Effects of antisense-mediated inhibition of 11β-hydroxysteroid dehydrogenase type 1 on hepatic lipid metabolism

Running title: ASO inhibition of 11β-HSD 1 and hepatic lipid metabolism

Guoping Li[1], Antonio Hernandez-Ono[1], Rosanne M. Crooke[2], Mark J. Graham[2], Henry N. Ginsberg[1*]

[1]Department of Medicine, Columbia University, New York, NY 10032, USA

[2]Isis Pharmaceuticals, Inc., 1896 Rutherford Road, Carlsbad, CA 92008-7326, USA

[*]Correspondence: hng1@columbia.edu, Phone: (212) 305-9562, Fax: (212) 305-3213.

The antisense used for this work were provided by ISIS Pharmaceuticals; no other support was provided. Two of the authors, Rosanne Crooke and Mark Graham, are employees of ISIS Pharmaceuticals, which has other antisense treatments in clinical trials. Henry Ginsberg has consulted for ISIS during the past 5 years, but not on anything related to 11beta HSD1.

Abbreviations: 11β-HSD1, 11β-hydroxysteroid dehydrogenase 1; ACC, acetyl CoA carboxylase; apoB, apolipoproteinB; ASO, antisense oligonucleotide; FAS, fatty-acid synthase; FFA, free fatty acids; FPLC, fast performance liquid chromatography; LDL-C, LDL cholesterol; QPCR, quantitative real-time polymerase chain reaction; SREBP, sterol regulatory element binding protein; TG, triglyceride; VLDL, very low density lipoprotein; WTD, Western-type diet.

ABSTRACT

11β-hydroxysteroid dehydrogenase 1 (11β-HSD1) converts inactive 11-keto derivatives to active glucocorticoids within tissues and may play a role in the metabolic syndrome. We used an antisense oligonucleotide (ASO) to knockdown 11β-HSD1 in livers of C57BL/6J mice consuming a Western-type diet (WTD). 11β-HSD1 ASO treated mice consumed less food, so we compared them to ad-libitum fed mice and to food-matched one receiving control ASO. Knockdown of 11β-HSD1 directly protected mice from WTD-induced steatosis and dyslipidemia by reducing synthesis and secretion of triglyceride (TG), and increasing hepatic fatty acid oxidation. These changes in hepatic and plasma lipids were not associated with reductions in genes involved in de novo lipogenesis. However, protein levels of both sterol regulatory element-binding protein (SREBP) 1 and fatty acid synthase (FAS) were significantly reduced in mice treated with 11β-HSD1 ASO. There was no change in hepatic secretion of apolipoprotein B (apoB) indicating assembly and secretion of smaller apoB-containing lipoproteins by the liver in the 11β-HSD1-treated mice. Our results indicate that inhibition of 11β-HSD1 by ASO treatment of WTD-fed mice resulted in improvements in plasma and hepatic lipid levels, reduced lipogenesis by posttranslational regulation, and secretion of similar numbers of apoB-containing lipoproteins containing less TG per particle.

Key Words:

88 Fatty acid/Oxidation
90 Fatty acid/Synthesis
162 Lipoproteins/Metabolism
Apolipoproteins
165 Liver
271 Steroid hormones
282 Triglycerides

INTRODUCTION

The phenotypic similarities between individuals with the metabolic syndrome (MS) and individuals with Cushing's syndrome have stimulated considerable interest in the role of endogenous glucocorticoids in the pathogenesis of MS (1). Insulin resistance appears to play a central role in the pathophysiology of MS, and glucocorticoids have been shown to induce insulin resistance in multiple tissues (2-4). However, whereas plasma concentrations of glucocorticoids are elevated in Cushing's syndrome, circulating glucocorticoid levels are normal in patients with the MS (3;4). Glucocorticoid action on target tissues depends not only on circulating hormone levels, but also intracellular glucocorticoid receptors and the activities of both 11β-HSD1 and 11β-hydroxysteroid dehydrogenase 2 (11β-HSD2) (5-9). 11β-HSD1 is an $NADP^+$/NADPH dependent oxidoreductase that reversibly interconverts inactive cortisone and 11-dehydrocorticosterone in humans and rodents, respectively, to active cortisol and corticosterone. In liver and adipose tissue, the direction of the reaction is from inactive to active glucocorticoids (3;4;7). 11β-HSD1 is widely expressed, most notably in liver, adipose, vasculature, brain and macrophages (3;4;7;10), where it increases intracellular cortisol levels. 11β-HSD1 does not participate in adrenal cortisol biosynthesis from cholesterol.

Genetic manipulations that either deleted 11β-HSD1 in the whole animal (6;11;12), or resulted in over-expression in either adipose tissue (5) or liver (13), indicated significant roles for this enzyme in the regulation of body weight, energy metabolism, hepatic glucose and lipid metabolism, and blood pressure regulation. Based on those studies, as well as the demonstration of increased 11β-HSD1 activity in adipose tissue of obese rodents and humans (3;4;14;15), inhibition of 11β-HSD1 has become a major therapeutic target for MS, type 2 diabetes mellitus (T2DM), and hypertension (6;16-20). However, results of many of these studies have been difficult to interpret because, systemic or adipose-specific inhibition of 11β-HSD1 activity appears to alter food intake. Specifically, on a C57BL/6J background, knockout of 11β-HSD1 expression increased food intake

(12) whereas adipose specific deactivation of 11β-HSD1 by overexpression of 11β-HSD2 decreased food intake (21).

In view of the expression pattern of 11β-HSD1 (3) and the previously described tissue distribution of other intraperitoneally administered ASO (22;23), we hypothesized that we would see relatively specific inhibition of both hepatic and adipose tissue 11β-HSD1 by the cognate ASO. Importantly, we conducted the present studies, which focus on the effects of ASO-mediated inhibition of 11β-HSD1 on hepatic metabolism, with both ad libitum-fed and pair-fed mice receiving control ASO: this enabled us to differentiate direct effects of reduced 11β-HSD1 activity on lipid and carbohydrate metabolism from effects secondary to decreased food intake and concomitant differences in body weight. We present here results demonstrating that inhibition of 11β-HSD1 results in protection from a Western-type diet (WTD)-induced hepatic steatosis by directly reducing hepatic de novo lipogenesis and increasing hepatic β-oxidation.

MATERIALS AND METHODS

Animals, Diets and ASO

We studied male C57BL/6J wide-type mice purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were maintained on a 12-h light/12-h dark cycle (light cycle was 7 am to 7 pm) and were fed a WTD TD.88137 (Harlan laboratories), comprising, by calories, 42% fat (anhydrous milk fat), 43% carbohydrate, 15% by protein. This diet has been used my many groups studying lipoprotein metabolism in mice (24). In our first set of experiments, we observed reduced food intake in 11β-HSD1 ASO-treated mice compared to control ASO-treated mice. Therefore, we added a food matched control ASO group (FMC). Both control and 11β-HSD1 ASO treated mice were fed ad libitum for 12 weeks, after which these two groups of mice were started on treatment with either an ASO to 11β-HSD1 or an in-house universal control. FMC group started receiving control ASO treatment one week later, as this group was delayed one week in order to match food consumption to the 11β-HSD1 ASO-treated group. Each ASO was injected for 12 weeks at a dose of 50 mg/Kg of body weight intraperitoneally (IP) twice a week. During the last four weeks of treatment we determined rates of TG and apoB secretion in vivo, rates of lipogenesis, beta-oxidation in primary hepatocytes, etc. Animal studies were approved by the Institutional Animal Care and Use Committee of Columbia University College of Physicians and Surgeons.

ASO to 11β-HSD1 (ISIS 146039) has the following sequence: 5'TGTTGCAAGAATTTCTCATG3'. The in-house universal control ASO (ISIS 141923) has the sequence: 5'CCTTCCCTGAAGGTTCCTCC3'.

Body Weight and Food Intake

Body weights were recorded once a week. Mice were housed individually and daily food intake was calculated as the difference between the food remaining and original food provided, divided by 7 days. For the food matched control ASO treatment group mice, food was provided every afternoon at 17:00.

Blood Metabolites and Hormones

Blood for various plasma metabolites and hormones was obtained around 13:00 after a 4-6 hour fast. Since limited food was provided for FMC mice and, therefore, they most likely ate all their food rapidly as soon as it became available, the fasting period of FMC was almost certainly longer than that of ad libitum control and 11β-HSD1 ASO treated mice. Plasma total cholesterol (TC), TG and free fatty acid (FFA) concentrations were measured enzymatically (Wako Pure Chemicals USA). Corticosterone concentrations were determined by radioimmunoassay (MP Biomedicals, Inc.).

Determination of Hepatic VLDL TG and ApoB Secretion Rates

TG and apoB were determined as described previously (25). Briefly, 4 h-fasted mice were injected intravenously with a mixture of 200 mCi of [^{35}S]methionine (1175 Ci/mmol, PerkinElmer Life Sciences) and 500 mg/kg Triton WR1339 (Sigma-Aldrich) in 0.9% sodium chloride and bled prior to injection and at 30, 60, 90, and 120 mins. Because plasma VLDL clearance is completely inhibited in mice under these conditions, the accumulation of plasma TG and apolipoproteins in plasma after injection of Triton can be used to estimate rates of secretion of VLDL into the plasma compartment. Under the experimental conditions, secretion is linear for at least 2 hrs and we used the difference between plasma TG at the 120 and 0 time points is indicative of the rate at which TG was secreted from the liver. ApoB secretion is estimated from the accumulation of [^{35}S]methionine-labeled apoB in plasma sample from the 120 min time point. ApoB100 and apoB48 were isolated by 4% SDS-PAGE, and appearance of newly secreted, labeled proteins estimated by autoradiography and scintillation counting of each band.

Hepatic Lipids Determination

Mice were fasted 6-7 hrs, and then sacrificed. Liver tissues were isolated, snap-frozen into liquid nitrogen, and then all the tissues were transferred to -80°C. These tissues were analyzed for hepatic lipids determination, quantitative real-

time PCR and immunoblots. Total liver lipids were extracted by a modification of the method of Folch and Lees (26).

Quantitative Real-time PCR

Total RNA samples were used for cDNA synthesis with oligo-dT primers with a commercial kit from Invitrogen. Quantitative real-time PCR was done using SYBR Green PCR Master Mix (Agilent Technology) in triplicate using the Mx3005p Multiplex Quantitative PCR system (Stratagene). Expression of each target gene was quantified by transformation against a stardard curve and normalized to β-actin expression. The primers used in the real-time PCR are shown in Table 1.

Immunoblots

Protein extracts were prepared by using T-per tissue protein extraction buffer (Thermo Scientific) containing a complete, EDTA-free protein inhibitor cocktail tablet (Roche Diagnostics Corp.). The primary antibodies used were anti-11β-HSD1 (Cayman chemical), anti-SREBP1 (provided by Jay D. Horton), anti-FAS and anti-MTP (BD Biosciences Pharmingen), anti-apoAI (Calbiochem), and monoclonal anti-β-ACTIN (Sigma-Aldrich). Densitometry was carried out with the National Institutes of Health Image J program (27).

Measurement of Hepatic de Novo Lipogenesis

The rate of hepatic de novo lipogenesis was determined by measuring the amount of newly synthesized FA present in the liver 1 h after intraperitoneal injection of $^{3}H_2O$ 1 mCi into 4 hr fasted mice as previously described (28). ^{3}H-Labeled fatty acids were isolated by saponification of liver samples in KOH. After extraction of nonsaponifiable lipids, and acidification with H_2SO_4, the ^{3}H-labeled fatty acids were extracted and separated by thin layer chromatography. The plate was stained with iodine; the FA "spot" was scraped off the plate, and the isolated FA was added to scintillation fluid (National Diagnostics) and counted in a liquid scintillation counter (LS6500 Beckman Coulter). The specific activity of body water was determined and used to calculate de novo lipogenesis as micromoles of $^{3}H_2O$ incorporated into FA/h/g liver protein.

Beta-oxidation in Primary Hepatocytes

Beta-oxidation in primary hepatocytes was measured as previously described (29) with modifications. Briefly, mice were fasted for 4 hrs before livers were perfused through the vena cava with perfusion buffer (oxygenated Hanks' balanced saline solution, buffered with 10 mM Hepes) pH 7.4, at 37°C, 5 mins. Then the perfusion buffer was changed to isolation buffer containing 30 mg of collagenase (Type I, 245 units/mg, Worthington) and 5 mm Ca^{2+}, pH 7.6, at 37°C for 18 mins. 5.0×10^5 hepatocytes were plated into a 25-ml flask with a center well in 2ml DMEM containing 0.1mM oleic acid (OA), 1.5% BSA, and [^{14}C]OA (0.5μCi/ml) (NEC317, PerkinElmer Life Sciences). The same amount cells were saved in -80°C for protein concentration. The flasks were sealed with a stopper and rocked for 2 hrs at 37°C with 5% CO_2. Prior studies in our laboratory showed that CO_2 secretion was linear for the first 2 hrs (data not shown). After 2 hrs, 200μl of 70% perchloric acid was injected into the bottom of the flask, 200μL of 1M KOH was injected onto filter paper, and the flasks were incubated for an additional 1 h at 37°C. Then the saturated filter paper containing trapped [^{14}C]CO_2 was put into vials with 5ml liquid scintillation and assessed for radioactivity in a liquid scintillation counter. The media was spun in a microcentrifuge tube for 2 mins at 12,000xG and 200μl of supernatant was counted to assess the amount of ^{14}C-labeled acid-soluble metabolites (ASM), which represent labeled ketone bodies.

Fast Performance Liquid Chromatography analysis of lipoproteins

4-h fasted plasma samples from 10 mice each group (500μl total) were pooled and subjected to FPLC analysis using two Superose 6 columns in series (GE Healthcare Bio-Sciences Corp.). Fifty-five 0.5-ml fractions were collected. Cholesterol determinations of each fraction were made by using commercial kits (Wako Pure Chemicals USA) adapted to 96-well microtiter plates.

Statistics

Values are expressed as mean ± S.E. The significance of the differences in mean values among three different treatment groups was evaluated by general linear model with treatment as the model factor and day as a covariate since the FMC group received their ASO-treatment one week later followed by post hoc analysis with Bonferroni's test. $P < 0.05$ was considered to be statistically significant.

RESULTS

11β-HSD1 ASO decreased hepatic 11β-HSD1 expression, food intake and body weight

All mice received a WTD comprised, by calories, of 42% fat (anhydrous milk fat), 43% carbohydrate, and 15% by protein, 0.2% cholesterol by weight (24). After 12 weeks on the WTD, mice were treated intraperitoneally (IP) with either control ASO or 11β-HSD1 ASO at 50 mg/kg body weight twice weekly for 8 weeks while continuing on the WTD. Compared to control ASO, IP-treatment with 11β-HSD1 ASO resulted in dramatic reductions of mRNA and protein levels of the enzyme in the liver (Supplemental Figures (S) 1A, 1B). 11β-HSD1 ASO also reduced average food consumption by about 12% (Figure S1C) and average body weight by about 11% (Figure S1D). The effects of 11β-HSD1 treatment on fasting plasma parameters are presented in Supplemental Table 1. 11β-HSD1 ASO treatment did not change circulating corticosterone levels, but decreased fasting concentrations of total cholesterol (TC), triglycerides (TG). There was no change in fasting plasma FFA concentrations.

11β-HSD1 ASO protected mice from WTD-induced hepatic steatosis by diminishing lipogenesis and increasing fatty acid oxidation.

Effects of 11β-HSD1 treatment on hepatic lipid metabolism are depicted in Supplemental Figure 2A-2G. Compared to control ASO-treated mice, ASO-mediated knockdown of 11β-HSD1 resulted in lower TG content (Figure S2A) without changes in hepatic TC mass (Figure S2B), and lower rates of hepatic TG secretion (Figure S2C) without changes in secretion of either apolipoprotein (apo)B48 or apoB100 (Figure S2D). Reductions in 11β-HSD1 were associated with reduced hepatic de novo lipogenesis, as determined indirectly by demonstration of lower sterol regulatory element-binding protein (SREBP) 1 (Figure S2E) and fatty acid synthase (FAS) protein (Figure S2F), despite the absence of change in either *SREBP1C* or *FAS* mRNA levels (Figure S2G). Microsomal triglyceride transfer protein (*MTP*) mRNA, *APOB* mRNA, and MTP

protein levels in the liver were not affected by 11β-HSD1 ASO treatment (data not shown).

Decreased food intake and/or weight loss accounted for the reductions in fasting plasma TG, but not plasma TC in 11β-HSD1 ASO-treated mice

Because we observed significant reductions in food intake and weight over the 8 weeks of 11β-HSD1 ASO treatment in preliminary studies, we carried out all of the experiments with two control ASO groups, one that was fed ad libitum (hereafter referred to as the control ASO group) and one that was pair-fed (hereafter referred to as the food-matched control or FMC ASO group) to match the food intake of the 11β-HSD1 ASO-treated group. The ASO-treatment period was from 8-12 weeks, depending on the specific studies performed. As in the first series of experiments, IP-treatment with 11β-HSD1 ASO decreased hepatic *11β-HSD1* mRNA by approximately 90% (Figure 1A) and 11β-HSD1 protein by almost 100% (Figure 1B) compared to either the control ASO or the FMC ASO group. Food restriction alone had no effect on either hepatic *11β-HSD1* mRNA expression or protein levels. 11beta-HSD1 ASO treatment also reduced mRNA levels of the enzyme in some, but not all adipose tissue depots, and in lung, heart, and kidney. Brain and muscle expression of *11β-HSD1* were not affected by ASO treatment. Liver had by far the greatest expression of *11β-HSD1* mRNA and the largest percent suppression (Figure S3). We will present the effects of 11β-HSD1 ASO on adipose tissue in a separate report.

Compared to control ASO, 11β-HSD1 ASO treatment was associated with about 5% reduction in food consumption and about 10% decrease in body weight. In the FMC ASO group, although food intake (Figure 1C) was matched to the 11β-HSD1 ASO-treated group, body weight decreased by only 5.5% (Figure 1D). Weights of mice in the 11β-HSD1 ASO-treated group were significantly lower than weights of mice in the FMC ASO group. Notably limited food supply caused lower body weight of FMC than that of control ASO group. Levels of ALT, measured after 8-12 weeks of ASO treatment, were in the normal range

(between 30-40 IU/L) in all three groups and there were no differences amongst the groups (data not shown).

Plasma corticosterone levels were similar in the control ASO and 11β-HSD1 ASO groups, but were significantly elevated in FMC ASO mice, a sign of stress that has been observed before in FMC mice (19) (Table 2). In accord with the data in ad lib fed animals (Supplemental Table 1), compared to the control ASO group, 11β-HSD1 ASO treatment significantly decreased fasting TC (47%) and tended to reduce TG levels (22%). There was no effect on 11β-HSD1 treatment on fasting plasma FFA concentration. However, when compared to FMC, 11β-HSD1 ASO treatment had no significant effect on fasting levels of TG, whereas the reduction TC concentration was still evident.

11β-HSD1 ASO treatment had direct effects on plasma lipoproteins

To determine the basis for the reduction in TC in 11β-HSD1 ASO-treated mice, we performed FPLC separation of plasma lipoproteins. FPLC results indicated that both low density lipoprotein (LDL) and high density lipoprotein (HDL) cholesterol levels were decreased in 11β-HSD1 ASO-treated mice (Figure 2A). Concomitant with lower HDL cholesterol levels, we found that apoAI concentrations in plasma were also reduced in 11β-HSD1 ASO-treated mice (Figure 2B).

A survey of the expression of hepatic genes involved in cholesterol, LDL, and HDL metabolism indicated that 11β-HSD1 ASO treatment reduced expression of *ABCA1*, *ABCG5*, *ABCG8* and *APOAI* compared to at least one of the two control groups (Figure 2C). Of note, *SREBP2*, *HMGCR*, *PCSK9*, and *LDLR* mRNA levels were not affected by 11β-HSD1 ASO treatment. LDLR protein in the liver was actually reduced in mice treated with 11β-HSD1 ASO compared to FMC and control ASO-treated mice (Figure S4). Together, these data indicate that although decreased food intake and or weight loss accounted for the reductions in fasting plasma concentrations of TG, 11β-HSD1 ASO treatment had direct

effects on LDL and HDL cholesterol concentrations. The mechanisms underlying these effects are unclear, although a direct effect of 11β-HSD1 ASO treatment on *apoAI* gene expression may be the basis for the reduction in HDL cholesterol level.

11β-HSD1 ASO treatment directly decreased hepatic TG, TG secretion and de novo lipogenesis, and increased hepatic fatty acid oxidation

Compared to control ASO-treated mice and FMC ASO treated mice, ASO-mediated knockdown of 11β-HSD1 resulted in 43% and 30% reductions in hepatic TG content (Figure 3A). ASO-mediated knockdown of 11β-HSD1 did not affect hepatic cholesterol compared to control ASO-treated mice. The FMC ASO treated mice did, however, have reduced hepatic cholesterol content (Figure 3B). There was a 26% decrease in TG secretion in mice with reduced 11β-HSD1 compared to either control group (Figure 3C), and this occurred without changes in hepatic apoB48 or apoB100 secretion (Figure 3D). Hepatic de novo lipogenesis, measured in vivo, fell by 25% versus either control group (Figure 3E). The lower rates of de novo lipogenesis were associated with decreased levels of both the precursor and mature forms of SREBP1 protein (Figure 3F) and reductions in FAS protein (Figure 3G), despite no effects of 11β-HSD1 ASO on mRNA levels of *SREBP1C* and *FAS* (Figure 3H). β-oxidation, measured ex vivo in primary hepatocytes isolated from each group of mice was increased by 81% in the 11β-HSD1 ASO-treated mice compared to control ASO mice and 55% compared to FMC ASO controls (Figure 3I). Neither *MTP* nor *APOB* mRNA levels, nor MTP protein levels were altered by 11β-HSD1 ASO treatment compared to controls (data not shown).

11β-HSD1 ASO treatment had variable effects on hepatic gene expression profile

The expression of most lipogenic genes, including *SREBP1C*, *FAS* (Figure 3H), *LXRα*, *DGAT1*, *DGAT2* and *SCD1* (Figure 4A), was not altered by 11β-HSD1 ASO treatment. *ACC1* expression increased in the FMC ASO group. Genes

involved in fatty acid oxidation were not uniformly affected either by 11β-HSD1 ASO treatment; *ACOX1* expression was increased but mRNA levels of both *MCAD* and *L-PBE* were decreased (Figure 4B). *PPARα* and *CPT1* levels were increased in the FMC ASO controls. *PPARγ2* mRNA levels were reduced in 11β-HSD1 ASO-treated mice, as were expression levels of several of its downstream targets, including *ADRP*, *CIDEA* and *FSP27* (Figure 4C). These changes were not observed in the FMC group. Key gluconeogenic genes, *PGC1α* and *PEPCK*, were significantly decreased, and *G6Pase* tended to be lower in the 11β-HSD1 ASO-treated mice, but no obvious differences were found in other genes in the gluconeogenic pathway, such as *ChREBP*, *CREB*, *GR* and *HES1* (Figure 4D). HES1 protein was not increased either (data not shown). Of note, there were no differences in mRNA levels of gluconeogenic genes between the control ASO and the FMC ASO groups.

DISCUSSION

Soon after the purification (30) and cloning (31) of 11β-HSD1, a series of papers from Seckl and colleagues highlighted the therapeutic potential of inhibiting this enzyme in adipose tissue and liver. Beginning with their first, brief report of the total body knockout in 1997 (6), and followed by more detailed examinations of the knockout mouse (11;12) as well as generation of mice overexpressing 11β-HSD1 in adipose tissue (5) and liver (13), interest has continued to increase. Indeed, oral inhibitors developed by several pharmaceutical companies have demonstrated beneficial effects on glucose (16;32;33) and lipid metabolism (17) and/or atherosclerosis (18), albeit almost exclusively in rodents.

Although these studies have clearly increased our knowledge in this area, several important issues regarding the role of 11β-HSD1 in energy metabolism remain incompletely characterized. For example, in several rodent species, inhibition of glucocorticoid activation either in the whole body (12;18;19) or adipose tissue (21) is associated with weight loss, often with reduced food intake.

Of note, increased 11β-HSD1 activity in adipose tissue (5) caused increased body weight and food intake whereas isolated overexpression of 11β-HSD1 in the liver had no effect on those parameters (13). 11β-HSD1 effects on food intake may occur via hypothalamic signaling (34). The importance of the food intake issue was acknowledged in previous studies (5;17;19), but the effects of reduced energy intake and/or decreased body weight has not, however, been consistently addressed in the published studies. Thus although decreased energy intake and weight loss would be beneficial clinical effects of pharmacologic inhibitors of 11β-HSD1, such effects observed in rodents may not be nearly as dramatic in humans (16), making demonstration of direct effects of 11β-HSD1 inhibition crucial. Additionally, while consistent benefits of 11β-HSD1 inhibition on glucose tolerance and insulin resistance have been reported (albeit usually with concomitant weight loss), effects of enzyme inhibition on hepatic lipid metabolism have been variable and seemingly dependent on the rodent model studied.

In the present study, after identifying an effect of 11β-HSD1 ASO-treatment on food intake and weight gain (see Supplemental data), we utilized food matched ASO controls to allow clear characterization of effects of 11β-HSD1 inhibition that are independent of food intake. It is important to realize that FMC controls are not, however, perfect controls. In our studies, we found that reduced availability of food in the FMC ASO control mice was associated with increased levels of plasma corticosterone (Table 2), reduced levels of hepatic cholesterol, increased expression of genes associated with hepatic fatty acid oxidation. Thus our interpretation of the effects of simultaneous knockdown of hepatic and adipose tissue 11β-HSD1 take into account the results in both the ASO control and the FMC ASO control mice.

We first examined the role of 11β-HSD1 in the regulation of plasma lipid levels. Dyslipidemia, with increased plasma TG levels, decreased HDL cholesterol levels, and variable concentrations of small, cholesterol-ester depleted LDL, is a

characteristic abnormality of the insulin resistant state in humans (35;36). Previous studies of the effects of deletion, knockdown, or inhibition of 11β-HSD1 have reported varying results. In the first studies of lipid metabolism in the 11β-HSD1 knockout mouse, Morton et. al. (11) noted that MF1/129 mice had lower ad lib plasma TG levels and increased HDL cholesterol concentrations on a chow diet; there were no food intake or weight data in that report. In later studies, after the knockout mice had been bred to a C57BL/6J background, these investigators found lower plasma TG levels on a high fat diet in association with less weight gain (12). HDL cholesterol levels on that same diet were not shown (12). Adipose-specific overexpression of 11β-HSD1 resulted in increased plasma TG levels associated with significantly greater weight gain and food intake (5). On the other hand, hepatic overexpression of 11β-HSD1, which did not affect food intake or weight gain, did not alter plasma TG levels on either low- or high-fat diets (13). In the latter studies, HDL cholesterol was higher in the hepatic 11β-HSD1 transgenic mice on a low-fat diet, but was lower on the high-fat diet. We found that plasma levels of fasting TG tended to fall in the 11β-HSD1 ASO-treated mice compared to control mice (Supplemental Table 1), but this appeared to be due completely to reduced food intake (Table 2). Our finding differs from those of Wang et al. (19), who observed lower TG levels in mice treated with a pharmacologic inhibitor of 11β-HSD1 even when compared with TG levels in pair-fed controls. They also differ from the results reported by Berthiaume et al. (17), who demonstrated a robust reduction of plasma TG with a pharmacologic inhibitor that did not affect food intake or weight. We did find that ASO-mediated inhibition of 11β-HSD1 was associated with lower levels of both LDL and HDL cholesterol concentrations independent of caloric intake. Examination of genes relevant to the regulation of LDL cholesterol and HDL metabolism revealed reduced expression of apoA-I and ABCA1; these changes are consistent with known effects of glucocorticoids on expression of these genes (37;38). Both of these changes could be directly involved in the reduced HDL levels we observed. Of note, short-term administration of glucocorticoids results in increased HDL cholesterol levels in humans (39). In summary, when reduced food intake is

accounted for, 11β-HSD1 inhibition does not affect plasma TG levels but does reduce plasma cholesterol in both LDL and HDL in C56BL/6J mice on a WTD. HDL cholesterol was reduced mainly due to a decrease in apoA-I synthesis, at least as judged by reduced *apoA-I* mRNA levels; if a similar decrease occurs in humans, this would limit the benefits of a strategy to lower *11β-HSD1* expression or activity for the treatment of metabolic diseases. We do not, at present, have an explanation for the reduction in LDL cholesterol observed with 11β-HSD1 ASO treatment. Relevant genes, including SREBP2, PCSK9, and the LDL receptor were not altered. LDL receptor protein was unexpectedly decreased. Additional studies of hepatic cholesterol flux to bile or to the circulation via lipoprotein secretion will be required to define mechanisms for this observation. In any event, reduced levels of LDL cholesterol, if demonstrated in humans, would be an additional positive effect of inhibiting 11β-HSD1.

We next characterized the effects of 11β-HSD1 inhibition on hepatic lipid metabolism. High levels of circulating glucocorticoids are associated with fatty liver in humans (40) and mice (41) although the mechanisms linking this association have not been fully characterized. We demonstrated that 11β-HSD1 ASO treatment protected C57BL/6J mice from hepatic steatosis induced by a WTD by decreasing de novo lipogenesis and increasing β-oxidation compared to both control ASO and FMC ASO groups. Of note, we did not observe changes in the expression of key genes involved in either lipogenesis or fatty acid oxidation. We did, however, find that FAS and SREBP1 proteins (both precursor and mature forms) were significantly reduced in 11β-HSD1 ASO-treated mice compared to both control and FMC ASO groups. Expression of *SREBP1C*, *FAS*, were also not different in 11β-HSD1 knockout mice in ad libitum fed and 24 hrs fasted state studied by Morton et al. (11). However these lipogenic genes (*SREBP1C* and *FAS*) were upregulated more in 11β-HSD1 knockout mice than in wild-type mice after a 24 hr fast followed by either 4-hour or 24-hour period of refeeding (11). Morton et al. (11) concluded that this result was due to enhanced insulin sensitivity in the 11β-HSD1 knockout mice. Our results cannot be directly

compared to those of Morton et al. because we did not conduct studies using the fasting-refeeding paradigm, but measured liver parameters after a 4-6 hr fast. On the other hand, Morton et al. (11) did not measure SREBP-1c of FAS proteins. Alhough the fasting-refeeding paradigm is not physiologic, we acknowledge that hepatic de novo lipogenesis is very sensitive to ambient nutrient flux and hepatic insulin signaling, and our results might have been different if we had studied the mice while they were eating.

In mice with hepatic overexpression of 11β-HSD1 (13) hepatic TG was increased with a chow diet but tended to be lower than wild type on a high-fat diet. On chow, overexpression of 11β-HSD1, which did not affect food intake or weight, was associated with increased expression of *FAS* but not *SREBP1C*; on the high-fat diet, transgenic expression of 11β-HSD1 was actually associated with lower expression of those genes. Lemke, et. al. (9) reported reduced steatosis in db/db mice on a chow diet after treatment with an adenovirus carrying shRNA for the glucocorticoid receptor; this did not affect food intake or weight gain. They also could not link reduced hepatic fat to changes in lipogenic gene expression, but did not measure either protein levels of FAS and SREBP1 or lipogenesis itself. Post-translational regulation of FAS (42) and SREBP1 (43) have been demonstrated under other circumstances; the mechanism for such an effect by glucocorticoids will require further investigation.

We also demonstrated increased fatty acid oxidation measured directly in hepatocytes isolated from mice treated with 11β-HSD1 ASO. We did not, however, find a clear pattern of changes in the expression of genes involved in hepatic fatty acid oxidation; mRNA levels for *PPARα* and *CPT1α* were slightly but not significantly higher compared to control mice, but reduced compared to levels in the FMC ASO controls. Furthermore, although *ACOX1* mRNA levels were increased in 11β-HSD1 ASO-treated mice compared to control mice, the expression of the other two fatty acid oxidation related genes, *MCAD* and *L-PBE* was decreased. Together, our findings support post-translational regulation of

fatty acid oxidation by glucocorticoids. In contrast, when the *11β-HSD1* gene was knocked out (11), hepatic expression of *PPARα, CPT-1*, and *ACO* was increased; there were no data on food intake or weight in this study, although it is likely that the knockout mice weighed less than the controls (12). A recent in vivo report demonstrated that pharmacologic inhibition of 11β-HSD1 activity, that did not affect food intake or body weight, significantly increased hepatic fatty acid oxidation: hepatic gene expression studies were also variable in that study, with no changes in *PPARα* or *CPT-1* mRNA levels but increased in some (but not all) of the mitochondrial oxidation genes (44). We cannot fully explain the differences in our findings, but overall, it seems clear that reductions in 11β-HSD1 activity results in increased fatty acid oxidation by the liver.

Lemke, et. al. (9), utilizing the alternative approach of knockdown of the glucocorticoid receptor in the liver, also observed increases in circulating levels of ketones as well as increased *CPT1α* and *ACAA2* mRNA levels in db/db mice. In addition to reducing the glucocorticoid receptor, rather than *11β-HSD1* expression, another major difference between the report by Lemke et. al. (9) and our studies is their identification of Hes1 as a key regulator of hepatic steatosis; *HES1* mRNA and protein were increased in their mice with shRNA knockdown of the glucocorticoid receptor, and were reduced in mice and hepatocytes treated with dexamethasone. Additionally, replenishment of Hes1 by adenoviral overexpression in db/db mice was associated with reductions in steatosis (9). *HES1* mRNA and protein levels (data not shown) were not affected by 11β-HSD1 ASO treatment in our study. We cannot explain these different results. In summary, our studies, done with proper controls for food intake, demonstrate significant direct effects of 11β-HSD1 inhibition on hepatic lipogenesis and oxidation that resulted in protection from WTD-induced steatosis in C57BL/6J mice.

We also demonstrated that inhibition of 11β-HSD1 was associated with reductions in TG secretion with unchanged apoB secretion; 11β-HSD1 inhibition

resulted, therefore, in the assembly and secretion of smaller VLDL particles. Our findings are concordant with those of Berthiaume, et.al. (17), who observed significant reductions in liver TG content and TG secretion during treatment of rats with a pharmacologic inhibitor of 11β-HSD1. Importantly, the pharmacologic inhibitor used by Berthiaume et al. did not affect food intake or weight (17). Lemke, et. al. (9) also demonstrated decreased secretion of TG concomitant with knockdown of the glucocorticoid receptor. Neither of those groups measured apoB secretion. It is well known that livers can secrete VLDL across a range of size and density by varying the quantity of TG secreted with each apoB (45). In particular, increased hepatic TG as a consequence of increased de novo lipogenesis has been linked to secretion of the same number of larger VLDL particles (46); our results suggest that dissociation of TG and apoB secretion also occurs when de novo lipogenesis is reduced.

A limitation of this study is the uptake of ASO not only by the liver, but also by adipose tissue (Supplemental Figure 3). Although *11β-HSD1* expression is much lower in adipose tissue compared to the liver, ASO treatment significantly reduced that expression, compared to FMC mice, in both epididymal and subcutaneous fat depots, and tended to reduce expression in mesenteric fat. There was no effect of 11β-HSD1 ASO treatment on either mesenteric or BAT expression of the enzyme. Furthermore, there was no effect of ASO treatment on *11β-HSD1* in muscle or brain. We did observe changes in adipose tissue metabolism and mass, and this will be the subject of a separate report. On the other hand, plasma levels of free fatty acids did not differ between any of the experimental groups.

In conclusion, this report demonstrates that selective 11β-HSD1 inhibition in the liver improves WTD induced obesity, dyslipidemia, and steatosis, independent of reduced food intake and weight loss, in the WTD model of obesity in C57BL/6J mice. Inhibition of 11β-HSD1 in the liver had direct effects on hepatic de novo lipogenesis, fatty acid oxidation, and VLDL secretion independent of food intake

and weight loss. These findings indicate that inhibition of the activation of glucocorticoids by 11β-HSD1 in liver should have beneficial effects in humans even if food intake and weight are unaffected. The mechanisms underlying the weight loss in 11β-HSD1 ASO-treated mice compared to FMC matched controls suggests greater energy expenditure in the former group; studies are underway to examine this possibility.

Acknowledgements: This work was supported by the following grants: NIH/National Heart, Lung, and Blood Institute R01 HL73030 (Ginsberg) and R01 HL55638 (Ginsberg).

Reference List

1. Walker,B.R., and Seckl,J.R. 2001. Cortisol metabolism. *International Textbook of Obesity*241-268.

2. Morgan,S.A., Sherlock,M., Gathercole,L.L., Lavery,G.G., Lenaghan,C., Bujalska,I.J., Laber,D., Yu,A., Convey,G., and Mayers,R.e.a. 2009. 11β-hydroxyteroid dehydrogenase type 1 regulates glucocorticoid-induced insulin resistance in skeletal muscle. *Diabetes* **58**:2506-2515.

3. Seckl,J.R., Morton,N.M., Chapman,K.E., and Walker,B.R. 2004. Glucocorticoids and 11β-hydroxysteroid dehydrogenase in adipose tissue. *Recent Prog Horm Res* **59**:359-393.

4. Morton,N.M., and Seckl,J.R. 2008. 11β-hydroxysteroid dehydrogenase type 1 and obesity. *Front. Horm. Res* **36**:146-164.

5. Masuzaki,H., Paterson,J., Shinyama,H., Morton,N.M., Mullins,J.J., Seckl,J.R., and Flier,J.S. 2001. A transgenic model of visceral obesity and the metabolic syndrome. *Science* **294**:2166-2170.

6. Kotelevtsev,Y., Holmes,M.C., Burchell,A., Houston,P.M., Schmoll,D., Jamieson,P.M., Best,R., Brown,R., Edwards,C.R., Seckl,J.R. et al 1997. 11β-hydroxysteroid dehydrogenase type 1 knockout mice show attenuated glucocorticoid inducible responses and resist hyperglycaemia on obesity or stress. *Proc Natl Acad Sci USA* **94**:14924-14929.

7. Paterson,J.M., Seckl,J.R., and Mullins,J.J. 2005. Genetic manipulation of 11β-hydroxysteroid dehydrogenases in mice. *Am J Physiol Regul Integr Comp Physiol* **289**:R642-R652.

8. Kotelevtsev,Y., Brown,R.W., Fleming,S., Kenyon,C., Edwards,C.R., Seckl,J.R., and Mullins,J.J. 1999. Hypertension in mice lacking 11β-hydroxysteroid dehydrogenase type 2. *J Clin Invest.* **103**:683-689.

9. Lemke,U., Krones-Herzig,A., Berriel,D.M., Narvekar,P., Ziegler,A., Vegiopoulos,A., Cato,A.C., Bohl,S., Klingmuller,U., Screaton,R.A. et al 2008. The glucocorticoid receptor controls hepatic dyslipidemia through Hes1. *Cell Metab* **8**:212-223.

10. Chapman,K.E., Coutinho,A.E., Gray,M., Gilmour,J.S., Savill,J.S., and Seckl,J.R. 2009. The role and regulation of 11β-hydroxysteroid dehydrogenase type 1 in the inflammatory response. *Mol Cell Endocrinol* **301**:123-131.

11. Morton,N.M., Holmes,M.C., Fievet,C., Staels,B., Tailleux,A., Mullins,J.J., and Seckl,J.R. 2001. Improved lipid and lipoprotein profile, hepatic insulin sensitivity, and glucose tolerance in 11β-hydroxsteroid dehydrogenase type 1 null mice. *J Biol Chem* **276**:41293-41300.

12. Morton,N.M., Paterson,J.M., Masuzaki,H., Holmes,M.C., Staels,B., Fievet,C., Walker,B.R., Flier,J.S., Mullins,J.J., and Seckl,J.R. 2004. Novel adipose tissue-mediated resistance to diet-induced visceral obesity in 11β–hydroxysteroid dehydrogenase type 1-deficient mice. *Diabetes* **53**:931-938.

13. Paterson,J.M., Morton,N.M., Fievet,C., Kenyon,C.J., Holmes,M.C., Staels,B., Seckl,J.R., and Mullins,J.J. 2004. Metabolic syndrome without obesity: Hepatic overexpression of 11β-hydroxysteroid dehydrogenase type 1 in transgenic mice. *Proc Natl Acad Sci USA* **101**:7088-7093.

14. Rask,E., Olsson,T., Soderberg,S., Andrew,R., Livingstone,D.E.W., Johnson,O., and Walker,B.R. 2001. Tissue-specific dysregulation of cortisol metabolism in human obesity. *J Clin Endocrinol Metab* **86**:1418-1421.

15. Walker,B.R., and Andrew,R. 2006. Tissue production of cortisol by 11β-hydroxysteroid dehydrogenase type 1 and metabolic disease. *Ann, NY Acad. Sci.* **1083**:165-184.

16. Rosenstock,J., Banarer,S., Fonseca,V.A., Inzucchi,S.E., Sun,W., Yao,W., Hollis,G., Flores,R., Levy,R., Williams,W.V. et al 2010. The 11–β-hydroxysteroid dehydrogenase type 1 inhibitor INCB13739 improves hyperglycemia in patients with type 2 diabetes inadequately cotrolled by metformin monotherapy. *Diabetes Care* **33**:1516-1522.

17. Berthiaume,M., Laplante,M., Festuccia,W.T., Cianflone,K., Turcotte,L.P., Joanisse,D.R., Olivecrona,G., Thieringer,R., and Deshaies,Y. 2007. 11β-HSD1 inhibition improves triglyceridemia through reduced liver VLDL secretion and partitions lipids toward oxidative tissues. *Am J Physiol Endocrinol Metab* **293**:E1045-E1052.

18. Hermanowski-Vosatka,A., Balkovec,J.M., Cheng,K., Chen,H.Y., Hernandez,M., Koo,G.C., Grand,L.C.B., Li,Z., Metzger,J.M., and Mundt,S.S.e.a. 2005. 11β-HSD1 inhibition ameliorates metabolic syndrome and prevents progression of atherosclerosis in mice. *J Exp Med* **202**:517-527.

19. Wang,S.J., Birtles,S., Schoolmeester,J.d., Swales,J., Moody,G., Hislop,D., O'Dowd,J., Smith,D.M., Turnbull,A.V., and Arch,J.R. 2006. Inhibition of 11β-hydroxysteroid dehydrogenasse type 1 reduces food intake and

weight gain but maintains energy expenditure in diet-induced obese mice. *Diabetologia* **49**:1333-1337.

20. Lloyd,D.J., Helmering,J., Cordover,D., Bowsman,M., Chen,M., Hale,C., Fordstrom,P., Zhou,M., Wang,M., and Kaufman,S.A. 2009. Antidiabetic effects of 11β-HSD1 inhibition in a mouse model of combined diabetes, dyslipidaemia and atherosclerosis. *Diabetes Obes. Metab.* **11**:688-699.

21. Kershaw,E.E., Morton,N.M., Dhillon,H., Ramage,L., Seckl,J.R., and Flier,J.S. 2005. Adipocyte-specific glucocorticoid inactivation protects against diet-induced obesity. *Diabetes* **54**:1023-1031.

22. Koizumi,M., Takagi-Sato,M., Okuyama,R., Araki,K., Sun,W., and Nakai,D. 2007. In vivo antisense activity of ENA oligonucleotides targeting PTP1B mRNA in comparison of that of 2'-MOE-modified oligonucleotides. *Nucleic Acids Symp, Ser* **51**:111-112.

23. Nagai,Y., Yonemitsu,S., Erion,D.M., Iwasaki,T., Stark,R., Weismann,D., Dong,J., Zhang,D., Jurczak,M.J., Loffler,M.G. et al 2009. The role of peroxisome proliferator-activated receptor gamma coactivator-1 beta in the pathogenesis of fructose-induced insulin resistance. *Cell Metab* **9**:252-264.

24. Hayek,T., Ito,Y., Azrolan,N., Verdery,R.B., Aalto-Setala,K., Walsh,A., and Breslow,J.L. 1993. Dietary fat increases high density lipoprotein (HDL) levels both by increasing the transport rates and decreasing the fractional catabolic rates of HDL cholesterol ester and lipoprotein (Apo) A-I. *J Clin Invest* **91**:1665-1671.

25. Siri,P., Candela,N., Ko,C., Zhang,Y., Eusufzai,S., Ginsberg,H.N., and Huang,L.S. 2001. Post-transcriptional stimulation of the assembly and secretion of triglyceride-rich apolipoprotein B-lipoproteins in a mouse with selective deficiency of brown adipose tissue, obesity and insulin resistance. *J Biol Chem* **276**:46064-46072.

26. Folch,J., and Lees,M. 1957. A simple method for the isolation and purifcation of total lipids from animal tissues. *J Biol Chem* **224**:497-509.

27. Rasband,W.S. 2009. "Image J", U.S. National Institutes of Health, Bethesda, Maryland, USA. http://rsb. *info. nih. gov/ij/*2009.

28. Zhang,Y.L., Hernandez-Ono,A., Siri,P., Weisberg,S., Conlon,D., Graham,M.J., Crooke,R.M., Huang,L.S., and Ginsberg,H.N. 2006. Aberrant hepatic expression of PPARgamma2 stimulates hepatic lipogenesis in a mouse model of obesity, insulin resistance, dyslipidemia, and hepatic steatosis. *J Biol Chem* **281**:37603-37015.

29. Erol,E., Kumar,L.S., Cline,G.W., Shulman,G.I., Kelly,D.P., and Binas,B. 2004. Liver fatty acid binding protein is required for high rates of hepatic fatty acid oxidation but not for the action of PPARalpha in fasting mice. *FASEB* **18**:347-349.

30. Lakshmi,V., and Monder,C. 1998. Purification and characterization of the corticosteroid 11 β–dehydrogenase component of the rat liver 11 β-hydroxysteroid dehydrogenase complex. *Endocrinology* **123**:2390-2398.

31. Agarwal,A.K., Monder,C., Eckstein,B., and White,P.C. 1989. Cloning and expression of rat cDNA encoding corticosteroid 11 β-dehydrogenase. *J Biol Chem* **264**:18939-18943.

32. Alberts,P., Nilsson,C., Selen,G., Engblom,L.O., Edling,N.H., Norling,S., Klingstrom,G., Larsson,C., Forsgren,M., and Ashkzari,M.e.a. 2003. Selective inhibition of 11 β-hydroxysteriod dehydrogenase type 1 improves hepatic insulin sensitivity in hyperglycemic mice strains. *Endocrinology* **144**:4755-4762.

33. Veniant,M.M., Hale,C., Hungate,R.W., Gahm,K., Emery,M.G., Jona,J., Joseph,S., Adams,J., Hague,A., and Moniz,G.e.a. 2010. Discovery of a potent, orally active 11β-hydroxysteroid dehydrogenase type 1 inhibitor for clinical study: identification of S-2-1S,2S4F-bicyclo{2.2.1} heptan-2-ylamino-5-isopropyl-5-methylthiazol-45H-one AMG221. *J Med Chem* **53**:4481-4487.

34. Densmore,V.S., Morton,N.M., Mullins,J.J., and Seckl,J.R. 2006. 11β-hydroxysteroid dehydrogenase type 1 induction in the arcuate nucleus by high-fat feeding: A novel constraint to hyperphagia? *Endocrinology* **147**:4486-4495.

35. Chahil,T.J., and Ginsberg,H.N. 2006. Diabetic dyslipidemia. *Endocrinol Metab Clin N Am* **35**:491-510.

36. Ginsberg,H.N., Zhang,Y.L., and Hernandez-Ono,A. 2005. Regulation of plasma triglycerides in insulin resistance and diabetes. *Archives Medical Research* **36**:232-240.

37. Hargrove,G.M., Junco,A., and Wong,N.C. 1999. Hormonal regulation of apolipoprotein A1. *J Mol Endocrinol* **22**:103-111.

38. Sporstol,M., Mousavi,S.A., Eskild,W., Roos,N., and Berg,T. 2007. ABCA1, ABCG1 and SR-B1: hormonal regulation in primary rat hepatocytes and human cell lines. *BMC Mol Biol* **8**:5.

39. Brotman,D.J., Girod,J.P., Garcia,M.J., Patel,J.V., Gupta,M., Posch,A., Saunders,S., Lip,G.Y., Worley,S., and Reddy,S. 2005. Effects of short-

term glucocorticoids on cardiovascular biomarkers. *J Clin Endocrinol Metab* **90**:3202-3208.

40. Itoh,S., Igarashi,M., Tsukada,Y., and Ichinoe,A. 1997. Nonalcoholic fatty liver with alcoholic hyalin after long-term glucocorticoid therapy. *Acta Hepatogastroenterol* **24**:415-418.

41. Vegiopoulos,A., and Herzig,S. 2007. Glucocorticoids, metabolism and metabolic diseases. *Mol Cell Endocrinol* **275**:43-61.

42. Kim,K.S., Park,S.W., and Kim,Y.S. 1992. Regulation of fatty acid synthase at transcripional and post-transcriptional levels in rat liver. *Yonsei Med. J.* **33**:199-208.

43. Horton,J.D., Goldstein,J.L., and Brown,M.S. 2002. SREBPs: activators of the complete program of cholesterol and fatty acidsynthesis in the liver. *J Clin Invest* **109**:1125-1131.

44. Berthiaume,M., Laplante,M., Festuccia,W.T., Berger,J.P., Thieringer,R., and Deshaies.Y. 2010. Preliminary report: pharmacologic 11beta-hydroxysteroid dehydrogenase type 1 inhibition increases hepatic fat oxidation in vivo and expression of related genes in rats fed an obesogenic diet. *Metabolism* **59**:114-117.

45. Fisher,E.A., and Ginsberg,H.N. 2002. Complexity in the secretory pathway: The assembly and secretion of apolipoprotein B-containing lipoproteins. *J Biol Chem* **277**:17377-17380.

46. Wiegman,C.H., Bandsma,R.H.J., Ouwens,M., van der Sluijs,F.H., Havinga,R., Boer,T., Reijngoud,D.-J., Romijn,J.A., and Kuipers,F. 2003. Hepatic VLDL production in ob/ob mice is not stimulated by massive de novo lipogenesis but is less sensitive to the suppressive effects of insulin. *Diabetes* **52**:1081-1089.

FIGURE LEGENDS

Figure 1. 11β-HSD1 ASO treatment decreased hepatic 11β-HSD1 transcript and protein, and reduced food intake and body weight. Food matched mice receiving control ASO had the exact same food intake, but higher body weight than 11β-HSD1 ASO-treated group

(A) The level of *11β-HSD1* mRNA, measured by QPCR, with mouse *β-ACTIN* as an internal control, in liver after 12 weeks of treatment with a control ASO (in both control and FMC mice) or an ASO against 11β-HSD1. **(B)** 11β-HSD1 protein levels determined by immunoblotting with mouse *β*-ACTIN as an internal control. The average values of densitometry scans from control ASO treated mice were set as 100%. A representative Western blot is depicted. **(C-D)** Food intake and body weight were determined during 12 weeks of ASO treatment. Food consumption was restricted in the FMC group according to the food consumption of 11β-HSD1 ASO-treated mice during the preceding week. Data are presented as means ± SEM (n=12-34 per group). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, 11β-HSD1 vs control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, 11β-HSD1 vs FMC; $^{\&}p < 0.05$, FMC vs control; $^{\&\&}p < 0.01$, FMC vs control.

Figure 2. 11β-HSD1 ASO treatment decreased fasting plasma levels of LDL cholesterol, HDL cholesterol, and plasma apoAI. Expression of apoAI and several ABC transporters was also reduced.

(A) Plasma samples from 10 mice in each treatment group were pooled for FPLC separation of lipoproteins using two Superose 6 columns in series. **(B)** Plasma apoAI was determined by immunoblotting with mouse *β*-ACTIN as an internal control. A representative Western blot is shown. **(C)** Genes related to cholesterol metabolism were determined using QPCR and normalized by *β-ACTIN* mRNA. Data are presented as means ± SEM (n=10-20 per group). $^{*}p <0.05$, $^{**}p < 0.01$, 11β-HSD1 vs control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, 11β-HSD1 vs FMC.

Figure 3. 11β-HSD1 ASO treatment significantly decreased hepatic TG content, TG secretion, lipogenic proteins and de novo lipogenesis, and increased beta-oxidation. Treatment with 11β-HSD1 ASO did not alter hepatic TC content, apoB secretion, or expression of lipogenic genes

(A, B) Total liver TG and TC. **(C)** TG Secretion. **(D)** Newly synthesized apoB secretion. **(E)** Hepatic de novo lipogenesis was measured *in vivo* with $^{3}H_20$. **(F, G)** Hepatic levels of the precursor (p-) and mature (m-) forms SREBP1 protein, as well as FAS protein. The average values of densitometry scans from control ASO-treated mice were set as 100%. **(H)** *SREBP1C* and *FAS* gene expression. **(I)** Beta-oxidation was measured *ex vivo*, in primary hepatocytes, using ^{14}Colieic acid. All data are presented as means ± SEM (n=5-14 per group). $^{*}p < 0.05$, $^{***}p < 0.001$, 11β-HSD1 vs control; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$, 11β-HSD1 vs FMC; $^{\&\&\&}p < 0.001$, FMC vs control.

Figure 4. 11β-HSD1 ASO treatment had variable effects on hepatic genes involved in TG synthesis and fatty acid oxidation but significantly decreased genes involved in hepatic lipid droplet formation and gluconeogenesis.

Expression of genes was determined using QPCR after 12 weeks ASO treatment and normalized with *β-ACTIN* mRNA. **(A)** Lipogenic genes. **(B)** Beta-oxidation genes. **(C)** Lipid droplet formation genes. **(D)** Gluconeogenesis genes. Expression of each gene in ad libidum-fed control mice was set at 100%. Data are presented as means ± SEM (n=14-20). $^{*}p <0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, 11β-HSD1 vs control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, 11β-HSD1 vs FMC; $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$, FMC vs control.

Table 1. Primer sequences of genes used for quantitative RT-PCR.

Gene	Forward (5'→3')	Reverse (5'→3')
11β-HSD1	AGTACACCTCGCTTTTGCGT	CTCTCTGTGTCCTTGGCCTC
BETA-ACTIN	GTATCCATGAAATAAGTGGTTACAGG	GCAGTACATAATTTACACAGAAGCA
SREBP1C	GGCACTAAGTGCCCTCAACCT	GCCACATAGATCTCTGCCAGTGT
FAS	CCTGGATAGCATTCCGAACCT	AGCACATCTGCAAGGCTACACA
SREBP2	CAGGGAACTCTCCCACTTGA	GAGACCATGGAGACCCTCAC
ABCA1	TCCGGATTCTTTGTCAGCTT	AGCCGTAGATGGACAGGATG
ABCG5	GGCTGCTTATTGGATCTGGA	TTGGATCCACCACAAGTGAA
ABCG8	CACCTTCCACATGTCCTCCT	GCAGGCACTATCCACAGGTT
HMG-CoA reductase	CCAGCTTGTGGTAGCTTTCAG	GTGGGAACTCTGGTTCTTTCC
PCSK9	ATGGACTCTTGCCACACACA	AGGTCCTTCAGAGCAGGTCA
LDLr	GCAAGGACAAGTCAGATGAGG	CATTGACGCAGCCGAGCTCG
APOA1	ACCCACCTGAAGACACTTGG	GGCCTTGTCGATCACACTCT
LXRα	GCTCTGCTCATTGCCATCAG	TGTTGCAGCCTCTCTACTTGGA
ACC1	GGAGGACCGCATTTATCGA	TGACCAGATCAGAGTGCCT
DGAT1	GTGCACAAGTGGTGCATCAG	CAGTGGGATCTGAGCCATC
DGAT2	AGTGGCAATGCTATCATCATCGT	AAGGAATAAGTGGGAACCAGATCA
SCD1	TGCGATACACTCTGGTGCTC	AGGATATTCTCCCGGGATTG
PPARα	AGGGTTGAGCTCAGTCAGGA	GGTCACCTACGAGTGGCATT
CPT1α	CATGTCAAGCCAGACGAAGA	TGGTAGGAGAGCAGCACCTT
ACOX1	CTTGGATGGTAGTCCGGAGA	TGGCTTCGAGTGAGGAAGTT
HMC-CoA synthase 2	TGAATCCTGGGTGTCTCTCC	CTGTGGGGAAAGATCTGCAT
MCAD	GAGCCCGGATTAGGGTTTAG	TCCCCGCTTTTGTCATATTC
L-PBE	CTACCTGAGGAGGCTGGTTG	CCATACATGGCAAAATGCAG
PPARγ2	AACTCTGGGAGATTCTCCTGTTGA	TGGTAATTTCTTGTGAAGTGCTCATA
ADRP	AGCATCGGCTACGACGACACC	CAGCATTGCGGAATACGGAG
CIDEa	CTCGGCTGTCTCAATGTCAA	CAGGAACTGTCCCGTCATCT
CIDEb	CCAAAGCAACAGGGAGAGAG	GCTGAGTTCCAGACCCTACG
FSP27	CAGAAGCCAACTAAGAAGATCG	TGTAGCAGTGCAGGTCATAG
PGC1α	GAGTCTGAAAGGGCCAAACA	ACGGTGCATTCCTCAATTTC
PEPCK	ATCTTTGGTGGCCGTAGACCT	GCCAGTGGGCCAGGTATTT
G6Pase	TCCTCTTTCCCATCTGGTTC	TATACACCTGCTGCGCCCAT
ChREBP	GTCCGATATCTCCGACACACTCTT	CATTGCCAACATAAGCATCTTCTG
CREB	ATGTTCCCTGGTTGCTTGTC	GGTTGCTCTGCAGAAAGGAG
GR	CCTCTCTGTCGGGGTAGCAC	ACAGACTTTCGGCTTCTGGA
HES1	AGCCACTGGAAGGTGACACT	GCCAATTTGCCTTTCTCATC

Table 2. Fasting plasma lipid and hormone levels after 8-12 weeks ASO treatment

	Control	FMC	11β-HSD1
Corticosterone (ng/ml)	120.5±7.0	156.4±8.5$^{\&\#}$	102.8±5.7
Total cholesterol (mg/dl)	209.2±6.0	194.4±7.9	111.5±8.4$^{***\#\#\#}$
Triglyceride (mg/dl)	33.4±3.1	26.3±1.8	26.1±2.5
Free fatty acid (mM)	0.503±0.033	0.485±0.030	0.506±0.038

Data are expressed as means ± SEM (n=12-14). $^{***}p < 0.001$, 11β-HSD1 vs control; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$, 11β-HSD1 vs FMC; $^{\&}p < 0.05$, control vs FMC.

Figures:

Figure 1.

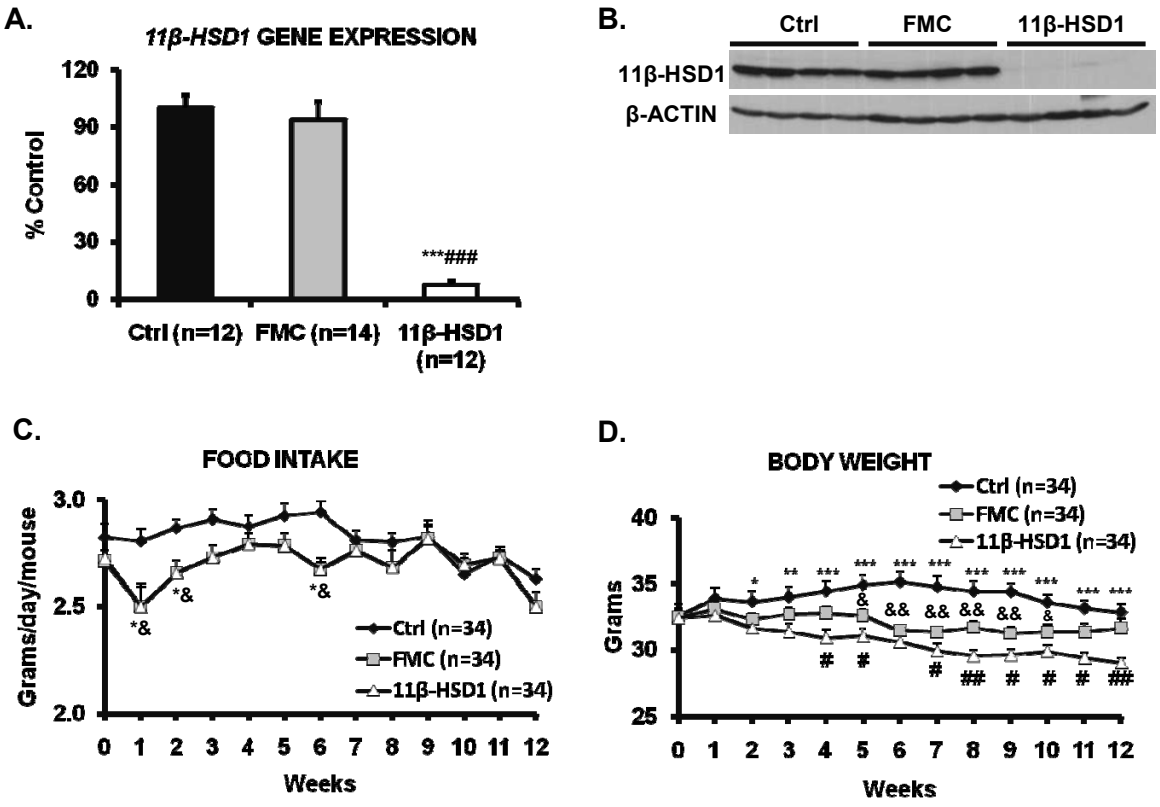

Figure 2.

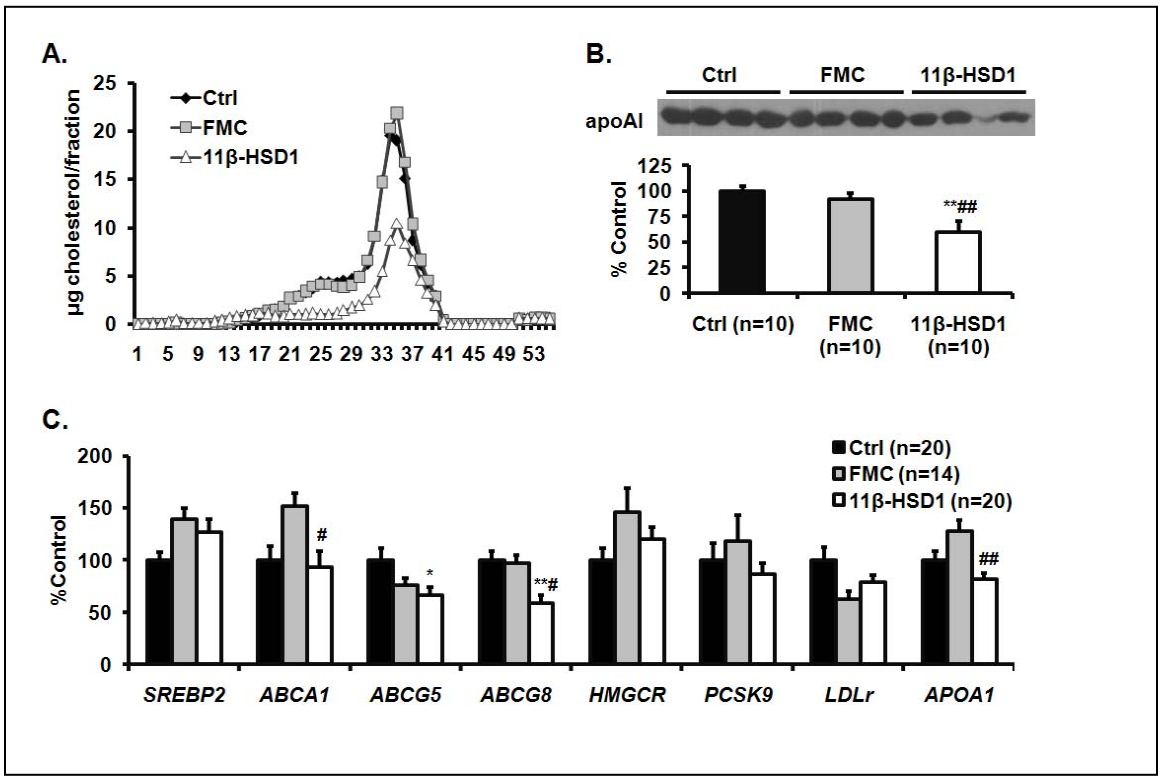

Figure 3.

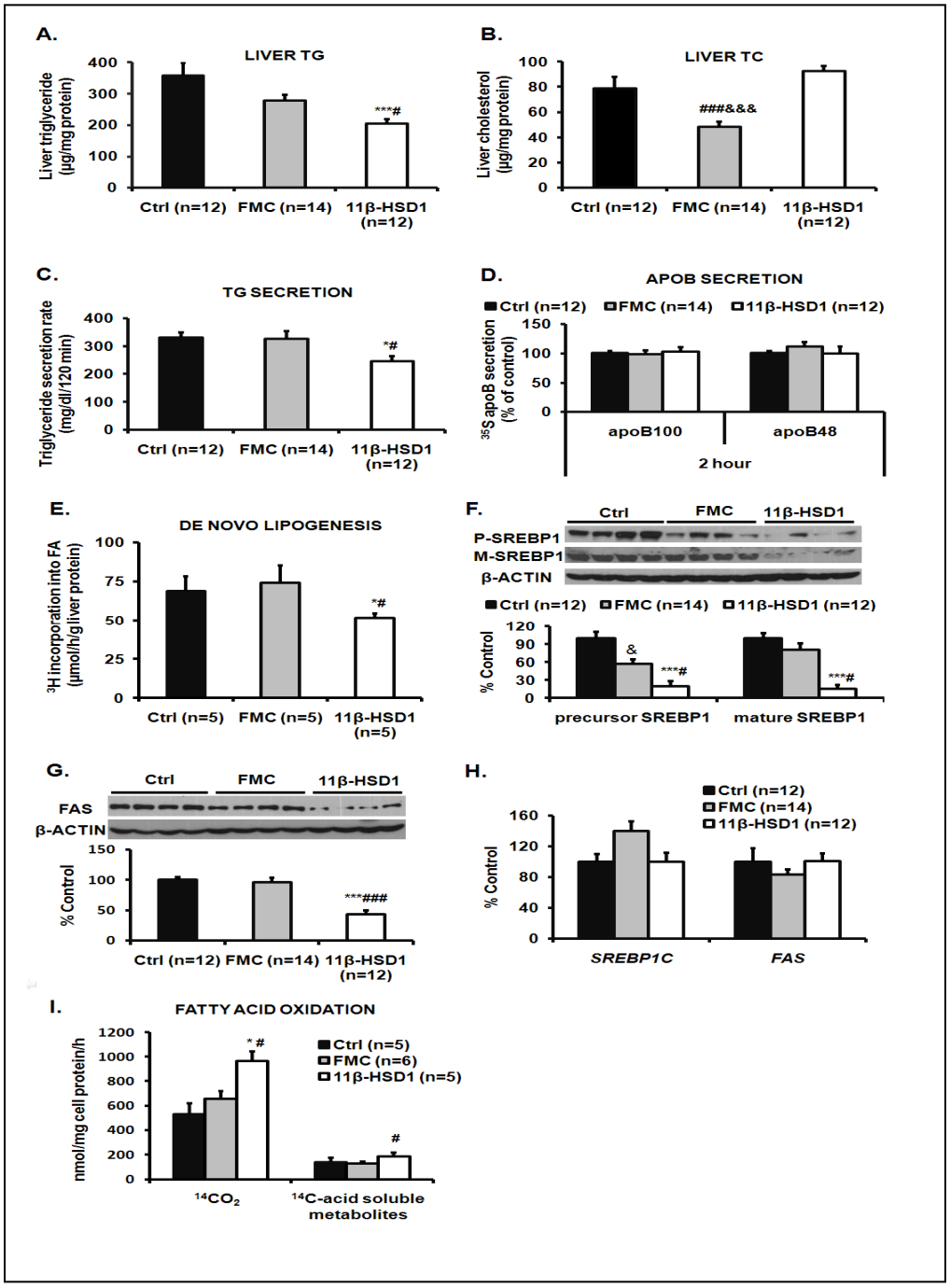

Figure 4.