The role of ATF2 in insulin action

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7

Summary and Discussion

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Insulin elicits complex responses in peripheral tissues like skeletal muscle, adipose tissue, the heart, the liver and brain to maintain glucose and lipid homeostasis. Insulin resistance and type 2 diabetes are associated with impaired insulin action in these target tissues. In order to understand the molecular mechanisms underlying insulin resistance and to develop novel (therapeutic) strategies to improve peripheral insulin sensitivity, it is crucial to understand the complex responses that are induced by insulin in its target tissues. Insulin action is initiated by binding of insulin to its receptor and eventually leads to activation of two major effector pathways: the PI-3K-dependent PKB/Akt-pathway and the Ras-dependent MAPK-pathway. Activation of the PKB/Akt pathway has been linked to the regulation of lipid and glucose metabolism, gene expression and cell survival. The MAPK-pathway contributes to activation of gene expression and cell proliferation. In contrast to the PKB/Akt-pathway, the role of the Ras/MAPK in physiological insulin action is less well defined.

We have identified the transcription factor activating transcription factor 2 (ATF2) as a downstream target of the Ras/MAPK signaling pathway and novel component of insulin signaling (1). In the research described in this thesis we have further characterized the role of ATF2 in insulin action by (i) detailing the mechanism of insulin-induced ATF2 phosphorylation, (ii) identifying genes whose expression depends on ATF2 and (iii) investigating whether the induction of ATF2-phosphorylation and ATF2-regulated genes is altered under conditions of insulin resistance.

Chapters 3 and 4 address the mechanism of ATF2-activation in response to insulin. The data reported in these chapters extend the previous findings by further detailing the mechanism with which insulin induces ATF2-phosphorylation in the genetic absence and presence of JNK. Where stresses seem to induce activation of ATF2 via simultaneous phosphorylation of the transactivating residues Thr69+71 dependent on one kinase, insulin (and mitogens in general) utilizes a Ras-dependent two-step mechanism employing two kinases to achieve this. The data presented in chapters 3 and 4 suggest the following model for the induction of ATF2-Thr69+71 phosphorylation in response to insulin in cultured cells (summarized in Figure 1): in both JNK-deficient and JNK-containing cells, insulin induces early activation of the ERK1/2 via the Ras-Raf-pathway. Active ERK1/2 then translocates to the nucleus and mediates ATF2-Thr71-phosphorylation. Minutes later, after Ras-RalGDS-Ral dependent activation of p38/JNK, nuclear translocation of active p38 (in JNK-deficient cells) or active JNK (in JNK-expressing cells) is responsible for the activation of ATF2 by phosphorylation of Thr71-phosphorylated ATF2 on Thr69. We propose that, in both JNK-/- and JNK-expressing cells, the difference between the nuclear appearance of ERK1/2 and the ATF2-Thr69-phosphorylating SAPK (p38 or JNK) is rate limiting for the two-step phosphorylation of ATF2-Thr69+71 in response to insulin.

Importantly, the data described in chapter 6 confirmed insulin-induced ATF2-phosphorylation in several mouse tissues *in vivo*. However, the question whether *in vivo* ATF2-phosphorylation is also regulated via a two-step mechanism dependent on two cooperating kinases remains unanswered. No evidence was found to support a two-step process, but as hepatic ATF2 was already found to be phosphorylated on both Thr69+71 at the earliest time-point examined (10 minutes after start of insulin infusion), analysis of earlier time-points may potentially reveal differential kinetics in phosphorylation of the

different ATF2 sites and/or correlating differences in MAPK-activation in response to insulin. Experimental procedures to measure in vivo ATF2 phosphorylation at these early time points are however difficult to perform. In adipose tissue and liver, we observed a weak and transient induction of p38 and ERK1/2 phosphorylation at the time-points examined. Although phosphorylation of JNK could not be detected on western blots, due to the presence of interfering background bands and a poor signal-to-noise-ratio, pharmacological inhibition of JNK (using SP600125), and not of p38 (with SB203580) severely reduced only the ATF2-Thr69 *in vitro* kinase activity, and not the ATF2-Thr71 directed activity present in lysates from insulin-treated mouse livers (BB/DMO, unpublished results). These preliminary data suggest a potential role for JNK in the *in vivo* Thr69-phosphorylation of ATF2 induced by insulin. Further experimentation, using cell-permeable inhibitors of JNK that have been described to be functional in whole animals (2;3) or JNK-knockout mice (4) can potentially be used to substantiate the involvement of JNK in the insulin-induced *in vivo* ATF2-phosphorylation. Alternatively, the *in vivo* regulation of ATF2-phosphorylation may differ from the mechanism found in cells, and other kinases can be involved in this process *in vivo*. For a more general identification of ATF2-kinases activated by insulin *in vivo*, anion-exchange chromatography of insulin-treated tissue lysates in combination with ATF2-directed kinase assays can be employed (1;5).

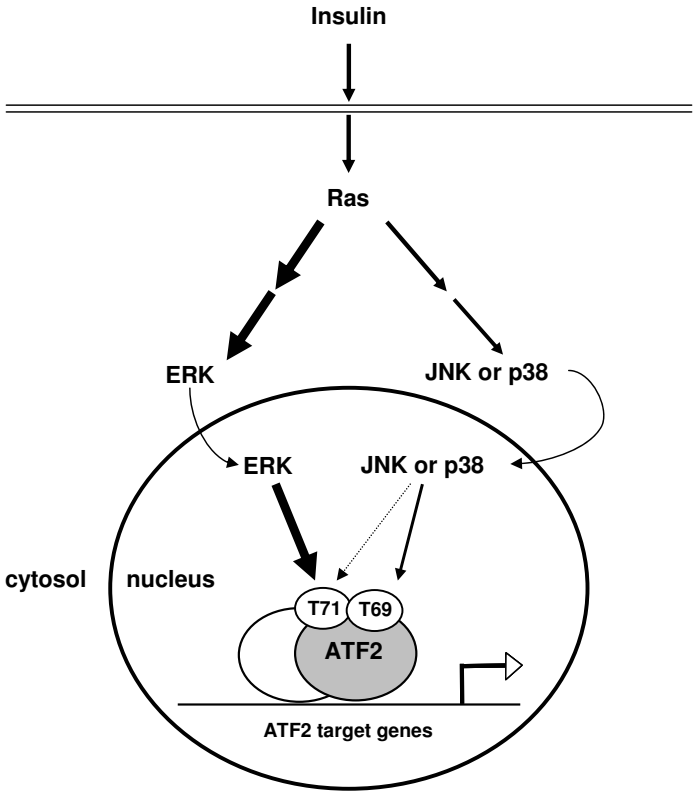

Figure 1. Proposed model for insulin-induced ATF2 activation in cultured cells. First, insulin induces Ras-dependent activation of ERK1/2. ERK1/2 subsequently translocates to the nucleus, where it mediates ATF2-Thr71 phosphorylation. Subsequently, JNK or (in JNK-deficient cells) p38 is activated. JNK (or p38) then translocates to the nucleus, and efficiently phosphorylates Thr71-phosphorylated ATF2 on Thr69, thus inducing transcriptional activation of ATF2.

To begin to detail the role of ATF2 in insulin action, we identified ATF2 target genes in insulin-sensitive cell lines and determined their hepatic expression levels in response to insulin-stimulation *in vivo*. In chapter 2, an overview is given on the potential role of ATF2 and ATF2-regulated genes in metabolic control and/or insulin action. Chapter 5 describes the identification of ATF3, *c-jun*, Egr1, MKP1 and SREBP1c as insulin-induced genes, whose expression is ATF2-dependent in both A14 fibroblasts and 3T3L1-adipocytes. Although *in vivo* insulin-inducibility could also be established for most of the identified ATF2-dependent genes in mouse liver (ATF3, *c-jun,* Egr1, and SREBP1c), a role for ATF2 therein was not formally proven (chapter 6). To obtain such evidence, adenovirus-mediated knockdown of hepatic ATF2 and/or Chromatin Immuno Precipitation (ChIP) studies for (phosphorylated) ATF2 (which is also possible in other tissues than liver) could be performed. Unfortunately, the ATF2 knockout mice or the phospho-dead $ATF2^{AA}$ expressing knock-in mice can not be used in studies on insulin action in adult animals, as they die shortly after birth (6;7). However, ATF2m/m mice, that express a mutant ATF2-isoform at low levels (7-9), have been employed to identify ATF2-dependent gene transcription and could therefore be suitable in further detailing the function of ATF2 in physiological insulin action. Other mouse-models include tissue-specific ATF2-knockout mice, which are currently being generated (10), or tissue-specific (inducible) expression of dominant negative forms of ATF2. Combination of these experimental setups with large-scale gene expression-profiling methods, such as microarray or solexa sequencing, could produce a higher yield of potential candidates and can provide more insight in the role of ATF2 in insulin-induced gene expression.

For some of the examined genes that were previously described as ATF2 targets, we could not establish ATF2-dependency in either one or both of our cell- or mouse-based studies. For example, we could not find any correlation between *in vivo* ATF2-phosphorylation and MKP1 expression in mouse livers, despite the fact that MKP1 expression was found to be ATF2-dependent in cultured cells stimulated with insulin (chapter 5) and expression of a number of MKPs (including MKP1) was reported to be dependent on intact ATF2-phosphorylation in embryonic livers (6). Apparently, other, potentially ATF2-independent, regulatory mechanisms govern the *in vivo* MKP1 expression in the adult animal.

Some of the other described ATF2-regulated genes (PEPCK, PGC1α, IL1β and TNFα) did not show any ATF2-dependency in response to insulin in cultured cells. In addition, *in vivo* insulin-treatment either lowered or had no effect on the expression of these genes in liver. For the cytokines IL1β and TNFα, it is not unthinkable that they are not expressed in the cell- or tissue-types and/or experimental conditions discussed here (see also below).

Several explanations can be given for the absence of regulation of the described ATF2 targets MKP1, PEPCK and PGC1α expression by insulin, both independent of and dependent on ATF2. Stimulus- and tissue-specific responses can underlie the apparent ATF2-independent regulation of these genes by insulin. The expression of most genes is regulated by a high number of factors and depending on the stimulus and cell- or tissue-type, other signaling pathways may be dominant over the ATF2-pathway. For example, the PKB/FOXO pathway, which is strongly activated by insulin in liver, could be crucial in the regulation of insulin-induced PGC1α expression (11).

Alternatively, expression of different ATF2-isoforms and/or ATF2-binding partners, some of which have been reported to repress transcription (e.g. ATF2d (12) and ATF3 (13)), can have opposing effects on ATF2-dependent gene regulation. Our

unpublished results suggest that several ATF2-isoforms are (co-)expressed in a tissue-specific manner (see figure 2). Differences in promoter-, co-factor- or dimer partner-specific characteristics would then determine which isoform of the ATF2 protein would be dominant in regulating a specific gene.

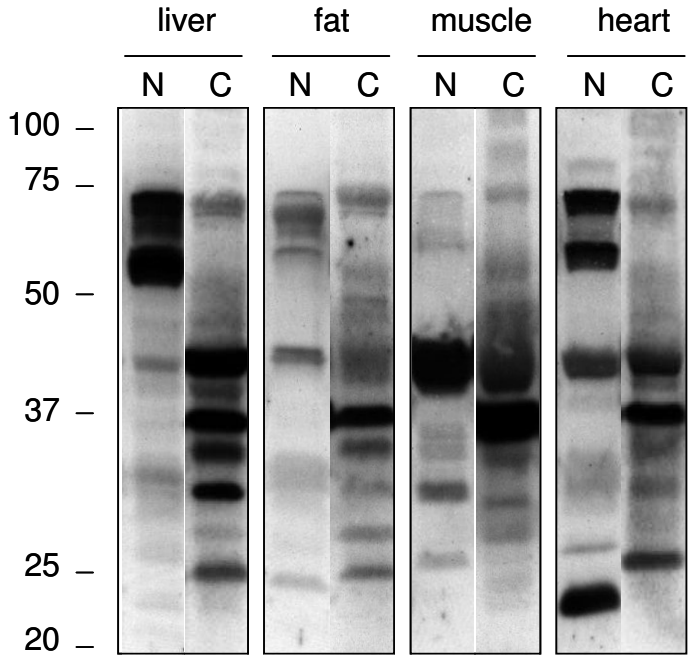

Figure 2. Expression of ATF2 isoforms in target tissues for insulin action. Lysates from mouse liver, adipose tissue, skeletal and cardiac muscle were analyzed by Western blotting using antibodies recognizing the first 96 (N) or last 19 (C) amino acids of ATF2.

A still unanswered question is how the identified ATF2-target genes impact on insulin action and metabolic control. Most of the positively identified insulin-induced ATF2-dependent genes encode transcription factors (e.g. Egr1, ATF3, *c-jun* and SREBP1c), whose targets have been postulated to be involved in several aspects of normal insulin action, like proliferation and differentiation of β-cells (for Egr1 and cJun (14-16)), control of hepatic fat and glucose metabolism (SREBP1, ATF3, cJun and Egr1 (13;17-19)). Further studies in relevant physiological models could be used to address their role in normal insulin action. One can think of ectopic expression of these factors in ATF2-'deficient' models (ATF2m/m mice (9) or (virus-mediated) knockdown of ATF2 in cellular model systems or in mouse liver), but not after a role for ATF2 in the regulation of these genes is established more firmly.

Finally, deregulation of a number of (potential) ATF2-target genes described in chapters 2 and 5 has also been associated with pathogenesis of insulin resistance, e.g. β-cell dysfunction and vascular complications found in type 2 diabetes (see chapter 2 for references). In addition, cytokine- or ER-stress-induced activation of one of the major ATF2 kinases: JNK, has been described to play a role in the development of insulin resistance in a number of tissues (reviewed in (20-22)). This suggests that next to a role in normal insulin action, ATF2 and its target genes could also be involved in the development of an insulin-resistant state and/or T2D(-associated complications).

To assess if insulin-resistance impacted on ATF2-phosphorylation, we examined ATF2-phosphorylation and expression of ATF2-dependent genes in tissues of high fat diet (HFD)-induced insulin-resistant mice. We found that already in the fasting state with low plasma concentrations of insulin, HFD induced increased ATF2-phosphorylation in livers and adipose tissue of insulin-resistant mice (chapter 6). As numerous studies have described elevated JNK-activity in HFD-induced insulin resistant states (4;23), the increased ATF2 phosphorylation found here, is probably (at least partially) mediated by JNK.

Correlating with the increased ATF2-phosphorylation found in these mice, HFD induced basal hepatic expression of the ATF2-dependent genes identified in chapter 5: ATF3, *c-jun*, Egr1 and SREBP1c. The described ATF2 target genes IL1-β and TNFα were also significantly induced by the HFD in liver, presumably produced by hepatic Kupfer cells or infiltrated macrophages activated by the HFD-induced state of chronic inflammation. The insulin-induced enhancement of all of these genes was severely reduced in the HFD-fed animals, correlating with the inability of insulin-administration to further increase ATF2 phosphorylation.

To ascertain the ATF2-involvement in this process, similar experiments as suggested above could be performed, e.g. comparison of (phospho-)ATF2-ChIPs in chow vs HFD-fed mouse tissues and/or analysis of the development of HFD-induced insulin-resistance in ATF2m/m mice or in mice with tissue-specific inactivation of ATF2 (10). Notably, as ATF2 is highly expressed in the brain (24;25) and there is increasing evidence for metabolic control via the brain (25-29), potential regulation of metabolism via central (neuronal) ATF2 should be considered. To further define the potential role of ATF2 in JNK-dependent HFD-induced insulin resistance analysis of the ATF2-phosphorylation status and ATF2-dependent gene expression in JNK-knockout mice (4;23;30;31) and/or combination of ATF2- and JNK-deficient mouse models could also be useful.

As a number of other genes related to metabolic control (such as PEPCK (32;33)) or the development of diabetes and/or its associated complications (for example the pro-inflammatory cytokine TNFα (9)) have been shown to be ATF2-dependent in several different experimental settings (see chapter 2), including these genes as targets in ChIP–on-chip experiments or larger scale expression profiling in several of the models described above could be particularly interesting.

In conclusion, we have identified ATF2 as a component of the cellular and *in vivo* insulin signaling systems. Insulin induced ATF2-phosphorylation in A14 fibroblasts, 3T3L1 adipocytes and several mouse tissues *in vivo*. In cell lines, the insulin-induced ATF2-phosphorylation was dependent on cooperation between two Ras-dependent MAPK-pathways: ERK and p38/JNK. Analysis of several described ATF2-target genes identified insulin-induced expression of Egr1, ATF3, *c-jun* and SREBP1c as being ATF2-dependent in cell lines. Quantitative PCR analysis showed increased mRNA expression of these genes in mouse livers correlating with hepatic ATF2-phosphorylation induced by insulin, but also in response to HFD-induced insulin resistance. Although the elucidation of the exact role of ATF2 activation under these conditions needs further experimentation, the data presented in this thesis suggest a potential dual function for ATF2 as a mediator of insulin action on the one hand and a putative regulator of the development or maintenance of insulin resistance on the other.

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References

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