Glucagon-like peptide-1 receptor agonism improves metabolic, biochemical, and histopathological indices of nonalcoholic steatohepatitis in mice

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Trevaskis JL, Griffin PS, Wittmer C, Neuschwander-Tetri BA, Brunt EM, Dolman CS, Erickson MR, Napora J, Parkes DG, Roth JD. Glucagon-like peptide-1 receptor agonism improves metabolic, biochemical, and histopathological indices of nonalcoholic steatohepatitis in mice. Am J Physiol Gastrointest Liver Physiol 302: G762–G772, 2012. First published January 19, 2012; doi:10.1152/ajpgi.00476.2011.—These preclinical studies aimed to 1) increase our understanding of the dietary induction of nonalcoholic steatohepatitis (NASH), and, 2) further explore the utility and mechanisms of glucagon-like peptide-1 receptor (GLP-1R) agonism in NASH. We compared the effects of a high trans-fat (HTF) or high lard fat (HLF) diet on key facets of nonalcoholic fatty liver disease (NAFLD)/NASH in Lepob/Lepob and C57BL6J (B6) mice. Although HLF-fed mice experienced overall greater gains in weight and adiposity, the addition of trans-fat better mirrored pathophysiological features of NASH (e.g., hepatomegaly, hepatic lipid, and fibrosis). Administration of AC3174, an exenatide analog, and GLP-1R agonist to Lepob/Lepob and B6 ameliorated hepatic endpoints in both dietary models. Next, we assessed whether AC3174-mediated improvements in diet-induced NASH were solely due to weight loss in HTF-fed mice. AC3174-treatment significantly reduced body weight (8.3%), liver mass (14.2%), liver lipid (12.9%), plasma alanine aminotransferase, and triglycerides, whereas a calorie-restricted, weight-matched group demonstrated only modest nonsignificant reductions in liver mass (9%) and liver lipid (5.1%) relative to controls. Treatment of GLP-1R-deficient (GLP-1RKO) mice with AC3174 had no effect on body weight, adiposity, liver or plasma indices pointing to the GLP-1R-dependence of AC3174’s effects. Interestingly, the role of endogenous GLP-1Rs in NASH merits further exploration as the GLP-1RKO model was protected from the deleterious hepatic effects of HTF. Our pharmacological data further support the clinical evaluation of the utility of GLP-1R agonists for treatment of NASH.

exenatide; steatosis; fibrosis

NONALCOHOLIC FATTY LIVER DISEASE (NAFLD) affects ~25% or more of the general population in many countries (1, 21, 43). NAFLD is highly associated with components of the metabolic syndrome, particularly obesity, diabetes, and insulin resistance (8, 24). This disease is also characterized by increased levels of lipid (primarily triglyceride) in the liver (steatosis), hepatomegaly and elevated plasma aminotransferase levels [usually alanine aminotransferase (ALT)] (32). In a percentage of individuals and through undetermined pathophysiology, NAFLD occurs as a more serious form of the disease, nonalcoholic steatohepatitis (NASH). NASH is diagnosed clinically via histological assessment of fibrosis, inflammation, apoptosis, and other features, such as hepatocyte ballooning and presence of Mallory-Denk bodies (6). Furthermore, up to 15–25% of people with NASH may progress to having cirrhosis over 10 to 20 yr (3, 25, 29). The current standard of care for NAFLD/NASH is limited to ameliorating components of the metabolic syndrome, primarily through weight loss and/or improving insulin sensitivity with lifestyle interventions or surgery (22, 30). Improving insulin sensitivity by the use of thiazolidinediones is being investigated in a growing number of trials (4, 36). However, this strategic approach appears to be inadequate as these agents display only modest and variable efficacy for NAFLD/NASH (27). Given the increasing prevalence of NAFLD/NASH and the lack of success implementing and sustaining lifestyle modifications, there remains a strong unmet need for effective pharmacologic agents.

GLP-1 receptor (GLP-1R) agonists (e.g., exenatide) are currently used to treat type 2 diabetes and may hold utility in NAFLD/NASH. These agents enhance glucose-dependent insulin secretion, regulate gastric emptying, decrease postprandial glucagon secretion, and reduce food intake/body weight. Recent studies in mice and rats have shown that GLP-1R agonism exerts beneficial effects on body weight, liver mass, and liver lipid, plasma ALT, and plasma triglycerides (5, 10, 31, 35, 39). In clinical studies, exenatide therapy lowered plasma ALT levels in patients with type 2 diabetes (17). An intriguing case study in an individual treated with exenatide for 44 wk documented a marked decrease in plasma ALT and hepatic steatosis (measured by spectroscopy) along with metabolic and diabetic improvements (42). Together, these data suggest a potential beneficial effect of GLP-1R agonism on NAFLD; however, no study has formally reported effects of GLP-1R agonism on fibrosis or NASH.

AC3174 is an analog of exenatide with leucine substituted for methionine at position 14 ([Leu(14)]exendin-4). AC3174 displays similar in vitro and in vivo efficacy as exenatide (14). Here, we report metabolic, biochemical, and histological improvements following AC3174 administration in mouse models of diet-induced NASH. First, we utilized a novel dietary model composed of a combination of high-fat (either in the form of trans-fat or lard), high fructose and high cholesterol to induce obesity, hepatomegaly, and steatosis, and, importantly, fibrosis. This model is essentially a variant on the model initially described by Tetri et al. (38). We explored the effects of these diets on the development of NAFLD/NASH in leptin-deficient mice (Lepob/Lepob), a model that is predisposed to develop hepatic steatosis even when maintained on standard, low-fat formulations of laboratory chow. As humans with...
NAFLD/NASH are more likely hyperleptinemic, rather than leptin-deficient, we also explored the effects of these diets as well as GLP-1R pharmacotherapy in wild-type (WT) C57BL6 mice.

Here we demonstrate the unequivocal development of hepatic fibrosis in the absence of circulating leptin and expand on the previously reported beneficial effect of GLP-1R agonism on body weight, liver size, and liver lipid (10, 31) by demonstrating a potential benefit of GLP-1R agonism on fibrosis (assessed both histologically and biochemically). We also demonstrate that benefits of GLP-1R agonism on NAFLD/NASH are largely, but not entirely, accounted for by weight loss. Interestingly, while intact GLP-1R expression is required for the pharmacologic effects of AC3174, mice that lack GLP-1R, are partially protected against developing NAFLD/NASH on these diets.

MATERIALS AND METHODS

Experimental animals and diets. All studies were approved by the Institutional Animal Care and Use Committee at Amylin Pharmaceuticals, in accordance with Animal Welfare Act guidelines. Animals were housed individually in standard cages at 22°C in a 12:12-h light-dark cycle. Male C57BL6 (B6) or Lepob/Lepob mice aged ~7 wk were purchased from The Jackson Laboratory (Bar Harbour, ME). Male GLP-1 receptor-deficient (GLP-1RKO) mice were derived from heterozygous mating pairs in a colony at Amylin Pharmaceuticals (San Diego, CA), initially derived from breeding pairs obtained from Dr. Daniel Drucker (34). These mice are a global knockout model in which the transgenic construct contains a PGK-neo cassette replacing two coding exons of the GLP1R gene in the same transcription orientation along with 4.8 kb and 3.5 kb of GLP1R sequence 5' and 3' to the PGK-neo sequence, respectively. The loss of these two exons equates to absence of the first and third transmembrane domains and intervening sequence (as described in Ref. 34). GLP-1RKO mice were genotyped at weaning by a commercial vendor (Taconic, Hudson, NY). Test diets were sourced from Research Diets (New Brunswick, NJ). To induce NASH, we tested two diets comprised of high fat (40% kcal) on either a low-fat diet (LFD), or high fructose diet (HTF). Body weight was measured at baseline (the day before pump implant) and at termination by NMR (Echo Medical), measured weekly. Body composition was measured at baseline (the vehicle or AC3174 (30 μg/kg/7 days) infused at a rate of 30 μg/kg/day) and at termination by NMR (Echo Medical) 1 week after pump implantation. Mice were age-matched between 8 and 10 wk for all experiments. Mice were weighed, snap-frozen in liquid nitrogen, and stored at −80°C until processed.

Histological and biochemical analyses of liver tissue. At termination, liver tissue was excised and fixed in 10 ml of 2.1 chloroform/methanol solution. The homogenate was filtered using fat-free paper and funneled into a preweighed 15-ml glass centrifuge tube. Next, an additional 5 ml of 2.1 chloroform/methanol solution was added followed by 2.5 ml of 0.9% NaCl. The filtered extract was subsequently mixed and centrifuged at 2,000 rpm, 10°C for 5 min. The aqueous layer was discarded, and the tube flushed with nitrogen until the lipid pellet was dry. The tube containing the lipid pellet was reweighed, and total lipid extracted per gram of starting liver was calculated.

For Western blot analysis, excised liver tissue was weighed, then snap-frozen in liquid nitrogen and stored at −80°C until processed. Protein was isolated from a fragment of each liver as follows. The frozen liver tissue was crushed on dry ice and then homogenized in lysis buffer with protease inhibitors (cat. no. 05892791001; Roche Complete Tablets; and 0.6 mM PMSF). Protein concentration of the cleared supernatant was measured with a BCA protein assay kit (Pierce, Rockford, IL). Liver tissue lysates were separated on 10% SDS-PAGE gels and transferred to PVDF membranes, following manufacturer’s protocols (Invitrogen, Carlsbad, CA). Membranes were cut between the 50- and 60-kDa markers and blocked with 5% milk plus 0.1% Tween-20. Membranes were incubated with horseradish peroxidase-conjugated anti-rabbit antibody, protein expression was detected with enhanced chemiluminescence (Pierce), and densitometry performed using a FluorChem System (Cell Biosciences, Santa Clara, CA).

Tissue gene expression. Livers were excised at termination, weighed, snap-frozen in liquid nitrogen, and stored at −80°C. Total RNA was extracted using TRI-reagent (Ambion, Austin, TX). RT reactions were performed with RETRscript RT-PCR reverse transcription system (Ambion). Quantitative real-time PCR (ABI PRISM 7900 Sequence Detection System; Applied Biosystems; Foster City, CA) for genes encoding murine collagen-1 (Col1a1; Mm00465519_g1), cluster of differentiation-68 (Cd68; Mm00801666_g1), cluster of differentiation-68 (Cd68; Mm00839636_g1), EGF-like module-containing mucin-like hormone receptor-like 1 (Emr1, F4/80 antigen; Mm01233105_g1), glucagon-like peptide-1 receptor (Glp1r; Mm01351009_g1), and β-actin, were performed using Taqman gene expression assays-on-demand and Universal PCR master mix (Applied Biosystems).

Plasma hormone and metabolite analyses. Plasma glucose, triglyceride, total cholesterol, ALT, and aspartate aminotransferase (AST) levels were measured using an Olympus AU400e Bioanalyzer (Olympus America Diagnostics, Center Valley, PA). Plasma samples were diluted 1:10 with PBS for detection of ALT and AST within the range of the standard curve. Total plasma adiponectin was measured using a commercially available ELISA according to manufacturer’s instructions (Millipore, Billerica, MA).

Statistical analyses. For comparison of two groups, unpaired Student’s t-test was used. Single point data was analyzed using one-way ANOVA, followed by Tukey’s test.
ANOVA, with Neuman-Keuls post hoc tests. Data comparing the effects of diet or drug treatment over time were analyzed using a two-(e.g., drug*time) or three-way (e.g., drug*genotype*time) ANOVA with repeated measures with Bonferroni post hoc tests to determine statistical differences. Significance was assumed for $P < 0.05$. Graphs and statistical analyses were generated using Prism 5 for Windows (Graphpad Software, San Diego, CA). All data points are expressed as means ± SE.

RESULTS

Exposure to a high trans-fat diet induced metabolic and hepatic characteristics of NASH that were attenuated by AC3174 treatment. We assessed the effects of a HTF or HLF diet on the induction of key facets of NAFLD/NASH in $\text{Lep}^{ob/ob}$ and B6 mice. Exposure of $\text{Lep}^{ob/ob}$ mice to either test diet for 8 wk resulted in significant body weight gain compared with mice maintained on LFD; however, weight gain was more pronounced in mice on HLF diet (Fig. 1A).

In contrast, B6 mice experienced greater weight gain in the lead-in period on the HTF diet compared with HLF diet (Fig. 1D). During the treatment phase, AC3174 significantly reduced body weight, regardless of strain, relative to vehicle controls (Fig. 1, B and E). Overall, AC3174-induced weight loss was greater in mice maintained on HTF diet compared with mice on HLF diet (weeks 2–4 of treatment for $\text{Lep}^{ob/ob}$ mice and weeks 2 and 3 of treatment for B6 mice; Fig. 1, B and E). AC3174 treatment significantly decreased fat mass relative to vehicle controls regardless of strain or diet. Significant reductions in lean mass relative to LFD and vehicle controls were noted in both strains of mice maintained on the HTF diet (Fig. 1, C and F). When normalized for body weight loss, however, the reduction in adiposity (% fat mass) remained significant, but the change in percent lean mass of all mice was not different relative to controls (data not shown).

![Fig. 1. Impact of diet and sustained glucagon-like peptide (GLP)-1 receptor agonism on body weight and body composition of $\text{Lep}^{ob/ob}$ and C57BL6 mice. Change in body weight during the dietary lead-in period (A and D) and testing period (B and E), and change in fat or fat-free mass (C and F) are shown for $\text{Lep}^{ob/ob}$ (A–C) and C57BL6 mice (D–F). LFD, low-fat diet; HTF, high trans-fat diet; HLF, high lard fat diet. For A and D, groups not sharing a superscript are significantly different. *$P < 0.05$ vs. LFD; $^*P < 0.05$ vs. vehicle control within diet; $^{+}P < 0.05$ vs. HLF-AC3174; $^#$ $P < 0.05$ comparing HTF and HLF vehicle control groups.](http://ajpgi.physiology.org/)

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In \textit{Lep}\textsuperscript{ob}/\textit{Lep}\textsuperscript{ob} mice, the HTF diet, but not HLF, increased body weight-adjusted liver mass relative to LFD and was significantly attenuated by AC3174 treatment (Fig. 2A). Intrahepatic lipid concentration was increased in \textit{Lep}\textsuperscript{ob}/\textit{Lep}\textsuperscript{ob} mice on both test diets relative to LFD, and was significantly reduced by AC3174 treatment relative to vehicle controls (Fig. 2B). In B6 mice, similar characteristics were observed. Body weight-adjusted liver mass was increased by both test diets, but to a greater extent by HTF diet, relative to LFD and was significantly reduced by AC3174 treatment relative to vehicle controls (Fig. 2C). Correspondingly, intrahepatic lipid was increased by both HTF and HLF diets, but significantly more so by HTF diet, relative to LFD and reduced by AC3174 treatment versus vehicle controls (Fig. 2D).

AC3174 treatment improved histopathological features of NASH induced by HTF diet. Histological examination confirmed that the HTF diet induced several key markers of NASH in \textit{Lep}\textsuperscript{ob}/\textit{Lep}\textsuperscript{ob} and B6 mice. Exposure to HTF diet significantly increased hepatic steatosis, evident primarily as macrovesicular steatosis in all hepatic zones, compared with animals maintained on LFD (Fig. 3, A–B, D–E). The presence of fibrosis in \textit{Lep}\textsuperscript{ob}/\textit{Lep}\textsuperscript{ob} mice was confirmed by trichrome staining; collagen was apparent in a perisinusoidal pattern characteristic of NASH (Fig. 3B). Western blot analysis revealed that collagen-1 protein was elevated approximately sixfold in animals exposed to HTF diet relative to \textit{Lep}\textsuperscript{ob}/\textit{Lep}\textsuperscript{ob} mice maintained on LFD (Fig. 3G), and \textit{Coll1a1} mRNA levels were also 5.9-fold higher in HTF vehicle mice compared with LFD controls (data not shown). Significant fibrosis was markedly less evident in B6 mice maintained on HTF diet for up to 16 wk despite the other features of NASH, with significant collagen-1 immunoreactivity detectable in only one sample (Fig. 3E and F). Furthermore, HLF diet did not induce fibrosis in \textit{Lep}\textsuperscript{ob}/\textit{Lep}\textsuperscript{ob} mice (data not shown). AC3174 treatment was associated with significant improvements in steatosis in both mouse strains on HTF diet (Fig. 3, C and F), and a nonsignificant trend for a reduction in collagen-1 protein (Fig. 3G), but not mRNA (remained 4.9-fold above controls) in \textit{Lep}\textsuperscript{ob}/\textit{Lep}\textsuperscript{ob} mice. In B6 mice, collagen-1 protein levels showed a small but significant decrease in AC3174-treated animals (Fig. 3H).

Development of hepatic inflammation was assessed in \textit{Lep}\textsuperscript{ob}/\textit{Lep}\textsuperscript{ob} mice exposed to LFD, HTF, or HLF treated with vehicle or AC3174. Mac-2 immunohistochemistry highlighted abundant Kupffer cell reactivity, generally associated with macrosteatosis, in both high-fat diet groups (data not shown). AC3174 treatment was not associated with an improvement in Mac-2 immunostaining (not shown). Hepatic gene expression levels of the macrophage markers \textit{Cd68} and \textit{Emr1} were assessed in \textit{Lep}\textsuperscript{ob}/\textit{Lep}\textsuperscript{ob} mice exposed to LFD, or HTF treated...
with vehicle or AC3174. Cd68 was upregulated by HTF diet (2.0 ± 0.3-fold increase relative to LFD, \( P < 0.05 \)) whereas Emr1 only tended to be increased (1.3 ± 0.4-fold relative to LFD). Both markers tended to exhibit reduced expression in AC3174-treated mice (1.7 ± 0.2-fold and 0.9 ± 0.1-fold, respectively) but were not significantly different from either LFD or HTF vehicle controls. In addition, mRNA levels of Glp1r tended to be reduced by HTF diet and were not altered by AC3174 treatment (fold change, LFD: 1.0 ± 0.4; HTF: 0.6 ± 0.1; HTF + AC3174: 0.5 ± 0.1).

Plasma and biochemical indices of NASH were improved by AC3174 treatment. In addition to histological endpoints, we assessed plasma markers associated with NASH, including ALT and AST, and circulating levels of triglycerides and cholesterol. HTF, but not HLF, tended to increase plasma levels of ALT, which were reduced ~50% by AC3174 treatment, in Lep\(^{ob}/\)Lep\(^{ob}\) mice (Table 1). In B6 mice, plasma ALT levels were increased ~10-fold by HTF and approximately fourfold by HLF diet, and were significantly reduced (by ~60%) by AC3174 treatment in HTF group only (Table 1). There were no differences in plasma AST levels in Lep\(^{ob}/\)Lep\(^{ob}\) or B6 mice (Table 1). Neither Lep\(^{ob}/\)Lep\(^{ob}\) nor B6 mice developed hypertriglyceridemia in the HTF and HLF diets. Plasma triglycerides were significantly lower in the AC3174-treated animals on HTF diet only (Table 1). Elevated levels of plasma cholesterol in Lep\(^{ob}/\)Lep\(^{ob}\) mice maintained on either the HTF and HLF diet were unaffected by AC3174 treatment. In B6 mice, AC3174 reduced the hypercholesterolemia induced by the HTF compared with mice on LFD (Table 1). Plasma total adiponectin levels were not significantly regulated by diet or AC3174 treatment (Table 1).

Fig. 3. Impact of HTF diet and sustained GLP-1 receptor agonism on steatosis and fibrosis assessed by histology. Representative sections from Lep\(^{ob}/\)Lep\(^{ob}\) (A–C) and C57BL6 mice (D–F) maintained on LFD control (A and D) or HTF diet and treated with vehicle (B and E) or AC3174 (C and F) for 4 wk. Lep\(^{ob}/\)Lep\(^{ob}\) mice (B) developed fibrosis, as seen by the blue staining emanating into the sinusoids in zone 3, while even on higher magnification no such staining could be seen in controls on HTF diet (E). The third column shows liver histology in mice on HTF diet following treatment with AC3174; in the Lep\(^{ob}/\)Lep\(^{ob}\) mice, the fibrosis was somewhat attenuated although it had not completely regressed (C); whereas improved steatosis and inflammation were noted in the HTF control mice following AC3174 (F). Western blot analysis for collagen-1 or GAPDH protein with corresponding densitometry analysis are shown for Lep\(^{ob}/\)Lep\(^{ob}\) (G) and C57BL6 mice (H). *\( P < 0.05 \) vs. LFD; ^\( P < 0.05 \) vs. HTF-vehicle. Staining: A, D, and F, hematoxylin and eosin; B, C, and E, trichrome.
GLP-1R agonism and fatty liver disease

Table 1. Metabolic plasma parameters of Lep"/Lep" or C57BL6 mice after 8- or 12-wk maintenance on HTF or HLF diet, respectively, followed by 4 wk of concomitant treatment with AC3174 (30 µg·kg⁻¹·day⁻¹) or vehicle, relative to mice maintained on LFD control

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>Diet/Drug</th>
<th>No.</th>
<th>Glucose, mg/dl</th>
<th>Triglycerides, mg/dl</th>
<th>Cholesterol, mg/dl</th>
<th>ALT, U/l</th>
<th>AST, U/l</th>
<th>Adiponectin, µg/ml</th>
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<tbody>
<tr>
<td>Lep&quot;/Lep&quot;</td>
<td>LFD</td>
<td>4</td>
<td>251 ± 20</td>
<td>73 ± 1</td>
<td>263 ± 11</td>
<td>811 ± 34</td>
<td>600 ± 36</td>
<td>9.9 ± 1.3</td>
</tr>
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<td></td>
<td>HTF/Vehicle</td>
<td>5</td>
<td>197 ± 13</td>
<td>62 ± 3</td>
<td>406 ± 27</td>
<td>1286 ± 340</td>
<td>856 ± 216</td>
<td>9.2 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>HLF/Vehicle</td>
<td>5</td>
<td>181 ± 20</td>
<td>62 ± 4</td>
<td>374 ± 14</td>
<td>792 ± 91</td>
<td>595 ± 65</td>
<td>9.1 ± 1.0</td>
</tr>
<tr>
<td>C57BL6</td>
<td>LFD</td>
<td>6</td>
<td>206 ± 3</td>
<td>73 ± 7</td>
<td>125 ± 2</td>
<td>18 ± 2</td>
<td>106 ± 22</td>
<td>ND</td>
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<tr>
<td></td>
<td>HTF/Vehicle</td>
<td>7</td>
<td>220 ± 11</td>
<td>64 ± 4</td>
<td>225 ± 13</td>
<td>176 ± 25</td>
<td>295 ± 28</td>
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<tr>
<td></td>
<td>HLF/Vehicle</td>
<td>7</td>
<td>174 ± 8</td>
<td>47 ± 4</td>
<td>147 ± 9</td>
<td>67 ± 13</td>
<td>223 ± 49</td>
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<td></td>
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<td>8</td>
<td>192 ± 21</td>
<td>59 ± 5</td>
<td>150 ± 20</td>
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<td>8</td>
<td>176 ± 10</td>
<td>57 ± 4</td>
<td>134 ± 10</td>
<td>54 ± 15</td>
<td>302 ± 60</td>
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ALT, alanine aminotransferase; AST, aspartate aminotransferase; LFD, low-fat diet; HTF, high trans-fat, high fructose, high cholesterol diet; HLF, high lard fat, high fructose, high cholesterol diet. ND, not determined. *a,b* Groups (within mouse strain) not sharing a superscript are significantly different from each other, \( P < 0.05 \). *P < 0.05 vs. LFD; \( P < 0.05 \) vs. vehicle control within diet.

GLP-1R-mediated improvements in indices of NASH are at least partly weight-independent. Sustained infusion of AC3174 induced significant reductions in body weight in these models. Weight loss per se is known to mediate benefits on NAFLD endpoints in rodents as well as humans (19, 28). To investigate whether the hepatic benefits conferred by sustained GLP-1R agonism were due solely to the induction of weight loss we assessed the liver phenotype of AC3174-treated Lep"/Lep" mice to those in a weight-matched control group (via caloric restriction). Although our regimen of caloric restriction did not elicit identical weight loss to that produced by AC3174-treatment during days 1–5, for the final 18 days of the study the AC3174-treated and weight-matched control groups were identically matched for mean body weight loss (Fig. 4A). Body composition analysis showed that both AC3174-treated and weight-matched animals exhibited similar significant reductions in whole body fat mass and fat-free mass relative to LFD and HTF-vehicle controls (Fig. 4B).

Body weight-adjusted liver weight was increased by HTF and was significantly reduced by AC3174 (by 14.2%; Fig. 4C), whereas normalized liver weight in weight-matched control animals was reduced by 9% and was not statistically different from vehicle. HTF diet likewise increased liver lipid relative to LFD controls, which was significantly reduced (by 12.9%; Fig. 4D) by AC3174 treatment. Weight-matched controls exhibited a trend for a reduction in liver lipid (by 5.1%), but this was not significant relative to vehicle controls. Overall, we estimate that weight loss mediated by AC3174 accounted for 63% of the improvement in liver weight and 39% of liver lipid levels respectively, pointing to improvements additionally attributable to sustained GLP-1R agonism.

Plasma ALT levels were significantly increased by HTF diet relative to LFD controls, and tended to be reduced by AC3174 treatment. ALT levels in weight-matched controls were not reduced and remained significantly greater relative to LFD controls and AC3174-treated mice (Fig. 4E). Plasma AST levels were not altered (Fig. 4F), and triglycerides were significantly lower in AC3174-treated animals compared with LFD controls, but not altered in weight-matched mice (Fig. 4G). Plasma total cholesterol was significantly higher in all mice maintained on HTF diet, with no effect of treatment or caloric restriction (Fig. 4H).

GLP-1Rs are required for the pharmacological effects of AC3174, although the absence of GLP-1Rs did not exacerbate development of NASH. We further characterized the impact of GLP-1R in NASH by examining the phenotype of WT or GLP-1RKO mice exposed to HTF diet for 12 wk and the impact of 4 wk of AC3174 treatment. During the fattening period (weeks 1–12; Fig. 5A), WT mice on HTF diet gained more weight compared with LFD WT controls. Likewise, HTF-fed GLP-1RKO mice also gained more weight relative to LFD WT controls. AC3174-treatment reduced body weight in WT mice compared with vehicle controls on either LFD or HTF diet, but had no impact on body weight in GLP-1RKO mice (data not shown). AC3174-treated WT mice gained more weight compared with LFD WT controls. Likewise, HTF-fed GLP-1RKO mice also gained more weight relative to LFD GLP-1RKO mice; however, weight gain in the GLP-1RKO mice was less than that of WT (Fig. 5A). During drug treatment (weeks 12–16), AC3174 significantly reduced body weight in WT mice compared with vehicle controls on either LFD or HTF diet, but had no impact on body weight in GLP-1RKO mice (Fig. 5, B and C). Food intake was significantly lower in AC3174-treated WT mice, but not GLP-1RKO mice, on either LFD or HTF during the first week of treatment only; no difference in food intake was noted between WT and GLP-1RKO mice (data not shown). AC3174-treated WT mice on LFD or HTF diet had significantly reduced adiposity (Fig. 5D), and lean mass (only in HTF-fed WT; Fig. 5E). Body composition was unchanged with AC3174 treatment of GLP-1RKO animals (Fig. 5, D and E).

On LFD, no effect of diet or drug was observed on body weight-adjusted liver mass (Fig. 5F). HTF-diet-maintained WT mice exhibited increased liver mass (~100%) relative to mice on LFD. Although HTF GLP-1RKO mice exhibited increased liver size (as % body weight) relative to GLP-1RKO maintained on a LFD (~60%), the increase was less than that observed in WT HTF controls. AC3174-treatment reduced liver weight in WT, but not GLP-1RKO, mice (Fig. 5F). Differences in liver weight were correlated with intrahepatic lipid concentration. In HTF-fed mice, AC3174 reduced liver lipid concentrations by ~28% in WT but not GLP-1RKO (Fig. 5G).

In LFD-fed mice plasma ALT levels were lower in GLP-1RKO compared with WT controls, but were not significantly altered in either WT or GLP-1RKO mice by AC3174 treatment (Table 2). There were no differences in plasma AST and triglycerides, and plasma cholesterol was lower in GLP-1RKO animals compared with WT controls, and was reduced by AC3174 treatment in WT relative to vehicle (Table 2). Similar
trends were observed in mice on HTF diet. Plasma ALT levels, elevated approximately fivefold relative to WT on LFD diet, tended to be reduced (by ~55%; Table 2), and also tended to be lower in GLP-1RKO animals; but neither relationship achieved statistical significance. There was no effect of genotype or diet on plasma AST levels. Both plasma triglycerides and cholesterol levels were significantly reduced by AC3174 treatment in WT mice but not altered in GLP-1RKO animals (Table 2).

**DISCUSSION**

Our findings provide several novel insights into the development of rodent models of NASH/NAFLD as well as strong evidence supporting the potential for GLP-1R-based therapeutics in this disease area. Animal models of NASH have been challenging to develop with no single reported model recapitulating the entire spectrum of the human disease (reviewed in Ref. 20). Traditional murine models have leveraged diets deficient in methionine and choline, or utilized toxins, such as carbon tetrachloride, to induce hepatic fibrosis; however, these agents are not representative of the clinical etiology of NASH. While these models have contributed to the current understanding of some of the molecular and cellular pathways activated in NASH, they are limited in their translational benefit. Animals maintained on these toxic diets are typically neither obese nor insulin resistant, and are usually in negative energy balance.
Recent advances include the formulation of diets high in trans-fat and fructose that appear to recapitulate many aspects of NAFLD/NASH in B6 mice. A notable example is the American Lifestyle-Induced Obesity Syndrome (ALIOS) model designed by Tetri et al. (38).

Our studies utilized a similar diet with several important modifications. First, we increased the content of cholesterol (2% by wt), and varied the source of the fat in the diet in the form of either trans-fat (Primex shortening; HTF) or lard (HLF). Fructose was also made available in the diet rather than...
drinking water. First, we observed important differences depending on whether trans-fat or lard was added to the diet. Inclusion of lard induced greater gains in weight and overall adiposity in Lep(ob)/Lep(ob) mice. However, with respect to modeling pathophysiologic features of NASH, trans-fat was a superior additive. HTF-fed Lep(ob)/Lep(ob) and B6 mice exhibited significant increases in liver size (as % body weight) and intrahepatic lipid, respectively. By contrast, the HLF diet elicited much more modest increases. These findings are in line with the ALIOS model previous used by Tetri et al. (38) and Sharma et al. (35) and support a key role of trans-fat in propagating NASH. A major difference in the HTF diet used in these experiments and the ALIOS model of trans-fat-induced NASH, described previously as not being a cause of fibrosis in normal B6 mice within 24 weeks (38), is the addition of 2% cholesterol to the HTF diet. Significant fibrosis was only observed in Lep(ob)/Lep(ob) mice on HTF diet; replacing trans-fat with lard fat did not result in fibrosis. This observation suggests that a high cholesterol/high fructose diet in the setting of other factors (obesity, insulin resistance, leptin insufficiency) may stimulate the genesis of fibrosis. The underlying mechanisms driving this effect are not yet known.

The development of fibrosis in leptin-deficient Lep(ob)/Lep(ob) mice induced by the HTF diet is a novel and important finding. Previous studies suggested that leptin activation of hepatic stellate cells was a necessary requisite for the induction of NASH (7, 15, 33). Our findings challenge this assertion; although our diet elicited some fibrosis in the B6 mice, the degree of fibrosis was greater in the leptin-deficient mice. The presence of fibrosis indicated that we had developed a favorable dietary murine model of NASH (steatosis, fibrosis, hepatomegaly, increased plasma ALT) in the context of metabolic disease (obesity, diabetes). Whereas B6 HTF mice may be a good model for evaluating anti-steatotic agents whose mechanisms of action may require intact leptin signaling, Lep(ob)/Lep(ob) mice on HTF diet might be more appropriate for exploring anti-fibrotic mechanisms and therapies, with the caveat that their mechanism of action be leptin-independent. To what extent an even more robust model of inducing NASH and fibrosis can be induced by maintaining these models on the HTF diet for a longer duration remains to be determined.

Recent studies have demonstrated that extended activation of GLP-1R in Lep(ob)/Lep(ob) mice maintained on standard lab chow where exendin-4 was administered for 60 days via twice-daily intraperitoneal injections (10) or in B6 mice on 42% fat high-fat diet with exendin-4 over-expressed via adeno virus (31), significantly reduced hepatic lipid content and improved plasma ALT and lipids. Consistent with these findings, infusion of the GLP-1R agonist AC3174 to mice maintained on our HTF diet significantly reduced weight and fat mass, liver mass, steatosis, and plasma ALT and lipids.

Our findings also extend upon these previous observations in several ways. First, our findings suggest that GLP-1R agonism can modestly improve the histological severity of fibrosis, a finding supported quantitatively by a reduction in liver collagen-I protein. Western blot analysis revealed that even in the livers of B6 mice, in which only low levels of collagen were visualized histologically, small but significant reductions in detectable protein were evident after AC3174 treatment. Hepatocyte ballooning and lobular inflammation are clinical histopathological observations indicative of human NASH. In animal models, the presence or absence (and relevance) of hepatocyte ballooning is debated. It has been previously observed in mice and toxin-induced rat models of NASH (9, 18), and in a recent elegant swine model of NASH (23); however, in our hands, as in others, the criteria according to the pathologist for ballooning were not met by simple but routine histochemical staining, agreeing with previous pathological examinations (40). A more detailed examination of hepatocyte ballooning, for example via K8/K18 immunostaining of liver sections, would be warranted to confirm this observation. Both HTF and HLF diets were associated with inflammation, as measured by Kupffer cell visualization (Mac-2 immunostaining) and expression of Cd68 and Emr1. The presence of GLP-1R have been demonstrated on mouse macrophages in apolipoprotein E knockout mice and GLP-1 treatment attenuated foam cell formation, aortic infiltration of macrophages, and ultimately suppressed the development of atherosclerotic lesions (26). There are no published reports on the effect of GLP-1R agonism on Kupffer cell function, but it is interesting to speculate that GLP-1R treatment may reduce the influx of macrophages to the liver and thereby mediate beneficial effects on NAFLD (16). While we did not observe such effects in our study, it may be that longer treatment is required, or these effects may be more visible in a preventive model or in GLP-1RKO mice that exhibited reduced steatosis.

Second, unlike previous findings, we clarify the contribution of weight loss to the observed improvements in hepatic endpoints. Whereas AC3174-treated animals exhibited reduced liver weight, liver lipid, and plasma triglycerides and ALT levels, weight-matched mice demonstrated only modest, non-significant reductions in liver weight and liver lipid, and no change in triglycerides and ALT levels. These data suggest that there are other aspects of GLP-1R-mediated signaling that elicit benefits on the liver beyond those achieved via calorie-restriction and maintenance of a reduced weight state.

The pharmacological weight-independent mechanism(s) by which GLP-1R agonism can improve NAFLD/NASH remain to be fully elucidated. GLP-1R is primarily expressed within pancreas and brain, but is also expressed in other tissues including muscle, adipose tissue, and liver where a direct effect of GLP-1 on hepatic tissues has been proposed. Functional GLP-1R has been localized on primary rat hepatocytes, immortalized mouse liver cell lines, and human hepatocytes (10, 13). GLP-1R agonism has been associated with reduced hepatic lipogenesis and enhanced fat oxidation in rodents (5, 10, 31, 37). More recently, a role for GLP-1R agonism in the regulation of endoplasmic reticulum (ER) stress and the unfolded protein response has emerged. Development of ER stress is associated with the development of diabetes and pancreatic β-cell loss (12). GLP-1R activation has been demonstrated to reduce ER stress and improve β-cell function in vivo in mice (41, 44), rat primary and insulinoma cell lines (44), and also in primary human hepatocytes (35). While we did not measure hepatic markers of lipid synthesis or oxidation or ER stress, the body weight-independent pharmacological effects of AC3174 treatment noted here may be the result of separate or combined modulation of these pathways.

Our studies additionally explored the impact of a lack of GLP-1R expression on the development of NASH. Recent work has shown that human liver tissue from subjects with NASH exhibited reduced levels of Glp1r mRNA as well as...
GLP-1 protein (37), and Lep <sup>ob</sup>/Lep <sup>ob</sup> mice on an HTF diet also demonstrated a trend for reduced levels of Glp1r mRNA in liver. It is not known whether reduced hepatic expression of Glp1r is a cause or consequence of NAFLD/NASH. In the absence of GLP-1R, mice were protected from the body weight-, hepatomegaly- and steatosis-inducing aspects of HTF diet, suggesting that the development of NAFLD/NASH precedes perturbation of Glp1r expression. There is precedence for paradoxical protective effects of GLP-1R deletion. For example, GLP-1RKO mice challenged with a high-fat diet failed to develop diet-induced obesity and maintained insulin sensitivity, attributable to compensatory increases in energy expenditure associated with increased locomotor activity; however, the precise mechanisms and pathways that regulate this remain to be determined (2). Compensation in transgenic models is common, and delineating the role of the hepatic GLP-1R in NASH will likely require the development of tissue-specific, inducible-knockout methodologies. Nevertheless, our pharmacological studies provide clear evidence that the therapeutic effects of the exenatide analog AC3174 were GLP-1R mediated, as neither body weight, body composition, or liver or plasma biochemistry were affected in the receptor knockouts.

Collectively, these studies contribute a new, diet-induced murine model of NAFLD/NASH and provide clear evidence for a beneficial role of an exenatide analog in the treatment of key aspects of this disease, including fibrosis. The precise mechanisms whereby this occurs remain to be determined; however, in our preclinical model it is apparent that pharmacological benefits beyond weight loss are contributory. Clinical trials determining the translational benefit of GLP-1R agonism in human subjects with NASH and associated metabolic disease are warranted.

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DISCLOSURES

J.L.T., P.S.G., C.W., C.D., M.E., J.N., D.G.P. and J.D.R. are employed by and/or own stock in Amylin Pharmaceuticals, Inc. B.A.N.-T. and E.M.B. have consulted for Amylin Pharmaceuticals, Inc.

AUTHOR CONTRIBUTIONS


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