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Exenatide Prevents Diet-induced Hepatocellular Injury in A CEACAM1-Dependent Mechanism

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Abstract

The Carcinoembryonic Antigen-Related Cell Adhesion Molecule 1 (CEACAM1) promotes insulin sensitivity by inducing insulin clearance and reducing de novo lipogenesis in the liver. Consistently, Cc1–/– mice with null deletion of Ceacam1 gene exhibit hyperinsulinemia and insulin resistance, in addition to steatohepatitis. They also exhibit early pericellular fibrosis. Redelivering Ceacam1 to the liver reverses the altered metabolism and histopathology of Cc1–/– mice. Exenatide, a long-acting glucagon-like peptide-1 receptor agonist, induces Ceacam1 transcription and consequently, reverses impaired insulin clearance and insulin resistance caused by high-fat intake. Additionally, it reverses fat accumulation in the liver. The current studies show that exenatide also restored the activities of alanine transaminase and aspartate aminotransferase, and reversed the inflammatory and oxidative stress response to high-fat diet in wild-type, but not in Cc1–/– mice. Exenatide also prevented diet-induced activation of the TGF$^{-/-}$ Smad2/Smad3 pro-fibrogenic pathways, and normalized the mRNA levels of pro-fibrogenic genes in wild-type, but not in Cc1–/– mice. Together, the data demonstrate that exenatide prevented diet-induced pro-fibrogenesis and hepatocellular injury in a CEACAM1-dependent mechanism.

Keywords: Fibrosis; Glucagon-Like Peptide-1; Insulin Clearance; Insulin Resistance; Steatohepatitis

Nonstandard Abbreviations

ALT	:	Alanine Transaminase
AST	:	Aspartate Aminotransferase
CEACAM1	:	Carcinoembryonic Antigen-Related Cell Adhesion Molecule 1
Ceacam1	:	Gene and mRNA Encoding Mouse CEACAM1 Protein
Cc1$^{-/-}$	:	Global Ceacam1 Null Mouse
Cc1$^{+/+}$	:	Wild-Type Littermate of Cc1$^{-/-}$
GLP-1	:	Glucagon-Like Peptide-1
RD	:	Regular Diet
HF	:	High-Fat Diet

Introduction

Nonalcoholic Fatty Liver Disease (NAFLD) ranges from benign steatosis to steatohepatitis and Non-Alcoholic Steatohepatitis (NASH) that also includes chicken-wire bridging fibrosis [1]. Uncontrolled, the disease can progress to adenocarcinoma to constitute a major risk factor for liver transplant [2].

The paucity of mouse models that replicate faithfully NASH human disease and in particular fibrosis [3], has limited our understanding of its molecular underpinning and consequently, has restricted progress in the development of effective pharmacologic interventions. Studies in our laboratory have identified a role for the Carcinoembryonic Antigen-Related Cell Adhesion molecule

1 (CEACAM1) in inducing hepatic insulin clearance to promote insulin sensitivity and reduce hepatic de novo lipogenesis to protect the liver against the high level of insulin in the portal circulation [4]. Accordingly, mice with global null deletion of Ceacam1 gene ($Cc1^{-/-}$) [5] and with liver-specific inactivation of CEACAM1 [6] develop impaired insulin clearance, followed by hyperinsulinemia and insulin resistance, in addition to fat accumulation in the liver, largely resulting from hyperinsulinemia-induced activation of the transcription of lipogenic genes [7,8]. With increased fat storage triggering changes in the inflammatory milieu [9,10], Ceacam1 mutants also develop steatohepatitis. Moreover, they develop a NASH-characteristic chicken-wire pattern of fibrosis on regular chow diet [5]. When fed a high-fat diet, these features of hepatocyte injury progress to include advanced chicken-wire fibrosis and apoptosis [11,12].

Recently, we have found that exenatide, a long-acting Glucagon-Like Peptide-I (GLP-I) receptor agonist and a synthetic analog of Exendin-4 that induces insulin secretion in part by inhibiting glucagon secretion [13,14], also induces CEACAM1-dependent hepatic insulin clearance [15]. Whereas exenatide reverses steatohepatitis in wild-type mice fed a high-fat diet, it fails to do so in $Cc1^{-/-}$ mice. The underlying mechanism involves the induction of Ceacam1 expression by binding directly to the peroxisome Proliferator-Activated Receptor Response Element (PPRE)/retinoid X receptor-α (RXRα) on the Ceacam1 promoter, and activating its transcription [15]. Thus, we aimed in the current study to investigate whether exenatide also ameliorates fibrosis, and whether this requires intact CEACAM1 expression.

Materials and Methods

Mice Maintenance

C57BL/6.$Cc1^{-/-}$ and $Cc1^{+/+}$ littermates (3 months of age) were fed ad libitum a standard (RD) or a High-Fat (HF) diet deriving 45:35:20% calories from fat: carbohydrate: protein (D12451, Research Diets) for 2 months [16]. In the last month of feeding, mice received an intraperitoneal injection/day of saline or exenatide (20ng/g BW/day) (507-77, California Peptide Research, Salt Lake City, UT) [15]. All procedures were approved by the Institutional Animal Care and Utilization Committee at the University of Toledo.

ALT and AST Colorimetric Assays

Per manufacturer's instructions (Abcam, Cambridge, MA), liver tissues (50mg) were homogenized in 200µl assay buffers, centrifuged (13,000xg, 10 min), and aliquots from the supernatant layer were added to 100µl of the reaction mix in the Alanine Transaminase (ALT) (ab105134) and Aspartate Aminotransferase (AST) (ab105135) kits. The products were read at OD570nm, and activities were measured in µM/mg.

Western Blot Analysis

Livers were lysed and proteins analyzed by SDS-PAGE followed by immunoprobing with polyclonal antibodies against phospho-Smad2$^{Ser465/467}$ and phospho-Smad3$^{Ser423/425}$ (Cell signaling, Danvers, MA). For normalization, proteins were reprobed with polyclonal antibodies against SMAD2 and SMAD3 (Cell signaling). Blots were incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG antibody (GE Healthcare Life Sciences, Amersham, Marlborough, MA) and proteins were visualized using ECL (Amersham).

Liver Histology

As described [12], Formalin-fixed, paraffin-embedded liver sections were stained with 0.1% Sirius Red stain (Sigma, Direct Red 80). Fibrosis was assessed on deparaffinized and rehydrated slides and scored using the Brunt Criteria [17,18].

Quantitative Real-time PCR Analysis

Perfect Pure RNA Tissue Kit (5 PRIME Inc.) was used to isolated total RNA and cDNA was synthesized by iScript cDNA Synthesis Kit (BIO-RAD), using 1µg of total RNA and oligo-dT primers (Table 1). cDNA was evaluated with qRT-PCR (Step One Plus, Applied Biosystems) and normalized to 18S. Results are expressed in fold change as the mean ± SEM.

Primer	Forward Sequence (5'-3')	Reverse Sequence (5'-3')
Col6-α3	GTCAGCTGAGTCTTGTGCTGT	ACCTAGAGAACGTTACCTCACT
α-Sma	CGTGGCTATTCCTTCGTTAC	TGCCAGGAGACTCCATCC
TGFβ	GTGGAAATCAACGGGATCAG	ACTTCCAACCCAGGTCCTTC
Smad7	GTTGCTGTGAATCTTACGGG	ATCTGGACAGCCTGCA
IFNγ	ATG AACGCTACACACTGCATC	CCATCCTTTTGCCAGTTCCTC
IL-6	CTTGGGACTGCCGCTGGTGA	TGCAAGTGCATCATCGTTGT
TNFα	CCACCACGCTCTTCTGTCTAC	AGGGTCTGGGCCATAGAACT
Nox1	TTACACGAGAGAAATTCTTGGG	TCGACACACAGGAATCAGGA
Nox4	TCCAAGCTCATTTCCCACAG	CGGAGTTCCATTACATCAGAGG

Gp91	TATGCTGATCCTGCTGCCAGT	TGTCTTCGAATCCTTGTCGAGC
18S	TTCGAACGTCTGCCCTATCAA	ATGGTAGGCACGGCGACTA

Table 1: Primer sequence of mouse genes used in quantitative Real-time PCR analysis.

Statistical Analysis

Data were analyzed by one-way ANOVA or the two-tailed Student-t-test using Graph Pad Prism 6 software. $P<0.05$ was considered statistically significant.

Results

Effect of Exenatide on Hepatic ALT and AST Activities

$Cc1^{-/-}$ mice exhibited higher hepatic ALT (Figure 1A) and AST (Figure 1B) activities than their age-matched $Cc1^{+/+}$ wild-type counterparts, as previously reported [19]. HF feeding for 2 months elevated hepatic ALT and AST activities in $Cc1^{+/+}$ and $Cc1^{-/-}$ mice (Figure. 1A and 1B, HF-S vs RD-S). Treating wth exenatide in the last 30 days of HF feeding reversed this increase in $Cc1^{+/+}$, but not $Cc1^{-/-}$ mice (Figure 1A and 1B, HF-Ex vs HF-S).

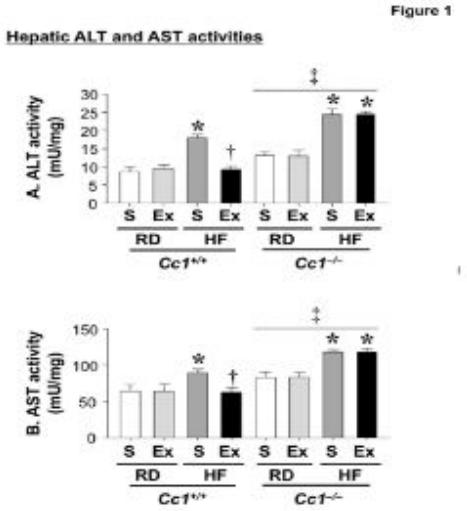

Figure 1: Effect of exenatide on hepatic ALT and AST activities. Mice were fed with a Regular Diet (RD) or a High-Fat Diet (HF) for 2 months and injected daily with either Saline (S) or Exenatide (Ex) in the last month of feeding. At the end of the feeding/treatment period, AST and ALT activities were measured in duplicate in liver lysates (n=5 mice/ genotype/ feeding/ treatment). RD-S (white), RD-Ex (light grey), HF-S (dark grey), and HF-Ex (black). Values are expressed as mean ± SEM. *P<0.05 Ex vs S/ feeding group; †P<0.05 HF vs RD/ treatment group, ‡P<0.05 Cc1−/− vs Cc1+/+ mice.

Effect of Exenatide on Oxidative Stress and Inflammation

qRT-PCR analysis revealed that HF diet induced hepatic mRNA levels of markers of oxidative stress, including Nox1, Nox 4 and Gp91, by ~2- to 3-fold in both groups of mice (Table 2, HF-S vs RD-S), as expected [20]. This was accompanied by a 2-fold increase in mRNA levels of markers of inflammation (Il-6, IFNγ and TNFα) (Table 2, HF-S vs RD-S). Exenatide treatment reversed the positive effect of HF diet on the hepatic mRNA levels of the inflammatory and oxidative stress markers in Cc1+/+, but not Cc1–/– mice (Table 2, HF-Ex vs HF-S). That exenatide reversed inflammation in Cc1+/+, but not Cc1–/– mice, is supported by our previously published H&E stain analysis [15].

	RD-S	RD-Ex	HF-S	HF-Ex
a) $Cc1^{+/+}$				
Fibrosis				
Col6-α3	2.11 ± 0.22	2.33 ± 0.20	6.11 ± 0.30*	2.37 ± 0.25†
α-Sma	3.02 ± 0.34	3.15 ± 0.31	6.99 ± 0.29*	3.19 ± 0.23†
TGFβ	2.36 ± 0.26	2.18 ± 0.21	6.16 ± 0.28*	2.13 ± 0.26†
Smad7	1.36 ± 0.04	1.41 ± 0.04	0.38 ± 0.06*	1.47 ± 0.04†
Oxidative stress				
Nox1	1.12 ± 0.07	1.21 ± 0.06	3.26 ± 0.12*	1.28 ± 0.11†
Nox4	1.08 ± 0.05	1.11 ± 0.07	3.01 ± 0.10*	1.22 ± 0.12†
Gp91	1.11 ± 0.12	1.16 ± 0.08	2.98 ± 0.17*	1.12 ± 0.13†
Inflammation				
Il-6	4.46 ± 0.18	4.21 ± 0.11	8.71 ± 0.21*	4.23 ± 0.19†
IFNγ	7.22 ± 0.18	6.87 ± 0.25	12.5 ± 0.25*	6.88 ± 0.15†
TNFα	3.07 ± 0.21	2.86 ± 0.22	6.86 ± 0.22*	2.73 ± 0.11†
b) $Cc1^{-/-}$				
Fibrosis				
Col6-α3	3.84 ± 0.18	4.07 ± 0.33	10.6 ± 0.41*	10.8 ± 0.41*
α-Sma	5.47 ± 0.30	5.68 ± 0.35	12.9 ± 0.44*	12.7 ± 0.42*
TGFβ	5.22 ± 0.26	5.47 ± 0.33	12.5 ± 0.47*	12.0 ± 0.49*
Smad7	2.88 ± 0.10	3.09 ± 0.07	1.74 ± 0.10*	1.76 ± 0.07*
Oxidative stress				
Nox1	1.34 ± 0.11	1.45 ± 0.15	3.87 ± 0.18*	3.58 ± 0.17*
Nox4	1.02 ± 0.12	1 1.13 ± 0.11	3.57 ± 0.22*	3.48 ± 0.20*
Gp91	1.36 ± 0.22	1.43 ± 0.21	3.03 ± 0.28*	3.11 ± 0.24*
Inflammation				
Il-6	7.13 ± 0.21	7.43 ± 0.14	13.4 ± 0.31*	12.9 ± 0.34*
IFNγ	7.22 ± 0.19	7.41 ± 0.18	14.3 ± 0.21*	14.1 ± 0.27*
TNFα	5.51 ± 0.23	5.74 ± 0.22	11.2 ± 0.27*	11.0 ± 0.21*

Male Mice (3-month-old) were fed RD or HF for 2 months. In the last 30 days of feeding, they were injected intraperitoneally once daily with Saline (S) or Exenatide (Ex) (20ng/g BW) (n=5/genotype/feeding/treatment). Hepatic qRT-PCR analysis was carried out in triplicate and normalized to 18S. Values are expressed as mean ± SEM. *P<0.05 HF versus RD/each of S or Ex treatment group; †P<0.05 Ex versus S/each of RD or HF feeding group

Table 2: Effect of Exenatide on the mRNA levels of genes in the liver of male mice.

Effect of Exenatide on Hepatic Fibrosis

qRT-PCR analysis showed higher mRNA levels of pro-fibrotic genes (Col6-α3, α-Sma and TGFβ) and lower mRNA levels of Smad7, an inhibitor of TGFβ activation, in the liver of untreated HF-fed relative to RD-fed $Cc1^{+/+}$ mice (Table 1, HF-S vs RD-S). Accordingly, Western blot analysis revealed induction of Smad2/Smad3 phosphorylation by HF diet in $Cc1^{+/+}$ mice (Figure 2A, HF-S vs RD-S). Sirius red staining showed periportal fibrosis in HF-S $Cc1^{+/+}$ mice (Figure 2 Bii and a Brunt score of 2-accompanying table). Exenatide treatment reversed these HF-induced profibrogenic parameters in $Cc1^{+/+}$ mice (Table 2 and Figure 2A, HF-Ex vs S-Ex, and Figure 2Biv with a Brunt score of 0). As expected from our previous studies [12], Sirius red staining revealed perivenular and/or peri cellular bridging chicken-wire pattern of collagen deposition in the liver of RD- and HF-fed $Cc1^{-/-}$ mice (Figure 2Bv-vi and a Brunt score of 3). This NASH-like fibrosis in $Cc1^{-/-}$ mice, whether spontaneously (under RD feeding conditions-Figure 2Bv) or in response to HF diet (Figure 2 Bvi) was not reversed by 4 weeks of exenatide treatment (Figure 2Bvii and Figure 2Bviii, respectively and a Brunt score of 3). Consistently, exenatide failed to normalize the mRNA levels of markers of fibrosis modulators under both feeding conditions (Table 2, RD/HF-Ex vs RD/HF-S), as well as it failed to reduce Smad2/Smad3 activation by HF diet in $Cc1^{-/-}$ mice (Figure 2A, RD/HF-Ex vs RD/HF-S).

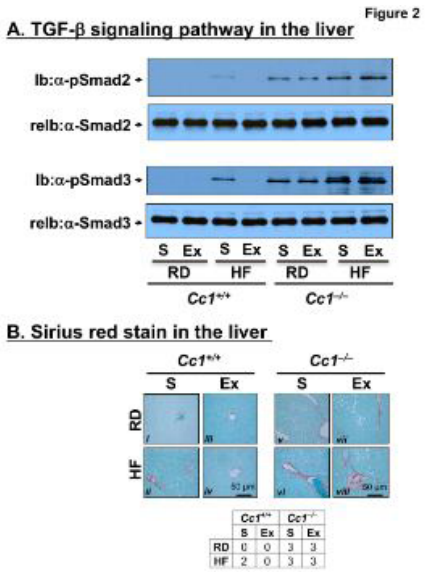

Figure 2(A-B): Effect of exenatide on hepatic fibrosis. **A.** Liver lysates were analyzed by immunoblotting with α-phospho-Smad2 (α-pSmad2) and α-phospho-Smad3 (α-pSmad3) antibodies followed by reimmuno probing (reIb) with antibodies against total Smad2 and total Smad3, respectively, for normalization. Gels represent more than 2 experiments performed on different mice/ feeding/ treatment group. **B:** Liver histology were analyzed by Sirius red staining to detect bridging fibrosis in $Cc1^{-/-}$ mice (n=7-8/ genotype/ feeding/ treatment). Representatives from S-treated RD-fed (i and v), S-treated HF-fed (ii and vi), Ex-treated RD-fed (iii and vii); and Ex-treated HF-fed mice (iv and viii) are shown. The degree of fibrosis was evaluated per Brunt Criteria, and scores are included in the accompanying table.

Discussion

$Cc1^{-/-}$ mice develop insulin resistance, steatohepatitis with spontaneous chicken-wire fibrosis that become more robust in response to high-fat feeding [12]. Liver-specific rescuing of CEACAM1 expression reverses hyperinsulinemia, insulin resistance, steatohepatitis and visceral obesity in $Cc1^{-/-}$ mice [21]. We have also shown that high-fat feeding for 21 days reduces CEACAM1 expression by >50% in C56BL/6 mice to cause insulin resistance and metabolically phenocopy the $Cc1^{-/-}$ mouse [16], but forced liver-specific overexpression [16] and adenoviral-mediated redelivery of CEACAM1 [22] prevents these metabolic abnormalities together with the rise in profibrogenic genes in HF-fed wild-type mice. Together, this suggests that loss of CEACAM1 in liver plays a critical role in the pathogenesis of metabolic and histological abnormalities detected in NAFLD. In support of this proposed CEACAM1-based mechanism, exenatide reverses insulin resistance and steatohepatitis in wild-type, but not $Cc1^{-/-}$ mice, via inducing Ceacam1 transcription [15]. The current studies showed that exenatide also reversed the other features of hepatocyte injury caused by high-fat feeding in $Cc1^{+/+}$, but not $Cc1^{-/-}$ mice. These include: activation of ALT and AST, oxidative stress as assessed by changes in the mRNA levels of associated genes, and of the TGFβ-mediated profibrogenic pathways in liver.

Normalization of ALT and AST activities, in addition to preventing the rise in inflammatory markers and inactivation of TGFβ pathways by exenatide is consistent with the ameliorating effect of IP118, a GLP-1 receptor agonist, on these markers of hepatocyte injury in high-fat diet-fed C57BL/6 mice [23]. Failure of exenatide to modulate these metabolic and pathological phenotypes in RD- and HF-fed $Cc1^{-/-}$ mice points to the key role of CEACAM1 in mediating the beneficial effects of exenatide. Thus, it is likely that by binding to the PPRE/RXR element on the Ceacam1 promoter [15], exenatide induces CEACAM1 expression in liver to prevent the advancement of hepatocyte injury, including the increase in fat accumulation and inflammation caused by high-fat diet [24].

In addition to basal steatosis and inflammation, $Cc1^{-/-}$ mice exhibit low levels of pericellular fibrosis when fed a regular chow diet [12]. The current studies show that this is accompanied by basal induction of Smad2/Smad3 in the TGFβ pathway, which similarly occurs in the liver of C57BL/6 mice fed a high-fat diet, likely resulting from the >50% decrease in hepatic CEACAM1 level by high-fat intake, as we have previously shown [16]. Reversal of

these pathways by adenoviral-mediated liver-specific redelivery of CEACAM1 demonstrates the anti-fibrogenic role of hepatic CEACAM1 in the liver [22] as well as in the white adipose tissue [25]. Inactivation of the TGFβ fibrogenic pathway by exenatide in $Cc1^{+/+}$, but not $Cc1^{-/-}$ mice, further assigns a critical role for hepatic CEACAM1 induction in the anti-fibrogenic effect of exenatide. Mechanistically, the loss of hepatic CEACAM1 impairs insulin clearance to cause hyperinsulinemia, followed by insulin resistance and elevated hepatic de novo lipogenesis [8], both being risk factors for fibrosis [26]. Increased fat accumulation can change the inflammatory microenvironment [9,10] in the liver to release profibrogenic factors [27], and proinflammatory cytokines [28] that modify hepatic inflammation and contribute to fibrosis [24].

Moreover, increase in hepatic lipid production yields redistribution of substrates to the white adipose tissue to cause visceral adiposity followed by associated induction of the pro-inflammatory state, including the release of leptin, which can exacerbate the fibrogenic effect of TNFα [29; 30], which causes oxidative stress [31] and reduces Smad 7 expression [32], leading to the activation of the TGFβ Smad2/Smad3 fibrogenic pathways. In addition to inducing the activity of TGFβ, high-fat diet also increases its hepatic level together with that of the pro-fibrogenic factor, IL-6 [33,34], but with a rise in the anti-fibrogenic IFNγ [34]. With exenatide inducing hepatic CEACAM1 production that contributes substantially to the decrease in visceral obesity [15], and subsequently, the pro-inflammatory state associated with high-fat feeding, it is conceivable that induction of CEACAM1 expression is required for the beneficial effect of exenatide not only in restoring the metabolic phenotype caused by high-fat intake, but also in limiting the progression of fibrosis.

Conclusion

Our data emphasize that induction of hepatic CEACAM1 by exenatide mediates its effect not only on insulin resistance, hepatic steatosis and visceral obesity [15], but also on the production of IL-6 and TNFα, which would, in turn limit their pro-inflammatory and pro-fibrogenic effect. The significance of this finding to human disease is highlighted by the reported low hepatic CEACAM1 levels in insulin resistance obese patients with fatty liver disease [35]. While by inducing CEACAM1 expression and promoting hepatic insulin clearance, exenatide maintains insulin levels at physiologic levels in the face of increased insulin secretion [15] in order to limit insulin resistance and steatohepatitis in animal models of NAFLD/NASH [36-38], the data supporting its clinical relevance and safety in the treatment of NASH remain limited [39] and warrant further investigations.

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