Role of Insulin in the Regulation of Proprotein Convertase Subtilisin/Kexin Type 9

Ji Miao, Praveen V. Manthena, Mary E. Haas, Alisha V. Ling, Dong-Ju Shin, Mark J. Graham, Rosanne M. Crooke, Jingwen Liu, Sudha B. Biddinger

Objective—Proprotein convertase subtilisin/kexin type 9 (PCSK9), which binds the low-density lipoprotein receptor and targets it for degradation, has emerged as an important regulator of serum cholesterol levels and cardiovascular disease risk. Although much work is currently focused on developing therapies for inhibiting PCSK9, the endogenous regulation of PCSK9, particularly by insulin, remains unclear. The objective of these studies was to determine the effects of insulin on PCSK9 in vitro and in vivo.

Approach and Results—Using rat hepatoma cells and primary rat hepatocytes, we found that insulin increased PCSK9 expression and increased low-density lipoprotein receptor degradation in a PCSK9-dependent manner. In parallel, hepatic Pcsk9 mRNA and plasma PCSK9 protein levels were reduced by 55% to 75% in mice with liver-specific knockout of the insulin receptor; 75% to 88% in mice made insulin-deficient with streptozotocin; and 65% in ob/ob mice treated with antisense oligonucleotides against the insulin receptor. However, antisense oligonucleotide–mediated knockdown of insulin receptor in lean, wild-type mice had little effect. In addition, we found that fasting was able to reduce PCSK9 expression by 80% even in mice that lack hepatic insulin signaling.

Conclusions—Taken together, these data indicate that although insulin induces PCSK9 expression, it is not the sole or even dominant regulator of PCSK9 under all conditions. (Arterioscler Thromb Vasc Biol. 2015;35:1589-1596. DOI: 10.1161/ATVBHA.115.305688.)

Key Words: diabetes mellitus ■ insulin ■ insulin resistance ■ LDL receptor

Low-density lipoprotein (LDL) receptors in the liver mediate clearance of >70% of LDL from the serum.1 Consequently, the LDL receptor (LDLR) is a key determinant of serum cholesterol levels and cardiovascular disease risk. Proprotein convertase subtilisin/kexin type 9 (PCSK9) has emerged as an important regulator of the LDLR. PCSK9 is a secreted protein which binds to the extracellular domain of the LDLR and targets it for degradation.2,3 Consequently, individuals with gain of function mutations of PCSK9, particularly by insulin, remains unclear. The objective of these studies was to determine the effects of insulin on PCSK9 in vitro and in vivo.

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protein, in parallel with \textit{Ldlr} mRNA. At 24 to 30 hours, however, insulin suppressed LDLR protein, and the amount of LDLR protein in cells treated with insulin for 24 hours fell below those observed in untreated cells (Figure 1B). In contrast, protein levels of fatty acid synthase, as well as transferrin receptor, were increased by insulin treatment at 24 to 30 hours (Figure 1B). Thus, insulin has a biphasic effect on LDLR protein, inducing it at early time points, and suppressing it at later time points.

The fact that insulin reduced LDLR protein but not \textit{Ldlr} mRNA at later time points suggested that insulin might increase LDLR degradation. To test this, we measured the stability of LDLR protein by treating cells with cycloheximide, which blocks protein synthesis. LDLR protein levels fell more rapidly in the presence of insulin than in the absence of insulin (Figure 1C). Quantification of the immunoblots of 5 independent experiments revealed that insulin reduced the half-life of the LDLR by almost 33% (Figure 1A and IB in the online-only Data Supplement). In parallel, pulse-chase studies also

Materials and Methods
Materials and Methods are available in the online-only Data Supplement.

Results
As expected, insulin increased mRNA levels of \textit{Ldlr} in rat hepatoma cells.\textsuperscript{12} It also induced the lipogenic enzyme fatty acid synthase (\textit{Fasn}) and suppressed the gluconeogenic enzyme phosphoenolpyruvate carboxykinase (\textit{Pck1}; Figure 1). However, the effects on LDLR protein were more complex. Insulin treatment for 4 to 12 hours increased LDLR protein, inducing it at early time points, and suppressing it at later time points.

![Figure 1. Biphasic regulation of low-density lipoprotein receptor (LDLR) by insulin. Rat hepatoma cells (A–C) or primary rat hepatocytes (D and E) were treated with insulin. Gene expression was measured by real-time polymerase chain reaction (A and D) and protein levels were determined by immunoblotting whole cell lysates (B, C, and E). A and B, Rat hepatoma cells were placed in serum-free medium 30 hours before harvest, and insulin was added for the indicated amount of time before harvest. C and E, Cells were treated with cycloheximide (CHX) for the indicated amount of time and insulin for a total of 12 hours before harvest. Primary rat hepatocytes were serum starved overnight and treated with insulin for 6 hours before harvest (D) or treated with CHX for the indicated amount of time and insulin for a total of 12 hours before harvest (E). In B, C, and E, representative gel images are shown on the left and protein quantifications on the right. Data represent mean and SEM of 3 to 5 independent experiments. In A and D, *P<0.05 compared with nonstimulated controls. In B, * and # denote P<0.05 versus nonstimulated controls for LDLR, fatty acid synthase (FASN), and transferrin receptor (TFRC), respectively. In C and E, *P<0.05 for insulin treatment and #P<0.05 for CHX treatment. A.U. indicates arbitrary units; and PCK1, phosphoenolpyruvate carboxykinase.](Image)
showed that insulin increased the degradation of the LDLR (Figure IC and ID in the online-only Data Supplement).

Primary rat hepatocytes are the most robust model system currently available for studying insulin action in vitro, particularly on lipid-related genes. In these cells, insulin increased mRNA levels of Ldr by 2-fold and fatty acid synthase by 7-fold; in parallel, insulin suppressed Pck1 by 30-fold (Figure 1D). At the same time, insulin increased LDLR degradation: in the absence of insulin, 44% of LDLR protein remained in the presence of insulin, only 16% of LDLR protein remained after cycloheximide treatment (Figure 1E).

PCSK9 and inducible degrader of the LDLR (IDOL) promote the degradation of the LDLR. In rat hepatoma cells, Idol mRNA was not detectable in either the absence or presence of insulin (data not shown). However, insulin did induce Pcsk9 mRNA, cellular PCSK9, and secreted PCSK9 (Figure 2A). To determine whether PCSK9 is necessary for the decrease in LDLR protein observed with prolonged insulin treatment, cells were treated with either a control adenovirus or an adenovirus encoding a shRNA against PCSK9, and then incubated in the presence or absence of insulin for 24 hours (Figure 2B). The shPCSK9 adenovirus effectively decreased PCSK9 protein in the cells and the media, both in the presence and absence of insulin (Figure 2B). In the absence of insulin, knockdown of PCSK9 increased LDLR as expected. Importantly, the ability of insulin to decrease LDLR protein was blunted by knockdown of PCSK9 (Figure 2B). Knockdown of PCSK9 did not, however, alter expression of fatty acid synthase or transferrin receptor (Figure 2B).

In primary rat hepatocytes, insulin produced an even more robust increase in Pcsk9. Pcsk9 mRNA levels were increased 3-fold, and secreted PCSK9 protein was increased 3-fold (Figure 2C and 2D). The effects of insulin were mediated at the transcriptional level, as insulin induced the activity

Figure 2. Proprotein convertase subtilisin/kexin type 9 (PCSK9) mediates insulin induced degradation of the low-density lipoprotein receptor (LDLR). Rat hepatoma cells (A and B) or primary rat hepatocytes (C and D) were treated with insulin. Gene expression was measured by real-time polymerase chain reaction (A and C) and protein levels were determined by immunoblotting whole cell lysates or medium (A, B, and D). A, Rat hepatoma cells were placed in serum-free medium 30 hours before harvest, and insulin was added for the indicated amount of time before harvest. B, Rat hepatoma cells were infected with adenovirus expressing a control shRNA (shControl) or an shRNA against PCSK9 (shPCSK9) and then treated with insulin for 24 hours. C and D, Primary rat hepatocytes were serum starved overnight and then stimulated with insulin for 6 hours (C) or 24 hours (D). D, PCSK9 protein in the medium was measured by immunoblotting; silver staining of the upper portion of the gel shows that similar amounts of protein were loaded. In A, B, and D, representative gel images and protein quantifications are shown. Data represent mean and SEM of 3 to 6 independent experiments. In A, *, #, and & denote P<0.05 versus nonstimulated controls for Pcsk9 mRNA levels, PCSK9 levels in cell, and medium, respectively. In B, *P<0.05 for the effects of insulin and #P<0.05 for the effects of Ad-shPCSK9. In C and D, *P<0.05 versus nonstimulated controls. A.U. indicates arbitrary units; FASN, fatty acid synthase; and TRFC, transferrin receptor.
of a PCSK9 promoter luciferase construct by 2-fold (Figure IIB in the online-only Data Supplement) and the ability of insulin to induce Pcsk9 mRNA in primary rat hepatocytes was entirely abolished by treatment with actinomycin D, which interferes with transcription (Figure IIA in the online-only Data Supplement). Moreover, insulin induction of the PCSK9 promoter was blocked by inhibition of the phosphoinositide 3-kinase signaling pathway, which mediates many of insulin’s metabolic effects (Figure IIC in the online-only Data Supplement); it was also blocked by mutation of the sterol response element and hepatocyte nuclear factor 1 (HNF1) binding sites, but not the Sp1 site (Figure IID in the online-only Data Supplement). We therefore examined expression of sterol regulatory element-binding protein-1a (Srebp-1a), Srebp-2, and Srebp-1c, as well as HNF-1α and HNF-1β, in response to insulin. In rat hepatoma cells, only Srebp-2 was increased at the mRNA level (Figure IIE in the online-only Data Supplement). In primary rat hepatocytes, Srebp-1c was increased 12-fold and Srebp-1a was increased 2-fold, whereas Srebp-2 and the HNFs were not changed (Figure II F in the online-only Data Supplement). Collectively, these data support the notion that insulin induces Pcsk9 transcription via the SREBPs, but do not rule out a role for other factors.

To determine the effects of insulin on PCSK9 in vivo, we used liver insulin receptor knockout (LIRKO) mice and their controls. LIRKO hepatocytes lack insulin receptors making them incapable of insulin signaling. Consequently, LIRKO mice are hyperglycemic and hyperinsulinemic. We have previously shown that LIRKO mice show decreased levels of SREBP-1, SREBP-2, and the SREBP target genes. Moreover, on an atherogenic Paigen diet (15% dairy fat, 1% cholesterol, 0.5% cholic acid), LDLR protein levels are markedly decreased.

We therefore measured PCSK9 and LDLR in LIRKO and control mice fed either a chow or atherogenic Paigen diet for 4 weeks (Figure 3). On the chow diet, LIRKO mice showed decreased levels of plasma PCSK9, decreased Pcsk9, and Ldlr mRNA levels, and normal LDLR protein levels (Figure 3A–C). These data suggest that insulin increases both LDLR synthesis and degradation, and is consistent with our in vitro data showing increased Pcsk9 and Ldlr mRNA in the presence of insulin.

The Paigen diet reduced Pcsk9 and Ldlr mRNA in both control and LIRKO mice. However, control mice maintained normal levels of LDLR protein on the Paigen diet (Figure 3E). LIRKO mice, however, showed a marked reduction in LDLR protein and developed severe hypercholesterolemia on the Paigen diet (Figure 3E and 3F). Why LDLR protein was decreased in the Paigen-fed LIRKO mice is still under investigation, but may be because of decreased Ldlr mRNA and increased Idol expression (Figure 3C and 3D).

Pcsk9 has also been previously shown to be decreased by fasting. To determine whether the effects of fasting on Pcsk9 were mediated by insulin, we subjected control and LIRKO mice to a 24-hour fast. Interestingly, fasting reduced Pcsk9 mRNA by 95% in control mice, and 80% to 90% in LIRKO mice (Figure 4A). Similar effects were observed on Ldlr mRNA, but the effects were more modest (Figure 4A). Conversely, refeeding (ie, feeding mice a high carbohydrate diet after a 24-hour fast) increased Pcsk9 mRNA by almost 60-fold in control mice, but only 30-fold in LIRKO mice (Figure 4B). In parallel, refeeding induced Ldlr 4-fold in control mice, but only 2-fold in LIRKO mice (Figure 4B). Idol mRNA, however, was again slightly higher in LIRKO mice, but unchanged by fasting or refeeding (Figure 4A and 4B). Interestingly, LDLR protein was similar in control

![Figure 3](http://www.ahajournals.org)
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One such factor could Pcsk9 mice indicates that other factors besides hepatic insulin action are important in the regulation of Pcsk9. One such factor could potentially be glucagon, which has previously been shown to suppress hepatic Pcsk9 mRNA in vivo in rats. Indeed, glucagon suppressed PCSK9 mRNA and protein levels by 50% in primary rat hepatocytes (Figure IIIA and IIC in the online-only Data Supplement). Glucagon also suppressed mRNA levels of Srebp-1c and Srebp-2 by 20% to 50%, whereas it increased expression of Pck1 9-fold (Figure IIIA and IIB in the online-only Data Supplement). More importantly, although glucagon decreased Ldlr mRNA by 20%, it increased LDLR protein by 2-fold (Figure IIIA and IIID in the online-only Data Supplement).

We also examined the effects of insulin in the context of insulin deficiency and selective insulin resistance. Selective insulin resistance is a key feature of type 2 diabetes mellitus, in which not all signaling pathways seem to become resistant to insulin—some seem to remain sensitive to insulin and are driven to excess by the hyperinsulinemia which coevolves with insulin resistance.14 As a model of insulin deficiency, we chose streptozotocin-treated mice, as streptozotocin destroys the β-cells of the pancreas. As a model of selective insulin resistance, we chose leptin-deficient, obese ob/ob mice. To compare directly the effects of insulin deficiency and selective insulin resistance, we studied in parallel lean, wild-type mice treated with vehicle (WT); lean, wild-type mice treated with streptozotocin (STZ); and ob/ob mice treated with vehicle (ob/ob). Prior studies have reported both STZ and ob/ob mice to be hyperglycemic and hyperglucagonemic, although STZ mice are hypoinsulinemic and lean or underweight and ob/ob mice are hyperinsulinemic and obese21 (see also Table II in the online-only Data Supplement).

Liver gene expression analysis by real-time polymerase chain reaction revealed that Pcsk9 was markedly decreased in streptozotocin-treated mice but not ob/ob mice (Table). In parallel, the SREBP transcription factors which drive Pcsk9, Srebp-1c,9 and Srebp-2,22 and their other targets, fatty acid synthase, stearoyl-CoA desaturase 1 (Scd1), 3-hydroxy-3-methylglutaryl-CoA reductase (Hmgcr), and farnesyl diphosphate synthase (Fdp1), were decreased by 44% to 98% in streptozotocin-treated mice. In ob/ob mice, however, these genes were generally unchanged, or in the case of Srebp-1c, Fasn, and Scd1, increased, consistent with prior studies.11,23 The changes in fatty acid synthase and stearoyl-CoA desaturase 1 were also evident at the protein level because they were markedly induced in ob/ob livers (Figure 5A). In contrast, both streptozotocin-treated mice and ob/ob mice showed increased expression of the gluconeogenic genes, glucose-6-phosphatase (G6pc), Pck1, and peroxisome proliferator-activated receptor gamma coactivator 1α (Ppargc1α) (Table), and were
Table. Effects of Diabetes on Hepatic Gene Expression

<table>
<thead>
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<th></th>
<th>WT</th>
<th>STZ</th>
<th>ob/ob</th>
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<tr>
<td><strong>Pcsk9</strong></td>
<td>1.00±0.07</td>
<td>0.12±0.03*</td>
<td>0.91±0.12†</td>
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<td><strong>Ldlr</strong></td>
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<td>0.49±0.06*</td>
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<td>0.12±0.03*</td>
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<td><strong>Fasn</strong></td>
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<td>0.21±0.03*</td>
<td>7.90±2.15†</td>
</tr>
<tr>
<td><strong>Scd1</strong></td>
<td>1.00±0.15</td>
<td>0.02±0.01*</td>
<td>17.56±3.83†</td>
</tr>
<tr>
<td><strong>Srebpl-2</strong></td>
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<tr>
<td><strong>Hmgcr</strong></td>
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<td><strong>Fdps</strong></td>
<td>1.00±0.09</td>
<td>0.18±0.06*</td>
<td>0.74±0.15†</td>
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<tr>
<td><strong>Idol</strong></td>
<td>1.00±0.09</td>
<td>0.74±0.05</td>
<td>1.27±0.17†</td>
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<td><strong>G6pc</strong></td>
<td>1.00±0.21</td>
<td>2.31±0.24*</td>
<td>4.47±0.35†</td>
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<td><strong>Pck1</strong></td>
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<td>1.61±0.14</td>
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<tr>
<td><strong>Ppargc1α</strong></td>
<td>1.00±0.08</td>
<td>6.60±0.34*</td>
<td>2.15±0.37†</td>
</tr>
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</table>

Vehicle-treated ob/ob mice (ob/ob) and their lean, wild-type controls treated with either vehicle (WT) or streptozotocin (STZ) were euthanized in the nonfasted state. Hepatic gene expression was measured by real-time polymerase chain reaction (n=5 per group). Fdps indicates farnesyl diphosphate synthase; Fasn, fatty acid synthase; G6pc, glucose-6-phosphatase; Hmgcr, 3-hydroxy-3-methylglutaryl-CoA reductase; Idol, inducible degader of the LDLR; Ldlr, low-density lipoprotein receptor; Pck1, phosphoenolpyruvate carboxykinase; Pcsk9, proprotein convertase subtilisin/kexin type 9; Ppargc1α, peroxisome proliferator-activated receptor gamma coactivator 1α; Scd1, stearoyl-CoA desaturase 1; Srebpl-1c, sterol regulatory element binding protein 1c; and Srebpl-2, sterol regulatory element binding protein-2.

*P<0.05 compared with WT mice.
†P<0.05 compared with STZ mice.

hyperglycemic (Table II in the online-only Data Supplement). Idol mRNA was slightly elevated in ob/ob livers (Table).

Plasma PCSK9 levels mirrored liver Pcsk9 mRNA levels (Figure 5B), with STZ mice showing a 70% reduction in plasma PCSK9 levels. However, both STZ and ob/ob mice showed increased LDL cholesterol, despite a tendency toward elevated LDLR protein (Figure 5A and 5C), likely because of increased secretion of very-low-density lipoprotein (VLDL), the precursor of LDL.24

To directly test the role of insulin signaling in the regulation of PCSK9 in ob/ob mice, we knocked down the insulin receptor using antisense oligonucleotides (ASO). Thus, ob/ob mice and their wild-type controls were injected weekly for 4 weeks with either a control ASO or an ASO against the insulin receptor. Such treatment would abolish the ability of insulin to act on the hepatocyte.

We have previously shown that antisense-mediated knockdown of the insulin receptor worsens hyperglycemia, but decreases levels of Srebpl-1c, its lipogenic targets, and hepatic steatosis in ob/ob mice.25 Consistent with this, treatment with ASO against the insulin receptor markedly decreased insulin receptor protein levels in the livers of both wild-type and ob/ob mice (Figure 5D).

Similar to farnesyl synthase protein, which was increased in the livers of ob/ob mice, was decreased by knockdown of the insulin receptor in mice of both genotypes (Figure 5D).

Knockdown of the insulin receptor decreased both Pcsk9 mRNA (Figure 5E) and plasma PCSK9 in ob/ob mice (Figure 5F), indicating that insulin promotes the expression of Pcsk9 in ob/ob mice. However, knockdown of the insulin receptor did not significantly alter Pcsk9 in lean mice. Knockdown of the insulin receptor also decreased Ldlr and Idol mRNA levels in ob/ob but not lean mice (Figure 5G and 5H); nonetheless, LDLR protein levels were not markedly different between groups (Figure 5D).

Discussion

PCSK9 has emerged as an important regulator of the LDLR and a novel therapeutic target. Here, we show that insulin promotes the degradation of the LDLR in vitro in a PCSK9-dependent manner. However, in vivo, decreased levels of PCSK9 in insulin-deficient states are generally not associated with an increase in LDLR protein; indeed, in LIRKO mice refed a carbohydrate diet for 6 to 12 hours, or LIRKO mice fed a Paigen diet, LDLR protein is decreased. These data point to the fact that insulin regulation of LDLR is complex, and suggest that in vivo, insulin may act through PCSK9-independent mechanisms to increase LDLR protein expression.

Our data in vivo support the notion that insulin can directly induce PCSK9 expression. Thus, LIRKO mice, ob/ob mice treated with ASO against the insulin receptor, mice treated with streptozotocin, and fasted mice all show decreased levels of Pcsk9. However, our data also point to the fact that insulin is not always a major regulator of PCSK9 in vivo. First, LIRKO mice, despite their inability to respond to insulin, still show an 80% reduction of PCSK9 on fasting. Consequently, in the fasted state, the effects of insulin receptor knockout on Pcsk9 expression are abolished. Second, in ob/ob mice, insulin clearly promotes the expression of PCSK9, as knockdown of the insulin receptor in these mice decreases PCSK9 expression. However, given that ob/ob mice are markedly hyperinsulinemic relative to their lean controls, it might be expected that PCSK9 levels would be supranormal. That they are not suggests that the effects of hyperinsulinemia are balanced by some other factor in the diabetic state which suppresses PCSK9. Finally, and perhaps most importantly, the acute knockout of the insulin receptor in lean, wild-type mice by ASO does not significantly decrease PCSK9 levels. Similarly, adeno viral-mediated delivery of Cre recombinase into mice carrying a floxed allele of the insulin receptor knockout on Pcsk9 expression are abolished. Second, in ob/ob mice, insulin clearly promotes the expression of PCSK9, as knockdown of the insulin receptor in these mice decreases PCSK9 expression. However, given that ob/ob mice are markedly hyperinsulinemic relative to their lean controls, it might be expected that PCSK9 levels would be supranormal. That they are not suggests that the effects of hyperinsulinemia are balanced by some other factor in the diabetic state which suppresses PCSK9. Finally, and perhaps most importantly, the acute knockout of the insulin receptor in lean, wild-type mice by ASO does not significantly decrease PCSK9 levels. Similarly, adenoviral-mediated delivery of Cre recombinase into mice carrying a floxed allele of the insulin receptor did not consistently decrease PCSK9 expression (data not shown). Why knockout of the insulin receptor in wild-type mice has little effect on Pcsk9, whereas LIRKO mice show markedly reduced Pcsk9 is not clear. However, LIRKO mice have chronic insulin resistance, starting in the perinatal period, which leads to peripheral insulin resistance.26 This peripheral insulin resistance may be necessary to unmask the effects of hepatic insulin signaling on the liver, perhaps by altering glucon signaling or whole body cholesterol homeostasis.

Our data showing that insulin promotes PCSK9 expression is also consistent with prior reports showing decreased Pcsk9 by fasting0,20 and increased Pcsk9 in mice subjected to a hyperinsulinemic euglycemic clamp.9,11 However, studies by Ai et al10 reported a 2-fold increase in Pcsk9 mRNA on knockdown of the insulin receptor in mice using an adenovirus encoding a shRNA. One important difference in the experimental design of this study is that Ai et al studied mice that had been fasted for 5 hours before euthanize. As shown in Figure 4B, a 24-hour fast abolishes the effects of insulin receptor knockout on Pcsk9. Indeed, even a fast of only 5 hours is sufficient to reduce Pcsk9 levels by 60% in wild-type mice (Figure IV in the online-only Data Supplement). This is
not surprising given the facts that nuclear levels of SREBP-1 and SREBP-2 are markedly decreased by 6 hours of fasting and that mice lacking both SREBP-1 and SREBP-2, because of knockout of SREBP cleavage-activating protein (Scap), show markedly reduced Pcsk9 levels. Thus, it is likely that in the studies by Ai et al, fasting abrogated the effects of insulin, unmasking the effects of other factors on Pcsk9. One such factor could be glucagon. Despite its hyperglycemic effects, glucagon has beneficial effects on the LDLR. Interestingly, it was shown 20 years ago that glucagon increases LDLR protein, but not mRNA levels in rats. Our data show that glucagon suppresses Pcsk9 expression in hepatocytes (Figure IIIA and IIIC in the online-only Data Supplement). Thus, fasting suppresses PCSK9 in 2 ways: it lowers insulin and raises glucagon. The increase in glucagon could account for the ability of fasting to decrease Pcsk9 mRNA even in the livers of LIRKO mice, which are unable to respond to insulin. Similarly, increased glucagon levels in ob/ob mice could potentially mitigate the effects of hyperinsulinemia on Pcsk9 in the liver.

Studies in humans show that PCSK9 levels are normal or increased in obesity/type 2 diabetes Mellitus. As diabetic patients are at high risk for cardiovascular disease, they are important candidates for PCSK9 therapy. Our data indicate that although insulin promotes the expression of PCSK9, other factors may play a dominant role. Defining these other factors and understanding how they interact with insulin in the control of PCSK9, the LDLR and cardiovascular disease risk will be important to developing better treatments for diabetic patients.

Figure 5. Proprotein convertase subtilisin/kexin type 9 (PCSK9) expression is decreased in mice with insulin deficiency, but not selective insulin resistance. Eight- to 12-week-old male mice were euthanized in the nonfasted state (n=4–6 mice per group). A–C, Vehicle-treated ob/ob mice (ob/ob) and lean, wild-type controls treated with either vehicle (WT) or streptozotocin (STZ) were studied in parallel. Protein levels were measured by immunoblotting (A), plasma PCSK9 levels were measured by ELISA (B), and low-density lipoprotein (LDL) cholesterol levels were measured by colorimetric assays (C). *P<0.05 compared with lean mice treated with vehicle and &P<0.05 compared with STZ-treated group. D–H, ob/ob mice (ob/ob) and their lean, wild-type controls (WT) were treated with control antisense oligonucleotides (ASO) or ASO against the insulin receptor (INSR) for 4 weeks. Protein levels were measured by immunoblotting liver extracts (D), hepatic gene expression was measured by real-time polymerase chain reaction (E, G, and H), and plasma PCSK9 levels were measured by ELISA (F). *P<0.05 compared with control ASO-treated mice and #P<0.05 compared with WT treated with the same ASO. In A and D, representative gel images (left) and protein quantifications (right) are shown. Data present the mean and SEM; control mice treated with vehicle or control mice treated with control ASO were set to 1. A.U. indicates arbitrary units; FASN, fatty acid synthase; IDOL, inducible degrader of the LDLR; LDLR, low-density lipoprotein receptor; and SCD1, stearoyl-CoA desaturase 1.
Acknowledgments
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Disclosures
None.

References
1. Dietschy JM, Turley SD, Spady DK. Role of liver in the maintenance of cholesterol and low density lipoprotein homeostasis in different animal species, including humans. J Lipid Res. 1993;34:1637–1659.

Significance
Proprotein convertase subtilisin/kexin type 9 is a promising therapeutic target. Here, we show that insulin induces proprotein convertase subtilisin/kexin type 9, although other hormones and factors are clearly involved. Dissecting the endogenous regulators of proprotein convertase subtilisin/kexin type 9 is an important step toward the rational utilization of proprotein convertase subtilisin/kexin type 9 inhibitors in patients with type 1 diabetes mellitus, type 2 diabetes mellitus, and obesity.
Role of Insulin in the Regulation of Proprotein Convertase Subtilisin/Kexin Type 9
Ji Miao, Praveen V. Manthena, Mary E. Haas, Alisha V. Ling, Dong-Ju Shin, Mark J. Graham, Rosanne M. Crooke, Jingwen Liu and Sudha B. Biddinger

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Supplemental Figure I. *Insulin shortens LDLR half-life in rat hepatoma cells.*

(A, B) Rat hepatoma cells were treated with cycloheximide in the presence or absence of insulin, and subjected to immunoblotting as described in Fig. 1C. (A) Immunoblots were quantified, and LDLR protein decay curves were plotted from five independent experiments. * p < 0.05 compared to non-stimulated controls at the same time point. (B) LDLR half-life was calculated as described in Supplementary Methods. (C, D) Rat hepatoma cells were infected with adenovirus expressing flag-tagged LDLR, and subjected to [35S]-methionine and cysteine pulse labeling for 30 minutes (0 time point), and chased for two to six hours, in the absence of insulin, or in the presence of insulin. Insulin treatment was initiated 18 hours before the pulse and continued until harvest. Autoradiography image is shown (C); after quantification, the amount of mature LDLR (expressed as a percentage of the total LDLR present at t=0) was calculated for this image and plotted (D); similar results were obtained in two additional experiments.
Supplemental Figure II

A. Pcsk9

- mRNA (A.U.)
- Insulin: □ Vehicle, ■ Actinomycin D

B. Pcsk9-luc

- Relative Luciferase Activity
- Insulin: □ - Insulin, ■ +Insulin

C. Pcsk9-luc

- Relative Luciferase Activity
- Vehicle, LY294002

D. Pcsk9-luc

- Relative Luciferase Activity
- WT, Sp1, SRE, HNF1
Supplemental Figure II

**E**

- **Srebp-1c**
  - mRNA (A.U.) vs. Insulin (h) graph
  - Data points showing a decrease in mRNA levels over time.

- **Srebp-1α**
  - mRNA (A.U.) vs. Insulin (h) graph
  - Data points showing an increase in mRNA levels over time.

- **Srebp-2**
  - mRNA (A.U.) vs. Insulin (h) graph
  - Data points showing a peak at 10 hours and a decrease thereafter.

- **Hmgcr**
  - mRNA (A.U.) vs. Insulin (h) graph
  - Data points showing a steady increase in mRNA levels.

- **Hnf1α**
  - mRNA (A.U.) vs. Insulin (h) graph
  - Data points showing a decrease in mRNA levels.

- **Hnf1β**
  - mRNA (A.U.) vs. Insulin (h) graph
  - Data points showing a decrease in mRNA levels.

**F**

- **Graph**
  - Comparison of mRNA levels in different conditions:
    - None
    - Insulin
  - Data represented with error bars indicating variability.
  - Significant differences marked with asterisks (p < 0.05).
Supplemental Figure II. Insulin increases Pcsk9 transcription in cells.

(A) Primary rat hepatocytes were treated with actinomycin D, in the presence or absence of insulin. Gene expression was measured by real time PCR. (B to D) Luciferase reporters driven by wildtype (WT) or mutant human PCSK9 promoters were transfected into rat hepatoma cells (B) or HepG2 human hepatocellular carcinoma cells (C, D); a β-galactosidase reporter was used to normalize for transfection efficiency. Cells were treated with or without insulin for 28 hours. In (C) cells were treated with a phosphoinositide 3-kinase inhibitor, LY294002, in addition to insulin. *p < 0.05 compared to non-stimulated controls. (D) Mutations in the Sp1, SRE and HNF1 binding sites of the PCSK9 promoter were studied. (E) Rat hepatoma cells were treated with insulin for the indicated times and gene expression was measured by real time PCR, as described in Figure 1. (F) Primary rat hepatocytes were serum starved overnight and treated with insulin for six hours; gene expression was measured by real time PCR. Data represent mean and the s.e.m. of technical replicates and are representative of three to four independent experiments.
**Supplemental Figure III.** *Glucagon decreases PCSK9 expression in hepatocytes.*

Primary rat hepatocytes were serum starved overnight and treated with glucagon for the indicated times. Gene expression (A, B) was determined by real time PCR and protein levels were determined by immunoblotting (C, D). *p < 0.05 compared to non-stimulated controls. In C and D, representative gel images are shown on the left and protein quantifications are shown on the right.
Supplemental Figure IV. Regulation of Pcsk9 and Ldlr.

Eight to twelve week old male C57BL/6J mice were sacrificed in the non-fasted (NF) state or after a five-hour fast (Fasted). Hepatic gene expression (A) (n= 6 per group) and protein levels (B) were determined by real time PCR and immunoblotting, respectively. *p < 0.05 compared to non-fasted mice. In B, representative gel images are shown on the left and protein quantifications are shown on the right.
### Supplemental Table I. Real time PCR primer sequences.

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<th>Sequence</th>
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<td>Mouse <em>Ldlr</em>-F</td>
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<tr>
<td>Mouse <em>Ldlr</em>-R</td>
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Supplemental Table II. Effects of diabetes on glucose, body and liver weight.

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<th>STZ</th>
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<tr>
<td>Glucose (mg/dL)</td>
<td>138.0 ± 9.0</td>
<td>489.4 ± 36.2 *</td>
<td>435.0 ± 66.9 * &amp;</td>
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<tr>
<td>Body weight (g)</td>
<td>27.0 ± 0.8</td>
<td>28.5 ± 0.4</td>
<td>45.4 ± 1.6 * &amp;</td>
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<tr>
<td>Liver weight (g)</td>
<td>1.088 ± 0.084</td>
<td>1.275 ± 0.001</td>
<td>3.028 ± 0.447 * &amp;</td>
</tr>
<tr>
<td>Insulin (ng/mL)</td>
<td>0.457 ± 0.091</td>
<td>0.0197 ± 0.040 *</td>
<td>18.321 ± 8.639</td>
</tr>
</tbody>
</table>

Vehicle treated ob/ob mice (ob/ob) and their lean, wildtype controls treated with either vehicle (WT) or streptozotocin (STZ) were sacrificed in the non-fasted state (n= 5 per group). *P < 0.05 compared to lean mice treated with vehicle, & P < 0.05 compared to STZ treated group.
Materials and Methods

Reagents

Cycloheximide, human insulin, dexamethasone, triiodothyronine, glucagon and actinomycin D were purchased from Sigma-Aldrich. RPMI-1640, William’s E media, penicillin-streptomycin, glutamine, fetal bovine serum, Lipofectamine 2000, Trizol reagent and silver staining reagent (SilverQuest) were purchased from Life Technologies. Protease inhibitors were purchased from Roche Applied Science (complete protease inhibitor tablets). Streptozotocin was purchased from Fisher Scientific. X-tremeGENE HP was purchased from Roche Applied Science and a luciferase assay kit (E1501) was purchased from Promega. LY294002 was purchased from Calbiochem.

Animal, Diets and Treatments

Animals were housed in a 12-hour light/dark cycle (7 a.m. to 7 p.m). They received standard chow and water ad libitum, unless otherwise indicated. Mice studied were male and sacrificed at eight to ten weeks of age, in the non-fasted state, at 2 p.m., unless otherwise indicated. All procedures were approved by the Institutional Animal Care and Research Advisory Committee at Boston Children's Hospital.

LIRKO (Cre+/−, IRlox/lox) mice and their littermate controls (Cre−/−, IRlox/lox) were generated as previously described. LIRKO mice were maintained on a mixed genetic background including 129/sv, C57BL/6, FVB and DBA. For fasting studies, mice were sacrificed in the ad libitum fed state or after a 24-hour fast. For the re-feeding time course experiment, mice were fasted for 24 hours, and then re-fed a high carbohydrate diet (TD88122) for various amounts of time. For the five-hour fasting experiments, C57BL/6J mice were fasted at 9 a.m. and sacrificed at 2 p.m. Non-fasted control mice were also sacrificed at 2 p.m.
In the Paigen diet study, mice were fed a chow or Paigen diet (TD09237, Harlan Teklad) for four weeks and sacrificed in the ad libitum fed state.

Ob/ob mice and their lean, wildtype C57BL/6J controls were purchased from Jackson Laboratories. They were treated with streptozotocin (180 mg/kg body weight) or vehicle (0.1 M citric acid, pH = 4.2) by intraperitoneal injection and sacrificed seven days later. Alternatively, mice were administered chemically modified anti-sense oligonucleotides (ASO, 50 mg/kg body weight) by intraperitoneal injection weekly for four weeks and sacrificed one day after the final dose. Control ASO (ISIS-141923, 5’-CCTTCCCTGAAGGTTCCCTCC-3’) and ASO against the insulin receptor (ISIS-401145 5’-GTGTTCATCATAGGTCCGT-3’) were diluted in normal saline before injection.

Construction of Plasmids and Adenoviral Vectors

Adenoviruses expressing control shRNA against human lamin (CTGGACTTCCAGAAGAACA) or shRNA against rat PCSK9 (GCCTGGAGTTTTATCGGAAGA) were constructed using the Block-it U6 adenoviral RNAi system (Life Technologies) followed by viral packaging and multiple rounds of amplification in 293A cells (Life Technologies). The resulting viruses were purified by cesium chloride gradient ultracentrifugation as previously described.

In vitro Studies

FAO cells

All cells were maintained at 37°C in a 5% CO2 mammalian cell culture incubator. Rat hepatoma cells (Fao cells, ATCC) were maintained in RPMI-1640 media containing penicillin-streptomycin and 10% fetal bovine serum (complete media). For insulin stimulation experiments, cells were grown in complete media until 90% confluent. 30 hours prior to collecting the cells, the complete medium was replaced with serum free medium (RPMI-1640 media containing penicillin-streptomycin). Insulin (100 nM) was
added to the cells at 30, 24, 16, 12, 8, or 4 hours prior to harvest, or not added at all (0 time point). The amount of time the cells remained in culture was therefore held constant. For adenoviral infection, cells were infected with adenovirus expressing shPCSK9 or shControl (shLamin) in RPMI-1640 containing penicillin-streptomycin and 10% fetal bovine serum for twelve hours, serum starved for twelve hours in RPMI-1640 containing penicillin-streptomycin, and then treated with or without 100 nM insulin for an additional 24 hours. For half-life measurements, cells were serum starved overnight. The next day, insulin (100 nM) was not added, or added twelve hours prior to harvest; cycloheximide (50 µg/ml) was not added, or added 2.5 or 5 hours prior to harvest.

**HepG2 cells**

HepG2 cells (ATCC) were grown in Dulbecco's modified Eagle's media containing penicillin-streptomycin and 10% fetal bovine serum. For transient transfection, cells were co-transfected with 250 ng of plasmids encoding either the wildtype or mutant PCSK9 promoter and 250 ng of pSV-β-galactosidase, for normalization. Transfection was performed using lipofectamine 2000 (Fao rat hepatoma cells) or X-tremeGENE HP (HepG2 cells) according to the manufacturers’ protocols. One day after transfection, the media was replaced with fresh media containing penicillin-streptomycin and 10% fetal bovine serum. Two days after transfection, the media was replaced with media containing penicillin-streptomycin, with or without 100 nM insulin and incubated for an additional 28 hours. Luciferase reporter assays were performed as previously described. Where indicated, cells were treated with 50 µM LY294002 for 30 minutes prior to and during insulin treatment.

**Primary Hepatocytes**

Primary hepatocytes were isolated from eight week old male Sprague-Dawley rats (Harlan) and treated as previously described with minor modifications. After isolation, cells were suspended in William’s E media containing penicillin-streptomycin, 100 nM glutamine and 10% fetal bovine serum; 1 X 10^6
cells were placed on rat tail collagen I (BD Biosciences) coated 6-well plates. Four hours later, cells were washed twice with PBS and incubated in William’s E media containing penicillin-streptomycin, 100 nM glutamine, 100 nM dexamethasone, 100 nM triiodothyronine and 1 nM insulin (fasting media) overnight. For insulin stimulation, cells were washed twice with PBS after overnight incubation in fasting media, and incubated in William’s E media containing penicillin-streptomycin, 100 nM glutamine, 100 nM dexamethasone and 100 nM triiodothyronine. 100 nM insulin was added for the indicated amount of times. For cycloheximide treatment, insulin (100 nM) was not added, or added twelve hours prior to harvest; cycloheximide (50 µg/ml) was not added, or added 4 hours prior to harvest. For actinomycin D treatment, cells were serum starved overnight. After pre-treatment with 0.5 µM actinomycin D for one hour, cells were treated with or without insulin for six hours. For glucagon stimulation, cells were starved overnight in fasting media. The next day, the fasting media was replaced with William’s E media containing penicillin-streptomycin, 100 nM glutamine, 100 nM dexamethasone; 100 nM glucagon was either not added or added 24 or 6 hours prior to harvesting the cells.

**Cycloheximide Treatment and LDLR Half-life Calculation**

To determine the half-life of LDLR, cycloheximide treated cells were immunoblotted for LDLR, and quantified using ImageJ (NIH). A serial dilution was included on each gel and used to determine the relative expression of LDLR at each time point and to confirm that all samples were within the linear range of the assay. For each experiment, the amount of LDLR at t = 0 was set to 100. The mean LDLR expression was calculated, plotted against time, and fitted to an exponential decay curve using Microsoft Excel, such that N(t)=N₀e⁻λt. The half-life (t_{1/2}) was calculated by the formula t_{1/2} = ln (2)/λ.

**Pulse-Chase Studies**

Pulse-chase experiments were carried out as previously described with some modifications. Fao cells were infected with Ad-C-terminal-Flag-LDLR. Twenty four hours later, cells were treated with vehicle
or insulin (100 nM) in serum free media for eighteen hours before pulse and chase; for insulin treated
cells, insulin was present throughout the pulse and chase as well. Cells then were then incubated in
methionine and cysteine-free medium for fifteen minutes followed by a pulse using EasyTag L-^{35}S-
methionine and L-^{35}S-cysteine (Perkin Elmer) for 30 minutes. For the chase, cells were washed four
times with PBS to thoroughly remove ^{35}S-labeled amino acids, and cultured in medium containing 10×
unlabeled methionine and cysteine for the indicated times. Cells then were collected and Flag-LDLR in
whole cell lysates was immunoprecipitated with a Flag antibody (Sigma) in RIPA buffer (50 mM Tris at
pH 7.5, 1 mM EDTA, 150 mM sodium chloride, 0.5% NP40, 0.1% sodium deoxycholate); radioactive
Flag-LDLR was detected by SDS-PAGE followed by autoradiography.

RNA Isolation and Gene Expression Analysis
Total RNA from cultured cells and liver tissues were isolated using Trizol reagent or an RNeasy kit
(Qiagen). Reverse transcription was performed according to the manufacturer’s protocol (Applied
Biosystems). The resulting cDNA was diluted ten-fold and used for real-time PCR analysis with SYBR
green (Thermo) in an Applied Biosystems 7900 HT or 7000 instrument. Results were normalized to the
house keeping genes, 36b4 (in vitro studies) or Tbp (in vivo studies). The value of the control group was
set to 1. Primer sequences are listed in Supplementary Table I. Gene expression studies were performed
with triplicate wells for all in vitro experiments.

Protein Isolation and Immunoblot Analysis
Whole cell lysates were prepared by collecting cells in lysis buffer A (50 mM Tris pH7.5, 150 mM
NaCl, 1 mM EDTA, 1% NP40, 0.5% sodium deoxycholate, 1.0% SDS, 2 mM NaF, 2 mM Na3VO4, and
supplemented with protease inhibitors) followed by sonication and centrifugation at 13,000 g for 10
minutes. Alternatively, 30 to 50 mg of liver was homogenized in lysis buffer B (50 mM Tris pH7.5, 150
mM NaCl, 1 mM EDTA, 1% NP40, 0.5% sodium deoxycholate, 0.2% SDS, 10 mM NaF, 10 mM
Na3VO4, 10 mM sodium β-glycerophosphate and supplemented with protease inhibitors) and centrifuged at 13,000 g for 10 minutes at 4°C. Protein concentration was measured by a bicinchoninic acid colorimetric assay (Thermo). Protein was loaded onto an SDS-PAGE gel and transferred to a PVDF membrane (Thermo). After one hour in SuperBlock blocking buffer (Thermo), membranes were incubated overnight with primary antibodies, and then horseradish peroxidase conjugated secondary antibodies (Thermo). Protein was visualized by chemiluminescent reagent (Thermo). Antibodies against the insulin receptor (sc-711), alpha-tubulin (sc-8305), beta-actin (sc-47778), transferrin receptor (sc-22597) and stearoyl CoA desaturase 1 (sc-14720) were purchased from Santa Cruz Biotechnology. Antibodies against fatty acid synthase (ab38844) were purchased from Abcam. PCSK9 antibody was generated by immunizing rabbits with mouse PCSK9 peptide (CDSHGTHLAGVVGSRDGVAK-GTSLHSLRVLNC) (21st Century Biotechnology). Antibody against the LDLR was a gift from Dr. Alan Attie. ImageJ was used to quantify bands on film.

**Plasma PCSK9 and LDL-cholesterol Measurements**

Plasma PCSK9 levels were measured by ELISA (MLB International) and plasma LDL-cholesterol levels were measured by colorimetric assays (Bioo Scientific) according to the manufacturer’s instructions.

**Statistical Analysis**

In all *in vitro* experiments, gene expression studies were performed using triplicate wells; reporter assays were performed using triplicate wells; western blotting was performed using duplicate wells. Unless otherwise indicated, representative results of two to five independent experiments are shown. Differences between groups were assessed by a two-tailed unequal variance Student’s t-test. Bars and error bars correspond to the mean and s.e.m., respectively.


