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Inhibition of 5α-reductases impairs androgen and glucocorticoid metabolism and induces insulin resistance in humans and rodents. The contribution of hepatic glucocorticoids to these adverse metabolic changes was assessed utilizing a liver-selective glucocorticoid receptor (GR) antagonist, A-348441.

Mice lacking 5α-reductase 1 (5αR1-KO) and their littermate controls were studied on a high fat diet, with or without A-348441(120 mg.kg^{-1}.day^{-1}). Male mice C57BL/6 (12 weeks) were also studied receiving dutasteride (1.8 mg.kg^{-1}.day^{-1} or vehicle) within a high fat diet, with or without A-348441.

In 5αR1-KO mice, hepatic GR antagonism improved diet-induced insulin resistance, but not more than controls. Liver steatosis was not affected by hepatic GR antagonism in either 5αR1-KO mice nor their littermate controls. In a second model of 5α-reductase inhibition using

dutasteride, hepatic GR antagonism with A-348441 attenuated excess weight gain brought about by dutasteride administration (7.03±0.5vs.2.13±0.4g; dutasteride vs. dutasteride+A-348441; mean±SEM, p<0.05) and normalized the associated hyperinsulinemia after glucose challenge (AUC: 235.9±17vs.329.3±16vs.198.4±25*ng.mL^{-1}min^{-1}; high fat, high fat+dutasteride, high fat+dutasteride+A-348441, respectively; *p<0.05). However, A-348441 again did not reverse dutasteride-induced liver steatosis.

Thus, overall hepatic GR antagonism improves insulin resistance but not steatosis induced by high fat diet. Moreover, it attenuates excessive insulin resistance caused by pharmacological inhibition of 5α-reductases, but not genetic disruption of 5αR1. Dutasteride might put patients at increased risk of type 2 diabetes mellitus and reduced exposure to glucocorticoids may be beneficial.

INTRODUCTION

The consequences of glucocorticoid excess are well established, with links to metabolic disease exemplified by patients with Cushing's syndrome. Glucocorticoids primarily mediate their actions through the glucocorticoid receptor (GR), activation of which can be regulated by pre-receptor metabolism within tissues. For example, the 11β-hydroxysteroid dehydrogenases types 1 and 2 have been shown to modulate GR and mineralocorticoid receptor activation respectively (1,2). The potential role of other enzymes such as the 5α-reductases (5αR), which are responsible for the rate-limiting step in glucocorticoid metabolism in modifying GR activation, is less clearly established.

There are two main isozymes of 5α-reductases which can metabolize many pregnene steroids: type 2 (5αR2) is highly expressed in the male reproductive tract where it amplifies androgen action, whereas type 1 (5αR1) is most highly expressed in the liver, but also in adipose tissue and skeletal muscle at lower levels (3,4). 5α-Reductase inhibitors such as finasteride (which inhibits type 2) and dutasteride (which inhibits both isozymes) are prescribed for many men with prostate disease (5). A recent population based cohort study found the risk of developing new onset type 2 diabetes was higher in men exposed to 5α-reductase inhibitors compared with those receiving tamsulosin (6). Furthermore, human studies showed combined inhibition of 5αR1 and 5αR2 (but not 5αR2 alone) impaired insulin sensitivity and resulted in increased hepatic fat content compared to before dutasteride treatment (7,8). Similarly, genetic disruption of 5αR1, but not 5αR2, in mice (5αR1-KO) also caused hyperinsulinemia, weight gain, increased hepatic triglyceride deposition and a predisposition to irreversible liver disease (4,9).

Given the abundance of 5αR1 in the liver and the characterized hepatic phenotype in 5αR1-KO mice and humans receiving 5αR inhibitors, it is likely that the liver is a crucial site of steroid accumulation following 5αR1 inhibition or deficiency. While accumulation of either androgens or glucocorticoids could be responsible for adverse metabolic effects, androgen accumulation appears a less likely mechanism; non-selective inhibition of 5α-reductases in obese Zucker rats induced fatty liver in both castrated and intact rats (4) and female 5αR1-KO mice are more susceptible to hyperinsulinemia than males (10). Therefore, we hypothesized that hepatic glucocorticoid excess underpins the adverse metabolic changes observed following 5αR1 inhibition or disruption, driving hepatic steatosis and insulin resistance.

Systemic GR antagonism with compounds such as mifepristone (RU486) improves insulin and glucose homeostasis in *ob/ob* mice (11), but the site of action is uncertain and likely multiple, with effects confounded by compensatory hyper-corticosteronemia. To test our

hypothesis that the metabolic phenotype in mice with disruption of 5αR1 is driven by excess glucocorticoid action in the liver we employed the liver-selective GR antagonist, A-348441. A-348441 is a cholic acid conjugate of the GR antagonist RU486 (12) that selectively acts in the liver to antagonize the GR. In leptin deficient *ob/ob* mice, A-348441 successfully improved glucose homeostasis, without elevated circulating corticosterone (13). Using euglycemic-hyperinsulinemic clamps, it was demonstrated to decrease hepatic glucose output and increased insulin sensitivity in *fa/fa* rats, a genetic model of obesity (13,14), making it a suitable model for our study.

Research Methods and Design

Chemicals were from Sigma (Poole, UK) and primers from Invitrogen Life Technologies (Paisley, UK), unless otherwise stated. All molecular biology reagents and kits were from Qiagen. Dutasteride was from AK Scientific (CA, USA) and A-348441 was a kind gift from KaroBio (Huddinge, Sweden). Diets were from Research Diets Inc. (New Jersey, USA), who also prepared custom high fat diets containing dutasteride and A-348441.

Animals

Male mice deficient in 5αR1 (5αR1-KO; n=8-11/group (housed as 2-4 mice/cage at 21°C), were bred in house by mating heterozygous mice on a mixed C57Bl6/SvEv/129 background (3,4) with corresponding mixed-strain wild-type littermates used as controls. C57BL6/J male mice were from Harlan (Bicester, UK). Animals were studied under UK Home Office license and had free access to drinking water and food. Animals were culled (0800-1030 h) by decapitation with trunk blood collected.

Insulin signaling analysis

Insulin signaling pathways were studied in a subset of C57BL/6 mice. These were fasted for 4 h (1000-1400 h) in clean cages and human insulin (0.75 U/kg body weight in saline) was given via intraperitoneal injection. These animals were culled via decapitation after 15 minutes. In all cases, tissues were dissected, wet weighed and snap frozen on dry ice or fixed in 10% formalin.

GR antagonism in 5αR1-KO mice

Male 5αR1-KO and corresponding littermate control mice (n = 8-11 per group; age 12 weeks) were randomly allocated to either: chow diet (11% fat, 0% sucrose, D12328); Western style high fat, high sucrose diet (HFD; 58% kcal fat, 13% kcal sucrose, D12331) alone; HFD containing A-348441 (120 mg.kg^{-1}.day^{-1}). After 9 weeks of experimental diet, mice were fasted for 6 h (0800-1400 h) before undergoing a glucose tolerance test (GTT, intraperitoneal injection, 2 mg.g^{-1}), with blood taken by tail venesection at 0, 15, 30, 60 and 90 min. One week later, blood was taken at 0800 h for basal corticosterone measurement and two days later (after 10 weeks of experimental diet) mice were culled as above. A-348441 was mixed within the high fat diet to achieve long term administration without causing chronic stress that would result from repeat injections/gavages. This is particularly desirable since disruption of 5αR1-mediated corticosterone clearance impairs the corticosterone stress response (15).

GR antagonism following pharmacological inhibition of 5αRs with dutasteride in C57BL/6 mice

C57BL6/J male mice (n = 8-12 per group; age 12 weeks) were randomly allocated to either: chow diet; HFD alone; HFD containing A-348441 (120 mg.kg^{-1}.day^{-1}), HFD plus dutasteride (1.8 mg.kg^{-1}.day^{-1}), or HFD plus dutasteride plus A-348441 (same doses) for 4 weeks. GTTs (as above) were performed after 3 weeks of experimental diet. Mice were culled one week later (after 4 weeks of experimental diet).

Laboratory Analyses

Biochemistry

Plasma hormones were measured by ELISA as follows: corticosterone (Enzo Life Sciences, Exeter, UK; RRID: AB_2307314) and insulin (Crystal Chem, IL, USA; RRID: 2783626). Glucose by hexokinase assay (ThermoFisher, Loughborough, UK) and triglycerides (ThermoFisher, Hemel Hempstead, UK) and nonesterified fatty acids (NEFAs) (Zen-Bio, NC, USA) spectrophotometrically. Liver triglycerides were quantified as reported previously (16).

Quantification of mRNAs by Real-Time Quantitative PCR

RNA was extracted as previously described (4). mRNA transcript abundances were quantified by real-time PCR as previously described and with primers and probes as detailed in (4,17,18). Transcript abundances for genes of interest were normalized for the mean abundance of the reference genes, *Tbp*, *Hprt* and *Actβ*, the expression of which did not differ between groups.

Western Blot Analysis

Snap-frozen liver (~40 μg) was homogenized with a rotor blade in ice-cold RIPA buffer (Santa Cruz, Dallas, USA). Western blots were performed as previously described (17). Specific primary antibodies used were: monoclonal rabbit anti-IR β (RRID: AB_2280448, 19), polyclonal rabbit anti-phospho-AKT (Ser473) (RRID: AB_329825, 20), polyclonal rabbit anti- AKT (RRID:AB_ 329827, 21), polyclonal rabbit anti- phospho-GSK-3β (Ser9) (RRID:AB_331405, 22), monoclonal rabbit anti-GSK-3β (RRID: AB_490890, 23) from Cell Signalling Technologies (Leiden, Netherlands); polyclonal anti-rabbit GR (RRID:AB_2155784, 24) from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany); monoclonal mouse anti-β-tubulin (RRID:AB_94650, 25), monoclonal mouse anti-IRS1 (RRID: AB_11214300, 26), monoclonal mouse anti-IRS2 (RRID:AB_10615782, 27) from Millipore Ltd (Watford, UK). Secondary antibodies were: IRDye 680RD goat anti-rabbit IgG (H+L) (RRID:AB_10956166, 28), IRDye 800CW goat anti-rabbit IgG (H+L) (RRID:AB_621843, 29) and IRDye 800CW goat anti-mouse IgG (RRID:AB_2782997, 30) from LI-COR Biosciences Ltd (Cambridge, UK). Phospho antibodies were diluted 1:500 in bovine serum albumin (BSA; Cohn Fraction V, 5 % w/v in TBST), while all total protein primary antibodies were diluted 1:1000 in BSA (5 % w/v in TBST). Secondary antibodies were diluted 1:10000 in BSA when looking at phosphorylation of proteins, or milk when looking at total protein. Band intensities were quantified using the Odyssey Imaging System (LI-COR Biosciences, Cambridgeshire, UK). Quantification of proteins of interest was calculated by dividing their integrated intensity by that of the loading control (β-tubulin). Phospho-proteins were normalized to their corresponding bands of β-tubulin and a ratio of (phospho-protein/β-tubulin)/(total protein/β-tubulin) was used in final analysis.

Statistical Analysis

Data are mean±SEM, analysed with GraphPad Prism 6 (San Diego, CA), using Student's t-test or 2-way ANOVA, with Sidak Post-hoc test as appropriate. Statistical significance was taken at $p<0.05$.

Results

Hepatic GR antagonism in mice with genetic disruption of 5αR1

10 weeks of high fat diet was sufficient to induce an adverse metabolic phenotype in the wild-type mice of mixed strain ("wild-type-HFD") background, causing excess weight gain of ~4 g compared to chow diet (9.0±1.1 vs 4.8±0.5 g; p<0.05). Furthermore, fasting insulin was

increased by high fat diet (2.8±0.4 vs 2.0±0.3 ng/mL, p<0.05) without raising fasting glucose (156.7±3.7 vs 151.6±9.9 mg/dL; HFD vs chow diet). Liver triglyceride levels were also increased by high fat diet (152.3±18.4 vs 36.97±9.4 μmol/g). The effects of A-348441 to attenuate the metabolic changes induced by 10 weeks of high fat diet were thus studied in 5αR1-KO ("5αR1-KO-HFD") and wildtype-HFD mice (the drug was not administered to animals receiving chow diet). Consistent with a lack of central effects and previous literature (12-14), basal circulating corticosterone was not altered in either genotype following administration of A-348441 (Table 1).

Weight gain induced by the high fat diet was attenuated by A-348441 in wild-type-HFD mice, but not in 5αR1-KO-HFD mice (Figure 1A, 1B, 1C). However, mass of specific adipose depots was not decreased by the drug of different by genotype (Table 1). A-348441 lowered fasting glucose in the wild-type-HFD mice only (Figure 1D, 1E, 1J), but had no effect on the overall glucose response to a GTT (Figure 1F). There was an overall effect of A-348441 to lower fasting insulin (Figure 1G, 1H, 1K) and insulin response to a GTT (Figure 1I) in mice of both genotypes. Similar to previously described (4), 5αR1-KO-HFD mice were predisposed to develop insulin resistance - with a trend to higher fasting insulin (Figure 1K) and increased insulin response to a glucose challenge compared to wild-type-HFD mice irrespective of the presence of A-348331 (Figure 1I). Surprisingly, 5αR1-KO-HFD mice overall had lower fasting NEFAs than the wild-type-HFD mice (Table 1). As a measure of adipose insulin sensitivity, NEFA suppression over the first 15 minutes of the GTT was measured. However, A-348441 did not influence NEFA suppression, but increased fasting NEFAs in the wild-type-HFD mice only (Table 1). Furthermore, as anticipated, 5αR1-KO-HFD mice overall had higher hepatic triglyceride levels than their wild-type controls but A-348441 did not lower hepatic or plasma triglyceride levels in either genotype (Figure 1L, Table 1).

Liver selective GR antagonism following dutasteride administration in C57BL/6 mice
Mice on a C57BL/6 background are predisposed to diet-induced obesity and were studied over a shorter time-frame. After only 4 weeks, high fat diet induced weight gain in excess of that in chow fed animals (6.3±0.4 vs 4.0.±0.1 g; p<0.05), accompanied by a doubling in fasting insulin of 2.1±0.2 vs 0.8 ±0.1 ng/mL (p<0.05) as well as an increase in hepatic triglyceride content (22.3±1.3 μmol/g vs 10.5±1.7 p<0.05). Similar to the littermate controls of the 5αR1-KO mice (Figure 1), C57BL/6 mice on A-348441 had reduced weight gain (Figure 2A), no changes in glucose tolerance (Figure 2D), but lowered insulin levels (Figure 2G).

Successful administration of dutasteride was confirmed by a reduction in prostate size (Table 2). Co-administration of dutasteride in the high fat diet exacerbated the adverse metabolic phenotype in C57BL/6. Body weight gain increased after 4 weeks compared to high fat diet alone (Figure 2C), with increased adipose weight in the omental depot only (Table 2). Dutasteride increased insulin response to a bolus of glucose during a GTT compared to high fat diet alone (Figure 2I), as well as fasting insulin (Figure 2K), without altering the glucose response to GTT or fasting glucose (Figure 2F, 2J). Compared with mice receiving high fat diet alone, mice fed dutasteride had increased hepatic triglyceride levels (Figure 2L) without differences in plasma triglyceride, fasting plasma NEFA or suppression of NEFAs following glucose challenge (Table 2). Food intake was not measured per animal, but on a cage basis (with limited power of n=3), appeared less in animals fed A-348441, irrespective of 5α-R status (16.9±0.8 vs 13.5±0.7 kCal/mouse/day)

In contrast with genetic deletion of 5αR1, co-administration of A-348441 with dutasteride reduced the excess weight gain (Figure 2B), in parallel with reduced expansion of visceral

adipose depots and lower fasting NEFAs (Table 2). Co-administration of A-348441 normalized the raised fasting insulin and insulin response to GTT caused by dutasteride (Figure 2I, 2K), again without affecting fasting glucose or glucose response during a GTT (Figure 2E, 2H). As in the 5αR1-KO mice, co-administration of A-348441 did not abrogate the raised liver triglyceride levels brought about by dutasteride (Figure 2L), nor alter NEFA suppression (Table 2).

Effects of A-348441 on glucocorticoid and bile-acid sensitive hepatic pathways following administration to C57BL/6 mice

As expected, dutasteride treatment increased the expression of glucocorticoid-sensitive genes such as *Per1* in the liver (Figure 3A). Interestingly, it also resulted in reduced expression of *Sgk* transcript, whereas *Gilz* was unaffected. Liver-selective GR antagonism did not alter expression of any of these glucocorticoid-sensitive transcripts (Figure 3A). Neither dutasteride nor A-348441 affected hepatic abundance of *Nr3c1* mRNA (Figure 3A).

Transcript abundance of genes involved in bile acid signaling was also investigated. A-348441 lowered *Cyp7a1* mRNA abundance and increased *Shp* abundance compared to high fat diet (Figure 3C). Dutasteride alone also lowered *Cyp7a1* mRNA abundance but not *Shp*. A-348441 administered in combination with dutasteride had no further effect on *Cyp7a1* transcript abundance compared to dutasteride administration alone.

Hepatic insulin signaling is unaffected by A-348441 administration

A-348441 did not alter total protein levels of IR, IRS2, AKT or GSK-3β in the liver (Figure 4A-C). A-348441 lowered *Gys2* transcript levels only in the presence of dutasteride. Although dutasteride decreased *Fas* mRNA levels, it had little effect on other hepatic metabolic transcripts (Figure 3B). A-348441 treatment increased transcript abundance of *Insr*, *Irs1*, *Ppargc1a, Fatp2*, with a trend (p=0.07) for higher *Irs2* mRNA transcripts (Figure 3B). When administered in combination with dutasteride, A-348441 lowered transcript abundance of *Gys2* only.

To better assess insulin signaling, we gave a subset of mice in each of the treatment groups a bolus of insulin following a 4 hour fast and collected tissue 15 minutes later. However, no changes were observed in pSer473AKT and pSer9 GSK-3β signal in liver (Figure 4D, 4E).

Discussion

Deficiency or inhibition of 5αR1 predisposes men and mice to insulin resistance and fatty liver (4,7-9). However, since 5αR1 can metabolize multiple steroids, notably both glucocorticoids and androgens, it has been unclear which hormone plays the more crucial role in the phenotype. Rodents with deficiency in 5αR1 develop hyperinsulinemia and hepatic steatosis even after androgen levels were decreased through castration (4), implicating glucocorticoids as the key hormone involved. Since 5αR1 is highly expressed in the liver, an organ crucial to metabolic balance, and glucocorticoid levels in this tissue are approximately doubled in 5αR1-KO (4), we therefore hypothesized that the predisposition to insulin resistance and fatty liver following 5αR1 deficiency was due to an increase in glucocorticoid action in the liver. Using A-348441, a liver-selective hepatic GR antagonist, we demonstrated in two models of 5αR1 deficiency, 5αR1-KO mice and administration of dutasteride (a dual 5α-reductase inhibitor), that glucocorticoids contribute to the phenotype.

To test this hypothesis, we initially used a genetic approach, utilizing 5αR1-KO mice. These are bred on a mixed genetic background (3) and differences in metabolic parameters between 5αR1-KO mice and their littermate controls develop slowly on high-fat diet (4,9). The intervention with A-348441 was commenced at 12 weeks of age for 10 weeks during the decline

in metabolic health. At this time point, 5αR1-KO mice on a high fat diet had increased insulin levels and greater insulin response during GTT compared with their littermate controls, but as expected, differences in weight were not yet observed (this aspect of the phenotype takes 6 months to manifest (4,9)). Interestingly, the predisposition to fatty liver in 5αR1-KO mice compared to their littermate controls was also already evident over this shorter timeframe and before overt changes in body weight between the genotypes was observed.

The outcome of treatment with A-348441 in the wild-type mice was similar to that observed in the *fa/fa* rats (13,14), with improved insulin sensitivity and attenuation of weight gain. Surprisingly, hepatic steatosis was unresponsive to hepatic GR antagonism. Previously genetic disruption of hepatic GR on a *db/db* background had attenuated hepatic triglyceride accumulation (31) and likewise, amplification of hepatic generation of glucocorticoids by over-expression of 11β-hydroxysteroid dehydrogenase 1 exacerbated steatosis (32). However, the lack of change seen here is in agreement with previous work from Macfarlane et al. (2014) where systemic glucocorticoid blockade with RU486 did not ameliorate excess liver fat in patients with type 2 diabetes mellitus (33). The beneficial metabolic changes brought about by A-348441 were less marked in 5αR1-KO mice compared with littermate controls. Whereas administration of A-348441 attenuated the excess weight gain induced by high fat diet in the littermate control mice, this was unaffected in 5αR1-KO mice. In terms of glucose disposal, A-348441 reversed the hyperinsulinemia to a similar extent between the 5αR1-KO mice and their littermate controls. Again, hepatic steatosis caused by the high fat diet was unresponsive to intervention and the 5αR1-KO maintained higher liver fat than their littermate controls on both diets. This is supported by the fact that A-348441 did not lower transcript abundance of *Fatp2* nor *Fatp5* in the liver.

Given the overall phenotype of the 5αR1-KO improved but remained different from wild-type, these observations suggest that GR activation plays a role in high fat diet-induced metabolic dysfunction on this mixed genetic background, but do not support the hypothesis that the it is solely glucocorticoid mediated, unless insufficient drug had been administered, which we believe unlikely. Had excess GR activation been singly responsible, the difference between genotypes would have been negated. Equivalent doses of RU486 to the dose of A-348441 used here and common in mouse models would be expected to achieve concentrations in the blood well in excess of those of corticosterone (~100 nM) and hence unlikely to be limiting in the liver (34,35).

To gain further insight, hepatic-selective GR antagonism was pursued in a second model, that of pharmacological inhibition with dutasteride, to better reflect the clinical therapeutic scenario. The 5αR1-KO mice have life-long deficiency in steroid metabolism, including during development, and may have undergone compensatory changes in steroid hormone action and metabolism. In a clinical setting, 5αR inhibition generally starts later in middle-aged adults. Dutasteride, a dual 5αR inhibitor, was administered to C57BL/6 mice, a strain which is predisposed to become obese, hyperglycemic and hyperinsulinemic due to a naturally occurring deletion in nicotinamide nucleotide transhydrogenase (36). Indeed, C57BL/6 mice were glucose intolerant and hyperinsulinemic after only 3 weeks of high fat diet, and thus this shorter time-frame of study was chosen, matching the insulin increment in wild-type mice in the previous experiment. The ability of 5αR inhibition to adversely affect metabolism was apparent after 4 weeks of treatment, reminiscent of studies in humans (4,7). Specifically, dutasteride exacerbated weight gain, increased fasting insulin and insulin response to a GTT and induced hepatic steatosis in mice fed high fat diet (4).

As with the wild-type controls of 5αR1-KO mice, high fat diet-fed C57BL/6 mice treated with A-348441 alone demonstrated an improved metabolic phenotype. Importantly, the exacerbation of metabolic dysfunction by dutasteride was also prevented by A-348441, highlighting a substantial role of hepatic glucocorticoids; weight gain was normalized to that of chow fed mice and insulin response to a glucose challenge and plasma triglycerides levels all decreased. Moreover, white adipose depots were reduced in weight. Systemic benefits may have been caused through hemocrine effects of hepatic metabolic mediators, as opposed to direct effects of the liver-selective drug. Indeed, other studies have demonstrated manipulation of metabolic signals in the liver such as FOXO1 and IR, as well as PI3K activity, can indirectly affect adipose tissue mass and systemic insulin sensitivity (37). Notably, however, hepatic steatosis was again not affected by hepatic GR antagonism, reinforcing the suggestion of a different etiology. Previous studies showed excess steatosis induced by pharmacological inhibition of 5αRs was also not reversed by castration, which suggests androgens alone are also not the sole cause (4). Either a complex interplay between glucocorticoids and androgens underpins the liver steatosis (38) or alternative hormonal pathways are involved. Nevertheless, we found most aspects of the observed adverse metabolic phenotype were responsive to GR blockade suggesting that excess hepatic glucocorticoid action plays a significant role in mediating many of the metabolic effects of dutasteride. To further confirm the contribution of increased hepatic glucocorticoid levels to changes in insulin sensitivity and hepatic steatosis, dutasteride could be administrated to liver-specific GR knockout mice on high fat diet.

To gain insight into the potential mechanism responding to hepatic GR antagonism, hepatic transcript abundance of genes involved in insulin signaling and the lipid metabolism were studied. The level of *Nr3c1* (GR) mRNA was unaffected by the drug. *Per1* mRNA was studied as a known GR-responsive transcript. The levels of *Per 1* were elevated by dutasteride, but did not suppress following GR antagonism. Indeed, very few changes in hepatic transcripts that might explain the phenotypic changes were seen in response to GR antagonism, with only some limited increase in genes associated with insulin signaling such as *Insr, Irs1* and *Ppargc1a* and a small reduction in *Gys2*. However, a similar lack of regulation of genes involved in gluconeogenesis or lipid metabolism was observed when hepatic glucocorticoid ligand availability was suppressed in mice with liver-selective disruption of 11β-hydroxysteroid dehydrogenase 1 (39). Protein expression from the insulin signaling pathway was also explored under insulin stimulation but A-348441 had no effect on the total or phosphorylated state of these proteins in the liver (40,41). Further studies using insulin clamps would permit greater insight and a greater difference might be observed if the tissues were collected after a longer duration of high fat diet and A-348441.

Given haemocrine mechanisms may underpin the improved phenotype, it is important to consider the tissue-selectivity of the antagonist. The drug had been developed to attenuate hepatic GR stimulation; pre-dosing with A-348441 antagonized glucocorticoid-induced up-regulation of hepatic TAT activity by 79% and suppressed glucocorticoid-induced hepatic glycogen formation by 59% (13). Furthermore, compared to RU486, a systemic GR antagonist, A-348441 had substantially less effects in non-hepatic tissues e.g. was 12-fold less potent in preventing glucocorticoid–induced up-regulation of glutamine synthetase in L6 skeletal muscle myocytes and 15-fold less potent in a glucocorticoid-stimulated model of adipocyte differentiation in 3T3-L1 cells (13). This highly liver-selective profile avoided adverse effects on HPA axis incurred through systemic actions or central GR antagonism. In line with previous studies, we were able to recapitulate that A-348441 did not affect circulating concentrations of

corticosterone suggesting that negative feedback vis GR within the brain was unaffected by the presence of an antagonist, suggesting minimal circulating levels (13).

GR is the most likely target for the drug, but being a conjugate of RU486 and cholic acid, A-348441 is hepato-selective through the presence of a bile acid. Bile acids can modulate insulin signaling pathways via nuclear hormone receptors such as farnesoid x receptor-α (FXRα) as well as GR (42,43). It was thought unlikely that A-348441 would have off target effects on the bile acid pathway as the Kd for binding of cholic acid to FXRα is exceedingly high - 36 mM versus a Ki of 0.27 nM for A-348441 to GR (12) and not anticipated with the given doses. However, we found that A-348441 lowered *Cyp7a1* and increased *Shp* mRNA abundance. Nonetheless, it is likely that the effects of GR and FXRα are linked - hepatic GR can regulate systemic bile acid homeostasis, with glucocorticoids inhibiting the transcriptional activity of FXR (43,44). Furthermore, SHP is a corepressor of the glucocorticoid receptor (45); increased mRNA of *Shp* would still have the end result of decreased GR action. Interestingly, *Cyp7a1* and *Shp* mRNA abundance in mice fed dutasteride were not significantly different from the mice given dutasteride along with A-348441. Altogether, A-348441 may influence hepatic bile acid signaling, but the effects of A-348441 in ameliorating the adverse phenotype caused by dutasteride were not through the same pathways. Had A-348441 lowered androgen levels and activation of the androgen receptor, this would confound our interpretation, but we see no evidence of this e.g. lack of reduction in prostate weight.

In summary, most but not all of the adverse metabolic effects of diet-induced obesity brought about by inhibition of 5αR1 with dutasteride can be reversed by liver-selective GR antagonism. Our data support a substantial role for increased hepatic GR activation in the adverse metabolic phenotype observed following pharmacological inhibition and a contributory role after genetic disruption of 5αR1, however the signaling pathways underpinning the changes were elusive. A-348441 proved a useful tool to dissect the role of liver GR in regulating metabolism and has revealed that many of the consequences of high fat diet in animal models, with the crucial exception of steatosis, can be reversed by targeting hepatic GR. The study consolidates the hypothesis that men receiving dual 5αR inhibitors are exposed to challenges to their metabolic health. Concomitant administration of therapies which attenuate glucocorticoid action, such as 11β-hydroxysteroid dehydrogenase 1 inhibitors (46,47) may prove to be of benefit.

Author Contributions

TCSM, DEWL and MN performed the experiments. The manuscript is guaranteed by RA. All authors contributed to design of the studies, analysis and interpretation of data and preparation and review of the manuscript.

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DISCLOSURE STATEMENT:
The authors have nothing to disclose.
conflict of interest
The authors declare no conflict of interest.

Data Availability

The datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

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Figure 1: Body weight gain and glucose homeostasis in 5α-reductase 1 deficient (knockout) mice and their wild-type controls following a high fat diet and administration of A-348441 for 10 weeks. Mice were fed high fat or high fat+A-348441 diet. A-348441 ameliorated the increase in body weight gain in the wild-type (A) but not knockout (B, C) mice. A-348441 did not significantly affect glucose levels in either genotype (D, E, F) in response to glucose tolerance tests, but had an overall effect to ameliorate the increase in insulin levels (G, H, I) (p=0.004). After 9 weeks of experimental diet, A-348441 lowered fasting glucose in the wild-type mice only (J), but ameliorated the increase in fasting insulin in both genotypes (K). Knockout mice overall had higher insulin (I) and hepatic triglyceride levels (L) than wild-type mice. Data are mean±SEM and presented by individual genotype for clarity, with analysis of all data by two-way ANOVA followed by Sidak post-hoc test ($p<0.05 vs matched genotype receiving high fat diet), n=8-11/group. NS = not significant, n=8-11/group. For overall ANOVA, * p<0.05, ** p<0.01, *** p< 0.001.

Figure 2: Body weight and glucose homeostasis in C57BL/6 mice following dutasteride and A-348441 administration for 4 weeks. C57BL/6 mice were fed high fat, high fat+A-348441, high fat + dutasteride or high fat + dutasteride + A-348441 diet. A-348441 ameliorated increased body weight in both mice fed high fat diet alone (A) or high fat + dutasteride diet (B). Mice on high fat diet + dutasteride had increased body weight gain versus high fat diet alone (C). Glucose tolerance was not changed by dutasteride or A-348441 administration (D, E, F). A-348441 lowered raised insulin levels during a GTT induced by both high fat diet alone (G) or in combination with dutasteride (H). Dutasteride increased insulin levels compared to mice on a high fat diet alone (I). A-348441 had no effect on fasting glucose (J) but normalized the increased fasting insulin caused by dutasteride upon high fat diet (K). With a high fat diet, there

was an overall effect for dutasteride to increase hepatic triglyceride levels (L), but A-348441 did not ameliorate this rise. Data are mean±SEM and presented by 5α-reductase status for clarity, with analysis of all data by two-way ANOVA at each time point followed by Sidak post-hoc test ($p<0.05 vs. high fat diet; #p<0.05 vs. high fat+dutasteride), n=8-12/group. NS = not significant, n=8-12/group. For overall ANOVA, * p<0.05, ** p<0.01, *** p< 0.001.

Figure 3: Response of hepatic transcripts to A-348441 in C57BL/6 mice after 4 weeks of experimental diet. mRNA abundances were quantified for *Nr3c1, Per1, Gilz, Sgk, Insr, Irs1, Irs2, Ppara, Pparg, Ppargc1a, Srebf1, Pepck, Gys2, Fatp2, Fatp5, Angpt12, Fas, Cyp7a1 and Shp.* Amongst glucocorticoid sensitive genes, dutasteride increased *Per1* and lowered *Sgk* (A), while A-348441 had no effect. Dutasteride also lowered *Fas* mRNA, while A-348441 increased transcript abundance for *Insr*, *Irs1* and *Ppargc1a*. When administered in combination with dutasteride, A-348441 lowered *Gys2* transcript levels (B). Both dutasteride and A-348441 lowered *Cyp7a1* abundance but only A-348441 alone increased *Shp* transcript levels compared to high fat diet (C). Data are mean±SEM and analyzed by two-way ANOVA followed by Sidak post-hoc test ($p<0.05 vs. high fat diet; #p<0.05 vs. high fat+dutasteride). For overall ANOVA, * p<0.05 effect of dutasteride, n=8-12/group, n=10-11/group.

Figure 4: Response of hepatic insulin signaling proteins to A-348441 in C57BL/6 mice. Total protein levels of hepatic IR (A), IRS2 (A), AKT (B) and GSK-3β (C) did not change in response to administration of A-348441. Phosphorylation status of hepatic [pSer473]AKT (D) and hepatic [pSer9]GSK-3β (E) following insulin stimulation did not change in response to the addition of A-348441. Data are mean±SEM and analyzed by Student t-test - *p<0.05 vs. high fat diet, n=6 (high fat) -12 (high fat + A-348441)/group.

Table 1 Indices of metabolism in wild-type and 5α-reductase 1 deficient (knockout) mice.

	Wild-type HF diet	Wild-type HF + A-348441 diet	Knockout HF diet	Knockout HF + A-348441 diet	Effect of A-348441 P value	Effect of genotype P value	Interaction P value
Total weight gain (g)	8.99±1.11	5.04±0.62*	8.83±1.37	7.61±0.55	<0.01	0.21	0.16
Tissues (% Body weight)							
Omental adipose	0.062±0.008	0.048±0.005	0.079±0.009	0.062±0.007	0.05	0.06	0.85
Liver	4.52±0.16	4.38±0.12	4.55±0.12	4.83±0.08	0.59	0.07	0.12
Gonadal adipose	3.91±0.32	3.33±0.21	4.14±0.36	3.83±0.13	0.11	0.19	0.61
Perinephric adipose	1.30±0.11	1.11±0.08	1.42±0.11	1.38±0.08	0.24	0.06	0.47
Mesenteric adipose	1.60±0.10	1.28±0.07	1.59±0.22	1.59±0.10	0.23	0.24	0.21
Subcutaneous adipose	2.08±0.26	1.77±0.12	2.54±0.34	2.01±0.18	0.08	0.14	0.65
Quadriceps muscle	1.14±0.06	1.19±0.03	1.05±0.05	1.23±0.02	0.20	0.10	0.76
Interscapular adipose	0.51±0.03	0.54±0.05	0.52±0.03	0.53±0.06	0.36	0.05	0.64
Biochemistry							
Corticosterone (nM)	35.2±2.2	37.9±2.1	35.9±3.5	38.5±3.3	0.10	0.45	0.82
Plasma TGA (mmol/L)	2.07±0.20	1.79±0.16	2.01±0.20	1.87±0.07	0.14	0.80	0.92
Fasting NEFA (mM)	1.58±0.12	1.91±0.14	1.26±0.08	1.38±0.10	0.06	<0.01	0.40
ΔNEFA (mM) (0-15min of GTT)	0.29±0.16	0.44±0.10	0.06±0.11	0.38±0.07	0.04	0.15	0.33

Mice were fed high fat (HF) or HF+A-348441 diet for 10 weeks. Data are mean ± SEM, compared by two-way ANOVA with Sidak post hoc tests. Codes of comparisons (p<0.05) within same genotype, * vs HF diet: n=8-11/group. TGA = triglycerides; NEFA = non-esterified fatty acids; GTT = glucose tolerance test.

Table 2 Indices of metabolism in C57BL/6 mice fed high fat (HF) diet, with or without A-348441 and/or without dutasteride.

	HF	HF + A-348441	HF + Dutasteride	HF + Dutasteride +A-348841	Effect of dutasteride	Effect of A-348441	Interaction
Total weight gain (g)	5.71±0.37	3.18±0.25*	7.03±0.52*	2.13±0.42†	0.73	<0.001	0.005
Tissue weight (% Body weight)							
Omental adipose	0.049±0.003	0.036±0.003	0.069±0.006*	0.043±0.008†	0.010	<0.001	0.195
Liver	4.89±0.08	4.71±0.12	5.16±0.12	4.56±0.28	0.704	0.016	0.187
Gonadal adipose	3.28±0.17	2.32±0.19*	3.46±0.17	2.07±0.18	0.838	<0.001	0.249
Perinephric adipose	0.99±0.05	0.56±0.06*	0.99±0.06	0.51±0.07	0.726	<0.001	0646
Mesenteric adipose	1.46±0.09	0.97±0.07*	1.56±0.09	1.04±0.05	0.354	<0.001	0.844
Subcutaneous adipose	1.53±0.09	1.02±0.06*	1.85±0.13	1.37±0.15†	0.004	<0.001	0.934
Quadriceps muscle	1.24±0.03	1.24±0.04	1.17±0.03	1.29±0.03	0.734	0.098	0.092
Interscapular adipose	0.38±0.03	0.42±0.03	0.44±0.03	0.32±0.02†	0.507	0.184	0.013
Prostate	0.049±0.005	NC	0.024±0.004*	0.034±0.003*	N/A	N/A	N/A
Biochemistry							
Corticosterone (nM)	86.23±10.9	106.6±23.4	32.69±3.9*	88.34±22.8	0.035	0.027	0.29
Plasma TGA (mmol/L)	2.03±0.14	1.56±0.13*	2.07±0.12	1.76±0.13	0.37	0.008	0.57
Fasting NEFA (mM)	2.13±0.11	1.98±0.15	2.08±0.01	1.54±0.15†	0.15	0.048	0.25
ΔNEFA (0-15min of GTT)	1.11±0.11	0.98±0.16	1.36±0.15	0.89±0.13	0.61	0.08	0.31

Mice were fed high fat (HF), HF+A-348441, HF+dutasteride or HF+dutasteride+A-348441 diet for 4 weeks. Data are mean ± SEM, compared with two-way ANOVA with Sidak post hoc tests, *$p<0.05$ vs HF diet alone, †$p<0.05$ vs HF + dutasteride. Prostate weight was compared by one-way ANOVA, p=0.002. n=8-12/group. NC is not collected; N/A is not applicable; TGA = triglycerides; NEFA = non-esterified fatty acids; GTT = glucose tolerance test.
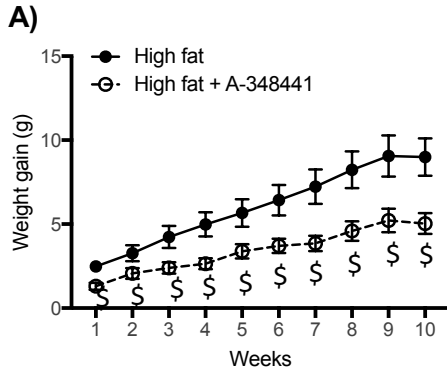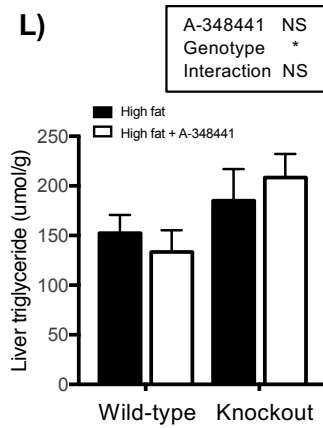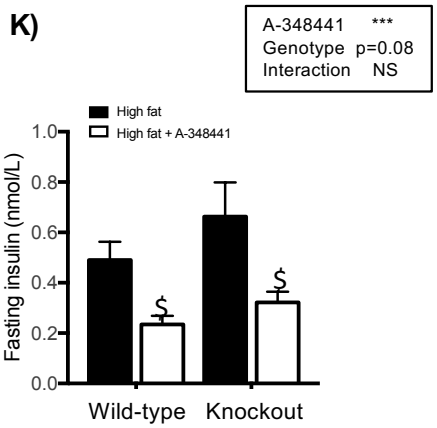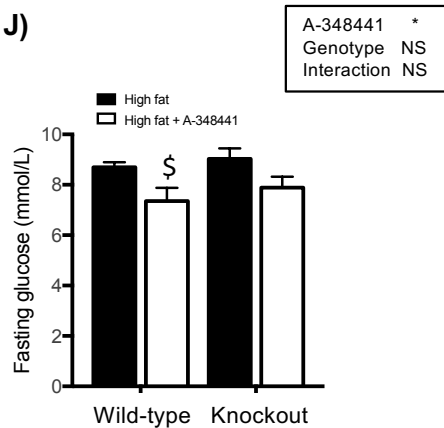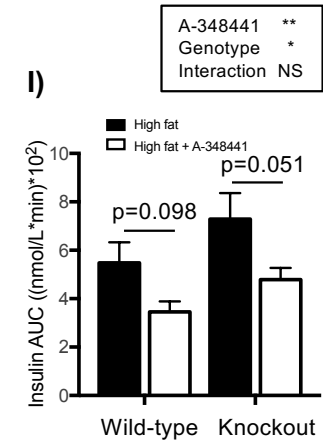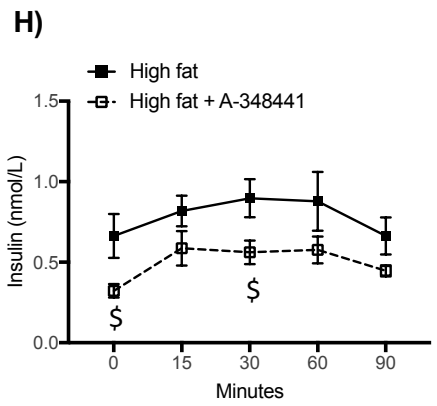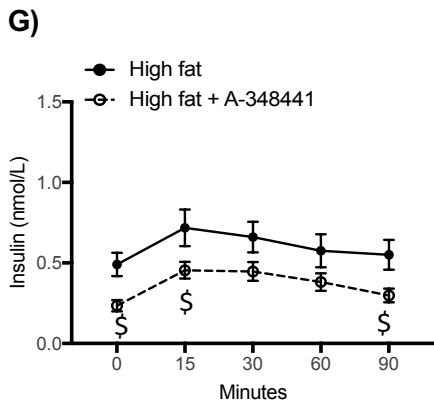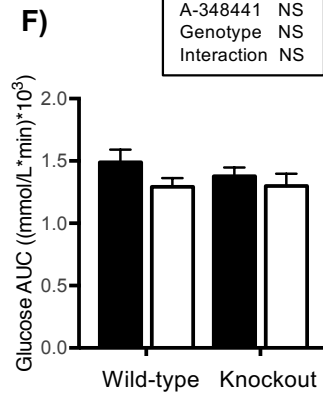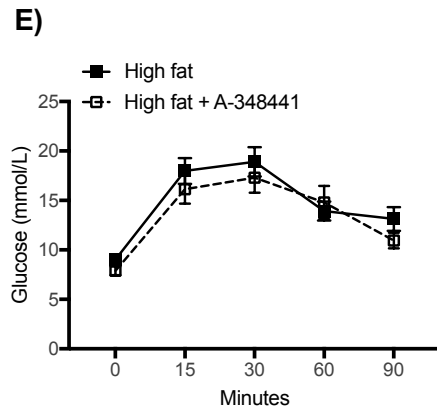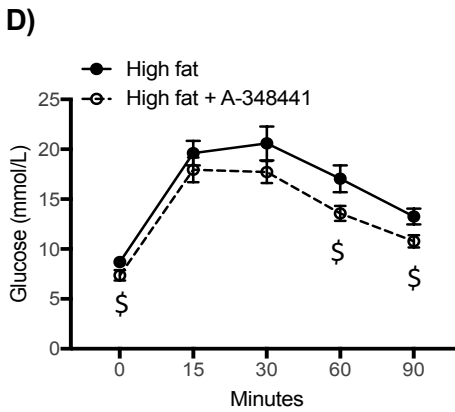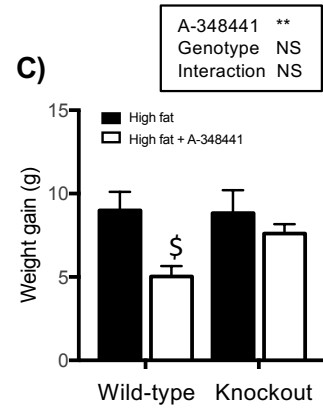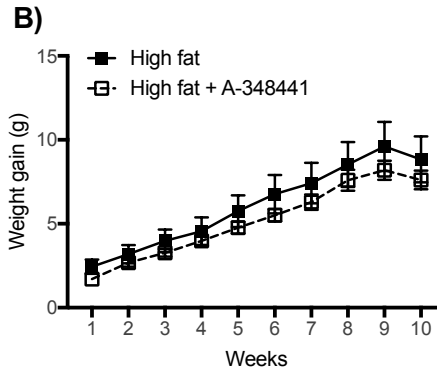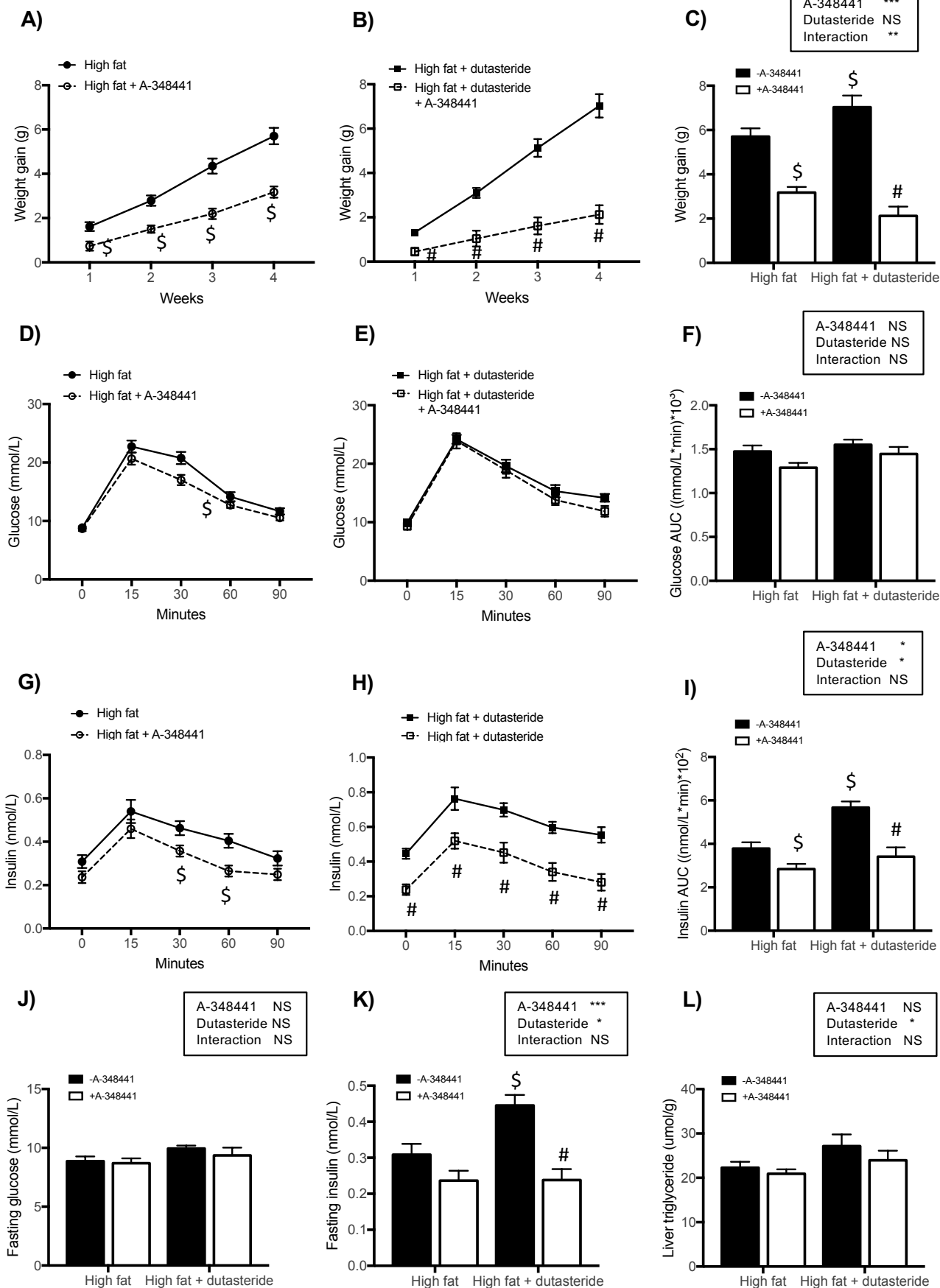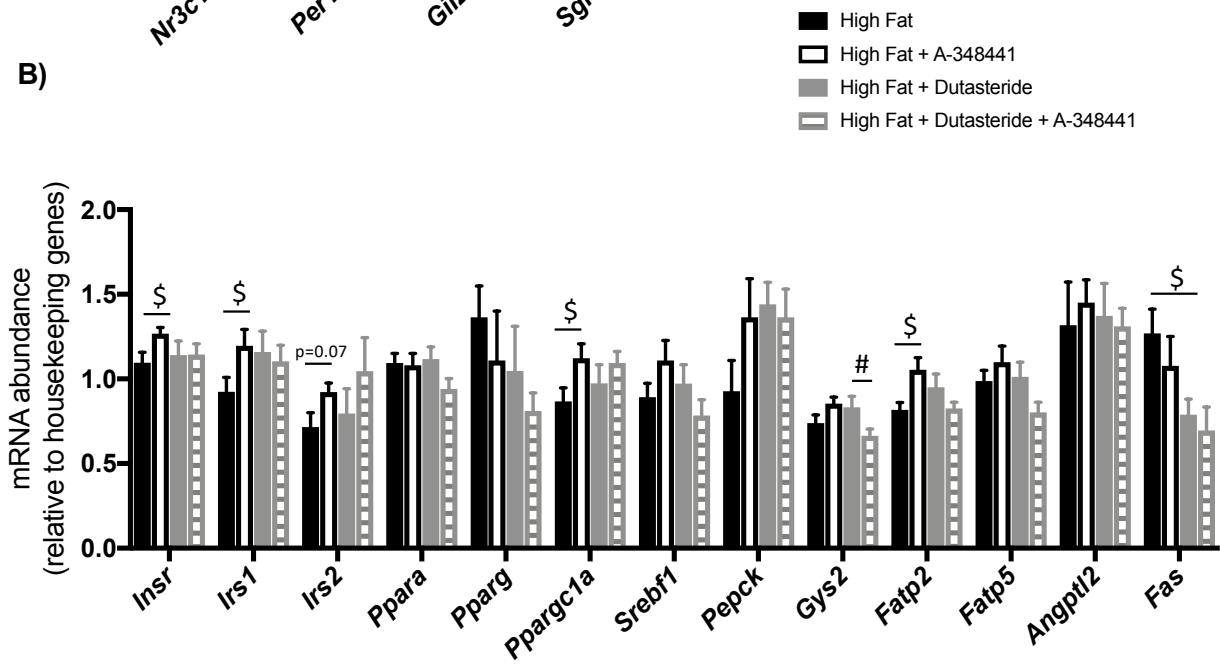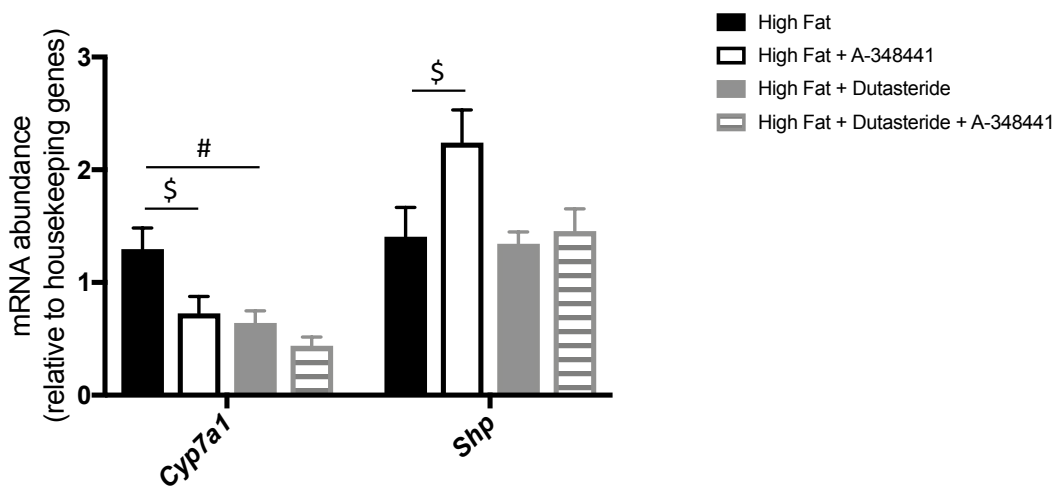
A) Liver IR and IRS2

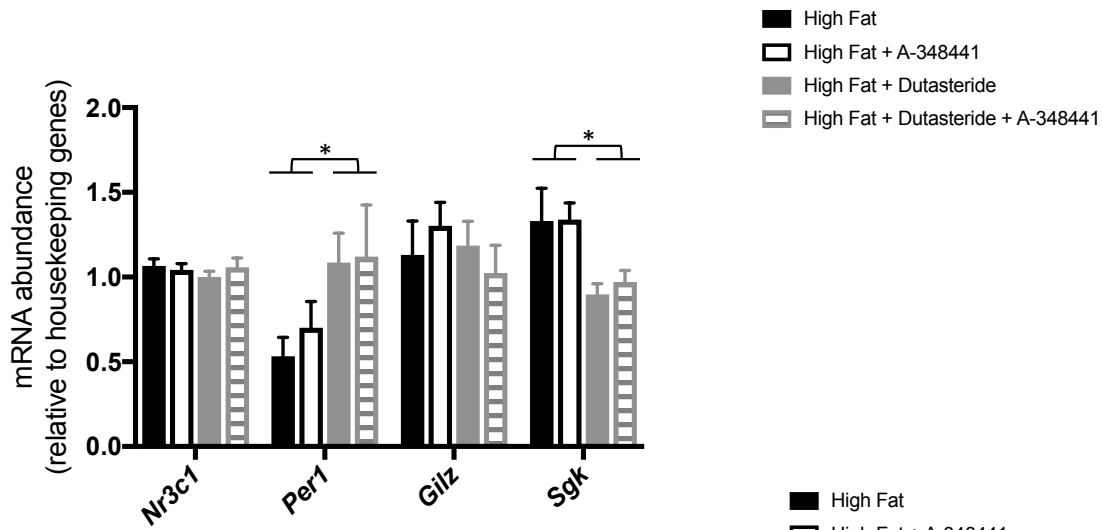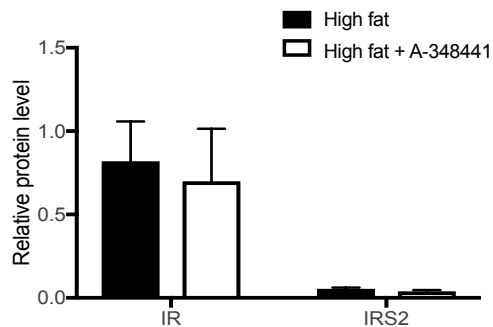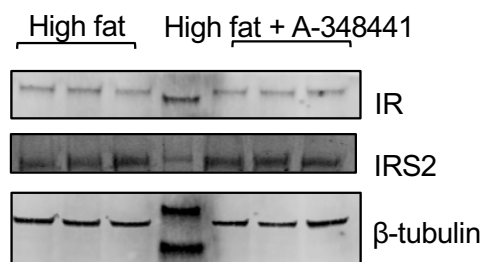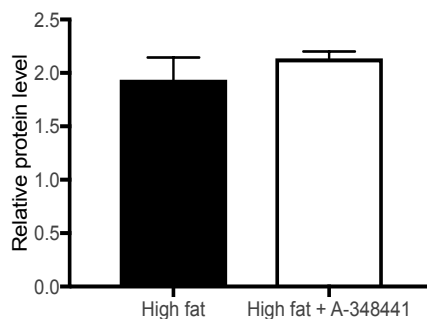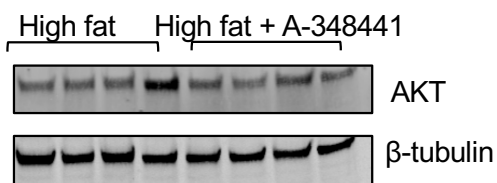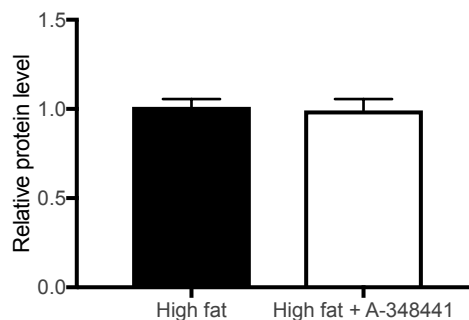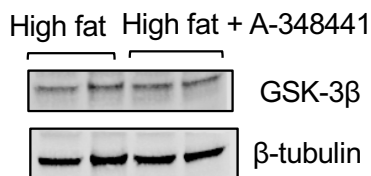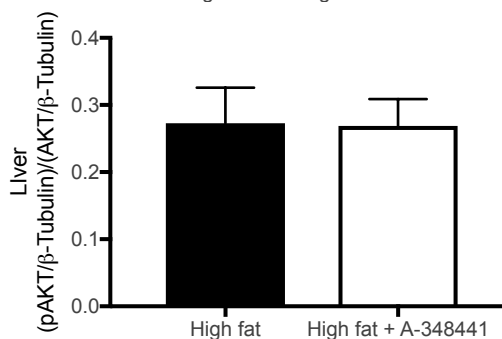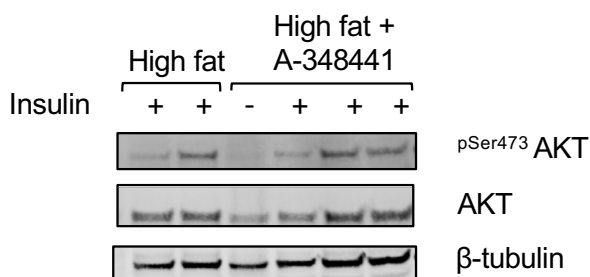**B)** Liver AKT

C) Liver GSK-3β

D) Liver pSer473AKT

E) Liver pSer9GSK-3β