EXPERIMENTAL WORKS

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VITAMIN D_3 REGULATES HEPATIC VEGF-A AND APELIN EXPRESSION IN EXPERIMENTAL TYPE 1 DIABETES

D. O. LABUDZYNSKYI[1]✉, I. O. SHYMANSKYI[1], O. O. LISAKOVSKA[1], A. O. MAZANOVA[1], L. V. NATRUS[2], M. M. VELIKY[1]

[1]Palladin Institute of Biochemistry, National Academy of Sciences of Ukraine, Kyiv;
[2]Bogomolets National Medical University, Kyiv, Ukraine;
✉e-mail: konsument3@gmail.com

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The deficiency of vitamin D is associated with the risk of various chronic diseases, including diabetes mellitus and its complications. Given the strong genomic action of vitamin D hormone-active form, its deficiency can lead to dysfunction of cytokine signaling pathways, including those dependent on vascular endothelial growth factors (VEGFs) and apelin. The present study was carried out to define the link between VEGF-A and apelin expression in liver, hepatocytes viability and vitamin D status at experimental type 1 diabetes in mice. We established that chronic hyperglycemia at streptozotocin-induced diabetes was accompanied by a 2.2-fold decrease in 25OHD content in the serum and increased hepatocytes apoptosis and necrosis. Vitamin D deficiency correlated with increased apelin and VEGF-A (8- and 1.6-fold respectively) expression. Almost complete restoration of circulatory 25OHD content in serum was achieved at vitamin D_3 treatment (800 IU/kg, per os, for 2 months) followed by reduced apelin and VEGF-A expression in liver and the decline of hepatocytes apoptosis. We conclude that vitamin D_3 can be involved in cell survival, angiogenesis and fibrogenesis by modulating VEGF-A and apelin dependent regulatory systems in diabetic liver.

K e y w o r d s: experimental type 1 diabetes, liver, angiogenesis, apoptosis, vitamin D_3, VEGF, apelin.

Type 1 diabetes mellitus (T1D) is associated with an active immune-mediated destruction of insulin-producing β-cells in the pancreas by lymphocytes infiltrating the islets that subsequently results in severe acute and chronic diabetic complications [1]. Growing evidence shows that metabolic alterations, inflammation and vascular dysfunction can be defined as common liver abnormalities associated with T1D [2]. Disruption of liver angiogenesis is closely related to the progression of fibrosis under conditions of chronic hyperglycemia, leading to increased formation of extracellular matrix and drastic changes in its quality [3].

Although angiogenesis is a highly complex and coordinated process, requiring multiple receptors and ligands produced by different cell types, the vascular endothelial growth factor A (VEGF-A), also called the vascular permeability factor, is a hypoxia-induced pro-angiogenic cytokine, which seems to be the most critical for growth and survival of the vascular endothelium in health and disease. VEGF-A belongs to a superfamily of secreted polypeptides with a highly conserved receptor-binding cystine-knot structure similar to that of the PDGF (platelet-derived growth factor). VEGF-A binds primarily to two endothelial transmembrane receptors (VEGFR1 and VEGFR2) of the receptor tyrosine kinase subfamily. Earlier studies revealed a fibrogenic effect of VEGF-A through multiple mechanisms, including promotion of inflammation, release of fibrosis-en-

hancing molecules from VEGF-activated endothelial cells as well as direct VEGF effects on hepatocytes survival [4].

Changes in liver remodeling, as well as concomitant cardiovascular and renal complications, may also be related to impaired expression of apelin, an endogenous vasoactive peptide ligand for apelin receptor (APJ, angiotensin-like-receptor 1) [5, 6]. The G-protein-coupled receptor APJ and its endogenous ligand are widely expressed in many peripheral tissues, including CNS, adipose tissue, skeletal muscles, kidney and liver. Apelin, through APJ-associated cell transduction cascades (ERKs, Akt, and p70 S6 kinase phosphorylation), activates proliferation of endothelial cells and formation of new blood vessels [7]. It has also emerged as the important regulator of insulin sensitivity, which stimulates glucose utilization and enhances brown adipogenesis in different tissues associated with diabetes [6]. Nevertheless, the biological role of apelin in the liver and its relation to VEGF-A function only are beginning to be explored.

Vitamin D is a secosteroid hormone primarily known for its important role in calcium homeostasis and bone metabolism. However, the finding of vitamin D receptor (VDR) expressed in various tissues has broadened the scope of biological effects of vitamin D in human health. Recent data suggest that vitamin D is involved in cell proliferation, differentiation, insulin sensitivity, angiogenesis, inflammation and apoptosis [8]. Its bioactive actions are predominantly mediated by binding to VDR on target cells and organs. Vitamin D deficiency has been frequently reported to be linked to chronic liver diseases, including non-alcoholic fatty liver disease (NAFLD) and chronic hepatitis C (CHC) virus infection [9]. As vitamin D may regulate cytokines production [10], the aim of the study was to assess the association of vitamin D status with the expression of VEGF-A and apelin in T1D-induced liver injury.

Materials and Methods

Animal experiments. The study was carried out on male C57BL/J6 mice (22 ± 3 g). We induced type 1 diabetes by 5 intraperitoneal injections of streptozotocin (Sigma-Aldrich, USA) at a dose of 40 mg/kg of body weight [11]. The selection of animals and the formation of experimental groups were performed by the method of "random numbers". Glucose level was measured fasting in the morning. After the development of stable hyperglycemia (20.4 ± 4.3 mmol/l), mice were treated with vitamin D_3 in an aqueous suspension form (DSM, Netherland; 800 IU/kg of body weight, per os) for 2 months. All experiments were conducted in accordance with the international recommendations of the European Convention for the Protection of Vertebrate Animals used for Research and Scientific Purposes (Strasbourg, 1986) and are ethically acceptable.

Elisa assay. We evaluated the vitamin D bioavailability by the level of serum 25-hydroxyvitamin D (25OHD) using immunoenzyme technique (ELISA kit, Immunodiagnostic Systems Ltd., USA) according to the manufacturer's instructions.

RNA isolation and RT-qPCR. Total RNA was extracted from the liver tissue using TRIzol reagent according to the manufacturer's instructions (Sigma-Aldrich, USA). The cDNA was synthesized from 1 μg of total RNA using random primers and Moloney murine leukemia virus reverse transcriptase (both from Life Technologies, USA) as previously described [12]. The forward and reverse primers for mouse were as follows: for apelin mRNA – 5′-CGAGTTGCAGCATGAATCTGAG-3′ and 5′-TGTTCCATCTGGAGGCAACATC-3′; for VEGF-A mRNA – 5′-GGACTTGTGTTGGGAGGAGG-3′ and 5′-GGCTCCCCAAACTTCTGGTT-3′, and for 18S – 5′-CGCCGCTAGAGGTGAAATTCT-3′ and 5′-CATTCTTGGCAAATGCTTTCG-3′. Real-time quantitative polymerase chain reaction analyses were performed using the Mx3005P Real-Time PCR System (Stratagene, USA). For each condition, we quantified gene expression in duplicate, and 18S rRNA was used as the endogenous control in the comparative cycle threshold (Ct) method. We expressed data as relative expression ratio.

Western blot analysis. We detected the level of VEGF-A in the total lysates from liver tissue by western blot analysis. Murine livers were lysed in RIPA buffer containing protease inhibitor cocktail (PIC, Sigma, USA). Protein concentrations in the liver lysates were determined as described previously (Lowry, 1951). Samples of 50 μg protein were loaded onto 10% SDS polyacrylamide gels. After SDS-PAGE, the proteins were transferred onto nitrocellulose membrane [13]. Immunoblotting was conducted with primary antibodies against VEGF-A (1:500; Santa Cruz Biotechnology, USA) and β-actin (1:20000; Sigma, USA). Primary-antibody-bound membranes were incubated with peroxidase-conjugated secondary antibodies – anti-rabbit IgG (H+L)-HRP conjugate (1:4000; Bio-Rad Laboratories, Inc., USA). The bands were visualized by enhanced

chemoluminescene agents *p*-coumaric acid (Sigma, USA) and luminol (AppliChem GmbH, Germany). Tissue levels of target proteins were normalized to β-actin. The immunoreactive bands were quantified with Gel-Pro Analyzer 32.

Hepatocytes isolation and apoptosis detection. Hepatocytes were isolated using collagenase perfusion technique and cell apoptosis was detected by GFP-Annexin V labelling as described [14]. Samples were analyzed using EPICS XLTM flow cytometer (Beckman Coulter, USA). The fluorescence of GFP-Annexin V was measured at λ_{ex} = 488 and λ_{em} = 540 nm.

Propidium iodide staining assay. Propidium iodide (PI; Sigma, USA), an intercalating dye, was used to determine necrotic cell death [15]. The cells (approximately 1×10^6 cells/ml) were incubated in Hank's balanced salt solution with PI (50 μg/ml) in the dark for 10 min at room temperature. Data acquisition was carried out on an EPICS XLTM flow cytometer (Beckman Coulter, USA) using the excitation/emission wavelengths of 530/645 nm.

Statistical analysis. All the data were expressed as mean ± SEM deviation of at least three independent experiments. Statistical differences between the various groups were compared by using Student's *t*-test and one-way ANONA. *P* values less than 0.05 were considered statistically significant.

Results and Discussion

The serum level of glucose in the animals with T1D increased almost 4-fold (to 21.4 ± 4.5 mmol/l) as compared with the control (5.5 ± 1.2 mmol/l). Hyperglycemia was associated with significant changes in vitamin D metabolism and bioavailability. The most reliable and informative biomarker of vitamin D availability is the 25OHD (25-hydroxyvitamin D) content in blood serum, which reaches a range of 100-150 nmol/l in humans under normal physiological conditions. Reduction of the serum 25OHD level below 75 nmol/l indicates the development of D-hypovitaminosis [10]. The data presented in Table show that diabetes causes a decrease in serum 25OHD level by 2.2 times compared with control mice. It may reflect the presence of severe vitamin D deficiency most likely due to inhibition of synthesis or/and enhanced catabolism of biologically active hydroxylated forms of vitamin D. In our previous works, we found essential changes in the functioning of vitamin D-metabolizing enzymes in liver tissue induced by T1D [16]. Vitamin D_3 supplementation partially normalized serum 25OHD without marked effect on blood glucose level (17.8 ± 3.9 mmol/l).

Liver disorder can be considered as one of the subacute complications of diabetes mellitus [2, 3]. To determine whether maintaining vitamin D insufficiency is associated with any liver damage induced by T1D, we isolated primary culture of hepatocytes from diabetic mice and investigated the level of cell demise. Our findings have shown that hyperglycemia and vitamin D deficiency correlated with an increase in the number of PI-positive dead (mostly necrotic) cells among isolated hepatocytes to 7.5% compared with 3.8% in the control (Fig. 1). Besides necrotic cells, PI-positive hepatocytes may include cells at late stage of apoptosis. Therefore, we concomitantly studied whether diabetes induces apoptosis in the liver. Indeed, T1D led to a 3.2-fold elevation of GFP-Annexin V labelling of hepatocytes compared with the control that corresponds to the increased number of apoptotic cells in diabetic liver, Fig. 2. The therapeutic administration of vitamin D_3 to animals suffered from diabetes significantly (by 1.3 times) diminished the number of PI-positive cells among isolated hepatocytes as compared with the diseased state, Fig. 1. In addition, Fig. 2 demonstrates that vitamin D_3 reduced the number of apoptotic hepatocytes 1.4 times compared with T1D.

It is likely that hyperglycemia, non-enzymatic glycation and oxidative stress can have negative ef-

Blood serum 25OHD and glucose levels in diabetes and after vitamin D_3 administration

Experimental groups	Glucose, mmol/l	25OHD	
		nmol/l	ng/ml
Control	5.5 ± 1.2	81.7 ± 4.12	32.68 ± 1.65
Diabetes	21.4 ± 4.5*	37.9 ± 2.12*	15.16 ± 0.85*
Diabetes + D_3	17.8 ± 3.,9	77.3 ± 5.48$^{\#}$	30.92 ± 2.19$^{\#}$

Values represent the means ± SEM for three experiments ($M \pm m$, $n = 6$) done in triplicate. *$P < 0.05$ vs. control, $^{\#}P < 0.05$ vs. diabetes

fects on hepatocytes function and survival, ultimately leading to augmented cell death observed in the present study. Previously, we have shown that vitamin D exerts hepatoprotective action by suppressing prooxidant processes in liver tissue. It can be assumed that vitamin D deficiency associated with elevated oxidative stress may be attributed to impaired VDR expression or inactivation. Furthermore, endothelial dysfunction is also associated with increased oxidative stress, pro-inflammatory response and altered angiogenic activity, which is a hallmark of many chronic diseases, including diabetes [17, 18]. As sufficient vitamin D levels and proper VDR expression could be fundamental for angiogenic and oxidative defense function of endothelial cells as well as for hepatocytes survival, we further demonstrated the possible role of angiogenic cytokines. We found 1.6-fold diabetes-induced increase in the hepatic expression of VEGF-A mRNA, which is a major angiogenesis and vascular permeability regulator, Fig. 3, *A*. These changes at transcriptional level also correlated with 1.7-fold increase in VEGF-A protein synthesis in diabetic liver as compared with the control, Fig. 3, *B*. VEGF-A is the predominant pro-angiogenic cytokine, but others may also be important. In particular, we revealed 9.5-fold overexpression of apelin mRNA in the liver of diabetic mice compared with the control group, Fig. 3, *C*. This bioregulator, in addition to being involved in angiogenesis, also has a crucial influence on fluid

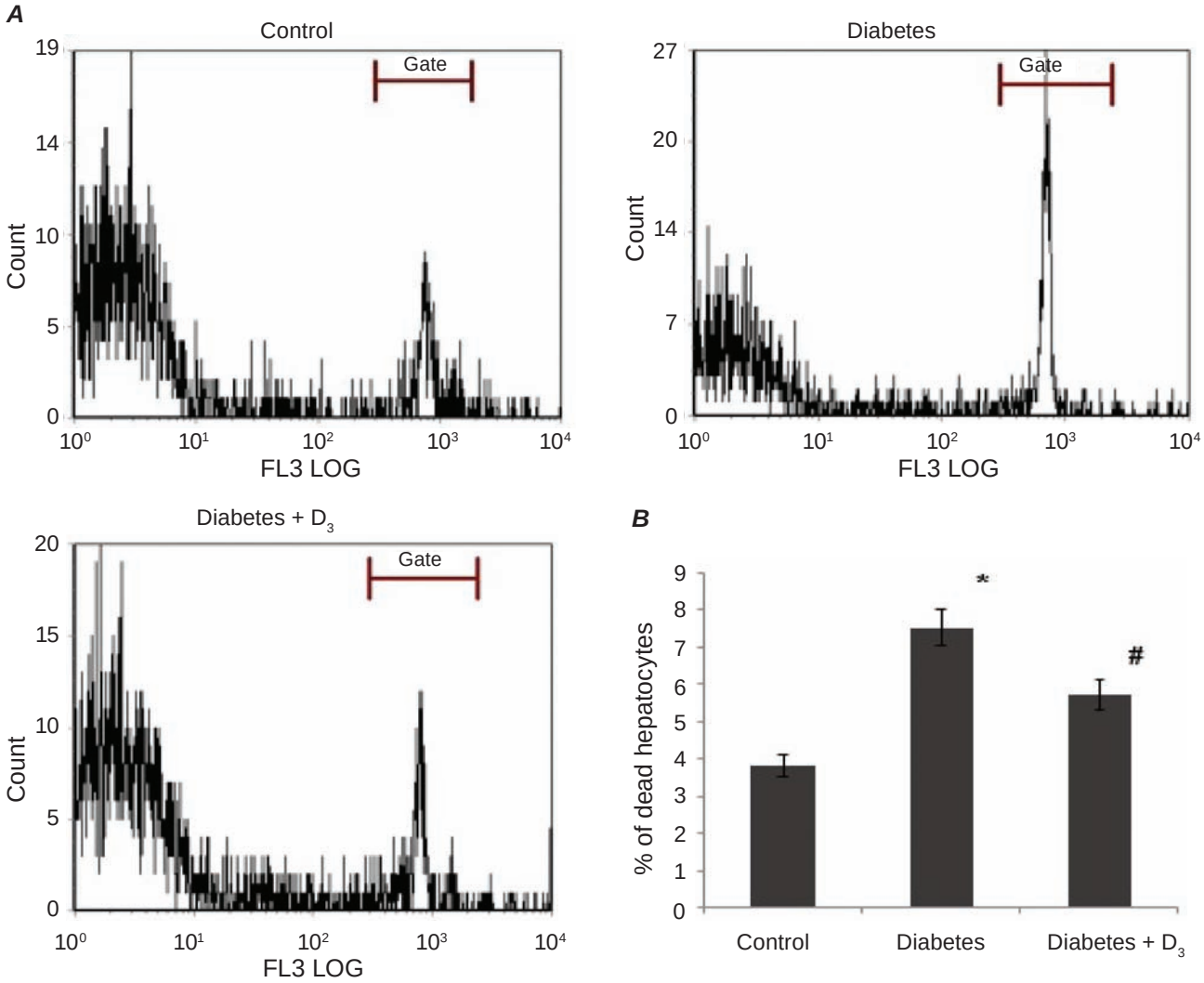

*Fig. 1. Propidium iodide accumulation by isolated hepatocytes in diabetes and after vitamin D_3 administration. (**A**) Representative histograms of PI accumulation (count – the number of events; FL1 LOG – fluorescence intensity); (**B**) quantification of PI fluorescence. Results are shown as mean ± SEM and representive of three independent experiments done in triplicate; *P < 0.05 vs. control, #P < 0.05 vs. diabetes*

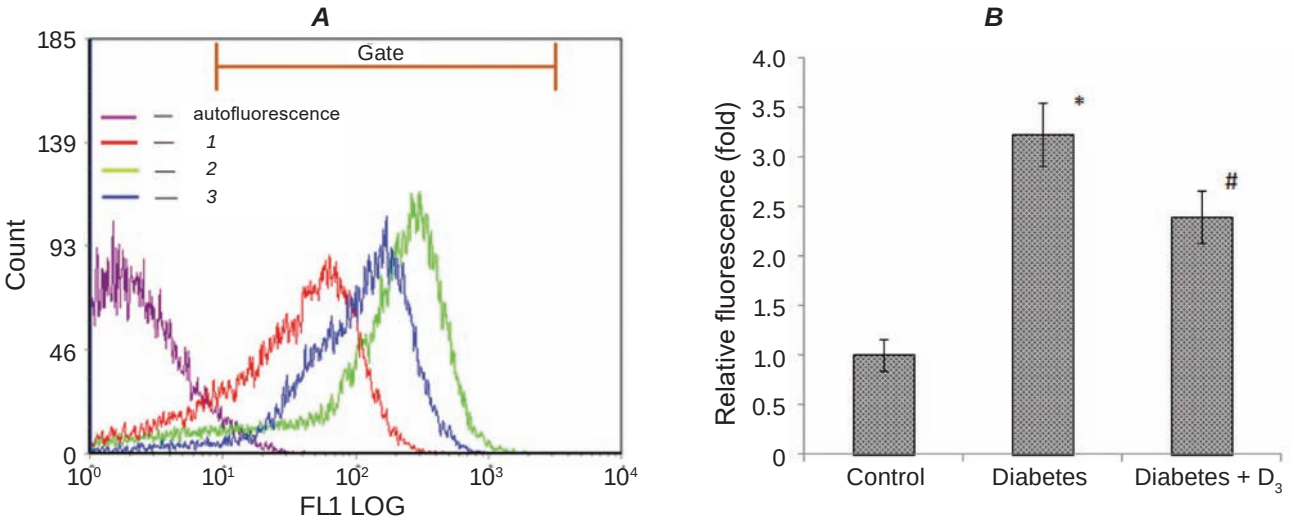

*Fig. 2. Apoptosis of isolated hepatocytes in diabetes and after vitamin D_3 administration. (**A**) Representative histograms of GFP-Annexin V accumulation (count – the number of events; FL1 LOG – fluorescence intensity); (**B**) quantification of GFP-Annexin V fluorescence. Results are shown as mean ± SEM and representive of three independent experiments done in triplicate; *P < 0.05 vs. control, #P < 0.05 vs. diabetes*

homeostasis, energy metabolism, and inflammation. Vitamin D_3 contributed to partial recovery of both cytokine levels. It suppressed the expression of VEGF-A and apelin mRNAs (1.8 and 1.7 times respectively), and significantly reduced the level of VEGF-A protein as compared with T1D, Fig. 3.

Our findings are in agreement with previous observations confirming sustained fibrous changes in the liver accompanied by a significant increase in the percentage of apoptotic phenomena in the tissues of this organ [3]. Furthermore, we detected a similar trend in increasing necrosis in the liver cell population. Inflamed and necrotic hepatocytes are known to secrete several types of chemical mediators that can activate stellate cells to produce certain factors of angiogenesis and connective tissue growth, as well as other biomolecules, such as enzymes and collagen, which cause rapid accumulation of extracellular matrix and favor fibrosis [19]. Notably, the processes of inflammation, excessive generation of reactive oxygen species (ROS) and angiogenesis are closely related [20]. Newly formed blood vessels provide recruitment of inflammatory cells that secrete various pro-angiogenic cytokines and growth factors that further enhance angiogenesis.

As a putative underlying mechanism of changes caused by T1D in liver tissue, we found a significant increase in the expression of VEGF-A and apelin. Liver lesions associated with diabetes most likely are not driven solely by the separate effect of each cytokine studied, but depend on the cross-talk of both. The initial activation of hepatic VEGF presumably occurs in response to hyperglycemia-induced hypoxia and pro-inflammatory processes. In turn, increased expression of apelin can result from the regulatory action of VEGF, which was previously shown to enhance the synthesis of APJ [21]. Moreover, apelin is able to induce the synthesis of VEGF and reinforce or replace the action of VEGF through VEGFR [22]. For instance, some authors have demonstrated that effects of apelin on ischemic neurovascular unit injuries are highly associated with the increase in VEGF binding to VEGFR-2, probably through the activation of the ERK and PI3K/Akt pathways [23].

Due to conflicting observational and experimental data, the role of apelin in organ inflammation, fibrosis and cell death remains controversial. The apelin-APJ axis has been found to be beneficial for diabetes-induced renal, cardiac and pulmonary fibrosis. Apelin interfered with diabetes-induced hypertrophy of the kidneys and cardiac hypertrophy associated with obesity [24]. On the contrary, this cytokine promoted retinal angiogenesis, thereby contributing to the development of diabetic retinopathy [25]. Furthermore, the apelin-APJ axis promotes liver inflammation and fibrosis mainly through elevated expression of collagen-II and platelet-derived growth factor receptor β (PDGFRβ) that can explain rather detrimental then protective role of apelin over-

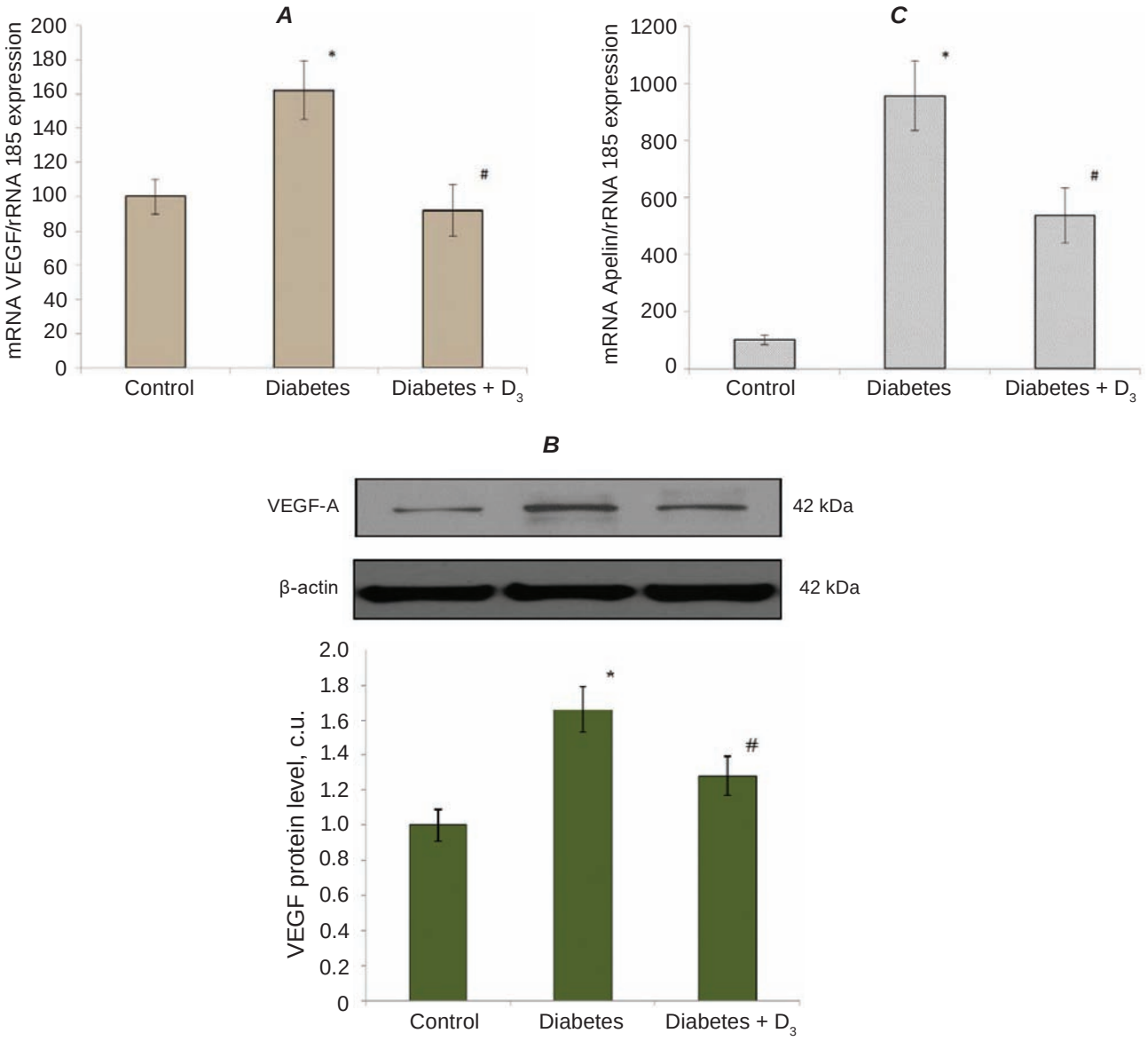

*Fig. 3. The VEGF-A (**A**), apelin (**C**) mRNA expression and VEGF (**B**) protein level in diabetes and after vitamin D_3 administration. Results are shown as mean ± SEM and representive of three independent experiments done in triplicate; *P < 0.05 vs. control, #P < 0.05 vs. diabetes*

expression found in the present study [26]. In the liver, the apelin/APJ system may participate in the formation of hepatic fibrosis or cirrhosis by inhibiting liver regeneration and promoting Fas-induced apoptosis [27]. Nonetheless, anti-apoptotic effects of apelin on vascular smooth muscle cells have also been observed [28].

An important aspect of the research was that all the described changes in the diabetic liver were accompanied by a severe vitamin D deficiency, since the level of 25OHD in the serum of diabetic mice was more than halved. Our results demonstrate that restoration of vitamin D bioavaolability in diabetic animals correlated with a sustained decline in the percentage of both apoptotic and necrotic hepatocytes, as well as a significant decrease in the hepatic expression of VEGF-A and apelin. These data are in line with previous reports suggesting vitamin D as a potent drug for the VDR-mediated prevention and treatment of angiogenic and inflammation-related disorders [29], but so far the relationship between circulatory 25OHD and altered function of VEGF-A and apelin has not been investigated. The studies published to date show that vitamin D plays a role in adipose tissue function and may be involved in the synthesis and modulation of adipokine produc-

tion, including apelin [30]. Besides affecting apelin-mediated regulation, hormonally active form of vitamin D, 1,25$(OH)_2$D, is able to have a direct effect on the expression of VEGF in different tissues [31, 32]. After the discovery of the vitamin D receptor, it became clear that a large number of genes are transriptionaly regulated by 1,25$(OH)_2$D via VDR [33]. Cardus et al. demonstrated direct binding of the VDR to two response elements in the VEGF promoter in vascular smooth muscle cells [34]. Like other steroid hormones, vitamin D can also trigger rapid responses that do not affect gene expression and appear to be mediated by the receptors located in cell plasmalemma. However, the nature of the receptor involved in the rapid actions remains largely undefined. It is noteworthy, that the success of vitamin D_3 in the inhibition of angiogenesis may be due not only to the down-regulation of vasoactive cytokine levels but also to the attenuation of diabetes-associated ROS formation, oxidative stress and inflammation [33]. We can summarize that effectively acting on the hepatic expression of VEGF-A and apelin, vitamin D_3 is involved in integrating a complex system of molecular processes in the liver, directly or indirectly modulating cell survival, angiogenesis and fibrogenesis related to T1D.

Overall, our findings suggest that a decrease in the bioavailability of vitamin D may be associated with the overexpression of VEGF-A and apelin, as well as an increased apoptotic and necrotic death of primary hepatocytes in type 1 diabetes. Vitamin D_3 supplementation has been helpful in altering angiogenic/inflammatory biomarkers and hepatoprotaction that broadens the understanding of the therapeutic potential of vitamin D for the treatment of diabetes-induced liver damage. Further studies incorporating gene knockouts and biochemical changes at the protein level are needed to confirm the requirement of VDR for VEGF- and apelin-dependent responses.

Conflict of interest. Authors have completed the Unified Conflicts of Interest form at http://ukrbiochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

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ВІТАМІН D_3 РЕГУЛЮЄ ЕКСПРЕСІЮ VEGF-A ТА АПЕЛІНУ В ПЕЧІНЦІ ЗА ЕКСПЕРИМЕНТАЛЬНОГО ЦУКРОВОГО ДІАБЕТУ

Д. О. Лабудзинський[1]✉, *І. О. Шиманський*[1], *О. О. Лісаковська*[1], *А. О. Мазанова*[1], *Л. В. Натрус*[2], *М. М. Великий*[1]

[1]Інститут біохімії ім О. В. Палладіна НАН України, Київ;
[2]Національний медичний університет ім. О. О. Богомольця, Київ, Україна;
✉e-mail: konsument3@gmail.com

Дефіцит вітаміну D_3 пов’язують із ризиком виникнення різних хронічних захворювань, у тому числі цукрового діабету та його ускладнень. З огляду на значну геномну дію гормональноактивної форми вітаміну D, його дефіцит може призвести до дисфункції сигнальних шляхів цитокінів, у тому числі залежних від фактора росту ендотелію судин (VEGF) та апеліну. Дослідження було проведено для визначення зв’язку між експресією VEGF-A та апеліном у печінці, життєздатністю гепатоцитів та статусом вітаміну D за експериментального діабету 1-го типу в мишей. Встановлено, що хронічна гіперглікемія за діабету, індукованого стрептозотоцином, супроводжувалася зменшенням вмісту 25OHD у сироватці крові в 2,2 раза і збільшенням апоптозу та некрозу гепатоцитів. Дефіцит вітаміну D корелював зі збільшенням експресії апеліну та VEGF-A (у 8 та в 1,6 раза відповідно). Практично повне відновлення вмісту 25OHD у сироватці крові було досягнуто у разі лікування вітаміном D_3 (800 МО/кг, *per os* протягом 2 місяців) із подальшим зниженням експресії апеліну та VEGF-A в печінці та зниженням апоптозу гепатоцитів. Дійшли висновку, що вітамін D_3 може бути залучений до виживання клітин, ангіогенезу і фіброгенезу за діабету через регуляторні системи VEGF-A та апеліну у печінці.

Ключові слова: експериментальний цукровий діабет 1-го типу, печінка, ангіогенез, апоптоз, вітамін D_3, VEGF, апелін.

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