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Dipeptidyl peptidase-4 impairs insulin signaling and promotes lipid accumulation in hepatocytes

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ABSTRACT

Dipeptidyl-peptidase 4 [DPP-4) has evolved into an important target in diabetes therapy due to its role in incretin hormone metabolism. In contrast to its systemic effects, cellular functions of membranous DPP-4 are less clear. Here we studied the role of DPP-4 in hepatic energy metabolism.

In order to distinguish systemic from cellular effects we established a cell culture model of DPP-4 knockdown in human hepatoma cell line HepG2.

DPP-4 suppression was associated with increased basal glycogen content due to enhanced insulin signaling as shown by increased phosphorylation of insulin-receptor substrate 1 (IRS-1), protein kinase B/Akt and mitogen-activated protein kinases (MAPK)/ERK, respectively. Additionally, glucose-6-phosphatase cDNA expression was significantly decreased in DPP-4 deficiency. Reduced triglyceride content in DPP-4 knockdown cells was paralleled by enhanced expressions of peroxisome proliferator-activated receptor alpha (PPARα) and carnitine palmitoyltransferase −1 (CPT-1) while sterol regulatory element-binding protein 1c (SREBP-1c) expression was significantly decreased.

Our data suggest that hepatic DPP-4 induces a selective pathway of insulin resistance with reduced glycogen storage, enhanced glucose output and increased lipid accumulation in the liver. Hepatic DPP-4 might be a novel target in fatty liver disease in patients with glucose intolerance.

1. Introduction

Obesity has become an epidemic in the Western world and despite recognition and counter-measures prevalence is still increasing worldwide [1]. It is highly associated with development of insulin resistance, type 2 diabetes (T2DM) and also non-alcoholic fatty liver disease (NAFLD) which is thought to be the hepatic manifestation of the metabolic syndrome [2].

In the past years several novel treatment strategies in type 2 diabetes have emerged including inhibition of renal sodium glucose transporters and enhancement of incretin action [3]. Latter is achieved by inhibition of dipeptidyl-peptidase IV (DPP-4) or by treatment with glucagon like peptide 1 (GLP-1) receptor agonists. DPP-4 inhibitors are widely used in type 2 diabetes treatment and have also been found to ameliorate diet induced hepatic steatosis in mice by improving mitochondrial carbohydrate utilization and modulating intrahepatic fatty acid metabolism [1]. From these encouraging results it remains to be determined whether beneficial hepatic effects result from direct cellular effects in the liver or systemic effects of DPP-4 inhibition leading to enhanced incretin action.

While systemic effects of DPP-4 have extensively been investigated, the role of cellular DPP-4 is less clear [4]. DPP-4 also known as CD26 is a 110 kDa membrane associated serine peptidase that is expressed in epithelial, acinar and endothelial cells, fibroblasts and lymphocytes [5,6]. DPP-4 is structured in a cytoplasmic domain, a transmembrane domain, a flexible stalk segment and an extracellular domain which contains the catalytic region [6]. After shedding from the cell surface it is released into the blood flow and circulates

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as soluble form cleaving several substrates including GLP-1, glucose-dependent insulinotropic polypeptide (GIP), neuropeptide Y (NPY) and stromal cell derived factor 1 (SDF-1) or interacting in a non-enzymatically way with adenosine deaminase (ADA) and other matrix proteins [4,6]. Cellular effects of DPP-4 have been described in adipose tissue, skeletal and smooth muscle where DPP-4 was found to impair cellular insulin signaling [2].

Aim of this study was to elucidate the cellular role of DPP-4 on hepatic glucose and fatty acid metabolism in order to better understand its role in pathophysiology of non-alcoholic fatty liver disease and associated disturbances in glucose homeostasis.

2. Material and methods

2.1. Cell culture model of DPP-4 knockdown

Human hepatoma cells HepG2 (ATCC, Manassas, Virginia, USA) were cultured in RPMI 1640 medium (Lonza, Basel, Switzerland). RPMI 1640 medium was supplemented with 10% foetal bovine serum (Life Technologies, Rockville, Maryland, USA), 5% L-glutamine (Lonza, Basel, Switzerland) and 1% penicillin/streptomycin (Lonza, Basel, Switzerland). The medium was changed every 2 days and cells were split at a ratio of 1:5 every 3–4 days.

Knockdown was achieved by a chemical, transient transfection of HepG2 cells with siRNA using the fast forward transfection method from Qiagen (HiPerfect, Qiagen, Hilden, Germany). HepG2 cells were either transfected by adding predesigned small interfering RNA (siRNA) targeting human DPP-4 (Hs_DPPIV_3 validated siRNA, Qiagen, Hilden, Germany) or non-silencing control siRNA (Qiagen, Hilden, Germany) at concentrations of 20, 50 or 100 nM. Transfected and control cells were maintained for 24, 48 or 72 h. Knockdown efficiency of hDPP-4 was determined by Western blotting.

2.2. Western blot analysis

HepG2 cell membranes were lysed using cytoplasmic lysis buffer (25 mM Tris-HCl, pH 7.4, 40 mM KCl, 1% Triton X-100) (Roche, Mannheim, Germany). After quantification by the Lowry's method [20] cell proteins were run on a 4%–15% sodium dodecyl sulphate–polyacrylamide gel (BioRad, Hercules, California, USA) and transferred to nitrocellulose membranes (Hybond-P, Amersham-Pharmacia, Austria). Proteins were detected using commercially available antibodies against human DPP-4 (R&D Systems, McKinley, Minneapolis, USA), proteinkinase B (AKT) (CellSignaling, Cambridge, New England), phospho-proteinkinase B Threonine308 (pAktthr308) (CellSignaling, Cambridge, New England), phospho-proteinkinase B Serine473 (pAktser473) (CellSignaling, Cambridge, New England), insulin receptor substrate 1 (IRS1) (CellSignaling, Cambridge, New England), phospho-insulin receptor substrate 1 Tyrosine608 (pIRS1 tyr608) (Millipore, Billerica, Massachusetts, USA), phospho-insulin receptor substrate 1 Serine307 (pIRS1ser307) (Novus, Minneapolis, USA), extracellular-signal related kinase (ERK) (Santa Cruz Biotechnology, Dallas, Texas, USA), phospho-extracellular-signal related kinase Tyrosine 204 (pERK tyr204) (Santa Cruz Biotechnology, Dallas, Texas, USA) and insulin receptor (IR) (CellSignaling, Cambridge, New England) and horseradish peroxidase conjugated (HRP) conjugated secondary antibody (Jackson ImmunoResearch, West Baltimore Pike, West Grove, PA, USA). Visualisation was performed by chemiluminescence (Amersham ECL Plus Western Blotting Reagent, GE Healthcare, Vienna, Austria). Target protein levels were controlled for β actin protein (Sigma-Aldrich, St. Lois, Missouri, USA) or by the stain free method [21].

2.3. Glycogen content

Basal and insulin-stimulated glycogen contents were measured in DPP-4 siRNA- and non-silencing siRNA-transfected cells in duplicates. For determination of insulin-stimulated glycogen content cells were preincubated with insulin [100 nmol/l] or PBS for 3 h at 37 °C. Glycogen content was quantified by a method described by Decker and Keppler [22,23]. Previously, cells were washed with ice-cold PBS and collected in 0.6 mol/l $HClO_4$, sonicated in ice water and neutralized with 1 mol/l $KHCO_3$. Aliquots of the homogenate were supplemented with 10 g/l amyloglucosidase in 0.2 mol/l acetate buffer and incubated for 2 h at 40 °C in the water bath. The reaction was stopped by addition of cooled 2 mol/l $HClO_4$. After centrifugation at 13200 rpm for 10 min at 4 °C glucose concentrations were quantified using a Cobas Mira analyser (Roche, Mannheim, Germany). Glycogen content was expressed as nmol glucose/mg protein.

2.4. Triglyceride content

Triglyceride (TG) content was measured in DPP-4 siRNA- and non-silencing siRNA-transfected cells and control cells in duplicates. After trypsinization and centrifugation the supernatant was removed, 0.1 mol/l NaOH was added and cells sonicated in ice water and centrifuged for 10 min at 13200 rpm. TG concentrations were determined by an enzymatic method using a Cobas Mira analyser (Roche, Mannheim, Germany). TG content was expressed as mg triglycerides/mg protein.

2.5. RNA isolation, reverse transcription and fluorescence-based real time PCR

Isolation of total RNA from HepG2 cells was performed with RNABee reagent (AMS Biotechnology, Abingdon, UK). After quantification and reverse transcription (Omniscript Reverse Transcription kit, Qiagen, Hilden, Germany) cDNA levels were assessed by the TaqMan fluorescence based real-time PCR method. Sequences of exon-intron boundaries spanning oligonucleotides are shown in Table 1. Oligonucleotides and probes were designed using the Primer Express software (Perkin-Elmer, Applied Biosystems, Warrington, UK). In short, 1.6 μl of each reverse transcription reaction mixture served as a template in 20 μl reaction mixture containing 10 μl TaqMan Universal Polymerase Mastermix (Applied Biosystems, Foster City, California, USA), 1.8 μl (10 nmol/ml) of each oligonucleotide primer (Microsynth, Balgach, Switzerland), 0.4 μl TaqMan Probe (Microsynth, Balgach, Switzerland) and aqua dest. Target cDNA levels were normalized to glyceraldehyde-3-phosphate dehydrogenase levels (Applied Biosystems, Foster City, California, USA). Results are expressed as target gene cDNA/GAPDH cDNA ratio.

2.6. Statistical analysis

Statistical analyses were performed using the statistical analysis software package (SPSS version 17.0; SPSS Inc., Chicago, IL, USA). Data are expressed as means ± SD. Distinctions between more than 2 groups were determined by using one-way ANOVA analysis. Normally distributed data were analyzed with unpaired *t*-test. Mann Whitney test with the Bonferroni correction was performed for analysis of non-parametric data. Statistical significance was inferred at a two-tailed p-value of less than 0.05.

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