundance of adiponectin and PPARα, as these metabolic genes are not expressed in A14 cells, and failed to obtain reproducible results for IGF2, IL1β, TNFα, and FOXO3a. Finally, whereas the insulin effects in 3T3L1 adipocytes were transient, the increases in gene expression in A14 fibroblasts were more linear in time (Figure 2).

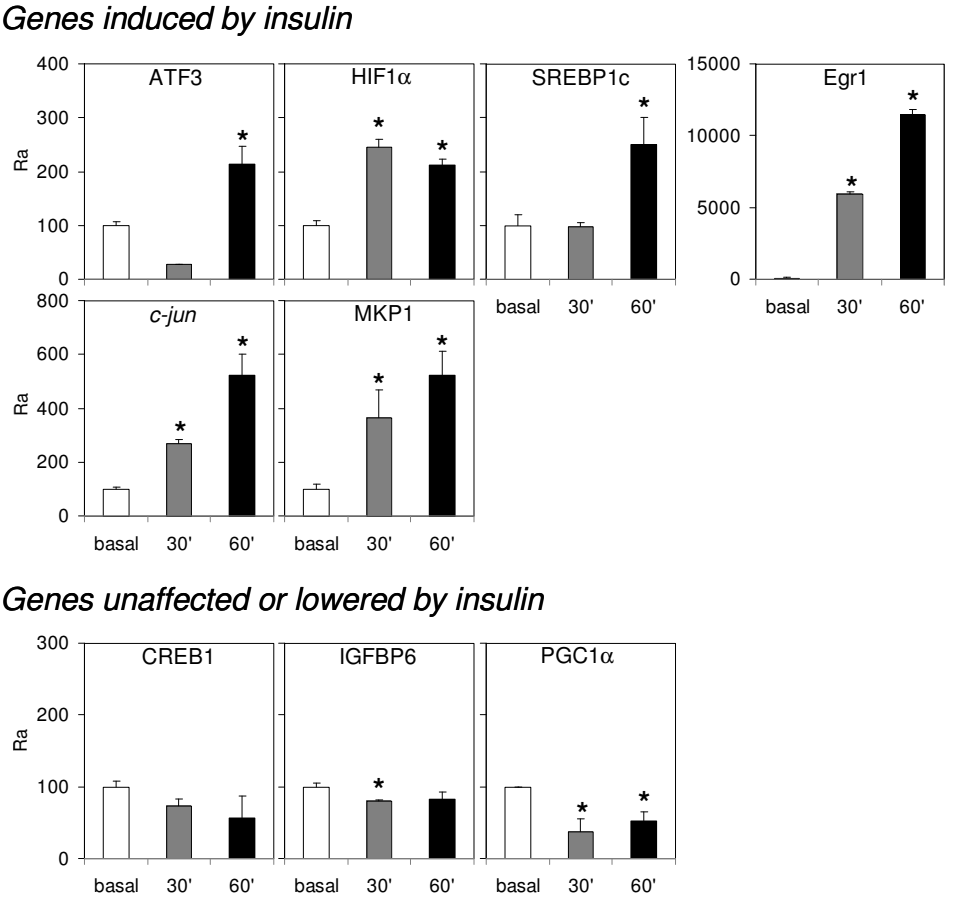

Figure 2. Regulation of gene expression by insulin in A14 fibroblasts. Serum-starved A14 cells were kept untreated (basal; open bars), or stimulated for 30 min (30'; grey bars) or 60 min (60'; black bars) with 10 nM insulin. Gene expression levels were determined by real-time PCR. The relative mRNA abundance (Ra) in untreated cells was set at 100. Data are mean ± SEM of 3 independent experiments performed in duplicate. *, $P<0.05$ versus basal.

Inhibition of JNK abrogates insulin-mediated gene induction in 3T3L1 adipocytes and A14 cells. We have previously shown that the JNK inhibitor SP600125 blocks insulin-mediated ATF2 activation (5). Therefore, we used SP600125 to ascribe the induction of the insulin-regulated genes to activation of the JNK/ATF2-pathway. As shown in Figure 3, SP600125 completely abrogated insulin-induced ATF2-Thr69 phosphorylation both in 3T3L1 adipocytes and A14 fibroblasts (Figure 3) without significantly affecting other components of insulin action, like PKB-S473 phosphorylation and the ERK1/2-dependent ATF2-Thr71 phosphorylation (Figure 3).

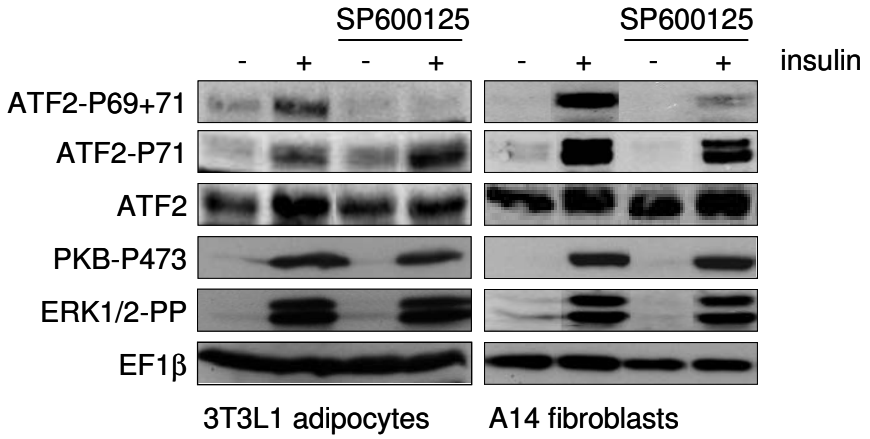

Figure 3. Effect of SP600125 on ATF2-phosphorylation and insulin signalling. Serum-starved 3T3L1 adipocytes and A14 fibroblasts were treated with DMSO or SP600125 (10 μM) for 30 min prior to stimulation with 10 nM insulin. Total cell lysates were prepared after 15 minutes and analyzed by Western blotting using phospho-specific ATF2-Thr71, ATF2-Thr69+71 and total ATF2 antibodies or phospho-specific antibodies directed against PKB-P473 and ERK1/2- P202/204. Equal loading was confirmed using an EF1β antibody.

In 3T3L1 adipocytes, SP600125 blocked the induction of adiponectin, ATF3, c-jun, Egr1, and MKP1, but not of IGFBP6 and PEPCK, mRNA by 30 min insulin treatment (Figure 4A). Similarly, in A14 cells, SP600125 significantly blunted the increase in c-jun, Egr1, MKP1, HIF1α and SREBP1c mRNA by 60 min of insulin exposure (Figure 4B). Thus, the insulin-mediated induction of ATF3, c-jun, Egr1, HIF1α, MKP1, and SREBP1c, but not PEPCK and IGFBP6, requires activation of the JNK/ATF2-pathway.

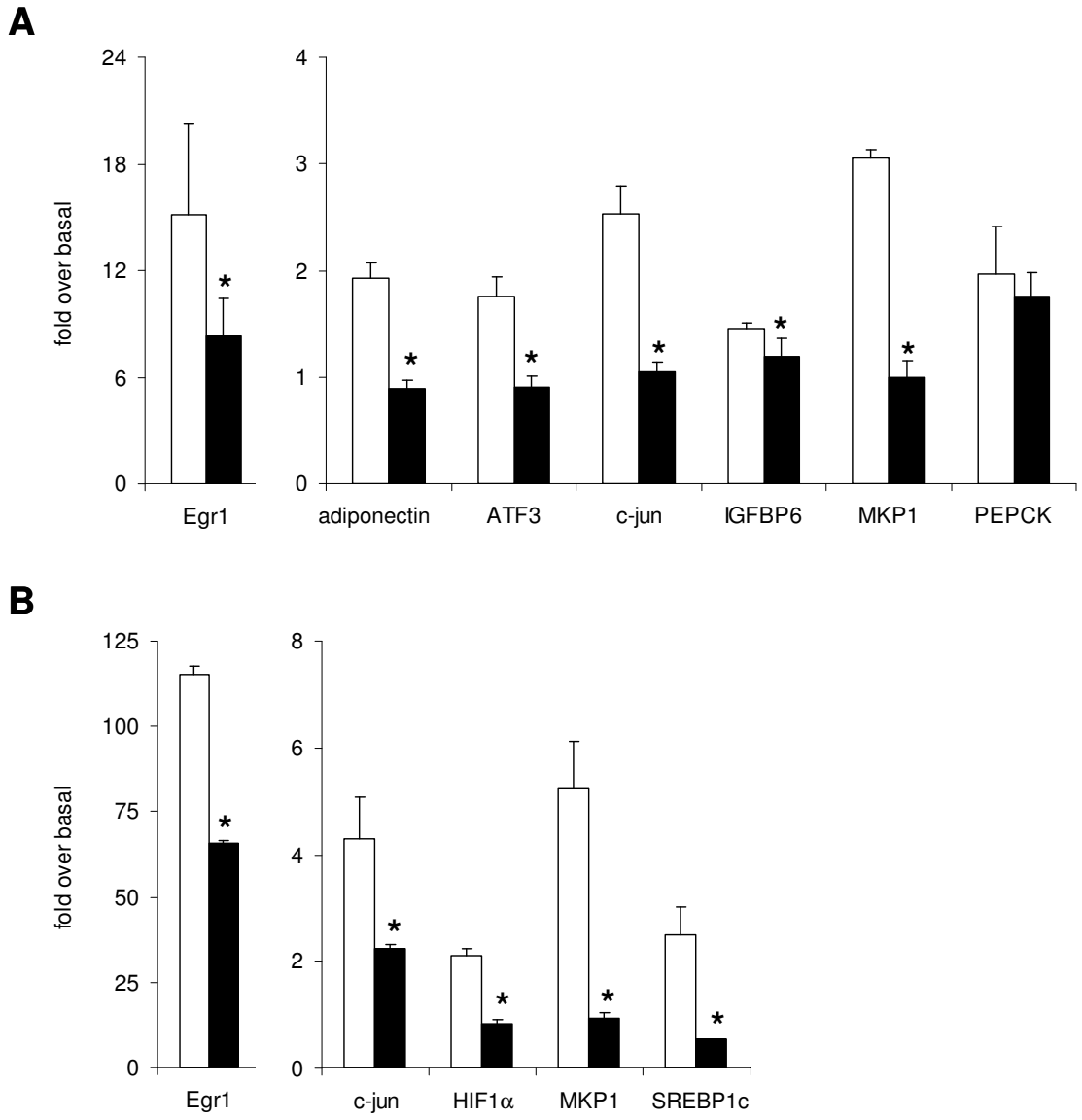

Figure 4. Inhibition of insulin-mediated gene expression by SP600125. Serum-starved 3T3L1 adipocytes (A) and A14 fibroblasts (B) were treated with DMSO (open bars) or with 10 μM SP600125 (black bars) for 30 min prior to stimulation with 10 nM insulin for 30 min (3T3L1 adipocytes) or 60 min (A14 cells). Gene expression levels were determined by real-time PCR. The effect of insulin is expressed as fold over basal. Data are mean ± SEM of 3 independent experiments performed in duplicate. *, $P<0.05$ versus DMSO.

ATF2 silencing and insulin-mediated gene induction in A14 fibroblasts. Next, we examined the effect of silencing of the ATF2 gene on insulin-mediated gene induction in A14 fibroblasts. Expression of mATF2#1 or mATF2#2 downregulated ATF2 mRNA levels by 70 and 82 %, respectively when compared to cells transfected with pSuper (Figure 5A). ATF2 silencing also resulted in an almost complete knock-down of ATF2 protein expression, but did not significantly interfere with the phosphorylation of PKB-S473 and ERK1/2 in response to insulin (Figure 5B).

Figure 5C shows that silencing of the ATF2 gene blunted the insulin-mediated induction of ATF3, Egr1, MKP1, and SREBP1c mRNA, and to a lesser extent *c-jun* mRNA. We did not observe induction of HIF1α by insulin in A14 cells transfected with pSuper (Figure 5C).

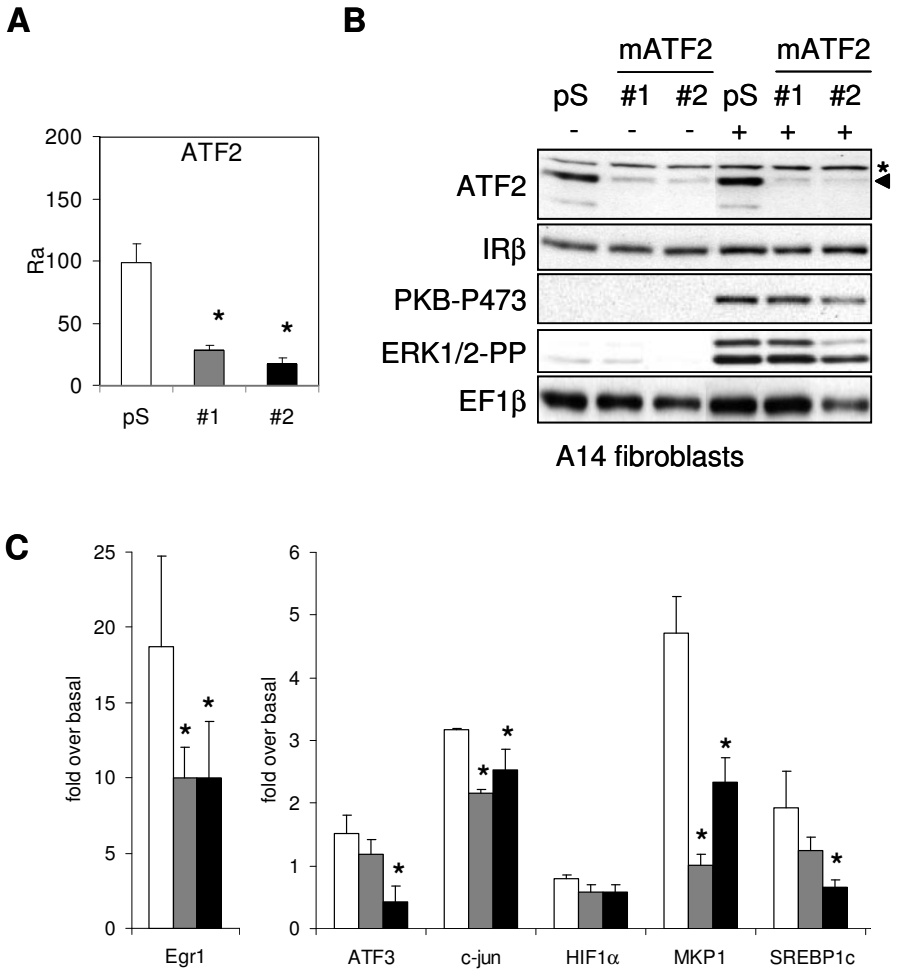

Figure 5. Effect of ATF2 silencing on insulin signalling and insulin-mediated gene expression. A14 cells were transfected with pSuper (pS) or ATF2-specific shRNA (#1 and #2). A. Effect of ATF2 silencing on relative ATF2 mRNA abundance (Ra). Data are mean± SEM of 2 independent experiments performed in duplicate. *, $P<0.05$ versus pSuper. B. Western blot analysis of cells kept untreated (-) or stimulated for 15 min with 10 nM insulin (+) using antibodies for ATF2, phospho-specific antibodies directed against PKB-P473 and ERK1/2- P202/204. Equal loading was confirmed using insulin receptor β-subunit and EF1β antibodies. * marks a background band C. Relative mRNA abundance (Ra) of ATF2 target genes in A14 cells transfected with pSuper (open bars), mATF2#1 (grey bars) or mATF2#2 (black bars). The effect of 60 min incubation with 10 nM insulin is expressed as fold over basal. Data are mean ± SEM of 2 independent experiments performed in duplicate. *, $P<0.05$ versus pSuper.

Thus, in A14 cells, ATF2 contributes to the induction of ATF3, *c-jun*, Egr1, MKP1, and SREBP1c mRNA by insulin.

Discussion

To detail the role of ATF2 activation in insulin action and metabolic control, insulin-regulated ATF2-dependent genes were characterized in both 3T3L1 adipocytes and A14 fibroblasts. The terminally differentiated 3T3L1 adipocytes display metabolic responses, like insulin-mediated glucose uptake, triglyceride synthesis, and inhibition of lipolysis (14-16), as well as secretion of adipokines (17). A limitation of 3T3L1 adipocytes is that both conventional gene transfer methods, like transient transfections, and lentivirus-mediated delivery of shRNA-constructs are inefficient in this cell type. To circumvent this problem, we also included A14 fibroblasts in this study. These cells respond to insulin in a more 'mitogenic' manner, such as induction of the immediate early genes *c-jun* and *c-fos*, and DNA synthesis (18;19). Both cell types were exposed to insulin for 30-60 min, not only to reduce secondary responses, but also because ATF2 activation and *c-jun* induction occur within 4-10 min and 30-60 min after the addition of insulin, respectively (5;8;19).

Potential ATF2 target genes were selected on the basis of a promoter analysis for CRE-binding sites, or previous studies (1;3;4;10;11;13;20;21). For a number of selected genes ATF3, *c-jun*, Egr1, MKP1, and SREBP1c, the mRNA levels were increased by insulin, and this induction was blunted both by the JNK-inhibitor SP600125 and by silencing of the ATF2 gene. In 3T3L1 adipocytes, we also found that the insulin-mediated induction of adiponectin was inhibited by SP600125. As this gene is not expressed in A14 fibroblasts, we could not validate this finding using shRNAs. Activation of luciferase reporter constructs fused to the adiponectin promoter by ATF2-VP16, a hybrid protein in which the potent transactivation domain of the viral transactivator VP16 is fused to the carboxyl terminus of ATF2 (22), or chromatin immunoprecipitation assays, could further detail a role for ATF2 in the regulation of adiponectin.

Although previous studies have identified PEPCK, PGC1α, IL1β, and TNFα (1;9-11;13;21) as ATF2-target genes, we could not confirm a role for ATF2 in the regulation of these genes by insulin in A14 cells and/or 3T3L1 adipocytes. In the case of IL1β and TNFα, we did not obtain reproducible amplification of these genes. It seems plausible that these genes are not expressed in these cell types under the experimental conditions used. Studies in murine tissues, including the liver, have demonstrated that the primer sets used to amplify these genes are functional (6).

Various possibilities may underlie the absence of regulation PEPCK and PGC1α expression by the insulin-ATF2 pathway. ATF2 binds to DNA either as homodimer, or as heterodimer with other ATF family members, like ATF3, ATF4, ATF6, and B-ATF, or *c-jun* family members(1-3). In addition, at least 21 distinct isoforms of mouse ATF2 have been identified so far. These various isoforms combined in different (hetero)dimer compositions confer a large repertoire of target genes on ATF2, depending on tissue-specific expression levels and the activating stimulus. In case of PEPCK, which was identified as an ATF2 target gene in rat hepatoma cells, but also in the fat-body, the fly equivalent of the mammalian liver and fat tissue (10;11;13), differences in the activating stimulus, rather than tissue-specific factors seem to underlie the absence of gene-regulation by the insulin-ATF2 pathway in the cell-types examined.

Both tissue- and stimulus-specific differences may contribute to the lack of PGC1α-induction by insulin in 3T3L1 adipocytes, and its downregulation in A14 fibroblasts, as PGC1α was characterized as target of the p38-ATF2 pathway in response to

β-adrenergic stimulation of brown adipose tissue (9). Other insulin-activated signaling pathways may be dominant over the ATF2-pathway in 3T3L1 and A14 cells. For example, the PKB/FOXO pathway, which is strongly activated by insulin, has been shown to lower the expression of PGC1α (23).

An important question arising from this study is how the identified target genes impact on insulin action and metabolic control. The gene most strongly induced by insulin in both 3T3L1 adipocytes and A14 fibroblasts, is the zinc-finger transcription factor Egr1. In β-cells, insulin-regulated induction of Egr1, like *c-jun*, regulates proliferation and differentiation (24-27), amongst others by Egr1-dependent induction of the homeodomain transcription factor pancreas duodenum homeobox-1 (PDX1), a key regulator of insulin expression and pancreatic β-cell development, function, and survival (25). In adipocytes, insulin-mediated regulation of Egr1 contributes to the induction of tissue factor and macrophage colony-stimulating factor, two cardiovascular risk factors (28;29). Furthermore, in endothelial cells, the insulin-induced Egr1-target genes, like platelet-derived growth factor, TNFα, intracellular adhesion molecule-1, monocyte chemotactic protein-1, plasminogen activator inhibitor-1, and vascular adhesion molecule-1, are strongly linked to the development of atherosclerosis (30;31) and also expressed in adipocytes. Intriguingly, both in adipocytes and endothelial cells, the induction of Egr1 by insulin was not altered under conditions of insulin resistance (29;31). Thus, whereas the function of Egr1 induction in adipocytes is unclear under normal conditions, deregulation of this response in type 2 diabetes may contribute to the development of vascular complications.

Also the role of ATF3, a transcription factor belonging to the same family as ATF2, in normal insulin action requires further studies. Although ATF3 has been implicated in the downregulation of PEPCK in the liver (32), other studies strongly link ATF3 to cellular damage and apoptosis. For example, the induction of ATF3 in response to pro-inflammatory cytokines, nitric oxide, hyperglycemia, high levels of palmitate, or endoplasmic reticulum stress precedes apoptosis in β-cells (11;33;34).

cJun is another member of the bZip-containing family of transcription factors and has been implicated in the regulation of cell growth and differentiation (2). cJun is an important dimer-partner of ATF2 (35). In addition, one of the major kinases phosphorylating both cJun and ATF2: JNK, is activated by insulin (5;36). Insulin-induced JNK-dependent cJun-phosphorylation has been reported in A14 cells ((7), BB and DMO, unpublished results). Thus, cJun could cooperate with ATF2 in regulating insulin-induced ATF2-dependent gene expression. However, it should be noted that, increasing evidence also supports a role for JNK in the development of insulin resistance in a number of tissues (reviewed in (37-39)) suggesting that cJun, perhaps in combination with ATF2, could also be involved in the development of insulin resistance.

MKP1 belongs to the MAP kinase phosphatase family of dual specificity phosphatases, which negatively regulate the activity of MAP kinases, like JNK, p38 and ERK1/2 (40). Interestingly, levels of phosphorylated ATF2 in insulin-treated cells remain elevated when the induction of MKP1 is abolished (DMO, unpublished data). This observation raises the possibility that MKP1 induction may serve to prevent sustained ATF2 activation by insulin and dampen a prolonged expression of detrimental ATF2-target genes. Accordingly, MKP1 has an inhibitory effect on the production of monocyte chemoattractant protein 1 (MCP-1), a chemokine highly associated with the development of adipocyte dysfunction in insulin resistance (41).

The transcription factor SREBP1c is a pivotal regulator of glucose and lipid metabolism, amongst others by inducing the expression of glucokinase and genes involved in lipogenesis (42). Insulin is a known inducer of SREBP1c in various tissues (43). Although SREBP1c was identified as ATF2-regulated gene before (4), analysis of the insulin-regulated part of the SREBP1c promoter did not (yet) identify putative ATF2-binding sites (43). It is therefore plausible that ATF2 regulates the SREBP1c promoter through the transcriptional co-activator p300/CBP (43;44). Like Egr1 and ATF3, however, deregulation of SREBP1c activity by high fat feeding, endoplasmic reticulum stress and hyperinsulinemia, contributes to the development of hepatic steatosis, which aggravates of insulin resistance (42;45).

Collectively, this study identifies ATF3, *c-jun*, Egr1, MKP1 and SREBP1c as insulin-regulated ATF2 target genes. Intriguingly, deregulation of (downstream targets of) these genes is highly linked to the pathogenesis of insulin resistance, β-cell dysfunction and vascular complications found in type 2 diabetes. Therefore, this study not only substantiates a role for ATF2 in insulin action, but also suggests that aberrant ATF2 activation under conditions of insulin resistance may contribute to the development of type 2 diabetes.

Materials and methods

Materials. Tissue culture media were obtained from Invitrogen (Carlsbad, Ca, USA), and SP600125 was from Biomol (Plymouth meeting, PA, USA). Antibodies recognizing phospho-T71-ATF2 (#9221), phospho-T69/T71-ATF2 (#9225), phospho-T202/Y204-ERK1/2 (#9101), and phospho-S473-PKB (#9271) were from Cell Signaling Technology (Beverly, MA, USA), anti-ATF2 (C19) and anti-insulin receptor β-subunit (C19) were from SantaCruz Biotechnology (Santa Cruz, CA, USA). Anti-elongation factor 1β (EF1β) has been described (46).

Generation of shRNA ATF2 constructs. To produce mouse-specific ATF2 shRNA constructs, we used the siRNA target finder to select oligonucleotides that were subsequently verified for specificity for mouse ATF2 (mATF2) by BLAST analysis of the mouse genome database. Sense and antisense versions of two silencing oligonucleotides, i.e. 5'-TGAGGAGCCTTCTGTTGTA-3' (mATF2#1), and 5'-GTGCCAAGCAGTCCACATA-3' (mATF2#2), corresponding to nucleotides 727-745 and 1685-1703, respectively, of the predicted mRNA ATF2 sequence in the mouse genome database were inserted into forward (5'-gatccccSENSEttcaagagaANTISENSEtttttggaaa-3') and reverse oligonucleotides (5'-agcttttccaaaaaSENSEtctcttgaaANTISENSEggg-3') purchased from Eurogentec (Liege, Belgium). Oligonucleotide pairs were annealed and phosphorylated and ligated into *Bgl*II- and *Hind*III-digested pSuperH1 (47). The pSuper-shATF2 constructs were verified by restriction and sequence analysis.

Cell culture and transient transfections. 3T3L1 adipocytes were purchased from the American Type Culture Collection (Manassas, VA, USA), and cultured and differentiated in adipocytes as described previously (48). A14 cells are NIH3T3 fibroblasts overexpressing the human insulin receptor, and grown in DMEM supplemented with 10% fetal bovine serum and antibiotics (19). For silencing experiments, A14 cells were transfected in 6-well plates with 500 ng of the pSuper ShRNA construct and 125 ng of pBabe-puro using Fugene 6 reagent (Roche Biochemicals, Indianapolis, IN). The next day, the cells were split into 2 6-well dishes, and selected with 3 μg/ml of puromycin (Sigma Aldrich, St. Louis, MO, USA) for 2 days, after which cells were serum-starved overnight and stimulated with insulin where indicated.

RNA extraction and real-time PCR analysis. Cells were homogenized in RLT-buffer (Qiagen, Venlo, The Netherlands), whereafter total RNA was extracted using RNeasy mini columns (Qiagen, Venlo, The Netherlands). DNase I digestion was performed to ensure complete removal of DNA. Purity and quantity of nucleic acids was determined by spectrophotometric analysis at 260 and 280 nm. Integrity of the RNA was verified by agarose gel electrophoresis. A total of 1.0 μg RNA was transcribed into complementary DNA using a Superscript™ first strand synthesis kit (Invitrogen, Breda, The Netherlands) using oligo-dT priming. mRNA abundance was measured using real-time PCR. Primers are listed in Table 2, and were designed using Primer Express version 3.0 software (Applied Biosystems, Foster City, CA, USA).

Table 2. Sequences of primers used for real-time PCR

Gene name	Accession number	Forward primer (5'->3')	Reverse primer (5'->3')
18S rRNA	NR_003278	gactcaacacgggaaacctc	agacaaatcgctccaccaac
adiponectin	NM_009605	ggaatgacaggagctgaagg	cgaatgggtacattgggaac
ATF2	NM_001025093	gactccaacgccaacaagat	aggtaaagggctgtcctggt
ATF3	NM_007498	aacacctctgccatc	ttatttctttctcgccgcctc
β-actin	NM_007393	agagggaaatcgtgctgac	caatagtgatgacctggccgt
c-jun	NM_010591	caacatgctcagggaacaggt	tgcgttagcatgagttggca
CREB1	NM_133828	ggagcttgtaccaccggtaa	gggctaatgtggcaatctgt
EF1α	NM_010106	aattggaggcattggcac	aaaggtaaccaccatgccagg
Egr1	NM_007913	aacactttgtggcctgaacc	aggcagaggaagacgatgaa
FABP4	NM_024406	aagaagtgggagtgggcttt	ctgtcgtctgcggtgattt
FOXO3a	NM_019740	ttcccatataccgccaagag	tgacgcaaggagttcagaga
HIF1α	NM_010431	cctggaaacgagtgaaagga	ctgccttgtatgggagcatt
IGF2	NM_010514	gagttcagagaggccaaacg	tagtgtgggacgtgatggaa
IGFBP6	NM_008344	cagagaccggcagaagaatc	ctcggaagacctcagtctgg
IL1β	NM_008361	caggcaggcagtatcactca	aggtgctcatgtcctcatcc
MKP1	NM_013642	ctcatgggagctggtcctta	gcgaagaaactgcctcaaac
PEPCK	NM_011044	tctgaggccacagctgctg	gggtcgcatggcaaagg
PGC1α	NM_008904	tttttggtgaaattgaggaatgc	cggtaggtgatgaaaccatagct
PPARα	NM_011144	caacccgccttttgtcatac	cctctgcctctttgtcttcg
SREBP1c	NM_011480	ggagccatggattgcacatt	cctgtctcacccccagcata
TNFα	NM_013693	gtccccaaagggatgagaag	cacttggtggtttgctacga

Reactions were carried out in the presence of 1x SYBR® Green PCR Master Mix (Applied Biosystems), 0.5μM of each forward and reverse primer on a StepOne Plus Real-time PCR system (Applied Biosystems) with the following cycle conditions: 95°C for 10 min followed by 40 cycles of 95°C for 15", 60°C for 30", and 72°C for 30". The threshold cycle number (C_t) was calculated using StepOne Plus software version 2.0 (Applied Biosystems) and an automated setting of the baseline. Data were analyzed using a comparative critical threshold (C_t) method in which the amount of target is normalized to the amount of endogenous control using the equation $R_a=2^{-\Delta Ct}$ in which R_a = the relative mRNA abundance, and $\Delta C_t=C_t$(gene of interest)-C_t(endogenous control), and C_t(endogenous control) is calculated as the geomean(C_t) of FABP4, ATF2, EF1α, and β-actin in 3T3L1 adipocytes, and geomean(C_t) of 18S rRNA, EF1α, β-actin in A14 fibroblasts, respectively.

The expression of the genes used as endogenous control was not affected by the experimental conditions used in this study.

Analysis of insulin signaling. Cells were washed twice with ice-cold PBS and lysed in 30 mM Tris.Cl, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate, 1% SDS, 1 mM Na_3VO_4, 10 mM NaF and protease inhibitors (Complete, Roche). Cell lysates were cleared by centrifugation (13.200 rpm; 15 min, 4^{o}C), and protein content was determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA). Expression and phosphorylation of proteins was studied by SDS-PAGE and Western blotting as described (5).

Statistical analysis. Data are expressed as means ± SE. Differences between groups were determined by unpaired students two-tailed *t*-test using SPSS version 16.0. $P<0.05$ was considered statistically significant.

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Chapter 5