Glucagon-like peptide-1 receptor activation stimulates hepatic lipid oxidation and restores hepatic signalling alteration induced by a high-fat diet in nonalcoholic steatohepatitis

Gianluca Svegliati-Baroni1, Stefania Saccomanno3, Chiara Rychlicki1, Laura Agostinelli1, Samuele De Minicis3, Cinzia Candelaresi1, Grazia Faraci1, Deborah Pacetti2, Marco Vivarelli2, Daniele Nicolini2, Paolo Garelli3, Alessandro Casini4, Melania Manco5, Geltrude Mingrone6, Andrea Risaliti3, Giuseppe N. Frega2, Antonio Benedetti1 and Amalia Gastaldelli7

1 Department of Gastroenterology, Polytechnic University of Marche, Ancona, Italy
2 Department of Food Science, Polytechnic University of Marche, Ancona, Italy
3 Transplant Center, Polytechnic University of Marche, Ancona, Italy
4 Nutrition Center and GI Unit, University of Florence, Florence, Italy
5 Pediatric Hospital ‘Bambino Gesù’, IRCCS, Rome, Italy
6 Department of Internal Medicine, Catholic University, Rome, Italy
7 Institute of Clinical Physiology, National Research Council (IFC-CNR), Pisa, Italy

Keywords
hepatic lipid oxidation – high-fat diet – GLP-1 receptor – NASH

Abbreviations
ACOX1, acyl-CoA oxidase; AMPK, AMP-activated protein kinase; CPT1A, carnitine palmitoyltransferase 1A; FFA, free fatty acid; GLP-1, glucagon-like-peptide-1; high-fat diet, high-fat/high-calorie diet; IR, insulin resistance; JNK, c-Jun NH2-terminal kinase; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; PKA, protein kinase A; PPAR, peroxisome proliferator-activated nuclear receptors.

Correspondence
Amalia Gastaldelli, Laboratory, Institute of Clinical Physiology, CNR via Moruzzi 1, 56100 Pisa, Italy
Tel: +39 050 3152679
Fax: +39 050 3152166
e-mail: amalia@ifc.cnr.it

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Hepatic insulin resistance is the main determinant of not only fasting but also postprandial hyperglycaemia. Subjects with nonalcoholic fatty liver disease (NAFLD) have been shown to be very insulin resistant at the level of the liver, even when they are lean and without the features of metabolic syndrome (1). NAFLD represents the hepatic manifestation of metabolic syndrome, leading to nonalcoholic steatohepatitis (NASH), cirrhosis and hepatocellular carcinoma (2). The prevalence of NAFLD and NASH, although not known, is increasing in western countries owing to high dietary fat intake and low physical activity and NAFLD/NASH is often associated with obesity and diabetes (3). Glucagon-like peptide-1 (GLP-1) analogues, recently approved for the treatment of diabetic hyperglycaemia (4), have also been proposed as a treatment for the liver injury associated with hepatic steatosis (5).

In this respect, GLP-1 is an incretin hormone secreted by enteroendocrine L-cells in response to ingested nutrients and is rapidly degraded by the dipeptidyl peptidase-4 (DPP-IV) enzyme (6). The first action of GLP-1 is
the potentiation of glucose-stimulated insulin secretion in β-cells, mediated by activation of its seven-transmembrane domain G-protein-coupled receptors (GLP-1r) (7). As a consequence, the GLP-1r selective agonist exenatide is now used as a novel antidiabetic therapy. A direct effect of GLP-1 on organs other than pancreas has also been postulated (8). GLP-1r has been found in pancreas, intestine, lung, kidney, breast, heart and brain (9) and is also expressed in nerves that innervate hepatocytes (10). Recently, GLP-1r expression has been found in cultured rodent hepatocytes and a GLP-1r agonist was able to reduce hepatic steatosis in ob/ob mice (11). In the present study, we demonstrated the presence of GLP-1r in human liver and in a human hepatoma cell line (HepG2), and that GLP-1r expression is reduced in patients with NASH. Starting from this result and using an experimental model of NASH in rats, we demonstrated that despite a reduction in expression, hepatic GLP-1 receptors could respond to treatment with exenatide that directly affects the expression of hepatic genes involved in glucose and lipid metabolism.

Material and methods

Patients
Normal human liver was obtained from six patients who underwent hepatic resection for focal nodular hyperplasia or hepatic adenoma. Pathological samples were obtained, in excess for diagnostic histological purposes, from 19 patients with biopsy-proven NASH. The protocol of data collection was part of the common clinical practice in the Clinica di Gastroenterologia, Università Politecnica delle Marche-Ospedali Riuniti Ancona. All subjects were asked to give their informed consent for the use of personal data, analyses and liver biopsy at the time of admission. This specific study was approved by the institutional review boards of Ospedali Riuniti Ancona, regulating noninterventional studies and by the Italian Ministry of Health. Diagnosis of NAFLD was performed according to the statement of the American Gastroenterological Association (12). The diagnosis of NASH and the degree of liver injury were determined according to Kleiner et al. (13).

Normal human pancreas, used as a positive control for GLP-1r expression, was obtained during pancreatectomy for pancreatic adenocarcinoma.

Animals
The experiment was performed following the guidelines of the local committee for care and use of laboratory animals. Male Sprague–Dawley CD rats (100–120 g body weight at the beginning of the treatment) (Charles River Laboratories, Como, Italy) were fed either a chow pellet diet (5% of energy derived from fat, 18% from proteins and 77% from carbohydrates; 3.3 kcal/g) (Harlan Italy, San Pietro al Natisone, UD, Italy) or a high-fat pellet diet (high-fat diet) (58% of energy derived from fat, 18% from protein and 24% from carbohydrates; 5.6 kcal/g) (Laboratorio Dottor Picioni, Gessate, Milano, Italy) (14, 15) twice weekly. Rats were housed in individual cages and the amount of food consumed was recorded to determine the amount of calories introduced. We have shown previously that this diet was able to induce the features of NASH (perivenular steatosis, hepatocellular injury and lobular inflammation) at 1 and 3 months of feeding, associated with an increased matrix deposition at 6 months (15). Rats were sacrificed at 1 and 3 months of treatment (n = 10 for each time point) after an overnight fasting. Portal blood was obtained before liver removal. After liver removal, the epididymal fat was removed to measure visceral adipose tissue, and its weight and volume were recorded (16). Hepatic lipids were extracted as described previously (17). The lipid extracts were resuspended in methanol and used for the evaluation of cholesterol and triglycerides levels by commercial kits from Sigma Chemical Co. (St. Louis, MO, USA). Plasma insulin and glucose were determined using the commercially available kit. Rat brain, used as a positive control for GLP1-r expression, was obtained at the time of sacrifice.

Reagents
Reagents were purchased from Sigma Chemical Co. unless otherwise indicated. The following antibodies were used: anti-diphosphorylated extracellular signal-regulated kinase (ERK)1/2 (pERK) (1:5000 final dilution), anti-Ser473 phosphorylated Akt (pAkt) (1:1000), anti-Thr183/Tyr185 phosphorylated JNK (pJNK) (1:800), anti-Thr272 phosphorylated AMPK (Cell Signaling Technology, Danvers, MA, USA) (pAMPK) (1:1000), anti-GLP-1r (1:2000, Alpha Diagnostic Intl, San Antonio, TX, USA) and anti-β-actin (1:10 000).

Cell preparation and treatment
Hepatocytes were isolated from high-fat diet rats between 1 and 3 months and cultured on Matrigel-coated six-well plates (0.5 × 10⁶ cells/well) in Iscove’s modified Dulbecco’s medium supplemented with 10% fetal bovine serum (FBS), 2 mmol/L glucose and 1% antibiotic–antifungal solution (IMDM) as described previously (18). After 4 h, cells were washed with phosphate-buffered saline (PBS) and incubated overnight with IMDM containing 0.5% FBS. HepG2 cells were kindly provided by Prof. D. Alvaro (University ‘La Sapienza’, Rome, Italy) and cultured in Dulbecco’s MEM (Amimed, Basel, Switzerland), containing penicillin/streptomycin (1%), nonessential amino acids (1%), sodium pyruvate (1%), l-glutamine (1%) and supplemented with 10% FBS. Before treatment, HepG2 were kept overnight in the same medium containing 0.5% FBS. Cells were incubated with exenatide (purchased from American Peptide Inc., Sunnyvale, CA, USA) at 10 and 100 nM (19) for the indicated period of time and treated as indicated below. In parallel experiments, cells were also pre-incubated for 30 min at 37 °C with either Rp-c-AMP (100 μmol/L, a protein kinase A (PKA) inhibitor), or wortmannin (100 nM, a PI3K inhibitor), or compound C (20 μM, an
AMPK inhibitor), or GW9662 (1 μM, 2 h pretreatment, a peroxisome proliferator-activated receptor γ (PPARγ) inhibitor), or MK886 [10 μM, 2 h pretreatment, a peroxisome proliferator-activated receptor α (PPARα) inhibitor] followed by incubation with exenatide (18–22). Rp-c-AMP and compound C were from Calbiochem (Milan, Italy), while wortmannin, GW9662 and MK886 were from Sigma Chemical Co.

Western blot
The cells were lysed in PBS containing 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 10 mg/ml PMSF, 30 μl/ml aprotinin and, 100 mM sodium orthovanadate. Twenty micrograms of protein was fractionated by electrophoresis on 10% (w/v) SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane, using the iBlotTM Dry Blotting System (Invitrogen, Life Technologies). The GLP-1r mRNA expression was assessed by reverse transcriptase-mediated polymerase chain reaction (RT-PCR) in human biopsies and rat tissues. Total RNA was extracted using the TRIzol® reagent according to the manufacturer's instructions (Invitrogen, Life Technologies). The integrity of the purified total RNA was confirmed by ethidium bromide staining after electrophoresis on agarose gel. First-strand cDNAs were synthesized from equal amounts of total RNA using random primers and M-MLV reverse transcriptase (Promega, Premier Biosoft International, Palo Alto, CA, USA). Primers for real-time PCR were designed using Beacon Designer software version 2.12, according to the parameters outlined in the BioRad iCycler Manual, using reference mRNA sequences accessed through Gene Bank and as shown in Table 1. Specificity of primers was confirmed by BLAST analysis. Real-time PCR for PPARα, PPARγ, acyl-coenzyme A oxidase 1-palmitoyl (ACOX1), liver carnitine palmityltransferase 1A (CPT1A) and GLP-1r mRNA was performed using the BioRad iCycler iQ Detection System (BioRad Laboratories Ltd, Hercules, CA, USA) with SYBR Green fluorophore. The change in fluorescence at every cycle was monitored and a threshold cycle (Ct) above background for each reaction was calculated. A melt curve analysis was performed following every run to ensure a single amplified product for every reaction. All reactions were carried out in at least duplicate for every sample. Duplicate negative controls (no template cDNA) were also run with every experimental plate to assess specificity and indicate potential contamination. Enolase 1α mRNA was constantly expressed under all experimental conditions and was then used as a reference gene for normalization as published previously (23). The comparative threshold cycle analysis method (GenEx software; Bio-Rad Laboratories Ltd) was used to assess the relative changes in gene expression compared with that of enolase 1α.

Expression of glucagon-like peptide-1 receptor in the liver
The GLP-1r mRNA expression was assessed by reverse transcriptase-mediated polymerase chain reaction (RT-PCR) in human biopsies and rat tissues. Total RNA was extracted using the TRIzol® reagent according to the manufacturer's instructions (Invitrogen, Life Technologies). The integrity of the purified total RNA was confirmed by ethidium bromide staining after electrophoresis on agarose gel. Total RNA was converted to cDNA with random primers using the M-MLV Reverse Transcriptase (Promega, Madison, WI, USA). PCR was performed using the Platinum Taq DNA Polymerase (Invitrogen, Life Technologies). Primers specific for rat and human GLP-1 receptor were designed as follows: sense 5′-TGTACCTGAGCATAAGGC TGG-3′ and antisense 5′-GCTCCCAGCTCTTCCGAAAC-3′; sense 5′-GAGTGCAGGAGTCCAAGC-3′ and antisense 5′-GGGTCTGATAAGGCCAGAGGAG-3′ respectively (Gene Bank accession number of rat and human mRNA RefSeq: NM_012728 and NM_002062 respectively). Rat brain and human pancreas biopsies were used as a positive control for rat and human GLP-1r respectively. Amplified products (453 and 142 bp respectively) were subjected to electrophoresis on 10% acrylamide gel, stained with ethidium bromide and then visualized on a UV transilluminator.

Glucagon-like peptide-1 receptor expression was also evaluated in cultured hepatocytes from high-fat diet animals by using a specific GLP-1r antibody (1:2000, Alpha Diagnostic Intl) by Western blot as described above.

**Real-time polymerase chain reaction**
Total RNA was extracted from hepatocytes and liver samples using the TRIzol® reagent (Invitrogen, Life Technologies) following the manufacturer's protocol. The integrity of the purified total RNA samples to be used in real-time PCR was confirmed by ethidium bromide staining after electrophoresis on agarose gel. First-strand cDNAs were synthesized from equal amounts of total RNA using random primers and M-MLV reverse transcriptase (Promega, Premier Biosoft International, Palo Alto, CA, USA). Primers for real-time PCR were designed using Beacon Designer software version 2.12, according to the parameters outlined in the BioRad iCycler Manual, using reference mRNA sequences accessed through Gene Bank and as shown in Table 1. Specificity of primers was confirmed by BLAST analysis. Real-time PCR for PPARα, PPARγ, acyl-coenzyme A oxidase 1-palmitoyl (ACOX1), liver carnitine palmityltransferase 1A (CPT1A) and GLP-1r mRNA was performed using the BioRad iCycler iQ Detection System (BioRad Laboratories Ltd, Hercules, CA, USA) with SYBR Green fluorophore. The change in fluorescence at every cycle was monitored and a threshold cycle (Ct) above background for each reaction was calculated. A melt curve analysis was performed following every run to ensure a single amplified product for every reaction. All reactions were carried out in at least duplicate for every sample. Duplicate negative controls (no template cDNA) were also run with every experimental plate to assess specificity and indicate potential contamination. Enolase 1α mRNA was constantly expressed under all experimental conditions and was then used as a reference gene for normalization as published previously (23). The comparative threshold cycle analysis method (GenEx software; Bio-Rad Laboratories Ltd) was used to assess the relative changes in gene expression compared with that of enolase 1α.

**Table 1. Primer sequences used for the real-time polymerase chain reaction**

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer sequences</th>
</tr>
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<tbody>
<tr>
<td>PPARα</td>
<td>Sense: 5′-CTC GGA GGC CTC TGT CAT C-3′</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5′-GGG ACT CAT TCG TAC TGG TGG-3′</td>
</tr>
<tr>
<td>PPARγ</td>
<td>Sense: 5′-CTC ACA ATG CCA TCA GGT TGG-3′</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5′-CTC GCA GAT CAG CAG ACT C-3′</td>
</tr>
<tr>
<td>ACOX1</td>
<td>Sense: 5′-GCA GAC AGC CAG GGT CCT GAT G-3′</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5′-ACT CGG CAG GTC ATT CAG GTA TG-3′</td>
</tr>
<tr>
<td>CPT1A</td>
<td>Sense: 5′-GCT TGG ACA GGT GGT TGG AC-3′</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5′-GGG GAT TTG GGG TTG GT-3′</td>
</tr>
<tr>
<td>Enolase 1α</td>
<td>Sense: 5′-TCA AGA TCC ATG CCA GAG AG-3′</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5′-GCC TCG TAG ATG CCA GTG-3′</td>
</tr>
<tr>
<td>Rat GLP1-r</td>
<td>Sense: 5′-ACC TCA ACC TGT TGG CGT CC-3′</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5′-AGC CCA GAG AGT CCT GAT ACG AG-3′</td>
</tr>
<tr>
<td>Human</td>
<td>Sense: 5′-GAGTGCAGGAGGAGTCCAAGC-3′</td>
</tr>
<tr>
<td>GLP1-r</td>
<td>Antisense 5′-GGGTCTGAAAGGCCAGAGGAG-3′</td>
</tr>
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ACOX1, acyl-coenzyme A oxidase 1-palmitoyl; CPT1A, carnitine palmitoyltransferase 1A; GLP1-r, glucagon-like peptide-1 receptor; PPARα, peroxisome proliferator-activated receptor α; PPARγ, peroxisome proliferator-activated receptor γ.
Measurement of protein kinase A activity
Protein kinase A activity was evaluated using the PepTag assay protein kinase kit (Promega). Effect of exenatide (EXE, 100 nM) on PKA, ERK, Akt and AMPK activity was evaluated in cultured hepatocytes isolated from rats with NASH. Isolated hepatocytes were cultured overnight in Iscove’s medium containing 0.5% FBS and then incubated with the same medium containing or not containing 100 nM exenatide for the indicated period of time. We also evaluated the effect of the PKA inhibitor Rp-c-AMP (Rp, 100 μmol/L), PI3K inhibitor wortmannin (W, 100 nM) and AMPK inhibitor compound C (CompC, 20 μM) on PPARγ, its downstream genes ACOX and CPT1A and PPARγ mRNA expression in isolated hepatocytes cultured overnight in Iscove’s medium containing 0.5% FBS and then incubated for additional 24 h with the same medium containing or not 100 nM exenatide. In parallel experiments, cells were also pre-incubated for 30 min at 37 °C with either Rp-c-AMP or wortmannin. Total RNA was extracted and real-time PCR was performed. Effect of exenatide on JNK phosphorylation was evaluated in hepatocytes isolated from rats with NASH, cultured overnight in Iscove’s medium containing 0.5% FBS and then incubated for additional 24 h with the same medium containing or not 100 nM exenatide. In parallel experiments, cells were also pre-incubated for 2 h at 37 °C with the PPARγ inhibitor GW9662 (20 μM). Protein lysates were obtained and Western blot was performed. The intensity of the bands of at least three experiments was then normalized to that of β-actin.

Measurement of total hepatic fatty acid content
The total amount of fatty fatty acid content was evaluated by gas chromatography as reported previously (22).

Statistical analysis
Result data are expressed as mean ± SD of fold increase compared with controls and represent at least three different experiments. Group means were compared by analysis of variance followed by the Student–Newman–Keuls test whether the former was significant. A P-value of < 0.05 was considered statistically significant.

Results
Glucagon-like peptide-1 receptor expression in human liver
The presence of GLP-1r in the liver has been questioned, especially in humans. Thus, we first evaluated the expression of GLP-1r in samples of patients with NASH and in normal livers. Using RT-PCR a single 142 bp band was detected both in human pancreas (used as positive control) and in all liver samples (Fig. 1A). A single 142 bp band was also detected in the human hepatoma cell line HepG2 as well as in pancreas (Fig. 1B). When quantified by real-time PCR, GLP-1r expression was reduced in the liver of patients with NASH compared with normal samples (Fig. 1B).

To confirm that hepatocytes express GLP1-r at the protein level, tissue and cell lysates were tested by Western blot. A single 53 kDa band was detected in cell lysate from

![Fig. 1](image-url). Assessment of presence of glucagon-like peptide-1 receptor (GLP-1r) mRNA in human livers. (A) GLP-1r mRNA expression was assessed by polymerase chain reaction (PCR) in human pancreas (positive control) and in the whole human liver. 1: pancreas; 2: liver; 3: no template product; 4: 100 bp ladder. (B) GLP1-r mRNA expression was assessed by PCR in human pancreas (positive control) and HepG2 cells. 1: 100 bp ladder; 2: pancreas; 3: no template product; 4: HepG2 cells. (C) Real-time PCR for GLP1r mRNA was performed in six normal human livers (white bar) and in nineteen patients with NASH (black bar). Data are expressed as mean ± SD of fold increase compared with controls. *P < 0.05 vs controls.
human pancreas and rat brain, used as a positive control, as well as from HepG2 cells, while no bands were observed when human serum albumin was used (Fig. 2A). Protein expression was then quantified in the liver of patients with NASH and in normal human samples, showing again a significant decrease in NASH (Fig. 2B and C).

Metabolic and histological hepatic changes induced by high-fat diet

To induce NASH in rats, we fed animals a high-fat or a chow diet, as described previously (15). Rats fed with high-fat diet consumed significantly more calories (98.4 ± 9.5 vs 75.2 ± 7.2, \( P < 0.001 \) vs controls) and increased body weight (1 month: 341.7 ± 18.2 vs 287.3 ± 15.0 and 3 months: 467.3 ± 14 vs 397.9 ± 24, \( P < 0.05 \) vs controls) in comparison to chow rats. The increase in weight was associated with hepatic steatosis (13.3 ± 0.8 and 16.5 ± 2.1 mg of triglycerides/g after 1 and 3 months of high-fat diet vs 3.2 ± 0.3 in controls, \( P < 0.05 \)), visceral obesity with higher epididymal fat weight (1.6 ± 0.4 vs 4.5 ± 0.7 g and 3.2 ± 0.5 vs 7.3 ± 0.8 g at 1 and 3 months, respectively, \( P < 0.05 \) vs controls) and development of insulin resistance, as shown by higher insulin (0.7 ± 0.07 and 0.7 ± 0.05 \( \mu \)g/L at 1 and 3 months, respectively, \( P < 0.05 \) vs controls) and glucose (147.1 ± 18.9 and 151.0 ± 15.7 vs 111.8 ± 12.3 mg/dL, \( P < 0.05 \)) values in the portal blood compared with chow rats. Thus, high-fat diet was able to induce visceral/hepatic fat accumulation and insulin resistance even in nongenetically modified animals.

No histological signs of liver injury were observed in rats fed the chow diet. After 3 months of high-fat diet, steatosis affected most of the hepatocytes, ballooning was diffusely present, and foci of mixed inflammatory cell infiltration and hepatocyte necrosis or apoptosis appeared throughout the lobules.

High-fat diet effect on GLP-1 receptor, peroxisome proliferator-activated receptors and lipid oxidation gene expression

To study the effect of high-fat diet on hepatic expression of GLP-1r, whole liver and hepatocytes were obtained from rats fed chow or high-fat diet up to 3 months. GLP-1r expression was evaluated in rat liver by PCR: a single 453 bp band was detected in rat brain (used as positive control) and in all liver samples (Fig. 3A). Real-time PCR showed that high-fat diet significantly decreased GLP-1r mRNA expression already after 1 month of high-fat diet confirming the association between NASH and reduced expression of GLP-1r (Fig. 3B).

To confirm that rat hepatocytes also express GLP1-r at the protein level, tissue and cell lysates were tested by Western blot. A single 53 kDa band was detected in cell lysates from rat brain, as well as from the whole liver and isolated rat hepatocytes (Fig. 3C). Protein expression was then quantified in the liver of chow- and high-fat diet-treated rats, showing again that GLP1-r expression is reduced in the presence of NASH (Fig. 3D and E). High-fat diet significantly reduced PPARγ expression in the whole liver (Fig. 4). Previously we have shown that high-fat diet decreased PPARγ activity, as shown by reduced nuclear translocation in the whole liver (15). Consistent with this, expression of PPARγ target genes...
Fig. 3. Assessment of glucagon-like peptide-1 receptor (GLP1-r) expression in rat. (A) GLP-1r mRNA band was visualized by polymerase chain reaction (PCR) in brain (2), whole liver (3) and isolated hepatocytes (4). (B) GLP-1r mRNA expression was quantified in controls (white bar) and high-fat diet-treated rats (black bars) by PCR. (C) The presence of GLP1-r protein was tested by Western blot in rat brain (1), rat whole liver (2) and in isolated hepatocytes (3). (D and E) To quantify the expression of GLP1-r protein, whole liver lysates from chow- and high-fat diet-fed rats were evaluated by Western blot and the intensity of the band was quantified by densitometry. White bar: chow-fed rats; black bars: high-fat diet-treated rats. Data are expressed as mean ± SD of fold increase compared with controls. *P < 0.05 vs controls.

Fig. 4. Effect of high-fat diet on mRNA expression in the whole rat liver of peroxisome proliferator-activated receptor γ (PPARγ), and of peroxisome proliferator-activated receptor γ (PPARγ) downstream genes acyl-coenzyme A oxidase 1-palmitoyl (ACOX1) and carnitine palmitoyltransferase 1A (CPT1A). Total RNA was extracted from the liver of control (white bars) and high-fat diet-treated rats (black bars) at the different time points, and quantified by real-time PCR. Data are expressed as mean ± SD of fold increase compared to controls. *P < 0.05 vs controls.
ACOX1 (a rate-limiting enzyme in peroxisomal FFA β-oxidation) and CPT1A (a key enzyme in mitochondrial β-oxidation of FFA) was decreased in high-fat diet compared with chow rats (Fig. 4).

Effect of exenatide on peroxisome proliferator-activated receptors and lipid oxidation modifications induced by high-fat diet

We investigated the role of GLP-1 on the alteration of intracellular signalling and gene expression in hepatocytes isolated from rats treated with high-fat diet. We firstly looked at expression of GLP-1r in hepatic parenchymal cells (i.e. hepatocytes). Using Western blot, the reactive band was evident in freshly isolated hepatocytes from high-fat diet rats, and was maintained up to 48 h in culture, thus indicating the possibility to respond to the specific ligand (Fig. 5A). When hepatocytes were isolated from high-fat diet rats, a decrease in PPARγ (−73.4 ± 5.2%, \( P < 0.05 \)), ACOX1 (−62.3 ± 7.1%, \( P < 0.05 \)) and CPT1A (−48.2 ± 4.6%, \( P < 0.01 \)) gene expression was observed compared with hepatocytes isolated from chow fed animals, thus resembling in parallel the observations in the whole liver. In hepatocytes isolated from high-fat diet fed rats, real-time PCR showed that 24 h incubation with 10 and 100 nM exenatide significantly increased PPARγ and PPARα expression, and mRNA levels of its downstream genes ACOX1 and CPT1A (Fig. 5B–E). A similar effect was observed when exenatide was incubated in hepatocytes isolated from rats fed the chow diet (data not shown). From this
point on, only 100 nM exenatide was used. No effect on gene expression was induced by exenatide incubation at earlier time points (i.e. 3–6 h of incubation). Furthermore, exenatide reduced by 30% the total amount of fatty acids in hepatocytes isolated from rats treated with high-fat diet (59.2 ± 12.5 vs 41.7 ± 10.6 mg fatty acids/g dry pellet, P < 0.05).

**Effect of exenatide on hepatic intracellular signalling**

Glucagon-like peptide-1 receptor belongs to the G-protein-coupled receptors that, upon ligand binding, induce PKA activation through cAMP. Incubation with exenatide of hepatocytes from rats with NASH resulted in a marked increase in active PKA formation, starting at 10 min of incubation (Fig. 6A and B). Exenatide did not affect ERK phosphorylation (Fig. 6C and D), while increased phosphorylation of Akt and AMPK starting at 30 and 10 min respectively (Fig. 6C and Fig. 6E and F). When NASH hepatocytes were incubated with exenatide for 30 min in the presence or not of specific inhibitors, Akt phosphorylation returned down to control value (Fig. 7A and B). No effect was induced on AMPK phosphorylation by exenatide, indicating that PI3K and AMPK pathways are independently activated by this GLP-1 analogue (Fig. 7A–C).

Next we evaluated the effect of PI3K and AMPK activation by exenatide on PPARs activity and expression. Both the PKA inhibitor Rp-c-AMP and the PI3K-inhibitor wortmannin reduced exenatide-stimulated PPARγ mRNA expression (Fig. 8A). Furthermore, both inhibitors reduced, ACOX1 and CPT1A gene expression, consistent with decreased PPARα activity, without

![Figure 6](https://example.com/figure6.png)

**Fig. 6.** Effect of exenatide on PKA (A, B), ERK (C, D), Akt (C–E) and AMPK (C–F) activity, in cultured hepatocytes isolated from rats with nonalcoholic steatohepatitis (NASH). Hepatocytes were isolated from high-fat diet-treated rats, cultured overnight and then incubated with the same medium containing (black bars) or not (white bars) 100 nM exenatide for the indicated period of time. Protein lysates were obtained and Western blot was performed. The intensity of the bands was normalized to that of β-actin and data are expressed as mean ± SD of fold increase compared with controls. aPKA, active PKA; iPKA, inactive PKA. ∗P < 0.05 vs controls.
affecting its mRNA level (Fig. 8B). Finally, the AMPK-inhibitor compound-C similarly reduced ACOX1 and CPT1A gene expression, and PPARγ mRNA levels, indicating that both the PI3K and the AMPK pathways are involved in GLP-1r signalling (Fig. 6C and D).

Previously we have observed a progressive increase in JNK1/2 Thr183/Tyr185 phosphorylation in the whole liver starting at 1 month of high-fat diet (15). Here we found that GLP-1r activation was able to reduce JNK phosphorylation in hepatocytes isolated from this in vivo model of NASH. This effect was mediated by PPARγ, as its specific inhibitor GW9662 abolished this effect, while the PPARα inhibitor MK886 had no effect (Fig. 9A and B).

Discussion

We have studied the effect of the GLP-1 activator, exenatide, on hepatocytes with NASH. NAFLD and NASH are characterized by impaired fatty acid oxidation and insulin resistance (1, 24, 25) and for such a reason they are now considered the hepatic feature of the metabolic syndrome (2). The mechanisms that lead to hepatic triglyceride accumulation and hepatic insulin resistance are complex and mostly unknown. At the moment no therapy for NAFLD/NASH has been established. One of the most promising approach is the use of agents that improve glucose tolerance and insulin sensitivity (26).

Glucagon-like peptide-1 analogues are new drugs approved for the treatment of diabetic hyperglycaemia. GLP-1 is an incretin hormone secreted by the intestine in response to a meal and rapidly degraded by the action of DPP-IV, resulting in an in vivo half-life of GLP-1 of approximately 2 min. The principal action of GLP-1 is the potentiation of glucose-stimulated insulin secretion in β-cells (6, 7), thus improving glucose disposal by increased insulin release. In type 2 diabetic and in obese subjects, GLP-1 concentrations are reduced (4, 27, 28). GLP-1 receptors have been found in pancreatic α- and β-cells, proximal intestinal tract, muscle (29), adipose tissue (30), nerve terminals in the hepatic portal bed (10), heart and brain (31). Several studies have found that GLP-1 can modulate hepatic glucose metabolism (10, 32–35) and that ob/ob mice treated with a recombinant adenovirus expressing GLP-1, exhibit reduced glucose production and reduced expression of enzymes related to gluconeogenesis and de novo lipogenesis (36). When exenatide was tested on glycaemic control, body weight and cardiometabolic markers in a population of patients with type 2 diabetes, a significant reduction in ALT values was also observed (5). As the expression of GLP1-r in the liver was questioned (7, 34), we firstly

![Fig. 7. Effect of exenatide, the protein kinase A (PKA)-inhibitor Rp-c-AMP (Rp, 100 μmol/L), and the PI3Kinhibitor wortmannin (W, 100 nM) on pAKT (A, B) and pAMPK (A, C) in cultured hepatocytes isolated from rats with nonalcoholic steatohepatitis (NASH). Isolated hepatocytes were cultured overnight and then incubated with the same medium containing (black bars) or not (white bars, control) 100 nM exenatide for 30 min. Protein lysates were obtained and Western blot was performed. The intensity of the bands was normalized to that of β-actin and data are expressed as mean ± SD of fold increase compared to controls. *P < 0.05 vs controls.](image-url)
evaluated the expression of GLP-1r in liver biopsy of patients with NASH and controls and found that, contrary to what was reported previously (31), GLP-1 receptor is present in the human liver, but its level (measured by both real-time PCR and Western blot) was decreased in patients with NASH (Fig. 1). Our data thus expand, in human patients, previous observations from Ding et al. (37), who found the receptor, at the protein level, in rat hepatocytes, and that treatment with exenatide ameliorates hepatic steatosis.

Our hypothesis is that GLP-1r could be implicated in hepatic insulin resistance and that long-acting GLP-1r activators, such as exenatide, could be used for the treatment of NASH/NAFLD. We used an animal model of diet-induced NASH to evaluate expressions of genes implicated in hepatic insulin resistance and fatty acid oxidation as the size of human liver biopsies does not allow the evaluation of the signalling cascade nor hepatocyte culture (15). NASH was obtained after 3 months of high-fat diet in nongenetically modified rats, as been shown previously (15). Expression of GLP-1r was significantly reduced already after 1 month of high-fat diet, similarly to what was observed in the liver of patients with NASH. This could be one of the causes of hepatic insulin resistance observed in patients with NAFLD/NASH (3) as also shown by a recent article from Ayala et al. (38) that found that mice lacking the GLP-1 receptor exhibit normal peripheral insulin sensitivity but hepatic insulin resistance.

One of the main causes of NASH is free fatty acids (FFA) overload towards the liver (either from peripheral and visceral lipolysis, de novo lipogenesis or high-fat diet) (24, 39, 40). If overload of FFAs to the organs do not stimulate their own oxidation, the surplus of fat is stored not only in white adipose tissue, but also as ectopic fat in liver, heart and muscle, promoting lipotoxicity and insulin resistance (24, 41). In subjects with NAFLD hepatic and total FFA oxidation has been found either similar or increased compared with subjects without NAFLD (25, 40) but correlated to liver fat content (40), indicating that the system is not responding adequately to the increased FFA flux, probably because of the saturation process. Several cellular mechanisms are involved in this aspect. We have shown previously that

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**Fig. 8.** Effect of the protein kinase A (PKA)-inhibitor Rp-c-AMP (Rp, 100 μmol/L), the PI3K-inhibitor wortmannin (W, 100 nM) and the AMPK inhibitor compound C (CompC, 20 μM) on peroxisome proliferator-activated receptor γ (PPARγ) (A), peroxisome proliferator-activated receptor α (PPARα) (B), its downstream genes acyl-coenzyme A oxidase 1-palmitoyl (ACOX1) (C) and carnitine palmitoyltransferase 1A (CPT1A) (D) mRNA expression in cultured hepatocytes isolated from rats with nonalcoholic steatohepatitis (NASH). Isolated hepatocytes were cultured overnight and then incubated for additional 24 h with the same medium containing (black bars) or not (white bars, control) 100 nM exenatide. In parallel experiments, cells were also pre-incubated for 30 min at 37°C with either Rp-c-AMP, wortmannin or compound C. Total RNA was extracted and quantified by RT-PCR. Data are expressed as mean ± SD of fold increase compared with controls. *P < 0.05 vs controls.
high-fat diet significantly reduced hepatic PPARγ expression (15) and this is confirmed in the present study where reduced expression of its downstream target genes ACOX1 (a rate-limiting enzyme in peroxisomal FFA β-oxidation) and CPT1A (a key enzyme in mitochondrial β-oxidation of FFA) was observed. In parallel, high-fat diet significantly reduced also PPARγ expression and increased JNK activation that is able to induce insulin resistance through Ser307 IRS1 phosphorylation (15). We thus took advantage from this in vivo model of NASH to show that these alterations were overall reversed by incubation of NASH hepatocytes with exenatide. Because PPARγ acts on FFA oxidation and PPARγ regulates lipid homeostasis and insulin sensitivity (42) we thus hypothesized that exenatide ameliorates the impairment in hepatic FFA metabolism and oxidation induced by high-fat diet. This was confirmed in our study by the observation that the total amount of fatty acids in hepatocytes from rats with NASH was reduced by 30% after GLP-1r activation. This result is also supported by the fact that pioglitazone (a PPARγ agonist) improves lipid metabolism as well as liver histology (43) and that FFA stimulation of GPR120, a G-protein-coupled receptor expressed abundantly by the intestine, promotes GLP-1 secretion and increases circulating insulin levels (44). In this regard, insulin resistance is associated to increased Ser307 JNK phosphorylation, which inhibits the insulin receptor signalling (15, 45). GLP1-r activation by exenatide was able to reduce the level of JNK phosphorylation in hepatocytes during NASH. This effect was PPARγ-dependent, but PPARγ-independent (Fig. 9). Also in agreement with our data, rosiglitazone (a PPARγ ligand) inhibits TNFα-induced JNK activation in adipocytes and enhances insulin sensitivity (46).

G-protein coupled receptors, such as GLP-1r, act through a cAMP-dependent activation of PKA (19). Increased cAMP levels have been observed in hepatocytes isolated from normal rats after incubation with exenatide (36). Active PKA represents a docking site for several downstream transduction pathways. Incubation with exenatide resulted in a marked increase in active PKA formation in hepatocytes isolated from high-fat diet-treated rats, from which signal diverged to activate AMPK and the ERK/PI3K pathway needed to transduce the message to PPARs. In agreement with our data, Gupta and colleagues recently used in vitro experiments to demonstrate GLP1-r internalization in hepatocyte cell lines. Following this, exenatide activated the docking protein PDK1 from which the signal diverged to phosphorylate Akt and PKC-ζ, making reasonable an effect on GLUT2 synthesis and decreasing glycogen and fatty acid synthesis (11). Thus, these and our data unequivocally demonstrate, by using different experimental approaches, that eventide is able to directly induce different intracellular signalling events that counteract the metabolic pathways altered in the course of NAFLD and NASH.

In summary, our experiments indicate that GLP-1r is expressed in human liver and its expression is decreased in patients with NASH. In hepatocytes isolated from rats with NASH, the signalling cascade involved in stimulation of hepatic fatty acid oxidation and insulin sensitivity is impaired and GLP1-r activation with exenatide ameliorated these defects. By activating specific intracellular kinases such as PI3K and AMPK, exenatide determined: (i) increased PPARγ activity, which induced transcriptions of ACOX1 and CPT1A (both involved in fatty acid β-oxidation) and reduced the total amount of fatty acids in hepatocytes; (ii) increased PPARγ expression that exerted its insulin-sensitizing action by reducing Ser307 JNK phosphorylation. Thus, our data indicate that GLP-1 analogues, currently used in the treatment of type 2 diabetes, are a promising treatment approach to improve hepatic insulin resistance and fatty acid β-oxidation also in patients with NAFLD/NASH.

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