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The Role of Vitamin D_3 in the Regulation of Mineral Metabolism in Experimental Type 1 Diabetes

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Abstract—Experimental streptozotocin type 1 diabetes in mice is characterized by a significant deficiency of vitamin D_3, detected by decreased level of serum 25(OH)D_3. This vitamin D_3 deficiency correlated with impairments of mineral metabolism in bone tissue, indicating the development of secondary osteoporosis. There was a decrease of the mass, length, and diameter (diaphysis, proximal metaphysis) in tibia of diabetic animals as compared to control. Hypocalcemia and hypophosphatemia, as well as increased levels of alkaline phosphatase activity and its isoenzymes were detected in serum of diabetic mice. In the liver of diabetic animals there was an altered expression of isoforms of vitamin D_3 25-hydroxylase, CYP27A1 and CYP2R1, which are the major enzymes responsible for cholecalciferol biotransformation into 25(OH)D_3, the immediate precursor of hormonally active form of vitamin D_3. Administration of vitamin D_3 normalized the serum level of 25(OH)D_3; this was accompanied by a significant improvement of the state of mineral metabolism compared to the untreated group of diabetic animals. Normalization of the total and ultrafiltration calcium, as well as inorganic phosphate concentration, a decrease in serum alkaline phosphatase activity and the increase in mass, length, and diameter (diaphysis, proximal epimetaphysis) of tibia in diabetic animals treated with cholecalciferol indicated a decrease in bone resorption process. Treatment of diabetic mice with cholecalciferol had a positive effect on expression of hepatic isoforms of vitamin D_3 25-hydroxylase (CYP27A1 and CYP2R1). Thus, impairments of mineral metabolism seen in mice with experimental diabetes mellitus are mainly determined by a deficiency of vitamin D_3 and its hormonally active forms.

Keywords: vitamin D_3, diabetes mellitus, secondary osteoporosis, bone resorption markers, CYP27A1, CYP2R1

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INTRODUCTION

Type 1 diabetes mellitus (DM1) is a multifactorial endocrine-metabolic disease, which is characterized by a genetically determined deficiency of the pancreatic hormone insulin. Insulin deficiency causes development of stable hyperglycemia, chronic inflammation, and metabolic impairments, leading to the development of secondary complications such as micro- and macroangiopathy, nephropathy, retinopathy, neuropathy [1, 2]. Recently, pathological changes in bone tissue have been also included in the group of chronic complications of diabetes. Convincing evidence exists that diabetes is characterized by a clear trend to impairments in bone remodeling and metabolism [3]. This results in reduction of bone mass and changes in its microarchitectural structure, which in most cases are diagnosed as secondary osteoporosis [4, 5]. At the molecular level remodeling dysfunction is mediated by intensification of non-enzymatic glycation (AGEs), prooxidant processes in bone tissue cells, impairments of RANKL/RANK/OPG signaling, Wnt signaling, and the cytokine regulatory system [6]. Reduced concentrations of calcium ions (Ca^{2+}), and phosphate anions (Pi), as well as the increase in total alkaline phosphatase activity in serum, and reducing metrics, ash and mineral components of bone in experimental animals are considered as conventional markers of bone resorption [7]. The secondary osteoporosis is characterized by impairments in the hormonal regulation of calcium metabolism, leading to changes in the functioning of regulatory system, which includes parathyroid hormone, calcitonin, and vitamin D_3 [8]. Vitamin D_3 is a potent regulator of calcium-phosphorus homeostasis; it plays an important role in maintaining bone health [9]. In particular, vitamin D_3 deficiency and impaired formation of its hormonally active forms lower absorption of ionized calcium in the intestine, elevation the level of parathyroid hormone and increase bone resorption and osteoporosis via decreased levels of calcitonin. Being a ligand for nuclear receptor NR1I1 (VDR), the hormonally active form of vitamin D_3 (1,25 $(OH)_2D_3$), is involved

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in regulation of the expression of several nuclear genes in osteoblasts and osteoclasts [10]. In target tissues VDR functions both in the cell nucleus (as a factor influencing transcription of about 3% of the human genome) and also in the plasma membrane (as a modulator of the activity of a number of important physico-chemical and biochemical processes). It should be noted that the possible pathogenetic role of impaired vitamin D_3 metabolism in the mechanism of the development of secondary osteoporosis in diabetes mellitus still requires (much) better understanding.

Hepatic vitamin D_3 25-hydroxylase, existing as cytochrome P450 isoforms, mitochondrial CYP27A1 and microsomal CYP2R1, is the most important enzyme of the first step of cholecalciferol biotransformation to 25-hydroxycholecalciferol ($25(OH)D_3$), and further to hormonally active forms of vitamin D_3. Polymorphism of the genes encoding vitamin D_3 25-hydroxylase or impairments of their expression are associated with $25(OH)D_3$ deficiency in the organism [11, 12].

Thus, the aim of this study was to investigate the relationship between supplementation of the organism with vitamin D_3 (evaluated by the $25(OH)D_3$ content), the expression of isoforms of vitamin D_3 25-hydroxylase (CYP27A1 and CYP2R1) and the state of mineral metabolism in experimental DM1.

MATERIALS AND METHODS

Studies were carried out using male C56Bl/J6 mice (22 ± 3 g). DM1 was induced by 5 sequential injections of streptozotocin (STZ, Sigma-Aldrich, USA) at a dose of 40 mg/kg. This mode of STZ administration is optimal for induction of experimental autoimmune DM1 in mice [13]. Animals were used in the study after 6 weeks of DM1 development; they had a blood glucose level of 20.4 ± 4.3 mmol/L. After the development of stable hyperglycemia mice were treated for 2 months with vitamin D_3 (DSM, Netherlands) administered per os as an aqueous suspension (800 IU/kg body weight, per os). Control animals were kept on a standard vivarium diet. During the period of adaptation (one week) and during the whole experiment the animals were in a vivarium at 18–22°C, 50–60% humidity, natural light mode "day–night" in standard plastic cages with free access to food and water. Selection of animals and group formation was performed by the random number method [14]. Euthanasia of animals was carried out under light ether anesthesia.

Experiments were carried out in accordance with international recommendations of the "European Convention for the Protection of Vertebrate Animals Used for Research and Scientific Purposes" (Strasbourg, 1986), "General Ethical Principles of Animal Experimentation," approved by the First National Congress on Bioethics (Kyiv, 2001).

Protein content was determined by the method of Bradford [15]. Supplementation of mice with vitamin D_3 was evaluated by serum levels of $25(OH)D_3$, determined by the ELISA method using an ELISA kit from Immunodiagnostic Systems Ltd. (USA) and an automated microplate reader ER-500 (Sinnova, China) for registration of results.

The level of synthesis of vitamin D_3 25-hydroxylase isoenzymes (CYP27A1 and CYP2R1) was determined by immunoblotting based on detection of the target proteins by specific immunoglobulins. Frozen liver samples were homogenized in a protein extraction buffer containing 20 mM Tris-HCl, pH 7.5, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1% sodium deoxycholate, and a mixture of protease and phosphatase inhibitors (1 μM aprotinin, 23 μM leupeptin, 1.5 μM pepstatin, 1 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine, 1 mM sodium orthovanadate) in a ratio of 1 : 10 (w/v) in an ice bath. The homogenate was additionally treated using a Labsonic M ultrasonic disintegrator (Sartorius, Germany) and incubated in ice for 20 min. The detergent insoluble fraction was pelleted by centrifugation at 14000 *g* for 20 min at 4°C. Protein lysates were separated by polyacrylamide gel electrophoresis (50–100 μg/well) in the Laemmli buffer system, transferred to a nitrocellulose membrane (Sigma) for 1 h at 350 mA in the buffer containing 25 mM Tris, 192 mM glycine, pH 8.3, 0.1% SDS, 20% methanol. Free binding sites were blocked for 1 h in 5% nonfat dry milk (Applichem, Germany) in phosphate buffer containing 0.05% Tween-20. After each incubation step, the membrane was washed three times (5 min each) in phosphate buffer containing 0.1% Tween-20. The membrane was incubated overnight at 4°C with polyclonal antiCYP2R1 (1 : 200) antiCYP27A1 (1 : 200) antibodies (Santa Cruz Biotechnology, USA) and then for 1 h at room temperature with a secondary horseradish peroxidase conjugated antibody (1 : 5000) (Santa Cruz Biotechnology). To control the content of proteins in the samples, the membrane was incubated with antiβ-actin monoclonal antibody (1 : 10000) (Sigma). The immunoreactive signals were detected by incubating the membrane with the coumaric luminol system (Sigma). The signal intensity on X-ray films was calculated using the GelPro32 program [16].

Serum calcium levels were determined using the Bio-Test kit (LaChema, Czech Republic) using 25 mM $CaCO_3$ dissolved in 1.7% HCl as a standard solution. The principle of the method is based on the ability of the calcium to form a red colored complex with glyoxal-bis-2(hydroxyanil) in alkaline medium, which can be determined photometrically. The content of serum inorganic phosphate was determined by the method of Dyce [17].

Activity of alkaline phosphatase (ALP; alkaline phosphohydrolase of orthophosphoric acid monoesters) was determined by its ability to cleave 4-nitro-

Table 1. The effect of vitamin D_3 administration on the serum levels of $25(OH)D_3$ and glucose in mice with experimental DM1

Experimental groups	$25(OH)D_3$ content		Blood glucose, mM
	nM	ng/mL	
Control	85.6 ± 4.11	34.2 ± 1.64	5.2 ± 1.1
DM	33.9 ± 1.91*	13.56 ± 0.76*	20.4 ± 4.3*
DM + vit D_3	81.2 ± 5.33#	32.48 ± 2.13#	14.5 ± 3.2*

Here and in other tables data represent mean ± SEM. Each group contained 6 animals. * Statistically significant differences compared to the control ($p < 0.05$) .# Statistically significant differences compared to the group of "diabetes" ($p < 0.05$).

Table 2. The effect of vitamin D_3 administration on levels of mineral components, activity of ALP and its isoenzymes in serum mice with DM1

Parameters studied	Control	DM	DM + vit D_3
Total calcium, mmol/L	2.28 ± 0.1	1.63 ± 0.08*	2.07 ± 0.6#
Protein bound calcium, mmol/L	0.29 ± 0.06	0.24 ± 0.045*	0.25 ± 0.034#
Ultrafiltration calcium, mmol/	1.99 ± 0.06	1.39 ± 0.03*	1.82 ± 0.05#
Inorganic phosphate, mmol/L	1.88 ± 0.08	1.31 ± 0.05*	1.83 ± 0.06#
Total ALP, U/L	238.1 ± 6.3	314.7 ± 9.2*	219.5 ± 5.1#
ALP, intestine isoenzyme, U/L	44.5 ± 1.68	64.7 ± 1.86*	48.3 ± 1.52#
ALP, bone isoenzyme, U/L	158.2 ± 5.4	264.3 ± 7.2*	181.6 ± 5.9#

phenylphosphate with formation of 4-nitrophenol and phosphate. The amount of liberated 4-nitrophenol, which was determined photometrically served as a measure of the catalytic activity of this enzyme. Activity of the bone (thermolabile) isoform of ALP was determined after incubation of the samples on a water bath at 55°C, and activity of the intestinal ALP isoform was determined using L-phenylalanine inhibitor [18].

The bone tissue ash content was determined by the method of dry mineralization at 500–600°C, after bone degreasing with hexane for 7 days and was calculated as percent of the bone tissue. The mineral components in the ashes were determined by the above described methods after ash dissolution in 0.5 mL of 0.1 M HCl. Osteometric measurements were performed on the tibia by standard methods; the following indicators were registered: bone mass, its length, thickness of proximal epimetaphysis and the diaphysis [19].

Statistical processing was performed using Microsoft Excel. The statistical significance of differences of mean values were carried out using a standard Student's *t*-test for non-correlated sets. Differences were considered as statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

Development of experimental diabetes in experimental animals was accompanied by stable hyperglycemia. The blood glucose level in diabetic mice increased to 20.4 ± 4.3 mmol/L compared to 5.2 ± 1.1 mmol/L in the control group. In diabetic animals treated with cholecalciferol the blood level was 14.5 ± 3.2 mmol/L (Table 1).

Results of recent studies demonstrate involvement of the bone tissue in endocrine regulation; molecular products of bone metabolism play a certain role in energy metabolism both in normal and also pathological conditions including DM [20]. Although impairments of calcium and phosphorus metabolism may be at different stages of development of DM, information on the mode and severity of these alterations are rather contradictory this clearly requires additional studies. Several authors reported about normocalcaemia in DM, while other studies showed increased levels of calcium in these patients [21, 22]. At the same time, clinical studies have shown some reduction in bioactive calcium [23, 24]. In DM1 patients it was also found a decrease in bone density and a diminution the level of osteocalcin and C-telopeptide (remodeling markers) [25].

The results of our biochemical studies listed in Table 2 show a significant decrease in the serum content of calcium and inorganic phosphate (1.4 times and 1.3 times, respectively). In diabetic animals, hypocalcaemia and hypophosphatemia correlated with a significant increase in serum alkaline phosphatase enzyme activity. Total ALP activity demonstrated a 1.32-fold increase in the diabetic group as compared with the control group. The magnification in total ALP activity occurred mainly due to changes

Table 3. The effect of vitamin D_3 administration on osteometric parameters and the content of mineral components in the bone tissue of mice with DM1

Parameters studied	Control	DM	DM + vit D_3
	Tibia		
Mass, mg	330 ± 11.55	240 ± 10.84 *	296 ± 9.48#
Length, mm	17.7 ± 0.2	16.8 ± 0.2*	18.3 ± 0.2#
Proximal epimetaphysis thickness, mm	2.1 ± 0.06	1.9 ± 0.05*	2.15 ± 0.06#
Diaphysis thickness, mm	1.6 ± 0.07	1.35 ± 0.05*	1.5 ± 0.06#
Ash, %	56 ± 2.4	47 ± 1.9*	52 ± 2.0#
Ca^{2+} content, %	37.6 ± 0.5	25.1 ± 0.7*	38.7 ± 0.6#
Pi content, %	16.2 ± 0.3	12.3 ± 0.6*	15.0 ± 0.5#

* Statistically significant differences compared to the control ($p < 0.05$). # Statistically significant differences compared to the group of "diabetes" ($p < 0.05$).

in the activity of its bone isoenzyme. For example, in DM activity of this isoform was 1.7-fold higher than in the control group thus indicating intensification of resorptive processes in the bone tissue. It should be noted that the intestinal isoform of ALP also demonstrated a 1.45-fold increase in serum of diabetic mice.

Osteometric parameters suggest a decrease in mass, length, and length of tibia, as well as decreased thickness of its proximal epimetaphysis in diabetic mice. Moreover, there was a 1.2-fold decrease in the tibia ash content; the latter was accompanied by a decreased in the content of both ash calcium (1.5-fold) and phosphorus (1.3-fold) (Table 3).

Long-term administration of vitamin D_3 to diabetic animals resulted in changes of mineral metabolism which approached towards the control group; this emphasizes an important role of vitamin D_3 in the process of bone remodeling. It should be also noted that after the course of vitamin D_3 administration, manifestations of hypocalcemia and hypophosphatemia decreased in diabetic mice and the concentration of Ca^{2+} and P ions reaches the control values. The animals in this group had basically normal ALK activity and activity of its isoforms corresponding to the control values.

Increasing volumes of current scientific evidence strongly suggest that inadequate supply with vitamin D_3, typical for the population of temperate latitudes, which is not exposed to sufficient solar radiation, is a factor that significantly increases the risk not only of bone diseases, but also a number of other common pathologies: cancer, cardiovascular, infectious, autoimmune, DM, and some others [10].

Since hypocalcemia and hypophosphatemia can be determined by impaired metabolism of vitamin D_3 (D-vitamin deficiencies and hyperparathyroidism), it was important to evaluated the D-vitamin status in diabetic mice. The study of the 25(OH)D_3 content, the main marker characterizing supply of the organism with vitamin D_3 and its hormonally active precursor forms revealed a decrease in the serum level of this 25-hydroxylated derivative to 33.9 nM; this is 2.4 times lower than in control animals (Table 1). Such a level of 25(OH)D_3 indicates a significant vitamin D_3 deficiency in DM; this deficiency can be caused by several factors.

For example, convincing evidence exists in the literature that congenital impairments in vitamin D_3 metabolism determined by chromosomal mutations or polymorphisms of genes of several cytochromes result in deficiency of 25(OH)D_3 and determine of predisposition of individuals to the development of autoimmune diseases. For example, there is a close correlation between reduced levels of circulating 25(OH)D_3 (due to decreased hydroxylation of vitamin D_3), determined by polymorphism in some regions of the CYP2R1 gene and the development of DM1 [26].

It is known that cholecalciferol conversion to 25(OH)D_3 involves two isoforms of cytochrome P450 (vitamin D_3 25-hydroxylase): and mitochondrial CYP27A1 and microsomal CYP2R1. Taking into consideration an important role of these enzymes of the vitamin D_3 25-hydroxylase system in vitamin D_3 metabolism, it was important to elucidate, whether the significant deficit in 25(OH)D_3 in DM may be associated with changes in the content of these two key cytochromes.

The content of the mitochondrial isoform CYP27A1 in the liver of diabetic animals decreased by 2.3 ± 0.11 times as compared to control animals (Fig. 1). This may be attributed to altered expression of the gene encoding this cytochrome P450 isoforms due to prolonged hyperglycemia induced by DM. Administration of vitamin D_3 to diabetic mice resulted in the 1.9 ± 0.08 fold increase in the expression level of CYP27A1 almost reaching the control values. This growth can be attributed, primarily, to the genomic effects of vitamin D_3, increased content of the hormonally active form, which could contribute to the activation of the CYP27A1 expression.

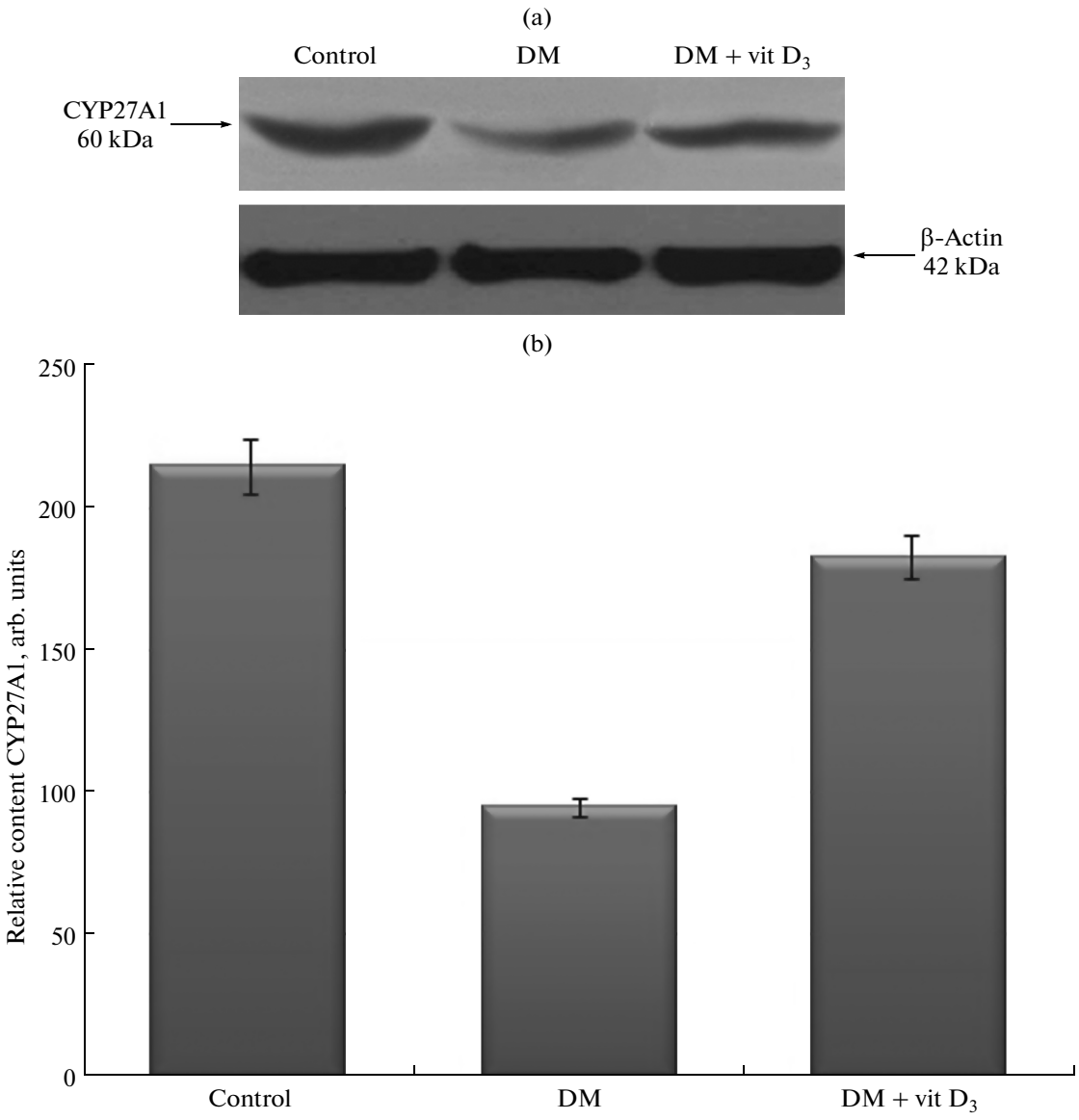

Fig. 1. The contents of the mitochondrial isoform CYP27A1 of hepatic vitamin D_3 25-hydroxylase in diabetic mice treated with vitamin D_3. (a) Immunoblot of CYP27A1 protein. (b) Relative CYP27A1 protein content in the liver of experimental animals (mean ± SEM, $n = 6$). * Statistically significant differences compared to the control ($p < 0.05$). # Statistically significant differences compared to the group of "diabetes" ($p < 0.05$).

Opposite changes were found in synthesis of the microsomal isoform of vitamin D_3 25-hydroxylase. In diabetic mice the hepatic content of CYP2R1 was 3.16 ± 0.14 times higher than in the control group (Fig. 2). It is known that CYP2R1 is characterized by higher affinity for cholecalciferol and its hydroxylase activity is higher than that of CYP27A1 [27]. CYP2R1 can efficiently convert picomolar concentrations of vitamin D_3, and this suggests preferential functioning of this isozyme operation at low cholecalciferol concentrations [28]. In the liver of diabetic animals treated with vitamin D_3 there was a significant (6.21 ± 0.29 fold) reduction in the content of CYP2R1 in comparison with the group of diabetic animals, which not treated with this vitamin. Our results demonstrate that CYP2R1 is probably the isoform of hepatic vitamin D_3 25-hydroxylase, which plays a compensatory role in DM, aimed at providing necessary conditions for the realization of physiological functions of vitamin D_3 levels of circulating 25(OH)D_3.

CONCLUSIONS

The experimental DM1 is accompanied by disorders in mineral metabolism in bone tissue including intensification of bone resorption processes, which eventually lead to secondary osteoporosis. This is evidenced by hypocalcemia and hypophosphatemia,

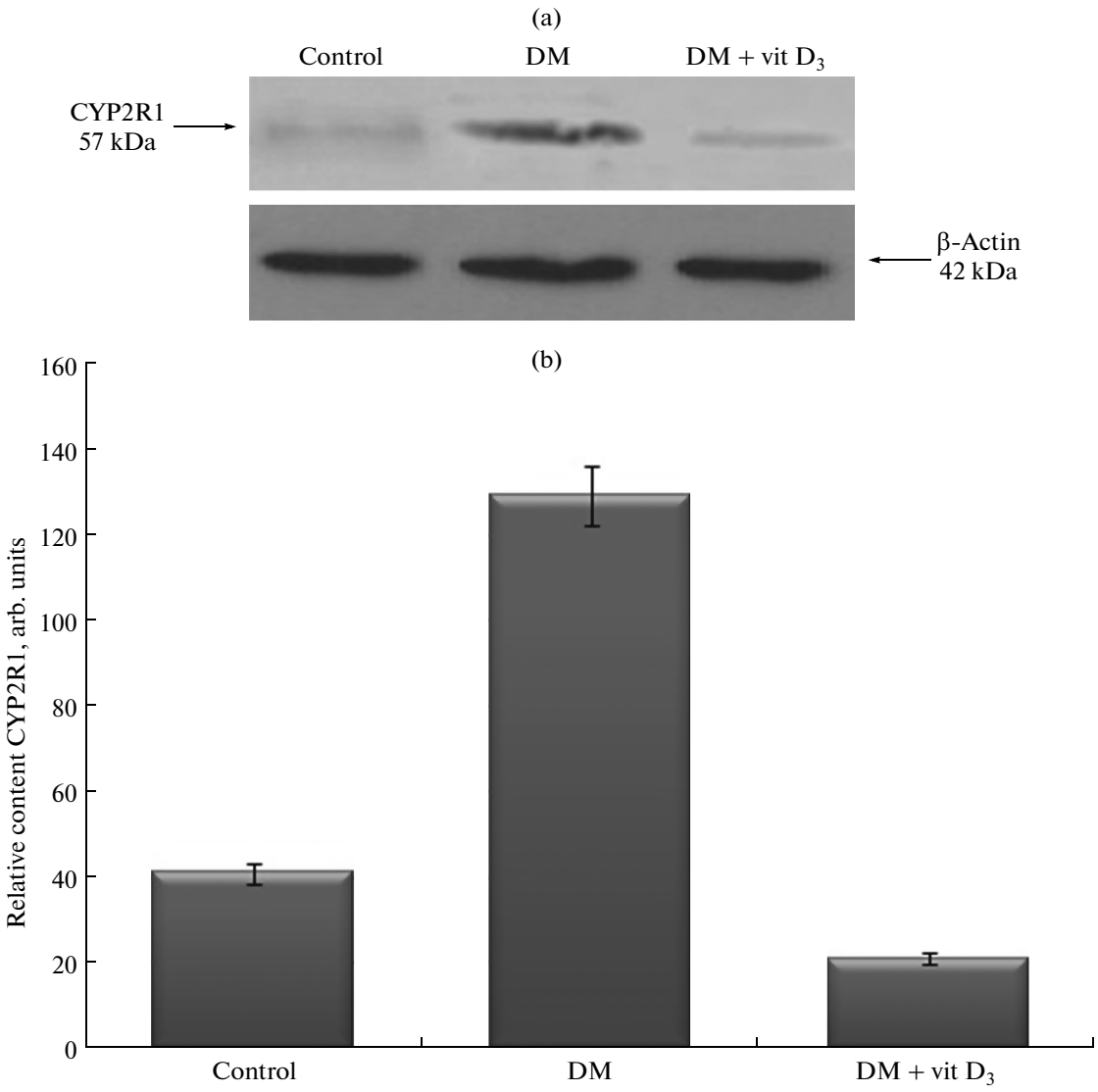

Fig. 2. The contents of the microsomal isoform CYP2R1 of hepatic vitamin D_3 25-hydroxylase in diabetic mice treated with vitamin D_3. (a) Immunoblot of CYP2R protein. (b) Relative CYP2R protein content in the liver of experimental animals (mean ± SEM, $n = 6$). * Statistically significant differences compared to the control ($p < 0.05$). # Statistically significant differences compared to the group of "diabetes" ($p < 0.05$).

increased enzymatic activity of serum ALP and its isoforms, altered bone metrics parameters, as well as bone content of the mineral components. These changes correlate strongly with decreased levels of $25(OH)D_3$ in diabetic animals. Our study confirmed the key role of the two isoenzymes of hepatic vitamin D_3 25-hydroxylase (mitochondrial CYP27A1 and microsomal CYP2R1) in vitamin D_3 metabolism. Diabetic animals were characterized by impaired synthesis of the investigated cytochromes with corresponding disregulation in expression of their genes. Prolonged administration of cholecalciferol to diabetic mice promoted normalization of serum $25(OH)D_3$ content. This treatment also caused normalization of biochemical and osteometric parameters of bone resorption, as well as expression of the hepatic isozymes of vitamin D_3 25-hydroxylase. The results indicate an important role of vitamin D_3 in the process of bone remodeling in DM. The observed deficiency of vitamin D_3 and impaired formation of its hormonally active forms provide experimental evidence for possible use of vitamin D_3 for prophylaxis and treatment of secondary osteoporosis as the diabetic complication.

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