Type 1 Deiodinase Regulates ApoA-I Gene Expression and ApoA-I Synthesis Independent of Thyroid Hormone Signaling

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Objective—Plasma levels of high-density lipoprotein cholesterol (HDL-C) and apolipoprotein A-I (ApoA-I) are reduced in individuals with defective insulin signaling. Initial studies using liver-specific insulin receptor (InsR) knockout mice identified reduced expression of type 1 deiodinase (Dio1) as a potentially novel link between defective hepatic insulin signaling and reduced expression of the ApoA-I gene. Our objective was to examine the regulation of ApoA-I expression by Dio1.

Approach and Results—Acute inactivation of InsR by adenoviral delivery of Cre recombinase to InsR floxed mice reduced HDL-C and expression of both ApoA-I and Dio1. Overexpression of Dio1 in InsR knockout mice had low expression of ApoA-I and reduced serum levels of HDL-C and ApoA-I. Treatment of C57BL/6j mice with antisense to Dio1 reduced ApoA-I mRNA, HDL-C, and serum ApoA-I. Hepatic 3,5,3′-triiodothyronine content was normal or elevated in InsR knockout mice or Dio1 knockout mice. Knockdown of either InsR or Dio1 by siRNA in HepG2 cells decreased the expression of ApoA-I and ApoA-I synthesis and secretion. siRNA knockdown of InsR or Dio1 decreased activity of a region of the ApoA-I promoter lacking thyroid hormone response elements (region B). Electrophoretic mobility shift assay demonstrated that reduced Dio1 expression decreased the binding of nuclear proteins to region B.


Key Words: apolipoprotein A-I ■ cholesterol, HDL ■ electrophoretic mobility shift assay ■ mice, knockout ■ response element ■ type 1 deiodinase

High-density lipoprotein cholesterol (HDL-C) and apolipoprotein A-I (ApoA-I) concentrations are strong predictors of risk for cardiovascular disease (CVD). The development of a well-tolerated agent that can significantly elevate serum HDL-C level remains a therapeutic target. Statins raise HDL-C only modestly, and it is not clear whether that effect plays a role in the success of statins in CVD reduction. Niacin increases HDL-C significantly, but 2 recent studies where niacin was added to statin treatment did not reduce CVD. Fibrates increase HDL-C moderately, depending on baseline triglycerides, but studies of fibrates and CVD reduction have produced mixed results. There is one large ongoing trial to determine whether CVD can be reduced by increasing HDL-C with inhibitors of cholesteryl ester transfer protein; three previous trials with this class of drug, however, have failed (Press Release: Eli Lilly and Co. October 12, 2015).

Although niacin may increase the production of ApoA-I, the other HDL-raising drugs do not. Relevant to this point, data from mouse models indicate that when ApoA-I synthesis is increased by genetic approaches, atherosclerosis is reduced. Importantly, short-term administration of ApoA-I has provided promising, but not definitive, evidence of beneficial effects on atherosclerotic lesions in animals and in humans.

Low levels of HDL-C are common in the people with defective insulin signaling, eg, metabolic syndrome and type 2 diabetes mellitus. Although the hypertriglyceridemia common to these disorders drives increased cholesteryl ester transfer protein–mediated exchange of HDL cholesteryl esters for triglycerides from very low-density lipoproteins and chylomicrons, those pathways only account for about half of the inverse relationship between triglycerides and HDL-C. Mouse models of defective insulin signaling, such as insulin receptor (InsR) liver-specific insulin receptor knockout (LIRKO) mice, PI3K double knockout mice and mice lacking hepatic Irs 1 and Irs 2, all have reduced HDL-C levels in the
Nonstandard Abbreviations and Acronyms

| ApoA-I | apolipoprotein A |
| ASO | antisense oligonucleotide |
| CVD | cardiovascular disease |
| Dio1 | type I deiodinase gene |
| D1KO | type I deiodinase knockout mouse |
| HDL-C | high-density lipoprotein cholesterol |
| InsR | insulin receptor |
| LIRKO | liver-specific insulin receptor knockout mice |
| SBs | specific bands |
| T3 | 3,5,3′-triiodothyronine |
| TREs | thyroid hormone response elements |

absence of cholesteryl ester transfer protein. The mechanism by which defective insulin signaling results in low levels of HDL-C remains to be determined.

To examine the role of insulin signaling in the regulation of HDL-C and ApoA-I levels, and based on our previous study in congenital LIRKO mice,21 we created acute LIRKO mice by injecting a Cre adenovirus into InsR(+/−) (Lox) mice. Microarray analyses indicated that concomitant with reductions in ApoA-I levels in serum, the mice had marked reductions in the hepatic expression of ApoA-I. In addition, and to our surprise, the largest change in gene expression was that of type I deiodinase (Dio1), which was reduced by 90% in the acute LIRKO mice. Here, we present results of a series of in vivo and in vitro experiments demonstrating that Dio1 can regulate ApoA-I gene expression at the level of the promoter, and that this regulation is independent of previously demonstrated effects of thyroid hormone on ApoA-I gene expression. Importantly, reduced Dio1 expression resulted in reduced rates of ApoA-I synthesis and secretion from hepatocytes. Our results demonstrate a completely novel mechanism for the regulation of ApoA-I gene expression and provide new insights on the link between states of defective insulin signaling and reduced levels of plasma HDL-C and ApoA-I.

Materials and Methods

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

Results

Acute and Chronic Loss of Hepatic InsRs Are Associated With Reductions in the Levels of HDL-C and ApoA-I, and the Expression of ApoA-I and Dio1

We reported previously that LIRKO mice have markedly reduced serum HDL-C levels.21 In this study, we produced acute liver InsR depletion by injecting 10-week-old InsR(fl/fl) (Lox) mice with either an adenovirus carrying the Cre recombinase with the liver-specific albumin promoter (Ad-Cre) or a control adenovirus carrying LacZ (Ad-LacZ). Levels of liver InsR levels were determined by Western blotting of hepatic samples from Ad-LacZ and Ad-Cre–injected mice at day 3, day 6, and day 12. By 6 days after injection of Ad-Cre, hepatic InsR expression levels were reduced by 90% (Figure 1A).

Fast protein liquid chromatography analysis revealed that both HDL-C and ApoA-I were significantly decreased in Lox mice infused with the Ad-Cre compared with Ad-LacZ injected mice (Figure 1B). As noted above, microarray analyses revealed that livers from the mice injected with Ad-Cre had marked reductions in the expression of ApoA-I and Dio1 (Figure 1A in the online-only Data Supplement); real-time quantitative polymerase chain reaction data confirmed these results (Figure 1C). Microarray analyses of other major genes involved in HDL-C metabolism, including SR-BI (scavenger receptor class B member 1), ABCAI (ATP-binding cassette transporter-A1), and ABCG1 (ATP-binding cassette transporter-G1), did not show significant changes in Lox mice treated with Ad-Cre (Figure 1A in the online-only Data Supplement). Other lipid or lipoprotein-related genes that were affected by acute loss of InsR included ApoB, ApoAII, ApoCIII, ApoE, LDLR (low-density lipoprotein receptor) and LCAT (lecithin–cholesterol acyltransferase); they were all downregulated in the microarray analysis (Figure 1B in the online-only Data Supplement) and, except for ApoCIII, by quantitative polymerase chain reaction as well (Figure 1C in the online-only Data Supplement). Based on these findings, we returned to LIRKO mice and confirmed the reduced HDL-C levels we had reported previously (Figure 1D).21 In addition, we found reduced ApoA-I levels in HDL fractions (Figure 1D). We also observed that ApoA-I mRNA and Dio1 mRNA levels were significantly decreased in LIRKO mice (Figure 1E). In our prior study, expression of constitutively active AKT reversed the low HDL-C in LIRKO mice22; we repeated this and demonstrated a marked increase in Dio1 mRNA levels in LIRKO after administration of an adenovirus carrying constitutively active AKT (Figure 2 in the online-only Data Supplement). These results are consistent with low levels of HDL-C and Dio1 mRNA levels present in AKT1/2 double knockout mice (Figure IIIA and IIIB in the online-only Data Supplement).

In mammals, Dio1 is highly expressed in liver and has been widely thought to be an important source of 3,5,3′-triiodothyronine (T3) in the euthyroid state. However, mice lacking the type I deiodinase enzyme (D1) have no evidence of hepatic hypothyroidism and have normal plasma T3 levels.23,24 Indeed mice lacking all 3 types of deiodinase activity have a normal hepatic T3 content.25 To test whether the reduced expression of hepatic Dio1 results in a reduced content of T3 in liver, we measured T3 content in the livers of LIRKO mice and their control Lox mice. There was no significant difference in liver T3 content between these 2 groups (Figure IVA in the online-only Data Supplement). Importantly, the normal hepatic T3 content in LIRKO mice suggested that any mechanistic association of defective hepatic insulin signaling, reduced Dio1 expression, and low plasma HDL-C and ApoA-I levels might not involve the well-described effects of T3 on ApoA-I gene expression.26,27

HDL-C, ApoA-I, and ApoA-I mRNA Levels Are Decreased in Both Type I Deiodinase Knockout Mice and C57BL/6J Mice Treated With Dio1 Antisense Oligonucleotide

The results presented above indicated that the effects of Dio1 on ApoA-I expression were downstream of defective hepatic insulin signaling. To determine whether Dio1 could regulate ApoA-I directly, we performed studies in type I deiodinase
knockout mice (D1KO) mice. In D1KO mice, both HDL-C and HDL ApoA-I levels were reduced compared with wild-type controls (Figure 2A). Concomitant with these differences in blood levels, hepatic ApoA-I mRNA levels were significantly reduced in D1KO mice (Figure 2B). Importantly, quantitative polymerase chain reaction of D1KO livers demonstrated that

Figure 1. Acute and chronic loss of hepatic liver-specific insulin receptors (InsRs) are associated with reductions in high-density lipoprotein cholesterol (HDL-C), ApoA-I, and the expression of ApoA-I and Dio1. A, InsR levels were determined by Western blots of livers from acute liver-specific insulin receptor knockout (LIRKO) mice and their controls generated by injecting 5×10⁹ viral particles of Ad-Cre or Ad-LacZ into 10-week-old Lox mice (n=3 per group). Livers were harvested at day 3, day 6, and day 11 after injections. B, Serum was obtained from 10-week-old Lox mice 6 d after injection with Ad-LacZ or Ad-Cre after a 6-h fast. 200 μL of pooled serum from mice (n=6 per group) was subjected to fast protein liquid chromatography (FPLC) to resolve lipoproteins and cholesterol was measured in each of the eluted fraction (top). The FPLC fractions representing HDL (fractions 27–33) were used for Western blotting of ApoA-I (bottom). The data shown are from 1 of 3 independent experiments. C, Livers (n=4–6 per group) were harvested 6 d after injection of Ad-LacZ or Ad-Cre into Lox mice. Real-time polymerase chain reaction (PCR) was used to verify microarray results showing significantly reduced hepatic ApoA-I and Dio1 expression levels in acute LIRKO mice. D, 200 μL of serum was pooled from 16-week-old LIRKO mice and Lox controls (n=5 per group) and lipoproteins were resolved by FPLC. Cholesterol and ApoA-I were determined as described in B. E, Liver ApoA-I mRNA and Dio1 mRNA were quantified by qPCR in LIRKO and Lox mice (n=10 per group). Bar graphs depict mean±SD. *P<0.05, **P<0.01.
mRNA levels of ApoAI, ApoB, ApoCIII, and ApoE, which were reduced in LIRKO mice, were not altered by direct loss of Dio1 expression (Figure V in the online-only Data Supplement). Liver T3 content was actually increased in the D1KO mice compared with wild-type mice (Figure IVB in the online-only Data Supplement), a result not unexpected based on our prior published studies in these mice.23,25 Because LIRKO mice have reduced Dio1 and ApoA-I mRNA levels in association with defective hepatic insulin signaling and insulin resistance, we measured fasting serum insulin levels in D1KO and wild-type mice: there were no differences between the 2 groups (Figure VI in the online-only Data Supplement).

To further understand the effect of Dio1 on normal mice, we knocked down Dio1 expression in C57BL/6J mice using a 16-mer cEt gap-mer antisense oligonucleotide (ASO) that was provided by Ionis Pharmaceuticals. Dio1 and control ASO were administered twice weekly at a dose of 12.5 mg/kg body weight to 10-week-old C57BL/6J. After 8 weeks of treatment, liver Dio1 mRNA was decreased by 95% (Figure VII in the online-only Data Supplement). The reduction in Dio1 expression was associated with a 50% decrease in ApoA-I mRNA (Figure 2C) in the mice treated with Dio1 ASO. Serum HDL-C levels and HDL ApoA-I concentrations were both significantly reduced in Dio1 ASO-treated mice compared with controls ASO (Figure 2D). The results from both the D1KO mice and the Dio1 ASO-treated mice indicate clearly that Dio1 deficiency results in reduced ApoA-I mRNA levels independent of hepatic insulin signaling and in the presence of normal or elevated hepatic T3 content.

Overexpression of Dio1 Can Restore HDL-C and ApoA-I levels, and Significantly Increase the Expression of ApoA-I in LIRKO Mice

The findings indicated a direct link between Dio1 and ApoA-I expression. To extend these results, we generated an adenovirus containing the cDNA for mouse Dio1 (Ad-Dio1) and
Knockdown of Either InsR or Dio1 Decreases ApoA-I Expression As Well As the Synthesis and Secretion of ApoA-I in HepG2 Cells

To determine the effects of reduced insulin signaling and Dio1 expression at the cellular level, we used siRNA to knock down either InsR or Dio1 in the human hepatoblastoma cell line, HepG2. Treatment of HepG2 cells with InsR/siRNA resulted in a dramatic decrease in receptors by Western blotting (Figure 1XA in the online-only Data Supplement) and a fall in InsR mRNA of >90% by quantitative polymerase chain reaction (Figure IXB in the online-only Data Supplement). These changes in InsR were associated with reductions of 40% and 60% in ApoA-I mRNA and Dio1 mRNA levels, respectively (Figure IXB in the online-only Data Supplement). When Dio1 expression was reduced >90% in HepG2 cells by direct siRNA treatment, ApoA-I mRNA levels were decreased to 50% of the levels in cells treated with control siRNA (Figure 4A). To demonstrate the effect of knockdown of either insulin signaling or Dio1 on the synthesis and secretion of ApoA-I, HepG2 cells treated with siRNAs for either InsR or Dio1 were labeled with [35S] methionine for 20 minutes. As shown in Figure 4B, compared with control cells (lanes 1 and 2), cells treated with siRNAs for InsR (lanes 3 and 4) or Dio1 (lanes 5 and 6) had reductions of newly synthesized cellular ApoA-I of 37% and 22%. Secretion of ApoA-I into the media was concomitantly reduced (Figure 4B, lanes 9 and 10 for InsR and lanes 11 and 12 for Dio1) by 52% and 30% compared with control cells (Figure 4B, lanes 7 and 8). When HepG2 cells that had been treated with siRNAs for either InsR or Dio1 were labeled with [35S] methionine for 20 minutes and then chased in media without [35S] methionine for 20 minutes, essentially identical results were obtained. Thus, during a 20-minute chase, cells in which either InsR or Dio1 had been knocked down synthesized 41% or 26% less ApoA-I (Figure 4C, lanes 3 and 4 for InsR and lanes 5 and 6 for Dio1), respectively, compared with control cells (Figure 4C, lanes 1 and 2). During the 20-minute chase experiment, InsR and Dio1 siRNA-treated cells (Figure 4C, lanes 9 and 10 and 11 and 12 respectively) secreted 50% or 32% less than control cells (Figure 4C, lanes 7 and 8). The similar reductions in synthesis and secretion in each experiment indicated that the reduction in ApoA-I secretion could be fully accounted for by decreased synthesis; there was no evidence of increased intracellular degradation of high-density lipoprotein cholesterol (HDL-C) and ApoA-I levels, and significantly increase the expression of ApoA-I in liver-specific insulin receptor knockout mice (LIRKO) mice. Twelve-wk-old LIRKO mice were injected intravenously with Ad-Dio1 (n=8) or Ad-GFP (n=6). After 12 d, liver Dio1 mRNA (A) and ApoA-I mRNA (B) were measured by quantitative polymerase chain reaction. C, Serum (200 μL) from mice with Ad-Dio1 or Ad-GFP was pooled and lipoproteins were resolved by fast protein liquid chromatography (FPLC), with cholesterol measured in each fraction (top). ApoA-I levels were examined by Western blot in FPLC fractions (27–33; bottom). Similar results were obtained in the 2 other experiments. Bar graphs depict mean±SD. *P<0.05, **P<0.01.
newly synthesized ApoA-I in the cells treated with either InsR or Dio1 siRNA.

**Dio1 Regulates Activity of the ApoA-I Promoter Through Region B, Which Does Not Contain T3 Responsive Elements**

The ApoA-I promoter region from −256 to −41 bp 5′ from the transcription start (+1) has been defined by some investigators as a liver-specific enhancer. It contains 3 transcription factor (TF)-binding regions designated as region A (−256 to −192), region B (−192 to −128), and region C (−128 to −41). We obtained pGL3-luciferase reporter plasmids including a construct containing ApoA-I gene promoter sequence from base pair −256 to +1 and two 5′ deletion constructs as gifts from Professor Bart Staels (France; Figure 5A). HepG2 cells were transfected with scrambled control siRNA or InsR siRNA for 24 hours, followed by transfection of ABC, BC, or C luciferase reporter constructs of the ApoA-I promoter for another 48 hours. As shown in Figure 5B, knockdown of InsR reduced the activity of the ABC ApoA-I promoter construct by ∼30% of the control level and the activity of the BC ApoA-I promoter construct to ∼50% of control promoter activity. In contrast, knockdown of InsR had no effect on the construct containing only region C of the promoter. These results suggested that region B contained response elements (REs) sensitive to defective insulin signaling. We extended these findings by generating additional ApoA-I promoter constructs containing only region A (A) or region B (B) (Figure 5C). Using the same experimental protocol as described above, we demonstrated that defective insulin signaling resulted in decreased ApoA-I promoter activity.
only through region B (Figure 5D). To confirm our earlier findings that defective insulin signaling reduced ApoA-I expression through reduced Dio1 expression, HepG2 cells were transfected with the 3 reporter constructs, A, B, or C, after siRNA-mediated knockdown of Dio1. As shown in Figure 5E, knockdown Dio1 reduced the activity of the B promoter construct by ≈35% of the control level, and there were no effects of Dio1 knockdown on either the A or C promoter constructs.

Romney et al10 had demonstrated that T3 increased ApoA-I gene expression at the level of transcription and Taylor et al13 identified region A to contain a positive T3 responsive element in the rat ApoA-I promoter. It was, therefore, important to determine which region(s) of the human ApoA-I promoter is/are responsive to T3 treatment. Because the actions of T3 are mediated by TRβ, HepG2 cells were cotransfected with TRβ and ApoA-I promoter constructs in the presence of T3 or vehicle. As shown in Figure X in the online-only Data Supplement, T3 treatment significantly increased region A promoter activity by ≈35%. Neither region B nor region C was responsive to T3 treatment. These results provide additional evidence that Dio1 is involved in the regulation of the expression of the ApoA-I promoter via effects on Region B, which does not respond to T3 treatment.

**Knockdown of Either InsR or Dio1 Reduces the Binding of Nuclear Proteins to Region B and Overexpression of Dio1 Restores That Binding**

Based on the results described above, we determined whether defective insulin signaling or decreased Dio1 expression induce changes in binding of TFs to region B of ApoA-I promoter. In these experiments, either InsR or Dio1 was knocked down in HepG2 cells, and nuclear proteins were extracted to do electrophoretic mobility shifted assay. The sequence of region B was labeled as a probe. The specificity of binding of nuclear proteins to the region B probe was confirmed by the addition of excess nonlabeled region B oligonucleotide (Figure 6, lane 2). As shown in lane 3, several nuclear proteins from HepG2 cells bound specifically to the DNA probe of region B, generating specific bands (SBs) of varying mobility; these bands were not affected by the overexpression of Dio1 in the cells (lane 4). However, the intensities of SB1, SB2, SB3, and SB4 were dramatically reduced in the cells treated with either InsR siRNA or Dio1 siRNA (lanes 5 and 7). Overexpression of Dio1 in the cells knocked down by InsR siRNA or Dio1 siRNA increased the intensities of SB1, SB2, SB3 (shown in lane 6 or lane 8) compared with those shown in lane 5 or 7. Overexpression of Dio1 did not affect the intensity of SB4, electrophoretic mobility shifted assay analysis indicated, therefore, that the specific binding of several nuclear proteins to a region B probe was clearly reduced when either InsR or Dio1 were knocked down when compared with control cells. Overexpression of Dio1 restored binding that had been reduced by knockdown of either InsR or Dio1. These results suggest that ApoA-I is transcriptionally regulated by Dio1 expression via Region B.

**Discussion**

ApoA-I, the major protein component of HDL, is expressed mainly in the liver. Interest in HDL as an antiatherogenic
lipoprotein led to extensive interrogation of the regulation of the ApoA-I gene by several groups >20 years ago. The proximal region of the ApoA-I promoter, from −256 to −41 bp 5' of the transcription start region (+1), which has also been called the liver-specific enhancer, was mapped in fine detail and found to contain 3 TF-binding regions that have been designated differently by each group of investigators.32 We have used the terminology of Vu-Dac et al,29 who divided the promoter into region A (−256 to −192), region B (−192 to −128), and region C (−128 to −41). Several TFs involved in regulating ApoA-I gene transcription have been identified that bind to REs in ≥1 of these regions.30,33–42 Most relevant to the present studies are the thyroid hormone receptors (TRs)31 that increase ApoA-I promoter activity by binding to REs in region A.

Despite the longstanding observation that HDL-C and ApoA-I levels are reduced in people with insulin resistance and type 2 diabetes mellitus, studies of the role of insulin signaling on ApoA-I expression have been limited. Elshourbagy et al43 found that insulin increased the expression of ApoA-I in cultured primary rat hepatocytes. In contrast, Levy et al44 did not find an effect of insulin on intestinal ApoA-I expression or synthesis, suggesting tissue-specific regulation. Haas et al45 demonstrated that hepatic ApoA-I mRNA levels were reduced in rats with streptozotocin-induced diabetes, and that insulin’s stimulatory activity occurred via binding of the TF, Sp1, to a GC-rich insulin response core element in the region of −411 to −404 bp of the ApoA-I gene promoter upstream from the initiation site.46 Our results complement those of Haas et al46 in that defective insulin signaling in LIRKO mice and in HepG2 cells results in reduced ApoA-I expression, synthesis, and secretion. However, we add 2 new and novel findings to prior studies: first, that the effect of absent or reduced hepatic insulin signaling occurs at the level of the ApoA-I promoter at region B of the liver-specific enhancer, between bases −128 and −192 from the transcriptional start region, and second, that the effect of absent or reduced insulin signaling is transmitted to the ApoA-I promoter via reduced expression of hepatic Dio1.

About the effects of absent or reduced hepatic insulin signaling, we have extended our initial finding of low HDL-C levels in LIRKO mice that was corrected by restoration of insulin signaling21 by demonstrating a similar phenotype in an acute model of InsR knockout. Furthermore, we reproduced these findings in HepG2 cells using a specific siRNA for InsR. Thus, InsR knockdown resulted in reduced expression of ApoA-I that led to lower rates of synthesis and secretion of Apo-AI from HepG2 cells. Finally, as noted above, we demonstrated that the loss of insulin signaling in HepG2 cells resulted in reduced activity of region B of the ApoA-I promoter, and this reduced promoter activity was associated with changes in the binding of nuclear proteins to region B of the ApoA-I promoter.

Our most important novel finding, however, was that the effects of absent or reduced hepatic insulin signaling on ApoA-I expression and promoter activity occurred via insulin signaling-mediated regulation of Dio1. Dio1 is expressed mainly in liver, kidney, thyroid, and pituitary gland, and is the source of D1, a selenoenzyme that can 5'-deiodinate thyroxine (T4) to T3. However, its preferred substrate is 3,3',5'-triiodothyronine (rT3). The D1 also has inner-ring or 5-deiodinase activity by which it can convert T4 and T3, usually as their sulfa-

![Figure 6](http://atvb.ahajournals.org/)

**Figure 6.** Knockdown of either InsR or Dio1 reduces the binding of nuclear proteins to region B and overexpression of Dio1 restores that binding. HepG2 cells were transfected with Control siRNA (Ctrl), InsR siRNA, or Dio1 siRNA. Six hours later, the cells were infected with Ad-Dio1 or control Ad-GFP for 48 h. Then nuclear extracts (NE) were prepared. The promoter sequence corresponding to region B (−192 to −142) was used to generate the DNA probe. Electrophoretic mobility shifted assay (EMSA) were conducted using a LightShift chemiluminescent EMSA kit. Lane 1: only labeled probe; lane 2: specific competitor+NE from the cells treated with Ctrl siRNA; lanes 3, 5, and 7: NE from the cells treated with Ctrl siRNA, InsR siRNA, and Dio1 siRNA, respectively, as well as Ad-GFP. Lanes 4, 6, and 8: NE from the cells treated with Ctrl siRNA, InsR siRNA, and Dio1 siRNA, respectively, as well as Ad-Dio1. All lanes include labeled probe. The results shown are representative of 6 experiments.
TREs in the promoter region. Recently, the TFs Forkhead box A1 and Forkhead box A2 were reported to regulate human Dio1 expression in the liver. Our present studies add hepatic insulin signaling to the list of regulators of Dio1 expression. Thus, both congenital and acute LIRKO mice have marked reductions in Dio1 expression, and the latter abnormality is reversed by delivery of constitutively active AKT. Severe insulin deficiency and metabolic derangements in rats with streptozotocin-induced diabetes mellitus are associated with marked reductions in both Dio1 expression and D1 activity that can be reversed by insulin treatment. In contrast, LIRKO mice have minimally altered metabolism, allowing a more precise demonstration of linkage between defective hepatic insulin signaling and reductions in Dio1 expression. The molecular mechanisms underlying the link between hepatic insulin signaling and downregulation of Dio1 expression will require additional experiments beyond the scope of the present work.

Not only is Dio1 gene expression markedly reduced in LIRKO mice but also seems to be central to the regulation of ApoA-I gene expression by hepatic insulin signaling. When we overexpressed Dio1 in LIRKO mice, ApoA-I expression and the levels of both plasma HDL-C and ApoA-I increased. Our studies with the D1KO mice and with Dio1 ASO in C57BL/6j mice confirmed the existence of a direct role for Dio1 in the regulation of Apo-A-I expression, as well as the levels of plasma HDL-C and ApoA-I. Critical to our model of a direct role of Dio1 in the regulation of ApoA-I expression, the D1KO mice have normal plasma insulin levels indicating normal hepatic insulin signaling. Finally, knockdown of Dio1 by siRNA in HepG2 cells produced the same pattern of nuclear protein binding in an electrophoretic mobility shifted assay as did knockdown of InsR.

Importantly, overexpression of Dio1 normalized the pattern of nuclear protein binding in region B in cells where expression of endogenous Dio1 or InsR had been knocked down.

It is well known that thyroid hormone status affects ApoA-I gene expression, and transcription factor binding proteins, NFY (nuclear transcription factor), or Sp1 interacts with the liver region A via TRs, resulting in increased promoter activity. Furthermore, propylthiouracil, a specific inhibitor of D1 activity, significantly decreased ApoA-I expression and ApoA-I synthesis in rat liver. There is, however, complexity in this pathway, as binding of the T3-TR complex to the other TRE, which is 3' to the TATA box in region C and close to the transcriptional start-region, leads to decreased promoter activity. In addition, in rodents, unliganded TRs can influence transcription of thyroid responsive genes in the opposite direction than the liganded TR. Our results add another level of complexity: the expression of Dio1 regulates ApoA-I expression at region B, which does not have canonical TREs. Our study that T3 treatment only significantly increased region A promoter activity in the presence of TRβ provided further evidence that region B is not responsive to T3. Additional support for a T3-independent mechanism derives from the finding that hepatic T3 content was normal in LIRKO mice and actually increased in the D1KO mice. These findings were not unexpected based on our previous published data showing a normal content of hepatic T3 in D1KO mice. In addition, both D1KO and C3H mice (which have very low D1 activity due to a naturally occurring mutation in Dio1) seem to be euthyroid in terms of their plasma T3 levels and the expression of several classical T3-responsive genes in the liver. The maintenance of normal or elevated hepatic T3 content in LIRKO and D1KO mice may result from increased delivery of plasma T3 to the liver and reduced conversion of T3 to inactive T2 metabolites when D1 activity is reduced or absent.

Although we have demonstrated that Dio1 acts on the ApoA-I promoter via region B, which does not contain a known TRE, and which is unresponsive to T3 in the presence of adequate TRβ, we have not as yet identified an alternative signaling pathway. Based on existing literature, we know that region B contains REs to which known TFs, including HNF-3β (hepatocyte nuclear factor 3, beta), C/EBP (CCAAT-enhancer-binding proteins), NFY (nuclear transcription factor), or Sp1 can bind. How a transmembrane enzyme might interact and activate one of these (or another) TFs that would then act on the ApoA-I promoter, will require significant additional work with approaches that include RNA Seq, transcriptomics, and bioinformatics analyses.

In summary, we have demonstrated that hepatic insulin signaling plays a significant role in the regulation of ApoA-I gene expression, as well as the synthesis and secretion of ApoA-I, through the regulation of Dio1. Of note, Dio1-mediated regulation of ApoA-I does not require defective hepatic insulin signaling, as shown by our studies in the D1KO mouse and with Dio1 ASO. Furthermore, loss of Dio1 expression reduces apoA-I expression without affecting expression of several other apolipoproteins that are also reduced when insulin signaling is lost. Our characterization of a novel pathway linking hepatic insulin signaling, Dio1, and ApoA-I, could have significant impact on the problem of low HDL-C in people with metabolic syndrome or type 2 diabetes mellitus. Recent negative CVD clinical trials with fenofibrate, niacin, and cholesteryl ester transfer protein inhibitors, coupled with early but promising results from studies where blood levels of ApoA-I were increased directly, provide impetus for additional studies focused on the molecular mechanisms involved in this pathway. More importantly, identification of the links between Dio1 expression and ApoA-I promoter activity could provide new and significant approaches to regulating plasma HDL-C and ApoA-I levels.

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Disclosures

Two of the authors (J.L. and H.N.G.) have filed a patent through Columbia University based on some of the work included in this article.

References


Acute or chronic loss of insulin signaling in the liver-specific insulin receptor knockout mouse resulted in significant reductions in plasma levels of high-density lipoprotein cholesterol, apoA-I, and apoA-IV gene expression. Type 1 deiodinase (Dio1) expression was also significantly reduced in acute or chronic liver-specific insulin receptor knockout mice and expression of Dio1 in liver-specific insulin receptor knockout mice restored plasma levels of high-density lipoprotein cholesterol and apoA-I, and apoA-IV gene expression. Dio1 knockout mice and mice treated with Dio1 antisense have low plasma levels of high-density lipoprotein cholesterol and apoA-I. Knockdown of Dio1 in liver cells results in reduced apoA-IV gene expression and apoA-IV synthesis. Dio1 regulates apoA-IV promoter activity in a region that is not affected by thyroid hormone and has no thyroid response elements.
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Materials and Methods

Animals
LIRKO mice were obtained by crossing C57BL/6J InsR (fl/fl) (Lox) mice ¹ (obtained from the Joslin Diabetes Center, Harvard Medical School) and C57BL/6J albumin-Cre (Jackson Labs). Acute LIRKO mice were made by infecting Lox mice with 5 x 10⁹ viral particles of albumin-Cre recombinant adenovirus (Ad-Cre) by vein infusion. At this dose, more than 80% of the liver hepatocytes can be transduced. Type I deiodinase deficient mice on the C57BL/6J background (D1KO) were generated by one of the authors². Male mice were used in all studies and were maintained in centrally ventilated cages in a barrier facility with 12 hours light cycle control and free access to food and water. Mice were on standard chow diet. All protocols were approved by the Columbia University Institutional Animal Care and Use Committee.

Delivery of Antisense Oligonucleotides Targeting Dio1 into C57BL/6J mice
4 different 16-mer cEt gap-mer ASOs targeting Dio1 were provided by Ionis Pharmaceuticals (Carlsbad, CA, United States). The scrambled oligonucleotide is used as a control. These ASOs were administered to 10 week C57BL/6J mice (n=2 per group) twice weekly with a dose of 12.5 mg/kg body weight for 8 weeks. Dio1 mRNA reduction was assessed by qPCR. qPCR results showed the most effective oligonucleotide sequence to be GCTTTATTTCTGGTG. After selection, 10 week C57BL/6J mice (n=6 per group) were injected with Dio1 ASO or Control ASO for 8 week with the same dose mentioned above.

Separation of Plasma Lipoproteinss: A 200µl of serum from several animals/group was subjected to fast-performance liquid chromatography (FPLC) analysis using two Superose 6 columns in series (ÄKTA, Amersham Biosciences). The columns were eluted with a buffer containing 0.15 M NaCl, 0.01 M Na₂HPO₄, 0.1 mM EDTA (pH 7.5), at a flow rate of 0.3 mL/min. Forty x 0.5-mL fractions were collected. The cholesterol content of each fraction was measured using the Cholesterol E enzymatic assay reagent kit (Wako Diagnostics). For ApoA-I analyses, equal volumes of FPLC fractions were subjected to SDS-PAGE and immunoblotted with antibody against ApoA-I (Santa Cruz).

Generation of Recombinant Adenovirus
We used an adenoviral vector construction system (AdEasy Adenoviral system; Stratagene) to generate recombinant adenoviral constructs. Mouse Dio1 cDNA (GenBank accession number NM_007860) was subcloned into a shuttle vector pAdTrack-CMV using XhoI and SalI (New England BioLabs). Next, the recombinant construct was linearized with PmeI and transformed, together with a supercoiled adenoviral vector pAdEasy-1 (Stratagene), into E. coli strain BJ5183 competent cells. Recombination of pAdEasy-1 and ligated pAdTrack-CMV through homologous recombination was detected by PacI digestion. For virus packaging, the recombinant adenoviral construct was cleaved with PacI and transfected into a 293 cell line. Virus stocks were amplified in HEK293 cells on 15 cm plates and purified using Vivapure AdenoPACK 100 Adenovirus Purification Kit (Sartorius Biotech). A control vector (Adv/GFP) carrying cDNA for green fluorescence protein was also prepared as described above. Virus titers were determined by spectrophotometric absorbance of viral particles (VPs) at 260 nm.

siRNA Transfection
Two predesigned Mission siRNA, targeted specifically to human InsR or human Dio1, were obtained from Sigma-Aldrich. The duplex sequences were:

*InsR* siRNA  
1) sense, 5’- GAAACUCUUCUCCACUUAU[dT][dT]3’,
   antisense, 5’AUAGUGGAAGAAGAGUUUC[dT][dT] 3’,
2) sense, 5’GAAACUGCAUGGUCGCCCA[dT][dT]3’,


antisense, 5'UGGGCGACCAUGCAGUUUC[dT][dT]3'.

*Dio1* siRNA  
1) sense, 5’GGAAGAAUUCCAGUUGUUA[dT][dT]3',  
antisense, 5’UAACAACUGGAUUCCUUC[dT][dT]3',  
2) sense, 5’GCAUUUAUGGAAUUGAUA[dT][dT]3',  
antisense, 5’UGAUCAAUCCAUUAAUG[dT][dT]3'.

HepG2 cells were seeded in six-well plates and maintained in complete media (DMEM plus 10% FBS) without antibiotics at ~50% confluence. A 30 nM pool of two *InsR* siRNAs or a 20nM pool of two *Dio1* siRNAs was transfected into HepG2 cells using a nanoparticle-based siRNA transfection reagent (N-TER™, Sigma-Aldrich). Mission siRNA Universal Negative Control (#1; Sigma-Aldrich) was used as a control. Cells were collected 72 h after transfection. Total RNA were extracted, then reverse transcription and qPCR were performed.

**Western Blot Analysis**

Mouse livers were snap-frozen in liquid nitrogen at day 3, day 6, and day 11 after adenovirus injection. 50ug of liver homogenate proteins were subjected to 8% SDS-PAGE and electrophoretically transferred to nitrocellulose membrane (Bio-Rad), the membrane was incubated with anti-mouse *InsR* β polyclonal antibody (Santa cruz) at 4 °C overnight. The blot was treated with HRP-conjugated goat anti-Rabbit IgG for 1 h and visualized by chemiluminescence (ECL; Thermo Scientific). 5 uls of FPLC fractions (27-33) were run on 12% SDS–polyacrylamide gel electrophoresis, Protein bands in the gel were transferred to nitrocellulose membrane (Bio Rad). Incubation of anti-mouse *ApoA-I* antibody (Novus Biologicals) with the membrane was performed at 4 °C overnight. The blot was treated with HRP-conjugated goat anti-Rabbit IgG for 1 h and visualized by chemiluminescence (ECL; Thermo Scientific).

**Quantitative Real-Time PCR**

Total RNA was isolated from mouse livers or HepG2 cell lysates using TRIzol reagent (Invitrogen). First-strand cDNA was synthesized from 4ug of total RNA using Oligo(dT) primers and SuperScript II reverse transcriptase (Invitrogen). Real time PCR was performed in 25ul of total volume with the use of Brilliant SYBR green qRT-PCR master mix (Agilent Technologies) using the ABI Prism 7700 Sequence Detection System (Applied Biosystems). Primers were obtained from Invitrogen. The mRNA levels were normalized by a housekeeping gene, cyclophilin. Primers (forward, reverse) used for this study were:

(h) - *InsR*, 5’GCTGAAGCTGCCCTGAGGA3'; 5’CGGCCACCGTCACATTCCCA3',  
(h) - *Dio1*, 5’AGCCACGACAACTGGATACC 3', 5’ACTCCCAAATGTTGCACCTC3',  
(h) - *ApoA-I*, 5’CAGACTGGGCAGTACCACGCAGCAAGGC3',  
5’TCACTGGGTGTTGGACCTTTCAGTGTA3',  
(h) - 18S, 5’CGCCGCCTGAGGCTAATTC3', 5’CATTCTTGCGCAAATGCTTTCG3',  
(m) - *InsR*, 5’GGCTGAAGCTGCCCTGAGGA3'; 5’GCTGCCACCGTCACATTCCCA3',  
(m) - *Dio1*, 5’GCCCTGGTTGGAAATTC3', 5’CTGCTGCGTGAGCTTCTTTC3',  
(m) - *ApoA-I*, 5’TGTGTATGTGGATGCGGTCA 3', 5’ATCCCAGAAGTCCGGTCA 3';  
(m) - cyclophilin, 5’GGAGATGGCACAGGAGGAA 3', 5’GCCCGTAGTGCTTCAGCTT3';

The thermal cycling protocol for reverse transcriptase-polymerase chain reaction (RT-PCR) amplification was initial denaturation for 1 minute at 94 °C, followed by 40 cycles of 30 sec at 94 °C, 30 sec at 60 °C, and 45 sec at 72 °C.

**Construction of Recombinant ApoA-I-A and ApoA-I-B**

We obtained pGL3-luciferase reporter plasmids containing DNA fragments, respectively, of the -256/+91(ABC), the -192/+91(BC), and the -128/+91(C) sequences of the human *ApoA-I* gene.
promoter from Professor Bart Staels. In addition, we created new deletion mutants containing -256 to -192/-41 to +91 (A), or -192 to -128/-41 to +91 (B) of the human ApoA-I promoter sequence. These were prepared by polymerase chain reaction amplification using ABC-P-pGL3 (-256/+91) construct as template and A primers and B primers. Two resulting PCR products were subcloned into Sall and Kpnl digested pGL3 vector (Promega) to create a fusion with the luciferase gene (-256 to -192/-41 to +91 pGL3 and -192 to -128/-41 to +91 pGL3). The primers A and B contain Sall and XhoI restriction sites to amplify the fragments -256 to -192 and -192 to -128 respectively. The primer P containing XhoI and Kpnl restriction sites is designed to amplify the fragment -41 to +91. The primers are listed below:

A, 5’ CAACGTGCACCCCGGAGACCT 3’
   5’CAACCTCGAGCAGGGGTCAAGGTTTCAG 3’

B, 5’CAACGTGCACCCCTGCAGCCCCCGCAGCTT 3’,
   5’CAACCTCGAGTCAGCTCTGTCCCTGGGGCT3’,

P, 5’CAACCTCGAGAGAGACTGCGAGAAGGAG 3’,
   5’TCTGGGTACCTAGAGGATC 3’,

The correct orientation of these deletion mutant constructs was confirmed by sequencing.

Transfection, Cotransfection, and Luciferase Assay

Cotransfection siRNA and promoter constructs: HepG2 cells were seeded in six-well plates and maintained in complete media (DMEM, 10%FBS) without antibiotics at ~40% confluence. A 30 nM pool of two InsR siRNAs was transfected into HepG2 cells using nanoparticle-based siRNA transfection reagent (N-TER™, Sigma-Aldrich). Mission siRNA Universal Negative Control (#1; Sigma-Aldrich) was used as a control. In experiments where the cells were transfected a second time after 24h with promoter constructs, we used the lipofectamine 3000 reagent (Life Technologies) according to the manufacturer’s instructions. In these experiments, cDNA encoding Renilla luciferase (Promega) was co-transfected with the promoter constructs as an internal control for transfection efficiency. Cells were harvested in 1× Passive Lysis Buffer (Promega). 72 h after transfection, luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity in each well to control for transfection efficiency.

Electrophoretic mobility shift assays (EMSAs)

InsR or Dio1 was knocked down in HepG2 cells using each of the siRNAs described above. siRNA for InsR, Dio1 or control (Mission siRNA Universal Negative) was purchased from Sigma. Nuclear extracts from these cells were isolated according to Noe et al. and then subjected to EMSAs, which were conducted using a LightShift chemiluminescent EMSA kit (Thermo Fisher Scientific) as briefly described below. Both sense and antisense strands of oligonucleotides (oligo) containing Region B sequences of the human ApoA-I promoter (-192 to -128) were commercially synthesized ((Life Technologies). Oligonucleotides were labeled with biotin onto the 3’ end of DNA strands of Region B using the Biotin 3’ End DNA Labeling Kit (Thermo Fisher Scientific). Then the labeled two DNA strands were annealed to generate double-stranded oligonucleotide probes. The Biotin-labeled probe (labeled Region B) was incubated with nuclear extracts in the presence or absence of competing unlabeled oligonucleotide (Region B sequences). After binding, DNA probe-protein complexes were separated by PAGE on a 6% non-denaturing gel. The probe-protein complexes were transferred to nylon membrane, which were cross-linked to stabilize them. Finally the bands with biotin-label were detected by chemiluminescence. Protein-bound probes will have different mobilities relative to the probe and thus present as “shifted bands” (SBs) compared to the probe alone.
De Novo ApoA-I synthesis and secretion in HepG2 cells
HepG2 cells transfected with either InsR or Dio1 siRNA for 48h (see above) were pre-incubated with methionine (Met)-free medium (Invitrogen) for 2 hrs. This was replaced by Met-free medium with $[^{35}\text{S}]$-Met (150uCi per well/6 well plate, PerkinElmer) and incubated for 20min. Then the cells and media were harvested and immunoprecipitation with ApoA-I antibody (Santa Cruz) was performed. Finally 12% SDS PAGE was used to separate ApoA-I. The gel was treated with Autofluor (National Diagnostics) and, after drying, exposed to x-ray film at –80°C.

For pulse chase experiment, the cells were labeled with $[^{35}\text{S}]$ méthionine for 20 minutes followed by 20min chase after knockdown of InsR and Dio1 with siRNA. The method for measuring labeled ApoA-I in cells and media is described above.

Statistical Analysis
All data are presented as mean ± standard deviation (SD). Statistical analysis was evaluated with two-tailed Student’s $t$ tests or 1-way ANOVA followed by the Bonferroni post hoc test when comparing two groups or more than two groups, respectively. $P < 0.05$ was considered significant. Statistical analysis was done using GraphPad Prism 5 software.

Reference:


SUPPLEMENTAL MATERIALS

Table of Contents

1. Detailed Methods
2. Figures and Legends
3. Supplemental References

Detailed Methods

MEEBO Microarray
The Mouse Exonic Evidence Based Oligonucleotide (MEEBO) contains 38,08370mer probes that were designed using a transcriptome-based annotation of exomic structure for genomic loci. Livers from acute LIRKO mice were sent to Ocean Ridge Biosciences (ORB) for a cDNA microarray analysis using MEEBO arrays. Spot intensities were transformed by taking the base 2 logarithm of each value. The log2-transformed and normalized spot intensities for the 18510 detectable probes were examined for differences between the treatment groups by 1-way ANOVA. The statistical significance was determined using the False Discovery Rate (FDR) method, which uses the proportion of false positives among all probes with $P$ values lower or equal to the $P$ value of the probes that we considered significant.

Restoration of insulin signaling in LIRKO mice
LIRKO mice were injected intravenously with adenovirus encoding either a constitutively active form of AKT (myr-AKT) or LacZ, with each dose of 5 X 10^9 viral particles. Livers and serum were obtained at day 6 after injecting.

Acute Liver (L)-AKT1/2 double KO mice:
Livers and serum from acute L-AKT1/2 double KO mice were provided by Dr. Morris Birnbaum (University of Pennsylvania). L-AKT1/2 double KO mice were generated from AKT1^loxp/loxpAKT2^loxp/loxp mice injected with Adeno-associated virus (AAV) expressing Cre recombinase under the control of thyroxine binding globulin (TBG) promoter which led to the liver-specific deletion of AKT1/2.

Measurement of serum insulin
Serum insulin levels were measured using sensitive rat insulin radioimmunoassay kit (EMD Millipore).

Measurement of Hepatic T3 content:
Hepatic T3 content was determined using a sensitive, nonequilibrium radioimmunoassay.

Cotransfection the vector containing cDNA for thyroid hormone receptor (pReceiverTRβ) and promoter constructs:
HepG2 cells were seeded in six-well plates and maintained in complete media (DMEM, 10% FBS) without antibiotics at ~80% confluence. The cells were cotransfected with pReceiverTRβ (GeneCopoeia) and ApoA-I promoter constructs using lipofectamine 3000 (Life Technologies).
*Renilla* luciferase is used as an internal control for correction of transfection efficiency. After 24 hour of incubation, 3nM T3 or vehicle was added to the medium. After a further 24 hour of incubation, the cells were harvested for measurement of Firefly luciferase activity and *Renilla* luciferase activity.
Supplemental Figures and Legends

Supplemental Figure I

A

B

[Graph showing relative expression of Dio1, ApoA-I, Scarb1, Abca1, Abcg1 with Ad-LacZ and Ad-Cre]

[Graph showing relative expression of ApoA-I, ApoA-II, ApoB, ApoC-III, ApoE, Lcat, Ldlr with Ad-LacZ and Ad-Cre]
Supp. Figure I: Acute LIRKO mice and their controls were generated by injecting adenovirus Ad-LacZ or Ad-Cre into 10 week old Lox mice (n=6 per group). Livers from acute LIRKO mice (Day 6) and their controls (n=3 per group) were sent to Ocean Ridge Biosciences (ORB) for a cDNA microarray analysis using the Mouse Exonic Evidence Based Oligonucleotide (MEEBO). Microarray analysis revealed several downregulated genes involved in lipid metabolism. A. Genes involved directly in HDL metabolism, B. Genes important in lipoprotein metabolism. C. Microarray data for apolipoprotein ApoA-I, ApoAII, ApoB, ApoCIII and ApoE were verified by qPCR. Bar graphs depict mean ± sd. * p < 0.05, ** p < 0.01.
Supp. Figure II: Restoration of insulin signaling significantly increased the expression of Dio1 in LIRKO mice. Liver Dio1 mRNA was measured at day 6 after injecting 5 x 10^9 viral particles of adenovirus containing constitutively active AKT1 or LacZ into 16 week old LIRKO mice (n=4 per group). Bar graphs depict mean ± sd. ** p < 0.01.
Supp. Figures III: Acute L-AKT1/2 double knockout mice were created by injecting AAV-Cre into Lox AKT1/2 mice. AAV-GFP was used as a control (n=4, per group). A. HDLC was measured by FPLC following 16 hr overnight fast. B. Liver Dio1mRNA was measured by qPCR. Bar graphs depict mean ± sd. * p<0.05.
Supp. Figure IV: Liver T3 content was measured in LIRKO mice and in D1KO by radioimmunoassay following extraction as previously described \(^1\). **A.** Liver T3 content in 16 week old LIRKO mice and Lox mice (n=6 per group). **B.** Liver T3 content in 12 week old D1KO mice and WT mice (n=6 per group). Bar graphs depict mean ± sd. * p < 0.05.
Supp. Figure V: Liver ApoA-II, ApoB, ApoCIII and ApoE mRNA were quantified by qPCR in D1KO mice (n=10 per group).
Supp. Figure VI: Serum insulin levels were determined by rat insulin RIA kit in 16 week old WT and D1KO mice (n=7-8 per group)
Supp. Figure VII: Antisense oligonucleotide reduced Dio1 mRNA in mice. 10 week old C57BL/6J mice were injected intraperitoneally with a dose of 12.5 mg/kg of Dio1 ASO or Ctrl ASO twice per week. Mice were sacrificed after 8 week treatment. Liver Dio1mRNA were measured by qPCR. Bar graphs depict mean ± sd. ** p <0.01.
Supplemental Figure VIII

A. 10 week old C57BL/6J mice were injected intravenously with Ad-Dio1 or Ad-GFP (n=4 per group). After 12 days, A. 200ul of serum was pooled from each group and lipoproteins were resolved by FPLC. Cholesterol was measured in each of the eluted fraction (upper panel). The FPLC fractions representing HDL (fractions 27-33) were used for western blotting of ApoA-I (lower panel). B. liver Dio1mRNA and ApoA-I mRNA were measured by qPCR.

Supplemental Figure IX
Supp. Figure IX: Knockdown of *InsR* decreases *ApoA-I* mRNA expression in HepG2 cells. A. Western blot analysis of InsR protein expression after knockdown of *InsR* with siRNA for 2 days in HepG2 cells. B. *InsR*, *Dio1*, and *ApoA-I* mRNA were analyzed by qPCR after knockdown of *InsR*. Bar graphs depict mean ± SD. *p < 0.05. **p < 0.01
**Supp. Figure X**: HepG2 cells were co-transfected with ApoA-I promoter constructs containing only Region A, or Region B or Region C and thyroid hormone receptor beta (TRβ). 24h later, the medium was changed and 3nm T3 or vehicle was added. The cells were harvested after another 24h. Values are expressed as percentage of control (set at 100%) for each condition and normalized to internal control Renilla luciferase activity. In all experiments, luciferase activity was determined using the Dual-Luciferase Reporter Assay System. Data represent the means ± sd of at least 3 independent experiments. Bar graphs depict mean ± sd. * p < 0.05

**Supplemental References**