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Control of liver glucokinase activity: A potential new target for incretin hormones?

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ABSTRACT

We tested the exendin-4 and des-fluoro-sitagliptin effects on fructose-induced increase in liver glucokinase activity in rats with impaired glucose tolerance and the exendin-4 effect on glucokinase activity in HepG2 cells incubated with fructose in the presence/absence of exendin-9-39. After 3 weeks of in vivo fructose administration we measured: (1) serum glucose, insulin and triglyceride levels; (2) liver and HepG2 cells glucokinase activity and (3) liver glucokinase and 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase mRNA and protein levels. Fructose fed rats had: hypertriglyceridemia, hyperinsulinemia and high liver glucokinase activity (mainly located in the cytosolic fraction) together with higher glucokinase and 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase mRNA and protein concentrations compared to control rats. Co-administration of either exendin-4 or des-fluoro-sitagliptin prevented serum and liver changes except glucokinase protein expression. Exendin-4 also prevented fructose-induced increase in glucokinase activity in cultured HepG2 cells, effect blunted by co-incubation with exendin-9-36. In conclusion exendin-4/des-fluro-sitagliptin prevented fructose-induced effect on glucokinase activity, mainly affecting enzyme activity modulators. Exendin 9-39 blunted in vitro protective exendin-4 effect on glucokinase activity, thus suggesting a direct effect of the later on hepatocytes through GLP-1 receptor. Alterations of glucokinase activity modulators could play a role in the pathogenesis of liver dysfunction, becoming a potential new treatment target for GLP-1 receptor agonists.

1. Introduction

Incretin hormones (glucagon-like peptide-1, GLP-1, and glucose-dependent insulinotropic polypeptide, GIP) exert multiple biological effects such as enhancement of glucose-induced insulin secretion, affect glucagon and somatostatin secretion, increment of β-cell mass, delay of gastric emptying and appetite inhibition [6]. Regarding their glucagon effect, while GLP-1 inhibits GIP stimulates its secretion [7]. The significant decrease of all these pleiotropic effects present in people with Type 2 diabetes (T2DM), has been mainly ascribed to a glucotoxic-induced down regulation of incretin-receptors rather than to a decrease in their circulating levels [6,21,30]. Currently, many of these people are treated with either GLP-1 and its analogs, or specific inhibitors of their degrading enzyme dipeptidyl peptidase IV (DPP-IV) [6,25,29].

Administration of a fructose-rich diet to normal rats induces insulin resistance and impaired glucose tolerance or diabetes, depending on treatment duration [23]. We have shown that development of these abnormalities as well as fat deposit in liver, are effectively prevented by co-administration of either exendin-4 or sitagliptin [24]. Supporting the latter effect, other authors also reported incretin effects on liver dysfunction in people with T2DM [20] and on glucose metabolism as well as on glucokinase activity in experimental diabetes [9,19]. Additionally, we previously showed that in fructose fed rats liver glucokinase activity is greatly increased due to the combination of enzyme translocation from nucleus to cytosol and its interaction with an increased amount of PFK2, a cytosolic positive modulator of enzyme activity [16]. The effect on glucokinase could be independent of insulin action and mediated through GLP-1 receptor [10].

Abbreviations: T2DM, type 2 diabetes; GLP-1, glucagon-like peptide-1; DPP-IV, dipeptidyl peptidase IV; des-F-sitagliptin, des-fluoro-sitagliptin; PFK, 26-phosphofructo-2-kinase/fructose-2,6-biphosphatase; CF, cytosolic fraction; DNF, nuclear fraction; qPCR, real-time PCR; GIP, gastric inhibitory peptide.

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Since little is known about the mechanism by which incretin affects liver glucokinase activity and the presence of GLP-1 receptor in hepatocytes is controversial [18,31], we decided to evaluate in vivo effects of exendin-4 and des-fluoro-sitagliptin (des-F-sitagliptin) on fructose-induced changes in liver glucokinase and mechanisms involved in its activation. In addition, we used HepG2 cells to test in vitro whether the incretin effect on fructose-induced changes in glucokinase activity depends on its general metabolic effect or on a direct effect on the liver cell acting either through or independently of GLP-1 receptor.

2. Materials and methods

2.1. Chemicals and drugs

Reagents of the purest available grade and β-actin antibody were obtained from Sigma Chemical Co. (St., Louis, MO, USA). Des-F-sitagliptin was kindly provided by Merck, Sharp and Dohme (Argentina). Glucokinase antibody (sheep anti-GST-glucokinase fusion protein antibody) was kindly provided by Dr. Mark Magnusson (Vanderbilt University, USA). This antibody and another from Santa Cruz Biotechnology Inc. (GCK N-19: sc:1980) were used to check the presence of glucokinase protein in HepG2 cells. PFK-2 polyclonal antibody (IgY-FBPase-2) was kindly provided by Prof. Sigurd Lenzen (Medizinische Hochschule, Hannover, Germany).

2.1.1. "In vivo" experiments

Normal male Wistar rats (180–200 g) were divided into two groups: animals fed a standard commercial diet (control, C) and the same diet plus 10% fructose (w/v) in drinking water for 3 weeks (F). C and F animals were randomly divided into three subgroups (10 animals each): untreated (C and F), treated with des-F-sitagliptin (115.2 mg/day/rat, premixed with the milled pellet at 0.6% [w/w]) (CS and FS) and treated with exendin-4 (0.35 nmol/kg body weight/ip twice a day) (CE and FE). We have previously shown that these doses exert significant effects in our model (Maiztegui et al., Ref. [24]).

All animals were housed in a room with controlled temperature (25 °C) and 12 h light/dark cycles. Water and food intake were measured daily, whereas individual body weight was recorded once a week.

Twenty-one days after this treatment, blood samples from 4-h fasted animals were drawn from the retroorbital plexus under light halothane anesthesia and collected into heparinized tubes to measure blood glucose, serum triglyceride and immunoreactive insulin levels. Afterwards, the animals were killed by decapitation and the same portion of liver (median lobe) was removed to perform all assays.

The protocols and procedures for the care and use of laboratory animals were reviewed by the Institutional Animal Care and Use Committee (IACUC) of the Facultad de Ciencias Médicas, Universidad Nacional de La Plata. Animal experiments and handling were performed according to the "Guide for the Care and Use of Laboratory Animals" (1996, National Academy Press, 2101 Constitution Ave. NW, Washington, DC 20055 USA).

2.1.2. "In vitro" studies

HepG2 cells were obtained from American Type Culture Collection (ATCC HB-8065) and held in 95 cm^2 flasks in nitrocellulose-filtered (0.22 μ pore size) Eagle's minimal essential medium with 5.5 mM glucose (MEM) plus 100 μg/ml streptomycin supplemented with 10% fetal-bovine serum. Cultures were then harvested with trypsin (0.25% w/v) in phosphate-buffered saline (PBS: NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4, 10.0 mM, KH_2PO_4 2.0; pH 7.4) and grown at 37 °C in a humidified atmosphere with 5% (v/v) CO_2 in air. The cultured cells were then incubated in serum-containing MEM until they reached the logarithmic growth phase and then washed and incubated for 72 h under the different experimental conditions: (a) control medium (C), (b) medium supplemented with 2 mM F, (c) 2 mM F plus 1 nM exendin-4 (FE4), (d) F plus 200 nM exendin-9-36 (FE9) and (e) F plus exendin-4 and exendin-9 (FE4/9).

2.2. Serum measurements

We measured the concentration of glucose (glucose-oxidase GOD-PAP method, Roche Diagnostics, Mannheim, Germany), triglyceride (enzymatic TG color assay GPO/PAP AA, Wiener, Buenos Aires, Argentina), and immunoreactive insulin (radioimmunoassay using an antibody against rat insulin, rat insulin standard [Linco Research Inc., IN, USA], and highly-purified porcine insulin labeled with ^{125}I).

2.3. Glucokinase activity assay

The liver portion removed from each animal was immediately homogenized in a hand-held homogenizer, suspended in ice cold phosphate saline buffer containing PMSF 0.1 mM, benzamidine 0.1 mM, DTT 2 mM, aprotinin 4 μg/ml and sucrose 0.3 M, adjusted to pH 7.4. Aliquots of these homogenates were centrifuged at different speed to isolate the nuclear and the cytosolic fractions (CF and DNF). Detailed description of the technique has been previously reported [26]. Glucokinase activity was finally measured in aliquots of both liver CF and DNF. The CF/DNF glucokinase activity ratio was also calculated.

For HepG2 cell, a pellet of 3 mg protein was re-suspended and disrupted by sonication in 50 μl of the same buffer used for liver homogenization and the product stored at −80 °C until enzyme activity measurement.

Rates of glucose phosphorylation in the 100,000 g soluble CF and in the DNF as well as in HepG2 samples were measured at 37 °C, pH 7.4 by recording the increase in absorbance at 340 nm in a well-established enzyme-coupled photometric assay [26,38] containing glucose-6-phosphate dehydrogenase, ATP and NADP. For each assay, five different experiments were done in triplicate. glucokinase activity was ascertained by subtracting activity measured at 1 mM glucose (hexokinase) from that measured at 100 mM glucose according to reported procedure [26,38]. Enzyme activities were expressed as mU per milligram of protein, one unit being defined as 1 μmol glucose-6-phosphate formed from glucose and ATP per minute at 37 °C.

2.4. Total RNA

Total liver RNA from control and treated rats was isolated using TRIzol Reagent (Gibco-BRL, Rockville, MD, USA). The integrity and purity of RNA isolated was checked by running it on 1% agarose-formaldehyde gel electrophoresis and by measuring the 260/280 nm absorbance ratio. DNA contamination was avoided by using DNase I digestion (Gibco-BRL). Reverse transcription-PCR was performed using SuperScript III (Gibco-BRL) and total RNA (50 ng) from FRD and C liver as a template.

2.5. Analysis of gene expression by real-time PCR (qPCR)

qPCR was performed with a Mini Opticon Real-Time PCR Detector Separate MJR (BioRad), using SYBR Green I as a fluorescent dye. 10 ng of cDNA were amplified in a 25 μl qPCR reaction containing 0.6 μM of each primer, 3 mM $MgCl_2$, 0.3 mM dNTPs, and 0.2 μl Platinum Taq DNA polymerase 6 U/μl (Invitrogen). Samples were first denatured at 94 °C for 3 min followed by 40 PCR cycles. Each cycle comprised a melting step at 94 °C for 30 s, an annealing step at 63 °C for 45 s, and an extension step at 72 °C for 30 s, followed