Novel concepts in insulin regulation of hepatic gluconeogenesis

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Barthel, Andreas and Dieter Schmoll. Novel concepts in insulin regulation of hepatic gluconeogenesis. Am J Physiol Endocrinol Metab 285: E685–E692, 2003; 10.1152/ajpendo.00253.2003.—The regulation of hepatic gluconeogenesis is an important process in the adjustment of the blood glucose level, and pathological changes in the glucose production of the liver are a central characteristic in type 2 diabetes. The pharmacological intervention in signaling events that regulate the expression of the key gluconeogenic enzymes phosphoenolpyruvate carboxykinase (PEPCK) and the catalytic subunit glucose-6-phosphatase (G-6-Pase) is regarded as a potential strategy for the treatment of metabolic aberrations associated with this disease. However, such intervention requires a detailed understanding of the molecular mechanisms involved in the regulation of this process. Glucagon and glucocorticoids are known to increase hepatic gluconeogenesis by inducing the expression of PEPCK and G-6-Pase. The coactivator protein PGC-1 has been identified as an important mediator of this regulation. In contrast, insulin is known to suppress both PEPCK and G-6-Pase gene expression by the activation of PI 3-kinase. However, PI 3-kinase-independent pathways can also lead to the inhibition of gluconeogenic enzymes. This review focuses on signaling mechanisms and nuclear events that transduce the regulation of gluconeogenic enzymes.

REGULATION OF HEPATIC GLUCOSE PRODUCTION

De novo synthesis of glucose in the liver from precursors such as lactate, gluconeogenic amino acids, and glycerol is a central mechanism to provide the organism with glucose in times of starvation (13, 48, 53). On the other hand, when glucose is directly available from external resources, gluconeogenesis is dispensable and consequently needs to be shut off. Integration of these events is complex and occurs through various hormonal and nutritional factors. The principal parameters affecting hepatic glucose output are the concentrations of the available gluconeogenic substrates and the activity of a few regulatory enzymes. The activity of the key gluconeogenic enzymes phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G-6-Pase) is regulated by transcriptional and nontranscriptional mechanisms, whereas the third key enzyme fructose-1,6-bisphosphatase (FBPase) is also regulated through competitive inhibition by fructose 2,6-bisphosphate.

Insulin is the most important hormone that inhibits gluconeogenesis. It acts predominantly by suppressing the expression of the genes for the key gluconeogenic enzymes PEPCK and G-6-Pase. PEPCK catalyzes one of the rate-limiting steps of gluconeogenesis, the reaction of oxaloacetic acid to phosphoenolpyruvate, whereas G-6-Pase catalyzes the final step of gluconeogenesis, the production of free glucose from glucose 6-phosphate. The expression of the genes for G-6-Pase and PEPCK is induced by glucagon during fasting, by glucocorticoids during periods of stress, or by catecholamines during exercise (20, 26, 35, 36, 59, 62, 67).

The initial stages of type 2 diabetes are characterized by insulin resistance (56). This leads to the inability of insulin to control the activity of gluconeogenic enzymes, thereby contributing to an increased hepatic glucose output and elevated blood glucose levels. In patients with type 2 diabetes, the rate of hepatic gluconeogenesis is considerably increased compared with control subjects, thereby contributing significantly to the fasting hyperglycemia in diabetes. Also, mice with an organ-specific insulin receptor knockout in the liver show, in addition to a severely impaired glucose tolerance, an increased hepatic glucose production with

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elevated G-6-Pase and PEPCK expression levels in the liver (41). Therefore, the signaling mechanisms that mediate the regulation of the gluconeogenic enzymes are of particular interest, because they are potential targets for pharmacological intervention to restore insulin sensitivity. For example, metformin, an antidiabetic drug with a poorly understood mechanism of action that has been in clinical use for more than 40 years, is known to decrease hepatic glucose production. There is evidence obtained with different methods, like NMR spectroscopy and stable isotopes, that this effect occurs mainly by reducing the rate of gluconeogenesis (30, 69), although it has also been debated that metformin may work by reducing glycogenolysis (18, 42).

Over the past few years, considerable progress has been made in the elucidation of intracellular signaling events of insulin (38, 57, 64, 73). This has led to a better understanding of the molecular mechanisms by which this hormone regulates the genes for PEPCK and G-6-Pase.

**SIGNALING PATHWAYS INVOLVED IN THE SUPPRESSION OF GLUCONEOGENESIS**

*Insulin-Regulated Signaling Pathways*

Stimulation of the insulin receptor results in the activation of two major pathways: 1) the mitogen-activated protein (MAP) kinase cascade and 2) the phosphatidylinositol 3-kinase (PI 3-kinase) pathway, which has been extensively studied in the context of the metabolic responses to insulin (38, 57).

The PI 3-kinase pathway. The insulin receptor is a tyrosine kinase that becomes activated after ligand binding. This leads to the phosphorylation of several intracellular substrates, including the family of insulin receptor substrate (IRS) proteins. Gene knockout studies have demonstrated that insulin decreases hepatic glucose production mainly via IRS-2, which suggests that this isoform has a predominant role in the signaling pathway that controls gluconeogenesis (34, 79). The tyrosine phosphorylation of IRS proteins results in the recruitment of the lipid kinase PI 3-kinase to the plasma membrane, where it phosphorylates PI-(4,5)-bisphosphate [PtdIns(4,5)P2] to generate PtdIns(3,4,5)P3, a central second messenger of several growth factor receptors. The activity of PI 3-kinase can be pharmacologically inhibited by wortmannin and LY-294002. These compounds abolish the suppression of both basal and cAMP- or dexamethasone-induced PEPCK and G-6-Pase gene expression by insulin (1, 20, 61).

In addition, overexpression of the catalytic subunit of PI 3-kinase is sufficient to markedly suppress PEPCK and G-6-Pase gene expression. Furthermore, the adenoviral overexpression of the dominant negative mutant of the PI 3-kinase regulatory subunit p85α, which lacks the binding site for the catalytic subunit, increases PEPCK and G-6-Pase gene expression as well as hepatic glucose production in vivo (43). These data led to the conclusion that the activation of PI 3-kinase is central for the regulation of gluconeogenesis by insulin.

The generation of PtdIns(3,4,5)P3 is known to increase the activity of 3-phosphatidylinositol-dependent kinase-1 (PDK1) (38, 58, 73). PDK1 phosphorylates and activates several members of the AGC kinase superfamily, which includes the group of protein kinase A (PKA)-, G (PKG)-, and C (PKC)-related kinases (66). Among these substrates are the protein kinase B (PKB/Akt), p70 S6 kinase, and the two isoforms of atypical protein kinase C (PKC)-α and -ζ (7). Because PI 3-kinase is essential for the regulation of PEPCK and G-6-Pase gene expression, several studies have investigated the role of the AGC kinases in the regulation of the PEPCK and G-6-Pase gene by insulin to identify the signaling mechanisms downstream of PI 3-kinase.

Overexpression of PKB in hepatoma cells and primary hepatocyte cultures decreases PEPCK and G-6-Pase gene transcription (1, 37, 61), and several proteins have been identified that are able to mediate the observed suppression of PEPCK and G-6-Pase gene transcription by PKB, such as Foxo1 and cAMP response element-binding protein (CREBP; see Forkhead transcription factors). Although the data show that activation of PKB is sufficient to mediate the regulation of PEPCK and G-6-Pase gene expression by insulin, it is at present unclear whether PKB activation is also necessary for this regulation. This question was addressed by the overexpression of dominant negative PKB mutants (33, 37). With this approach, controversial results have been obtained regarding the ability of these constructs to suppress PEPCK gene expression. This might arise from the different experimental procedures to monitor the PKB regulation or to overexpress the dominant negative PKB mutant. The transient overexpression of PKB in hepatoma cells mimics the insulin-dependent regulation of G-6-Pase promoter activity only in part (61), whereas overexpression of constitutively active PI 3-kinase constructs results in a strong inhibition of G-6-Pase promoter activity (20). This indicates that other PI 3-kinase-dependent signaling pathways besides the activation of PKB are necessary for the regulation of G-6-Pase gene expression by insulin. Indirect evidence for a participation of PKB-β in the regulation of gluconeogenesis in vivo comes from data obtained in the PKB-β knockout mouse model (14). This mouse is insulin resistant and hyperglycemic because of an increased hepatic glucose production. It is tempting to speculate that this might be due to an impairment in the regulation of PEPCK and G-6-Pase gene expression, although this has not yet been demonstrated. In contrast to the PKB-β knockout mouse, the disruption of the gene for PKB-α has not been found to impair glucose homeostasis (15). The development of specific compounds to inhibit PKB enzymatic activity (like wortmannin or LY-294002 for PI 3-kinase activity) may therefore potentially contribute to the understanding of the exact role of PKB and its isoforms in the regulation of PEPCK and G-6-Pase gene expression.

Activated PKB is also able to phosphorylate and thereby to inhibit glycogen synthase kinase-3 (GSK-3),
and specific inhibitors of GSK-3 are known to increase hepatic glycogen synthesis. However, these compounds also decrease hepatic glucose production in fasted fa/fa rats (16). From these observations, it has been concluded that GSK-3 inhibitors might be useful agents for the treatment of diabetes (17, 22, 39). The reason for the decreased hepatic glucose production in vivo after inhibition of GSK-3 might be a suppression of PEPCK and G-6-Pase gene expression (39), which has been described after pharmacological inhibition of GSK-3 activity in hepatoma cells. GSK-3 inhibitors suppress the expression of PEPCK and G-6-Pase to a lesser extent than insulin. In addition, overexpression of GSK-3 does not prevent the regulation of the G-6-Pase gene by insulin (39). These data therefore suggest that insulin and inhibitors of GSK-3 might suppress the expression of PEPCK and G-6-Pase by two different mechanisms. The molecular events underlying the regulation of PEPCK and G-6-Pase gene expression by GSK-3 are unclear. GSK-3 phosphorylates and regulates the transcription factors c-Jun and c-Fos (17). One could therefore hypothesize that GSK-3 also regulates a yet-to-be-identified transcription factor that plays a role in the regulation of the genes encoding PEPCK and G-6-Pase.

In hepatoma cells, PKC-λ is the predominant isoform of atypical PKCs (33). After administration of insulin, PKC-λ is activated in a PI 3-kinase-dependent way. However, overexpression of PKC-λ does not suppress the promoter activity of a reporter construct under the control of the PEPCK gene promoter (33). Similar results have been obtained using G-6-Pase promoter constructs. These data therefore suggest that the activation of atypical PKC isoforms does not significantly contribute to the effect of insulin on the expression of gluconeogenic enzymes.

Insulin also stimulates the activation of p70 S6-kinase, a cytosolic kinase that phosphorylates S6, a ribosomal protein, in a PI 3-kinase-dependent manner. However, rapamycin, an immunosuppressive drug specifically inhibiting activation of p70 S6-kinase, does not affect the regulation of PEPCK and G-6-Pase gene expression by insulin (20, 37, 70). Therefore, p70 S6-kinase does not appear to be involved in the suppression of these genes by insulin.

MAP kinase. Insulin is able to stimulate the Raf/MEK/ERK1/2 pathway. Furthermore, the activation of this pathway is sufficient to mediate the suppression of PEPCK and G-6-Pase gene transcription by PMA (25, 60). However, neither pharmacological inhibitors of this pathway such as PD-98059, PD-184352, and U-0126 nor the overexpression of dominant negative forms of ras or MEK affects the regulation of PEPCK and G-6-Pase gene expression by insulin (1, 25, 60, 71). PMA stimulates ERK2 activity ~20 times more strongly than insulin in hepatoma cells (60). With that, insulin stimulation of ERK1/2 might apparently be too weak to significantly contribute to the overall regulation of the G-6-Pase gene. ERK1 and -2 are known to stimulate gene transcription via transcription factors of the Ets family (77). However, the molecular events leading to a suppression of PEPCK and G-6-Pase gene expression after stimulation of ERK1 or -2 have not yet been elucidated.

When we consider all of the evidence together, we find that the Raf/MEK/ERK pathway is a redundant mechanism for the regulation of gluconeogenic enzyme activity and that the physiological significance of this pathway in the context of hepatic glucose production is largely unclear.

AMP-Activated Protein Kinase Pathway

AMP-activated protein kinase (AMPK) is a heterotrimeric serine/threonine kinase that is widely expressed in a variety of organs including the liver (65). Activation of AMPK has been described to stimulate glucose uptake in muscle and fatty acid oxidation and ketogenesis in liver (78). Furthermore, the kinase inhibits cholesterol synthesis and lipogenesis. AMPK is activated by conditions like glucose starvation, and the enzyme is therefore regarded as a sensor of the cellular energy charge. A pharmacological activator of AMPK is the AMP analog 5-aminoimidazole-4-carboxamide-riboside (AICAR). AICAR is a prodrug, which is metabolized in cells to ZMP, the compound that allosterically activates AMPK. Treatment of H4 rat hepatoma cells with AICAR was found to mimic the effect of insulin on G-6-Pase and PEPCK gene expression, indicating that the activation of AMPK is able to suppress G-6-Pase and PEPCK gene expression (40). Furthermore, the adipocyte-derived hormone adiponectin, which probably plays an important role in the regulation of glucose and lipid metabolism, suppresses PEPCK and G-6-Pase gene expression by the activation of AMPK (80). Also, the drug metformin might exhibit some of its antidiabetic effects by the activation of AMPK and subsequent downregulation of gluconeogenesis (83) and increased glucose uptake in skeletal muscle (44).

To date, there is no evidence for a direct regulation of AMPK by insulin, suggesting that AMPK is part of an independent signaling pathway controlling the expression of gluconeogenic enzymes. The mechanism by which AICAR may promote insulin-like effects on the level of signal transduction is currently under debate. Recent evidence suggests that AMPK can phosphorylate IRS-1 on Ser789 in C2C12 myotubes and that this phosphorylation correlates with an increase in insulin-stimulated IRS-1-associated PI 3-kinase activity (31) and an augmentation of insulin signaling. In two other reports, forkhead transcription factors have been identified as downstream targets of AMPK. Yang et al. (81) describe the inhibition of AFX (now called Foxo4)/c-dependent reporter activity through AICAR. Other data suggest that activation of AMPK can repress G-6-Pase gene expression and therefore inhibit gluconeogenesis in hepatoma cells by reducing the cellular level of FKHR (now called Foxo1), presumably mediated by a specific degradation of the protein (5).
TRANSCRIPTIONAL REGULATION

The transcriptional regulation of the genes encoding for G-6-Pase and PEPCK involves complex interactions of a variety of transcription factors and other proteins. The promoters of these genes contain both glucocorticoid-responsive and cAMP-responsive structures that have been characterized in the context of transactivation by the glucocorticoid receptor and related proteins as well as by CREBP. Knowledge of the factors and mechanisms involved in the insulin-dependent inhibition of both genes has been largely speculative, although considerable progress has been made over the last few years.

Insulin-Regulatable Transcription Factors Involved in the Regulation of Gluconeogenesis

Forkhead transcription factors. The G-6-Pase and PEPCK promoters are known to contain so-called insulin-responsive sequences (49, 51). These sequences [T(G/A)TTTT] have been functionally characterized, and the transcription factors that bind to these regions have been unknown for some time. Interestingly, both the G-6-Pase and the PEPCK promoters have hepatic nuclear factor-3 (HNF-3) binding sites, and members of the HNF-3 family of proteins have been found to be involved in the glucocorticoid-stimulated expression of both genes (50, 75). Furthermore, in the PEPCK promoter, an HNF-3 binding site is located in close proximity to the IRS with a partial overlap of both structures. Therefore, HNF-3 has been discussed as a transcription factor mediating the insulin response on the PEPCK promoter. However, mutational analysis of the promoter provided evidence that proteins of the HNF-3 subclass are not involved in the insulin responsiveness of the PEPCK promoter.

The forkhead transcription factor DAF-16 has been identified by a genetic approach in Caenorhabditis elegans as an insulin-sensitive factor located downstream of the PI 3-kinase homolog AGE1 (52). In mammalian cells, the DAF-16 homologs Foxo1 (previously known as FKHR), Foxo3 (previously FKHRL1), and Foxo4 (previously AFX) have been found to be phosphorylated by PKB at three conserved serine or threonine residues within PKB consensus phosphorylation sites (RXRXXS/T), namely Thr24, Ser256, and Ser319 in human Foxo1. Phosphorylation in these three places results in transcriptional inactivation and nuclear export of the proteins by a 14-3-3-dependent mechanism (4, 8, 9, 27, 32, 55, 72). Electromobility shift assays provided evidence for Foxo1 and Foxo3 binding to the insulin-responsive sequences in the G-6-Pase promoter, and reporter gene analysis demonstrated transactivation of the PEPCK and G-6-Pase promoter in vitro (3, 28, 61). In addition, overexpression of Foxo1 markedly increased the expression of the endogenous G-6-Pase transcript in H4 rat hepatoma and LLC kidney epithelial cells (4, 46). The overexpression of dominant negative Foxo1 mutants that are not regulated by PKB impairs the suppression of G-6-Pase gene transcription by insulin in vitro (61). Furthermore, recent evidence obtained from gene knockout studies has linked Foxo1 to the control of glucose homeostasis and hepatic gluconeogenesis by insulin in vivo (45). Haploinsufficiency of Foxo1 was found to correlate with normalized blood glucose and insulin levels in an insulin-resistant mouse model. This is at least in part due to an improved insulin sensitivity, which leads to a decrease in G-6-Pase and PEPCK gene expression. In addition, the overexpression of a constitutively active Foxo1 mutant, which is resistant to inactivation by insulin, increased PEPCK and G-6-Pase expression and resulted in elevated fasting blood glucose concentrations (45).

Sterol response element-binding protein. Members of the sterol response element-binding protein (SREBP) family were initially characterized as transcription factors that become activated by low cholesterol levels in the cell (76). However, the SREBP-1c isoform in the liver has been demonstrated to be regulated by nutritional stimuli such as a carbohydrate-rich diet in vivo, and insulin has been found to rapidly stimulate the transcription of the SREBP-1c gene in hepatocytes and in adipose and muscle tissue (24). This effect of insulin on the expression level of SREBP-1c protein in hepatocytes appears to be mediated through activation of PI 3-kinase. In addition, studies with a conditionally regulatable version of PKB in hepatoma cells provided evidence that activation of PKB is sufficient to mimic this biological response of insulin (23). This suggests that the effect of insulin on SREBP-1c gene expression is mediated via activation of PKB. The precise mechanism and the transcription factors involved in the insulin-dependent expression of the SREBP-1c gene are still discussed and largely unknown. The adiponoviral-mediated overexpression of SREBP-1c leads to a downregulation of PEPCK gene expression in vivo (12) and in vitro (6) studies and decreases hyperglycemia in diabetic mice, whereas the expression of the G-6-Pase gene is not directly affected by SREBP-1c (24). However, transgenic mice overexpressing nuclear SREBP-1c in adipose tissue showed a lipodystrophic phenotype and a marked insulin resistance, with inappropriate gluconeogenesis and diabetes mellitus (63).

Liver X-activated receptor. The liver X-activated receptor (LXRα and LXRβ) belong to a nuclear receptor superfamily (21). Whereas LXRβ is expressed ubiquitously, LXRα is expressed in a tissue-specific manner, with highest levels of expression in liver, kidney, and intestine. LXRα form heterodimers with the retinoic acid receptor-α and bind to hormone response elements within their target genes. The endogenous activating ligands of LXRα are oxysterols, such as 24(S),25-epoxy-cholesterol, and the LXR/RXR dimers transactivate the expression of genes controlling the synthesis of lipids, bile acid formation, and cholesterol transport, such as fatty acid synthase, cholesterol 7α-hydroxylase, and ABCA1, respectively. However, synthetic agonists of LXR decrease blood glucose levels in rodent models of diabetes, suggesting that LXR may also be involved in physiological mechanisms of the homeostatic control of glucose metabolism (10). This decrease in blood glucose concentration after treatment with LXR agonists is at
least in part to a suppression of the genes for the key gluconeogenic enzymes PEPCK, FBPase, and G-6-Pase (10, 68). The fact that the LXR agonists failed to regulate these genes in LXR knockout animals suggests a participation of LXR in the transcriptional regulation of these genes (68). However, it is not clear whether the downregulation of the gluconeogenic enzymes by LXR agonists involves the direct binding of LXR/RXR heterodimers to the respective gene or is mediated by indirect mechanisms. For example, LXR is known to activate the expression of SREBP-1c, which has been reported to affect the expression of PEPCK (see above discussion). Because insulin induces the expression of LXR itself (21), it has also been speculated that LXR might contribute in part to the regulation of gluconeogenesis by this hormone (10, 68).

### Insulin-Regulatable Coactivator Proteins

**PGC-1 and CREBP.** G-6-Pase and PEPCK gene expression is regulated by the recruitment or modification not only of transcription factors, but also of coactivator proteins, such as peroxisome proliferator-activated receptor-γ coactivator-1 (PGC-1) (29, 82). The expression of PGC-1 by itself is induced by glucagon via cAMP. This leads to an increased expression of the genes encoding PEPCK and G-6-Pase, probably by the interaction of PGC-1 with HNF-4 and the glucocorticoid receptor. In addition, this also suggests that glucagon may stimulate gluconeogenesis via PGC-1. Furthermore, in several models of type 2 diabetes that are associated with increased gluconeogenesis, PGC-1 gene expression is also elevated. Although under these conditions the glucagon level is probably also elevated, this might be the first evidence that insulin is able to suppress the expression of gluconeogenic enzymes by inhibiting the action of PGC-1. In two very recent reports, the relationship between PKB, PGC-1, and Foxo1 has been elucidated. Daitoku et al. (19) found in HepG2 cells that PGC-1 promoter activity is induced by coexpression of Foxo1 via the insulin-responsive sequences within the PGC-1 promoter. Furthermore, coexpression of PKB was found to mimic the inhibitory effect of insulin on PGC-1 promoter activity. Puigserver et al. (54) provided evidence that PGC-1 serves as a critical coactivator, which directly interacts with Foxo1 and thereby increases the Foxo1-dependent transcriptional activation of the G-6-Pase and PEPCK promoters. On the basis of their data, they propose a model in which gluconeogenic gene expression is inhibited by insulin via a PKB-dependent disruption of this complex (54).

In addition to PGC-1, insulin might also target transcriptional coactivator proteins of the p300/CREBP family, which play a central role in the integration of cellular signaling processes (11). For example, evidence has been provided that p300 is phosphorylated by PKB at Ser1834 which inhibits the transactivation by CCAAT/enhancer-binding protein (C/EBP)β (27). In addition, C/EBPβ has been described to be involved in the control of PEPCK and G-6-Pase gene expression (2). However, it has not been demonstrated so far that p300 is a direct target of PKB in vivo or that the mutation of Ser1834 diminishes the insulin regulation of the PEPCK and G-6-Pase genes. In addition, the inhibitory effect of the overexpression of NF-κB on PEPCK gene expression has been attributed to a tethering of CBP by this transcription factor (74). Although NF-κB is activated by insulin in a PI 3-kinase-dependent manner, a direct participation of this transcription factor in the inhibition of PEPCK gene expression by insulin has not yet been shown. Furthermore, pp90RSK has been described to bind to CREBP after stimulation of the Ras pathway, thereby preventing CREB-mediated transcription (47). However, this mechanism is most likely not involved in the downregulation of cAMP-induced PEPCK expression by insulin, because this regulation is not affected either by the overexpression of dominant negative mutants or by pharmacological inhibitors of the Ras/MEK/ERK pathway (1, 25, 71).

### CONCLUSIONS

Over the recent years, considerable progress has been made in understanding the mechanisms and molecular details involved in the regulation of hepatic glucose production and its impairment in diabetes. A spectrum of different experimental approaches, ranging from genetic manipulations like knockout models and use of gain or loss of function-mutated constructs in cellular models to the development and application of selective pharmacological compounds, has provided evidence for the convergence of multiple signaling pathways in particular on the level of the transcriptional regulation of PEPCK and G-6-Pase gene expression, the key gluconeogenic genes in liver cells. Analogous to multiple braking systems in cars or motorbikes, the redundancy in the regulation of hepatic glucose production emphasizes the critical importance of this process in the glucose homeostasis of the organism. On the other hand, our increasing knowledge in this field has made it possible to identify a group of promising pharmacological targets, therefore providing a solid basis for the development of new and more potent anti-diabetic drugs.

We apologize to the many researchers in the field who were not cited because of constraints on the number of references.

### DISCLOSURES

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