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Aging and a long-term diabetes mellitus increase expression of 1 α-hydroxylase and vitamin D receptors in the rat liver

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ABSTRACT

Diabetes mellitus (DM) is a metabolic disorder associated with serious liver complications. As a metabolic chronic disease, DM is very common in the elderly. Recent studies suggest ameliorating effects of vitamin D on metabolic and oxidative stress in the liver tissue in an experimental model of DM. The aim of this study was to investigate the expression of vitamin D receptors (VDRs) and 1α-hydroxylase, the key enzyme for the production of active vitamin D form (calcitriol) in the liver during long-term diabetes mellitus type 1 (DM1) in aging rats. We performed immunohistochemical analysis of liver expression of 1α-hydroxylase and VDRs during aging in long-term streptozotocin-induced DM1. 1α-Hydroxylase was identified in the monocyte/macrophage system of the liver. In addition to the nuclear expression, we also observed the expression of VDR in membranes of lipid droplets within hepatocytes. Aging and long-term DM1 resulted in significant increases in the number of 1α-hydroxylase immunoreactive cells, as well as the percentage of strongly positive VDR hepatocytes. In conclusion, the liver has the capacity for active vitamin D synthesis in its monocyte/macrophage system that is substantially increased in aging and long-term diabetes mellitus. These conditions are also characterized by significant increases in vitamin D receptor expression in hepatocytes. The present study suggests that VDR signaling system could be a potential target in prevention of liver complications caused by diabetes and aging.

1. Introduction

Diabetes mellitus (DM) is a chronic metabolic disease very common in the elderly (Chow et al., 2014) and represents a growing health problem (Wild et al., 2004). Oxidative stress and inflammation play important roles in mechanisms underlying aging and complications of diabetes (Halter et al., 2014), that are commonly associated with severe liver complications (George et al., 2012; Hamden et al., 2009). Recent studies suggested beneficial effects of vitamin D in age-associated pathologies related to cardiovascular diseases, DM, cancer and the autoimmune diseases (Haussler et al., 2011; Hayes, 2010; Najmi Varzaneh et al., 2013), as well as in reducing the effects of metabolic and oxidative stress in the liver during experimental DM (George et al., 2012).

Metabolic activation of vitamin D begins in the liver by synthesis of 25-hydroxyvitamin D_3 ($25(OH)D_3$), the major circulating form of vitamin D (Dusso et al., 2005; Haussler et al., 2013). The $25(OH)D_3$ is a substrate for the mitochondrial enzyme 25-hydroxyvitamin D_3-1α-hydroxylase (1 α-hydroxylase, 1α-OHase, CYP27B1), that catalyzes conversion of $25(OH)D_3$ to calcitriol ($1,25(OH)_2D_3$), the most active vitamin D metabolite (Dusso et al., 2005; Haussler et al., 2013). Although 1α-OHase is primarily renal enzyme, it has also been detected in different extra-renal tissues such as the lymph nodes, skin, breast, prostate, colon and cells of the immune system (Townsend et al., 2005).

Calcitriol's actions are mediated through its interaction with vitamin D receptor (VDR), a member of the nuclear hormone receptor superfamily (Haussler et al., 2013; Nagpal et al., 2005). Liganded VDRs form heterodimers with the retinoid X receptors (RXRs), which subsequently bind to VDR-responsive elements in the promoter region of genes whose expression is directly controlled by vitamin D (Dusso et al., 2005; Haussler et al., 2013; Marshall, 2008). Calcitriol can also exert its bioactivity through a rapid non-genomic response system mediated by receptors associated with the plasma membrane (Dusso et al., 2005).

Although the presence of VDR in liver cells is confirmed (Barchetta et al., 2012; Ding et al., 2013; Gascon-Barre et al., 2003; Segura et al., 1999), the mechanisms underlying the ameliorating role of vitamin D in hepatic complications of DM (George et al., 2012) are poorly

Abbreviations: DM, diabetes mellitus; VDR, vitamin D receptor; 1α-OHase, 1 α-hydroxylase; DM1, diabetes mellitus type 1; RXR, retinoid X receptors; STZ, streptozotocin; DAPI, 4′,6-diamidino-2-phenylindole; PBS, phosphate buffer saline; SD, standard deviation; GFAP, glial fibrillary acidic protein; Iba1, ionized calcium-binding adapter molecule 1; VEGF, vascular endothelial growth factor; MPO, myeloperoxidase; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling; LD, lipid droplets; αSMA, alpha-smooth actin; HSC, hepatic stellate cell; PPAR, peroxisome proliferator-activated receptor.

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understood. The presence of 1α-OHase in the liver is known, but there are no data on the cellular distribution of the enzyme in the hepatic tissue.

The aim of our study was to determine the distribution and localization of VDR and 1α-OHase in the hepatic tissue of rats during long-term streptozotocin-induced DM1, and to investigate their association with aging and pathological changes in the liver cells caused by diabetes. In the present study, for the first time, we have demonstrated the presence of 1α-OHase in the monocyte–macrophage system of the liver, and varied strongly with aging and diabetes.

2. Materials and methods

2.1. Ethics

The experimental protocol was approved by the Ethical Committee of the University of Split, School of Medicine. All experimental procedures followed the EU Directive (2010/63/EU).

2.2. Experimental animals

Thirty male 8-week-old Sprague–Dawley rats weighing ~200 g were used. They were raised under controlled conditions (22.1 °C temperature and 12/12 h light schedule) at the University of Split's Animal Facility. Standard laboratory chow (4RF21 GLP, Mucedola srl, Settimo Milanese, Italy) and water were given ad libitum. The intake of vitamin D was 1260 IU/kg of diet and the intake of calcium 9163 mg/kg of diet.

DM1 was induced by intraperitoneal administration of streptozotocin (STZ; 55 mg/kg), freshly dissolved in citrate buffer (pH 4.5), after overnight fasting. The control group received pure citrate buffer solution. Five groups of rats were included: 3-month-old control (c-3m; N = 4), 8-month-old control (c-8m; N = 7) and diabetic groups (dm-8m; N = 7); and 14-month-old control (c-14; N = 7) and diabetic groups (dm-14; N = 6). Duration of diabetes was 6 or 12 months for dm-8 or dm-14 group, respectively.

Plasma glucose levels were measured with a glucometer (One-133TouchVITa, LifeScan, High Wycombe, UK) once a month. DM1 rats with glycemia below 300 mg/dl were excluded from the study. Diabetic rats received injections of 1 U of long-acting insulin (Lantus Solostar; Sanofi-Aventis Deutschland GmbH, Frankfurt, Germany) to prevent ketoacidosis.

2.3. Tissue collection and immunohistochemistry

Rats were anesthetized with isoflurane (Forane, Abbott Laboratories, Queenborough, UK) and sacrificed by decapitation. The liver was removed and blocks of tissue were post-fixed in Zamboni's fixative, dehydrated and embedded in paraffin wax.

Histological sections (5 μm thick) were made. After deparaffinization and rehydration, sections were heated in Dako Target Retrieval Solution (S2367 DakoCytomation, Carpinteria, CA, USA) for 12 min at 95 °C in a microwave oven. Sections were incubated overnight with primary antibodies (Table 1) using standard immunohistochemical methods. Detection was performed using an appropriate combination of secondary antibodies (Table 1) for 1.5 h. The nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Slides were mounted, air-dried, and cover slipped (Immu-Mount, Shandon, Pittsburgh, PA, USA). Alternatively, after deparaffinization and rehydration, endogenous peroxidase activity was blocked by incubation with 3% H_2O_2 for 15 min, followed by heating as described above. Slides were incubated overnight with primary VDR antibody. Detection was performed by using LSAB + System-HRP (K0690) and diaminobenzidine (DAB) reaction (Liquid DAB + Substrate chromogen system K3468; both DakoCytomation, Carpinteria, CA, USA). The nuclei were counterstained with hematoxylin. Sections were then mounted for light microscopy (Olympus BX40, Tokyo, Japan).

Control staining included omission of primary antibody from the staining procedure, which resulted in no staining of the tissue. Positive staining control included staining of paraffin sections of the rat kidney tissue (data not shown).

2.4. Data acquisition and analysis

Liver sections were viewed and photographed using a microscope (BX61) equipped with cooled digital camera (DP71; both Olympus, Tokyo, Japan). ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used for further analyses. Each microphotograph of liver section taken at ×40 objective magnification was divided into squares of 100 × 100 μm. Five randomly selected fields were analyzed in each of five microphotographs analyzed. Cells were considered as strongly VDR positive when more than half of cytoplasm exhibited intensive immunoreactivity.

The number of VDR-immunoreactive nuclei was counted on three photographs taken at ×40 objective magnification. The 0.12 mm^2 of the section area was analyzed in order to quantify a number of hepatocytes and mean hepatocyte diameter, which was calculated as mean value from longest and shortest cell diameter. Since significant differences in number and size of hepatocytes were observed, the results were corrected for the number of hepatocytes per section area. A mean number of hepatocytes in c-3m group was taken for normalization, and it was divided by a mean hepatocyte number of the other groups to get specific correction factor for every group. Then, the results were multiplied by the correction factor and final corrected results were expressed as the number of VDR-immunoreactive nuclei of hepatocytes per mm^2.

1α-OHase immunoreactive cells were counted as the number of cells per unit area. The whole cut surface of each liver section was photographed in toto at ×10 objective magnification, and the number of immunoreactive cells expressed per mm^2.

Table 1
Primary and secondary antibodies.

	Antibody	Code no.	Host	Dilution	Source
Primary	Anti-vitamin D receptor	ab350	Rabbit	1:300	Abcam plc. 330 Cambridge, CB4 OFL, UK
	Anti-GFAP-antibody	ab7260		1:50	
	Anti-VEGF antibody	ab46154		1:200	
	Anti-human myeloperoxidase	A 0398	Mouse	1:300	DakoCytomation, DK-26000 Glostrup, Denmark
	Alpha smooth actin	M0851		1:300	
	Anti-murine 25-hydroxyvitamin D3-1 αhydroxylase	PC 290	Sheep	1:100	The Binding Site Group Ltd., Birmingham, UK
	Anti Iba1	019-19741	Rabbit	1:2000	Wako Pure Chemical Industries, Osaka 540-8605, Japan
Secondary	Anti-rabbit IgG-rhodamine	711-295-152	Donkey	1:300	Jackson Immuno Research Laboratories, Inc., Baltimore, PA, USA
	Anti-mouse IgG-rhodamine	715-295-151		1:300	
	Anti-goat IgG-rhodamine	705-295-003		1:300	
	Anti-rabbit IgG-FITC	711-095-152		1:200	
	Anti-mouse IgG-FITC	715-095-150		1:200	
	Anti-sheep IgG- FITC	713-095-147		1:200	