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Research paper

The protective effect of pentoxifylline versus silymarin on the pancreas through increasing adenosine by CD39 in a rat model of liver cirrhosis: Pharmacological, biochemical and histological study

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ABSTRACT

Impaired glucose homoeostasis due to insulin resistance and decrease sensitivity of pancreatic β-cells is a feature of liver disease and results into hepatogenous diabetes. Decrease expression of CD39 was linked to inflammation and occurrence of diabetes. Therefore, we performed this study to explore the protective effect of pentoxifylline (PTX) and silymarin administration on the β-cells of the pancreas in a rat model of thioacetamide induced liver cirrhosis. Biochemical, histological and immunohistochemistry studies of the liver and pancreas were performed and provided an evidence on the protective effect of PTX to pancreatic β-cells compared to silymarin. Also, silymarin induced a significant improvement of liver cirrhosis compared to PTX. In conclusion, the potential protective effect of PTX against β-cells deterioration could be attributed to increasing pancreatic CD39 expression and the subsequent increase of adenosine.

1. Introduction

The liver plays an important role in controlling blood glucose level. In liver cirrhosis, glucose intolerance occurs frequently due to insulin resistance (IR) and the detrimental effect of liver failure on pancreatic islets thus affecting insulin secretion as well (Barthel and Schmoll, 2003; Postic et al., 2004). This disturbed homeostasis results into hepatogenous diabetes (HD) with an incidence of 30% to 60% of cirrhotic patients (Hickman and Macdonald, 2007).

In the early stages of cirrhosis, HD may be subclinical and oral Glucose Tolerance Test (OGTT) may be the most relevant marker. With the progression of liver disease, diabetes becomes clinically manifested. Therefore, HD can be used as a marker for deterioration of liver functions (García-Compeán et al., 2016).

In liver cirrhosis, released cytokines mediate inflammation and damage of pancreatic cells with potential for increased release and accumulation of extracellular nucleotides that in turn trigger the release of pro-inflammatory cytokines from activated macrophages and endothelium (Apte et al., 1999; Liu et al., 2015). The hydrolysis of extracellular nucleotides is mediated by ectonucleotidases such as Cluster of Differentiation 39 (CD39) which is expressed by hepatic vascular endothelium, hepatic stellate cells (HSCs), pancreatic stellate cells, vascular pericytes and leukocytes (Beldi et al., 2008). The expression of CD39 is regulated by several other factors including pro-inflammatory cytokines (Deaglio and Robson, 2011). CD39 catalyzes the hydrolysis of γ and β phosphate residues of ATP and ADP respectively thus producing AMP (Enjyoji et al., 1999) and its action is accomplished by CD73 which dephosphorylates AMP into the anti-inflammatory adenosine (Yegutkin, 2008). It has been demonstrated that the susceptibility of mice to streptozotocin-induced diabetes was affected by the level of expression of CD39. CD39 knockout mice become diabetic faster and with a higher overall incidence than wild type animals (Antonioli et al., 2014).

Pentoxifylline (PTX) is known by its anti-cell proliferation, anti-inflammatory and anti-fibrotic actions (Raetsch et al., 2002; Vege et al., 2015). It is a competitive nonselective phosphodiesterase inhibitor (Essayan, 2001) which raises intracellular cAMP which was found to increase CD39 expression (Baek et al., 2013).

Silymarin was known by its hepatoprotective effects (Fraschini et al., 2002). Recent evidence indicated that silymarin at low concentrations decreased the activity of cAMP phosphodiesterase thus increasing cAMP (Meng et al., 2016).

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The treatment of HD is complex due to the hepatotoxic effect of oral hypoglycemic drugs (Tolman and Dalpiaz, 2007). We postulated a pancreatic protective effect for pentoxifylline and silymarin through increasing pancreatic CD39 expression and subsequent increase in adenosine. Therefore, we conducted this study to evaluate the potential protective effect of the two drugs in decreasing the progression of chronic pancreatitis in a rat model of thioacetamide (TAA) induced liver cirrhosis.

2. Material and methods

2.1. Animals and grouping

All animal procedures were approved by the Institutional Animal Ethics Committee for Ain Shams University, Faculty of Medicine.

Twenty four male Wistar rats (weighing 150 to 200 g) purchased from National Research Institute (Cairo, Egypt) were housed in an animal room with temperature (22 °C) and lighting (12 h (light)–12 h (dark) cycle) control. They were fed on ordinary chow diet (Meladco for Animal Food, Egypt). An adaptation period of 1 week was allowed before initiation of the experimental protocol.

The rats were randomly distributed equally among 4 groups:

(i) The control naïve group (1 mL saline every three weeks, i.p.).
(ii) The thioacetamide (TAA) treated group; received TAA (200 mg/kg three times/week) for 12 weeks (Said et al., 2013).
(iii) The pentoxifylline (PTX) treated group; received TAA (200 mg/kg three times/week) for 12 weeks + PTX [100 mg/kg/day p.o. (Luo et al., 2015) for the last 4 weeks of the experiment].
(iv) The Silymarin treated group: received TAA (200 mg/kg three times/week) for 12 weeks + silymarin [50 mg/kg/day p.o. (Salama et al., 2012) for the last 4 weeks of the experiment].

2.2. Chemicals and drugs

Thioacetamide (TAA) was purchased from Bio-Research Products, pentobarbital and silymarin were purchased from the Sigma Chemical Company, Cairo, Egypt, and the PTX was purchased from Sanofi-Aventis, Cairo.

2.3. Experimental procedures

Liver cirrhosis was induced by intraperitoneal injection (i.p.) injection of TAA (200 mg/kg) three times per week for 12 weeks. Treatment was started at the end of week 8 for 4 weeks. At the end of the experiment retro-orbital blood samples were taken from each rat after 8 h fasting for biochemical analysis.

Liver and pancreas were collected for histological and immune–histochemical analysis (Salama et al., 2012) and for evaluation of the expression of hepatic TGFβ1 and pancreatic CD39 by real time PCR and measurement of adenosine levels in pancreatic tissues.

2.3.1. Samples processing for biochemical assays

2.3.1.1. Serum samples. Serum was separated from blood and kept frozen at −80 °C until used in the determination of fasting blood glucose (FBG), alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALK) and total and direct bilirubin using Synchron cx5 autoanalyzer (Beckman, USA). Serum amylase was measured using rat ELISA kit (Uscnlife), C-peptide was measured using rat C-peptide ELISA kit (LifeSpan Biosciences, Seattle, WA, USA) and fasting insulin was measured using rat insulin ELISA kit (RayBio, Norcross, GA, USA). Insulin resistance, steady state beta cell function (%β) and insulin sensitivity (%S) were calculated using HOMA-2 calculator (university of Oxford, website; http://www.dtu.ox.ac.uk/homacalculator/index.php, Rudenski et al., 1991).

2.3.1.2. Tissue samples. RNase inhibitor (Thermo Scientific Ribolock RNase inhibitor) was added to hepatic and pancreatic tissue samples used in the evaluation of TGFβ1 and CD39 expression respectively by real time PCR prior to storage at −80 °C.

For processing of pancreatic tissue samples used in the measurement of adenosine, we followed the procedure of Zhang et al., 1991 which prevents the potential production of adenosine from the degradation of endogenous adenine nucleotides. We pulverized 40 mg of each sample in a 400 μL solution of 99% pure methanol containing 5 μM erythro-9-(2-hydroxy-3-nonyl)-adenine to inhibit adenosine deaminase and 0.1 M HCL. By centrifugation, we separated the supernatant to which we added 20 μL of 100% trichloroacetic acid to precipitate proteins followed by another centrifugation step. We immediately added 40 μL of 3.3 M KOH to 300 μL of supernatant for neutralization. Then, we precipitated the adenine nucleotides by adding 200 μL of 1 M zinc sulfate and 100 μL of saturated barium hydroxide followed by vortex-mixing for 10 s and centrifugation. The separated supernatant was stored at −80 °C till used in the measurement of adenosine. All centrifugations were carried out at 4 °C and 14,000*g* for 5 min.

2.3.2. Real-time PCR

We used high pure RNA tissue kit (Roche Diagnostics, Mannheim, Germany) for extraction of total RNA. We performed real-time PCR on LightCycler 1.5 system (Roche Applied System, Mannheim, Germany). We used LightCycler-RNA amplification kit SYBR Green I (Roche Applied System, Mannheim, Germany). The final volume of each reaction mixture was 20 μL containing 1 μL of extracted RNA, 4 μL $MgCl_2$, 0.4 μL LightCycler RT-PCR Enzyme mix, 4 μL LightCycler RT-PCR Reaction Mix SYBR Green, 0.6 μL resolution solution, 1 μL each of either TGFβ1 or CD39 forward and reverse primers and 8 μL nuclease free water. Primer sequences of TGFβ1: Forward, 5′-TGCTTCAGCTCCACA GAGAA-3′ and Reverse, 5′-TGGTTGTAGAGGGCAAGGAC-3′, GenBank: NM_021578.2, CD39: Forward, 5′-CCAGAATCACATGGCATAATTG-3′; Reverse, 5′-CGGCTGTCCTTTGTACCACA-3′, GenBank: XM_006227692.3). Primer sequences of the reference gene (β-actin): Forward, 5′-AGATTACTGCCCTGGCTCCT-3′; Reverse 5′-ACATCTGCTGGAAGGTG GAC-3′, GenBank: NM_031144.3. The reverse transcription was performed at 55 °C for 10 min. Denaturation was performed at 95 °C for 30 s. 45 cycles of PCR were performed as follows: 95 °C, 57 °C for 10 s and 72 °C for 25 s. The expression of the target genes is presented as fold expression calculated using the $2^{-\Delta\Delta Ct}$ equation.

2.3.3. Measurement of adenosine

We measured adenosine concentration in pancreatic tissue by HPLC (Maguire et al., 1986). The sample volume injected was 20 μL, the pump speed was 1.0 mL/min, the detection wavelength was 210 nm and the elution buffer was a gradient of 0.02 M potassium dihydrogen phosphate buffer, pH 5.5 and 60:40 (vol/vol) mixture of methanol and water. Peak areas were determined and the concentration of adenosine was determined from a standard curve constructed using adenosine purchased from Sigma-Aldrich (Saint Louis, MO, USA) as a standard.

2.4. Histological study

At the end of week 12, the liver and the pancreas were extracted immediately. The right lobe of liver and the pancreas were divided into two halves for the following:

2.4.1. Light microscopic (LM) study

One half was cut into thin slices and fixed in 10% neutral buffered formalin then processed to get paraffin blocks for LM. The paraffin blocks were cut into five microns thick sections for H&E and Mallory's trichrome stains. Immunostaining was performed using an avidin biotin-peroxidase technique for detection of α-SMA antibody (purchased from Lab vision, CA, USA) for liver and pancreas fibrosis at a dilution of 1:800 for one and half hour, proliferating cell nuclear

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