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The link between vitamin D status and NF-κB-associated renal dysfunction in experimental diabetes mellitus

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ABSTRACT

Background: Type 1 diabetes (T1D) is accompanied by numerous side effects, including renal dysfunction. Mounting evidence suggests that overactivation of nuclear factor κB (NF-κB) is one of the key triggers of diabetes-associated chronic kidney disease. Vitamin D_3 is considered as a strong modulator of a number of transcription factors, including NF-κB. The purpose of our study was to assess the contribution of NF-κB to type 1 diabetes (T1D)-induced kidney dysfunction and to determine if vitamin D_3 supplementation can correct the changes associated with T1D.

Methods: The following animal groups were used: control, diabetic (induced by single i.p. injection of streptozocin at dose 55 mg/kg b.w.), T1D group treated with vitamin D_3 (600 IU/kg b.w.), T1D group treated with NF-κB inhibitor – BAY 11-7082.

Results: Diabetes led to a decrease in serum 25(OH)D that was accompanied by down-regulation of vitamin D receptor (VDR) expression and up-regulation of hydroxylases CYP24A1 and CYP27B1 synthesis in the kidneys. Diabetes activated the transcription factor NF-κB and increased cleaved (p17) caspase-3 level in renal tissue. Restoration of vitamin D status normalized vitamin D-endocrine system, decreased NF-κB activation and caspase-3 protein level in the kidneys of diabetic animals. BAY 11–7082 partially mimicked the effects of vitamin D_3.

General significance: Vitamin D_3 supplementation counteracts diabetes-induced kidney damage, most likely through VDR-mediated inhibition of NF-κB activation.

1. Introduction

Diabetes mellitus is considered as a global non-infectious epidemic and by 2030 this endocrine-metabolic disease will become the seventh cause of mortality worldwide [1,2]. Diabetes-induced nephropathy is one of the major complications of both type 1 and type 2 diabetes often leading to end-stage renal disease with poor prognosis and quality of life for patients [3]. Diabetes-induced kidney dysfunction is associated with the development of glomerular sclerosis and tubulointerstitial fibrosis because of metabolic disorders and hemodynamic changes induced by diabetes. The disease combines the entire complex of lesions of arteries, arterioles, glomeruli and tubules of the kidneys [4].

Despite controlling metabolic risks such as hyperglycemia, dyslipidemia and hypertension, the number of patients with diabetes who ultimately develop chronic kidney disease remains high. This is most likely due to the complexity of the mechanisms involved, which include, among others, oxidative stress, inflammation, and pathological angiogenesis. Emerging evidence is increasingly emphasizing renal inflammation as a critical pathogenic factor in the progression of diabetes-induced kidney failure [5,6]. Although the exact cellular mechanisms are generally poorly defined, it has been shown that advanced glycation end product receptor (RAGE), nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 4 (NOX4), oxidative stress, nuclear factor kappa B (NF-κB) and pro-inflammatory cytokines (i.e., TNF-α, IL-1β, and IL-6) are involved in the development of inflammation [7,8]. In this connection, a new therapeutic approach for chronic kidney disease in diabetes should go beyond the control of traditional risk factors such as hyperglycemia and hypertension, with a special emphasis on counteracting inflammation.

It is becoming increasingly apparent that patients with type 1 diabetes (T1D) generally demonstrate a vitamin D insufficiency/deficiency [9], which may be one of the reasons for the development of diabetes-

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associated pathologies, in particular diabetes-induced kidney failure [10]. Both animal studies and clinical trials have confirmed an inverse correlation between low vitamin D levels and the risk of developing diabetes-induced kidney damage [11]. Vitamin D_3 (cholecalciferol) is now recognized as a promising renoprotective therapeutic agent. Its therapeutic use has been shown to inhibit the renin-angiotensin system, improve endothelial cell damage, reduce proteinuria, attenuate renal inflammation and fibrosis, and, as a result, delay the progression of diabetes-induced kidney failure [12]. Vitamin D_3 exerts its pharmacological effect mainly through the vitamin D receptor (VDR), the activation of which can have a beneficial effect on the diabetes-related oxidative stress and inflammatory response in the kidney tissue [13]. VDR is revealed in most tissues, not just those participating in the classic actions of vitamin D such as gut, kidney, and bone [14]. The non-classic actions are, therefore, potential targets for the hormonally active metabolite of vitamin D, $1\alpha,25(OH)_2D_3$ (calcitriol), which is formed in two consecutive reactions of hydroxylation at 25th and 1st position catalyzed by enzymes of the cytochrome P450 family – vitamin D-25-hydroxylase (CYP27A1 and CYP2R1 isoforms), and vitamin D 1α-hydroxylase (CYP27B1) [14–17]. Pre-inactivation of vitamin D active metabolites occur in the kidneys by mitochondrial CYP24A1 [18]. Few mechanistic studies have demonstrated that pathways relevant to VDR-mediated anti-inflammatory effects may include decreased macrophage infiltration, blockage of NF-κB and NF-κB-dependent formation of various pro-inflammatory mediators.

The vitamin D receptor is thought to mediate the effects of cholecalciferol on the activity of such important and common cellular transcription factor as NF-κB [19]. NF-κB controls a wide variety of cellular functions, thereby regulating the immune response and the development of inflammation, apoptosis, bone remodeling, etc. [20]. This transcription factor affects different genes involved in the immune, acute phase and chronic inflammatory responses. In different cells, the most common form of NF-κB consists of a large subunit, RelA (p65), which forms either a homodimer or a heterodimer with a structurally related p50 protein. Since the activation of NF-κB-associated transcription is one of the key events that mediate gene expression of various regulatory peptides and proteins, including cytokines, and is one of the markers of the intensity of diabetes-related pro-inflammatory processes [21], our goal was to evaluate transcriptional activation of NF-κB in the kidney tissue depending on the bioavailability of vitamin D and the functional state of the renal vitamin D-auto/para/endocrine system.

2. Materials and methods

2.1. Animals

Male Wistar rats (140 ± 5 g) were injected intraperitoneally with streptozotocin (STZ; Sigma, USA) at a dose of 55 mg/kg in 50 mM citrate buffer (pH 4.5). Two weeks after the injection, blood glucose was tested and the animals were randomly divided into groups, each of which included six rats: (1) the diabetic group (D); (2) the group of diabetic animals, received vitamin D_3 (Sigma, USA) at dose 600 IU/kg (per os, daily for 30 days) (D + vit D_3); (3) the group of diabetic animals, received BAY 11–7082 (Sigma, USA), dissolved in saline at a dose of 1 mg/kg (i.p., daily for 10 final days) (D + BAY) [22]. The control group (C, $n = 6$) of animals did not receive any treatment. At the end of the study, animals were fasted overnight, anaesthetized and sacrificed by cervical decapitation. All animal procedures were performed in accordance with EU Directive 2010/63/EU on the protection of animals used for scientific purposes; General Ethical Principles of Animal Experimentation, approved by the First National Congress on Bioethics (Kyiv, 2001). Ethics of all experimental procedures with animals were approved by Ethics committee for controlling the rules of research work with experimental animals of Palladin Institute of Biochemistry, National Academy of Sciences of Ukraine, Kyiv, Ukraine (approval № 1/8–208).

2.2. Measurement of blood biochemical parameters

The concentrations of urea and creatinine were measured by colorimetric assay (NPP Filisit-Diagnostika LLC, Ukraine) according to the manufacturer's instructions. Serum 25(OH)D was determined using in-house developed ELISA kit, as was described previously [23,24].

2.3. Isolation of peripheral blood mononuclear cells and ROS-determination

Peripheral blood mononuclear cells of experimental animals were isolated using Histopaque-1083 reagent (Sigma, USA) according to the manufacturer's instructions. Generation of reactive oxygen species was determined by using 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA; Sigma, USA) as we described previously [25]. Briefly, $0.5{\cdot}10^6$ mononuclear cells were treated with 10 μM DCFH-DA solution in dimethyl sulfoxide (DMSO; Lubnypharm, Ukraine) followed by incubation for 30 min at 37 °C. The fluorescent signal was recorded using excitation/emission wavelengths of 485/530 nm on an EPICS XLTM flow cytometer (Beckman Coulter, USA). The results were analyzed using the FCS Express V3 software and expressed as the relative fluorescence of the samples compared to the control.

2.4. Kidneys morphometric analysis

Paraffin sections from each group were selected for conventional deparaffinization and the H&E staining. The images were observed and photographed under a light microscope (400×) (Olympus BX 51).

2.5. Western blot analysis

Protein levels in kidney tissue were examined by Western blotting. Briefly, total lysates were prepared from 100 mg of kidney tissue according to the previously described procedure [26]. Protein concertation was measured by Lowry method. All samples (50 μg protein) were separated in 10% SDS-PAGE and then transferred to nitrocellulose membranes. For protein signal detection, the samples were blocked with a phosphate-buffered saline containing 5% fat free milk and 0.05% Tween-20 for 1 h at room temperature. The membranes were than incubated with primary antibodies against VDR (1:200; Santa Cruz Biotech., USA), CYP2B1 (1:200; Santa Cruz Biotech., USA), p65 subunit of NF-κB (1:250; Thermo Fisher, USA), phosphorylated at Ser 311 NF-κB (1:200; Santa Cruz Biotech., USA), IκB-α (1:500; Santa Cruz Biotech., USA), cleaved caspase-3 (1:500; Santa Cruz Biotech., USA), β-Actin (1:10000; Sigma, USA) and secondary peroxidase-conjugated antibodies: anti-mouse IgG (Fab Specific)-Peroxidase (1:2500; Sigma, USA), anti-rabbit IgG (H + L)-HRP conjugate (1:4000; Bio-Rad Laboratories, Inc., USA) or anti-goat IgG (H + L) (1:2500; Invitrogen, USA). For specific signal detection chemiluminiscent method with p-coumaric acid (Sigma, USA) and luminol (AppliChem GmbH, Germany) was used. The immunoreactive bands were quantified with GelPro Analyzer 3.2.

2.6. mRNA isolation and real-time quantitative PCR (RT-qPCR) analysis

InnuPREP RNA mini kit (Qiagen, USA) was used for mRNA isolation from kidney samples (20 mg). Procedure was performed according to the manufacturer's protocol. Quantity and purity of the RNA were examined by NanoDrop DeNovix DS-11 (USA). Synthesis of cDNA was carried out using a Maxima H Minus First Strand cDNA Synthesis Kit (ThermoFisher, USA) as described by the manufacturer. RT-qPCR was performed with Maxima SYBER Green/ROX qPCR Master Mix (2×) (ThermoFisher, USA) during 50 cycles. The primer sequences were designed using the Primer BLAST program as follows: *Lrp2 (megalin)* (5'-TGGCCCTGGACTTTGATAGA-3′ sense, 5′-GACAAACAGGCAGTCCAAGA-3′ antisense), *Cubn (cubilin)* (5'-GGTTGGCAGGGAAATGGTTA-3′ sense, 5′-CTGAAACCTGCTGGACAGTT-3′ antisense), *Vdbp* (5'-TGGCCCTGGACTTTGATAGA-3′ sense, 5′-

GACAAACAGGCAGTCCAAGA-3′ antisense), *Vdr* (5′-GGATACGACCCTG AAAACCC-3′ sense, 5′-TTTTGTCCTCAGTCGTTCCG-3′ antisense), *Cyp27b1* (5′-TGGGTGCTGGGAACTAACCC-3′ sense, 5′-TCGCAGACTGATTCCACCT C-3′ antisense), *Cyp24a1* (5′-TTCGCTCATCTCCCATTCGG-3′ sense, 5′-TTGCTGGTCTTGATTGGGGT-3′ antisense) *RelA* (p65 subunit of NF-κB) (5′-TTTGATAACCGTGCCCCCAA-−3′ sense, 5′-GCCAGGTCCCGTGAAATACA-3′ antisense) and *Gapdh* (5′-TGAACGGGAAGCTCACTGG-3′ sense, 5′-TCCACCACCCTGTTGCTGTA-3′ antisense). Data were calculated using the ΔΔCt method.

2.7. Statistical analysis

All results are presented as mean ± SEM. The Shapiro-Wilk test was used for testing on normal distribution. Statistical differences between the various groups were compared by using the one-way ANOVA. Tukey post hot test was used for comparison between different groups. Differences were considered significant when $p \leq 0.05$. All statistical analysis was performed using Origin Pro 8.5 (OriginLab Corporation, Northampton, MA, USA).

3. Results

It was found that the development of type 1 diabetes after six weeks of STZ administration was accompanied by a 5.0-fold increase in blood glucose level and a 1.5-fold decrease in serum 25(OH)D ($p \leq 0.05$) level compared with the control group (Table 1). Experimental T1D led to significant weight loss of animals (1.4-fold, $p \leq 0.05$), whereas kidney mass increased (1.25-fold, $p \leq 0.05$). The kidney to body weight ratio (KW/BW) in diabetic animals was 1.6-times higher in comparison with the control group. We also showed an increase in the concentration of urea, blood urea nitrogen (BUN) and creatinine in the blood of diabetic rats (Table 1), which confirms the development of renal disorder induced by T1D. In addition, we demonstrated a 1.9-fold ($p \leq 0.05$) elevation of DCFH-DA-sensitive reactive oxygen species in peripheral blood mononuclear cells of animals with experimental T1D, indicating generalized oxidative stress (Fig. 1).

Histological examination of the kidneys from diabetic animals showed the preservation of the general structural organization of the renal cortex and medulla (Fig. 2A and B). We revealed single hemorrhages and erythrocyte stasis of blood vessels in the kidneys of the T1D group. The proximal convoluted tubules of the renal cortex also demonstrated a statistically significant increase in cross-sectional area (Fig. 2A). Single tubules included areas with signs of tubulonecrosis. In the renal medulla, we can notice the presence of microvessels with erythrocyte stasis, as well as swelling of epithelial cells in the walls of the collecting tubules (Fig. 2B). The proximal thin tubules did not undergo significant changes in structural integrity. However, morphometric analysis revealed a clear increase in the area and perimeter of their cross-sections.

Table 1
Blood biochemical parameters in diabetes and after vitamin D_3/BAY 11-7082 treatment.

	Control	Diabetic Rats	Vitamin D_3-treated diabetic rats	BAY-treated diabetic rats
Body weight (BW), g	365.8 ± 14.9	252,5 ± 10.6*	255.5 ± 6.4	281.5 ± 16.3$^{\#}$
Kidney weight (KW), g	2.4 ± 0.1	3.0 ± 0.3*	2.7 ± 0.1	2.8 ± 0.5
KB/BW ratio*1000	6.8 ± 0.3	11.1 ± 0.4*	10.9 ± 2.1	10.8 ± 0.5
Blood glucose, mmol/l	4.7 ± 0.4	23.4 ± 1.4*	20.6 ± 1.6	22.8 ± 0.9
Serum 25(OH)D, nmol/l	96.3 ± 3.2	63.1 ± 1.1*	77.3 ± 2.2$^{\#}$	59.1 ± 1.4*
Blood urea, mg/dl	40.2 ± 2.3	62.4 ± 3.5*	56.0 ± 1.4$^{\#}$	57.1 ± 1.1$^{\#}$
Blood urea nitrogen (BUN), mg/dl	18.8 ± 1.0	30.8 ± 1.5*	26.1 ± 0.6$^{\#}$	27.3 ± 0.5$^{\#}$
Blood creatinine, mg/dl	0.30 ± 0.05	1.19 ± 0.06*	0.61 ± 0.04$^{\#}$	0.79 ± 0.02$^{\#}$

Results are expressed as mean ± SEM *$p \leq 0.05$ vs. control; $^{\#}p \leq 0.05$ vs. diabetes, $n = 6$.

Vitamin D_3 treatment attenuated morphological signs of structural abnormalities in different nephron segments (Fig. 2A and B). In particular, we established a statistically significant decrease in the morphometric parameters of the thick and thin tubules of the nephrons, as well as reduced prevalence of damaged tubules and stasis of blood vessels. Morphometric analysis of the kidneys using light microscopy in different experimental groups of rats is presented in Table 2.

Normalization of the kidney histological structure after treatment with vitamin D_3 contributed to a decrease in both urea and BUN, and creatinine in the blood of animals with experimental T1D (Table 1), and was also accompanied by a decrease in ROS production in peripheral blood mononuclear cells (Fig. 1). Pharmacological inhibition of NF-κB by BAY 11–7082 also clearly correlated with a partial mitigation of the biochemical parameters of diabetes-induced renal disorder (Table 1).

To assess the severity of impaired renal reabsorption at the molecular level, we determined the mRNA expression of multiligand endocytic receptors – megalin (*Lrp2/megalin*) and cubilin (*Cubn/cubilin*), and their major ligand – vitamin D-binding protein (*Vdbp*). We found a strong suppression of *Vdbp* mRNA synthesis in the kidney tissue of diabetic rats, which was completely corrected by the administration of vitamin D_3 (Fig. 2C). Type 1 diabetes down-regulated *Lrp2/megalin* and *Cubn/cubilin* mRNA expression in kidney tissue, and vitamin D_3 partially reversed their levels to control values (Fig. 2C).

The kidneys are known to be responsible for the synthesis of the hormonally active form of vitamin D for its subsequent transport to the peripheral organs. Furthermore, kidney tissue also provides active inactivation of calcitriol due to the presence of catabolic enzymes of vitamin D, primarily CYP24A1. Fig. 3A shows that the observed lowering of serum 25(OH)D most likely leads to a compensatory up-regulation of both mRNA and protein levels of vitamin D 1α-hydroxylase (1.3-fold, $p \leq 0.001$ and 1.5-fold, $p \leq 0.002$, respectively) in the kidney tissue of diabetic animals. Meanwhile, we also demonstrated a 4.0-fold rise in *Cyp24a1* mRNA synthesis compared to control ($p \leq 0.001$) (Fig. 3B). Next, we looked at how vitamin D_3 regulates VDR expression at transcriptional and translation levels in kidney tissue. It was found that the levels of both VDR mRNA and its protein were significantly lower in the kidneys of diabetic rats compared to the control group (1.8- and 3.5-fold respectively, $p \leq 0.001$) (Fig. 3C).

Vitamin D_3 supplementation to diabetic animals caused a partial restoration of VDR biosynthesis, and a decrease in mRNA and protein levels of both studied hydroxylases – CYP27B1 and CYP24A1. In turn, it was the normalization of the vitamin D-endocrine system functioning that most likely contributed to an increase in the blood content of 25(OH)D as compared with the diabetes group (Table 1).

Our next step was to investigate whether NF-κB plays a potential role in regulating the expression of VDR and CYPs. Pharmacological inhibition of NF-κB by BAY 11–7082 in diabetic rats resulted in effects similar to those of vitamin D_3. We revealed that a 10-day treatment with BAY 11–7082 up-regulated VDR mRNA and protein levels in kidney tissue, while down-regulated CYPs biosynthesis. However, inhibitor administration did not affect the concentration of serum 25(OH)D compared with diabetes (Table 1).

Since we found that inhibition of NF-κB by BAY 11–7082 or vitamin D_3 affects the synthesis of components of the vitamin D-auto/para/endocrine system, we additionally assessed the level of expression of this transcription factor and its natural inhibitor, IκB, in the kidneys of experimental animals. The results of our study indicate a significant up-regulation of both *RelA*/NF-κB mRNA and p65/NF-κB protein biosynthesis in T1D kidneys (2.45-fold and 1.48-fold respectively vs. control, *p*

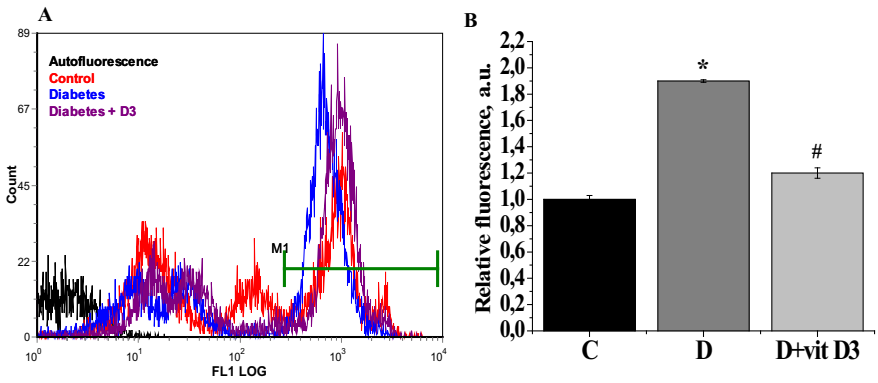

Fig. 1. DCF-sensitive ROS formation in blood peripheral mononuclear cells of diabetic animals and after vitamin D_3 supplementation. Representative histograms (count – the number of events; FL1 LOG – intensity of fluorescence) (A); quantification of DCF oxidation in gated peaks, which correspond to peripheral mononuclear cells of the studied experimental groups, using flow cytometry (B). ANOVA with Tukey post hot test was used to analyze differences between groups. Statistically significant differences are expressed as mean ± SEM of three experiments ($n = 3$) in duplicate. *$p \leq 0.05$ vs. control; #$p \leq 0.05$ vs. diabetes.

≤ 0.001) (Fig. 4A). To establish the transcriptional activation of NF-κB in diabetes, we determined the level of its form phosphorylated at Ser 311 and showed that it was significantly higher (1.2-fold, $p \leq 0.002$) in diabetic rats compared with the control group (Fig. 4B). In addition, we demonstrated a 1.5- fold ($p \leq 0.001$) enhancement of IκB-α protein level in the kidneys of diabetic animals compared to the control group (Fig. 4C). T1D animals treated with BAY 11–7082 showed a significant lowering of both total and phosphorylated forms of NF-κB, as well as IκB-α in the kidneys (Fig. 4). Vitamin D_3 supplementation resulted in a marked down-regulation of *RelA*/NF-κB mRNA and p65/NF-κB protein levels in the kidney tissue compared with diabetes (Fig. 4). We also found that vitamin D_3 significantly reduces diabetes-associated NF-κB activation in the kidneys by directly decreasing the level of phosphorylated NF-κB (at Ser 311) protein without any effect on IκB-α level.

Considering that excessive activation of NF-κB can cause impaired renal cell function and even apoptotic/necrotic death, we finally identified the p17 caspase-3 fragment as one of the reliable marker peptides for execution-phase of cell apoptosis. Its level in the kidneys of animals from the T1D group was established to be significantly higher than in the control (2.0-fold, $p \leq 0.003$) (Fig. 5). Diabetic rats treated with vitamin D_3 and BAY 11–7082 showed a slight decrease in the level of the p17-cleaved caspase-3 protein. It should be noted that the hypoglycemic effect of both vitamin D_3 administration and pharmacological inhibition of NF-κB BAY 11–7082 was negligible (Table 1).

4. Discussion

About one third of all patients diagnosed with type 1 diabetes mellitus have manifestations, to one degree or another, of chronic kidney disease, and our data additionally confirm the prevalence of this complication in an experimental model of T1D. Within the framework of the present study, we observed an increase in the concentration of urea, urea nitrogen and creatinine in the blood of animals with type 1 diabetes, indicative of diabetic-induced kidney dysfunction, that is generally consistent with the severity and duration of STZ-induced diabetes previously described in the literature [27]. Diabetes-associated kidney damage is known to be characterized by distinct histological changes in all types of renal cells, namely mesangial cells, podocytes and tubulointerstitial cells [28]. The abnormalities we demonstrated in the structure of diabetic kidneys, such as those found both in the renal cortex (an increase in the cross-sectional area, hemorrhage, stasis of blood vessels and tubulonecrosis), and in the medulla of the kidneys (microvessels with erythrocyte stasis), as well as swelling of epithelial cells in the walls of the collecting tubules are likely to be caused by glucose toxicity and an overproduction of growth factors (e.g., TGF-β) due to severe hyperglycemia [29]. However, one of the most obvious pathological changes in the histological structure of the kidneys induced by T1D is the hypertrophy we have established herein. There was also a pronounced protective effect of vitamin D_3 on structural changes in the kidneys associated with T1D.

The proximal tubules of the kidneys are responsible for the reabsorption of a number of substances, in particular vitamins and hormones, in complex with specific carrier proteins, which, in turn, are recognized by multiligand endocytic receptors – megalin and cubilin [30]. Vitamin D binding protein appears to be a ligand for both megalin and cubilin and has been shown to play a critical role in the efficient reabsorption of vitamin D. It is noteworthy that megalin and cubilin, as endocytic receptors, promote the formation of $1\alpha,25(OH)_2D$ after reabsorption of the VDBP/25(OH)D complex. Disruptions in the synthesis of both *megalin/cubulin* and *Vdbp* mRNAs in the kidneys of experimental animals are in line with the data of several experimental and clinical studies, according to which the loss of VDBP in the urine may be a secondary process in relation to the decrease in megalin content/function that may correlate with proteinuria in T1D [31]. The detected changes in the system of endocytotic receptors, causing the probable loss of VDBP with urine, can be attributed, at least partially, to harmful structural defects of renal tissue associated with diabetes. As we have demonstrated, vitamin D_3 therapy resulted in partial restoration of *megalin/cubilin* and *Vdbp* RNA transcripts, which can be explained by the fact that $1\alpha,25(OH)_2D_3$ is able to stimulate the expression of megalin and retain 25(OH)D in the body for further hydroxylation [32].

When activated, vitamin D is known to undergo a two-step hydroxylation process. The formation of 25(OH)D occurs in the liver, followed by its conversion to the hormonally active form $1\alpha,25(OH)_2D$ in the kidneys, and is considered the classical pathway. In our previous work, we have shown that experimental T1D caused disruption of vitamin D_3 hydroxylation in the liver due to impaired functioning of the CYP2R1 and CYP27A1 [17]. A similar trend towards dysfunction of the first step of vitamin D_3 hydroxylation has been shown on in vivo models of Zucker diabetic fatty rats and high-fat diet-fed (HFD) obese mice [33,34]. The decrease in the synthesis of 25(OH)D, as well as its transport in the body of animals with diabetes mellitus, is attributed by the authors of these works to ROS-modulated disturbances in the synthesis of vitamin D hydroxylation enzymes in the liver, as well as to abnormal synthesis of hepatic VDBP, which is critical for the transport of cholecalciferol. At the same time, in a mouse model of chronic kidney disease induced by long-term HFD, a significant decrease in such key components of the vitamin D auto/para/endocrine system as VDR, CYP27B1 and retinoic X receptor (RXR)α was demonstrated, while CYP24A1 level remained elevated [35].

In the present study, we also demonstrated a dramatic increase in vitamin D 1α-hydroxylase (CYP27B1), the expression of which can be regulated in several ways, including direct signaling of calcitriol via the VDR [36,37]. Recent studies have shown that vitamin D levels in the body normally correlate negatively with CYP27B1 levels and positively correlate with CYP24A1 levels; the latter is known to be the most important enzyme of vitamin D catabolism. However, a significant elevation of CYP24A1 biosynthesis has been shown in T1D, especially in

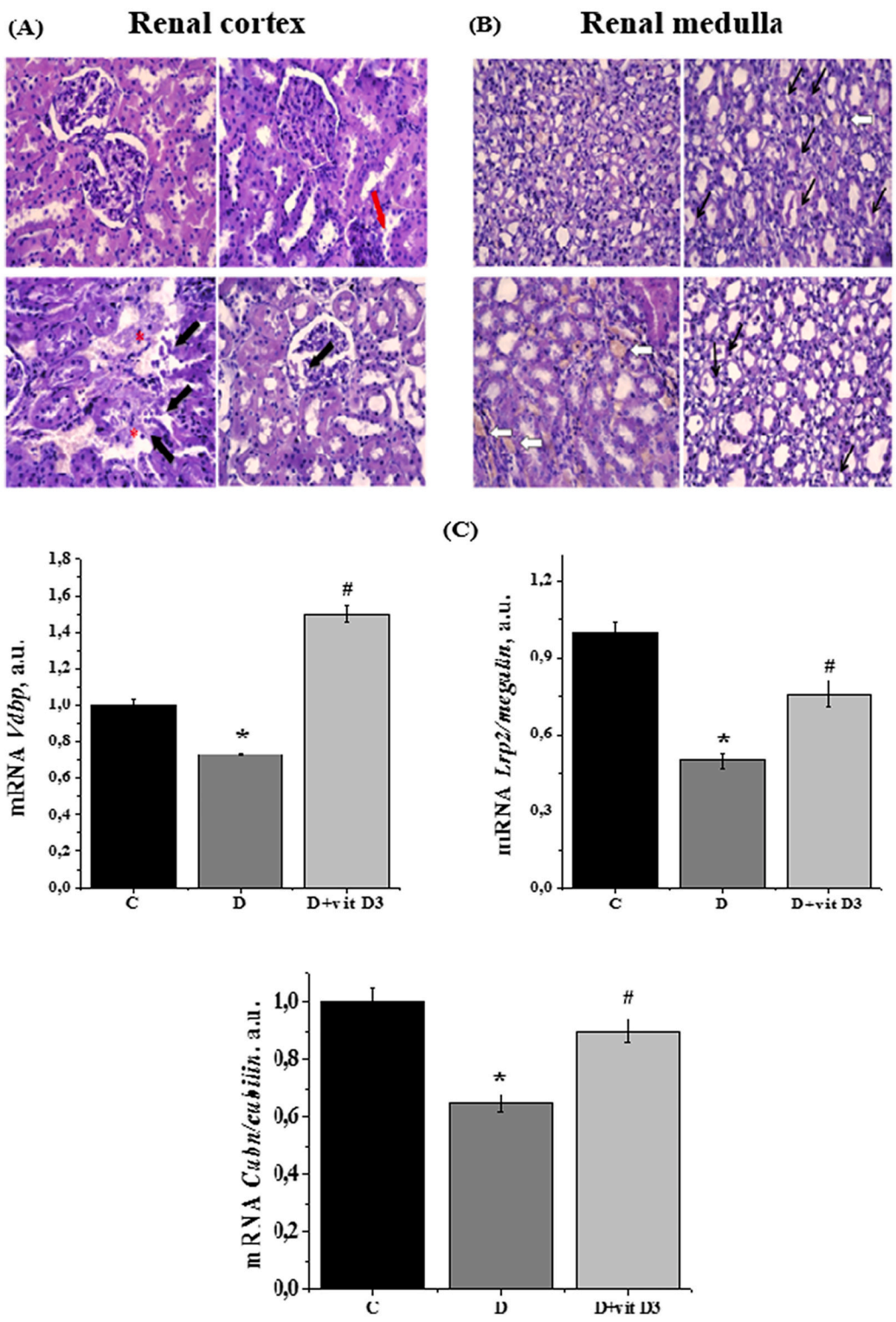

Fig. 2. Structural changes in (A) the proximal convoluted tubules (renal cortex) [* - necrosis in nephron tubules; ⬅ - globular (protein) deposits in the area of dystrophic changes; ⬅ - areas of chronic inflammation infiltrated by leukocytes] and (B) in the excretory tubules (renal medulla) [⇦ - stasis of blood vessels; ⬅ - globular (protein) deposits in the area of dystrophic changes] of diabetic animals and after vitamin D_3 supplementation (hematoxylin – *eosin* (H&E), 40×): 1 – control (C); 2,3 - diabetic rats (D); 4 - vitamin D_3-treated diabetic rats (D + vit D_3); (C) mRNA levels of *Vdbp* and its endocytotic receptors in the kidney tissue of diabetic animals and after vitamin D_3 supplementation. ANOVA with Tukey post hot test was used to analyze differences between groups. Statistically significant differences are expressed as mean ± SEM of three experiments ($n = 3$) in duplicate. *$p \leq 0.05$ vs. control; $^{\#}p \leq 0.05$ vs. diabetes, $^{\#\#}p \leq 0.01$ vs. diabetes.

the renal proximal tubules [37]. It is noteworthy that this elevation occurred at a low concentration of 25(OH)D in blood serum. These findings were additionally supported by our data showing a strong elevation of the *Cyp24a1* mRNA expression and could explain the pronounced downregulating effect on the circulatory 25(OH)D level, which was almost halved in experimental type 1 diabetes. Such a significant increase in the expression of vitamin D catabolic enzyme suggests that inactivation of $1\alpha,25(OH)_2D$ may be the primary mechanism aimed at limiting the anti-inflammatory physiological effects of calcitriol under conditions of acute or chronic inflammatory process. Our data are

Table 2
Morphometric analysis of kidneys by light microscopy in different experimental groups.

	Control	Diabetic Rats	Vitamin D_3-treated diabetic rats
Cross-section square of the renal corpuscles, μm^2	24,653,8 ± 1743,3	22,547,5 ± 1256,0	21,750,5 ± 1234,8*
Cross-section perimeter of the renal corpuscles, μm	587,1 ± 22,5	504,9 ± 25,0	570,3 ± 8,9
Cross-section square of kidney proximal tubules, μm^2	3420,6 ± 279,4	5263,0 ± 258,7**	3768,0 ± 198,3##
Cross-section perimeter of the kidney proximal tubules, μm	232,6 ± 20,4	263,4 ± 6,5	221,6 ± 6,1##
Cross-section square of the kidney thin tubules, μm^2	375,9 ± 23,9	617,3 ± 29,0***	395,5 ± 22,3###
Cross-section perimeter of the kidney thin tubules, μm	75,8 ± 2,4	102,9 ± 2,4***	76,8 ± 1,8###

*p ≤ 0.01 vs. control; #p ≤ 0.01 vs. diabetes; **p ≤ 0.003 vs. control, ##p ≤ 0.003 vs. diabetes; ***p ≤ 0.003 vs. control, ###p ≤ 0,001 vs. diabetes.

consistent with those already reported in the literature, indicating that abnormal function of vitamin D hormonal system in kidney disease is associated with enhanced renal inflammation [37,38].

Diabetes-related suppression of VDR mRNA and protein biosynthesis in renal tissue found in our study indicates impaired signaling through the $1\alpha,25(OH)_2D$/VDR complex. At the same time, we observed the restoration of the balanced functioning of the renal endocrine system of vitamin D in diabetic animals treated with vitamin D_3 or BAY 11–7082. When explaining the biological effects of $1\alpha,25(OH)_2D$ in the renal tissue, first of all, its modulating role in the regulatory binding sites of those genes that encode enzymes of vitamin D metabolism, as well as its possible participation in the regulation of NF-κB activity, deserve attention. Notably, the effects of the selective NF-κB inhibitor (BAY 11–7082) were similar to those of vitamin D_3, which may indicate the following: 1) activation of NF-κB-mediated signaling pathways plays an important role in deregulating the functional state of vitamin D endocrine system; and 2) the normalizing effects of vitamin D_3 may include the inhibitory action of the calcitriol/VDR complex on NF-κB directly or via associated signaling pathways. Although regulation of CYPs expression by NF-κB is plausible and generally proven [39,40], and

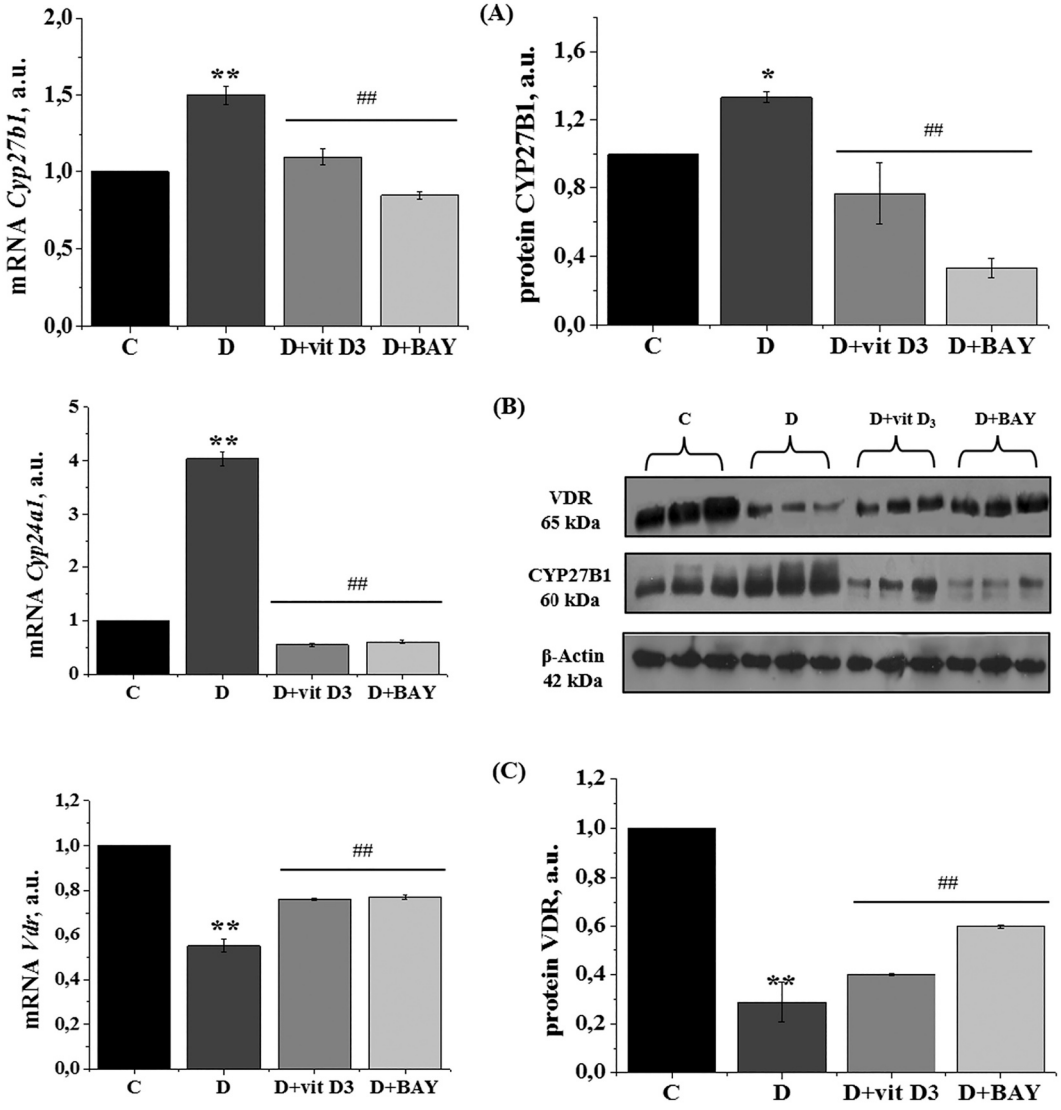

Fig. 3. Auto/para/endocrine signaling system of vitamin D in the kidneys of diabetic animals with or without vitamin D_3/BAY 11–7082 supplementation. Transcript level (mRNA) and protein abundance of CYP27B1 (A), transcript level of *Cyp24a1* (B), and transcript level (mRNA) and protein abundance of VDR (C). ANOVA with Tukey post hot test was used to analyze differences between groups. Statistically significant differences are expressed as mean ± SEM of three experiments ($n = 3$) in duplicate. *$p \leq 0.002$ vs. control; #p ≤ 0.002 vs. diabetes, **$p \leq 0.001$ vs. control; ##p ≤ 0.001 vs. diabetes.

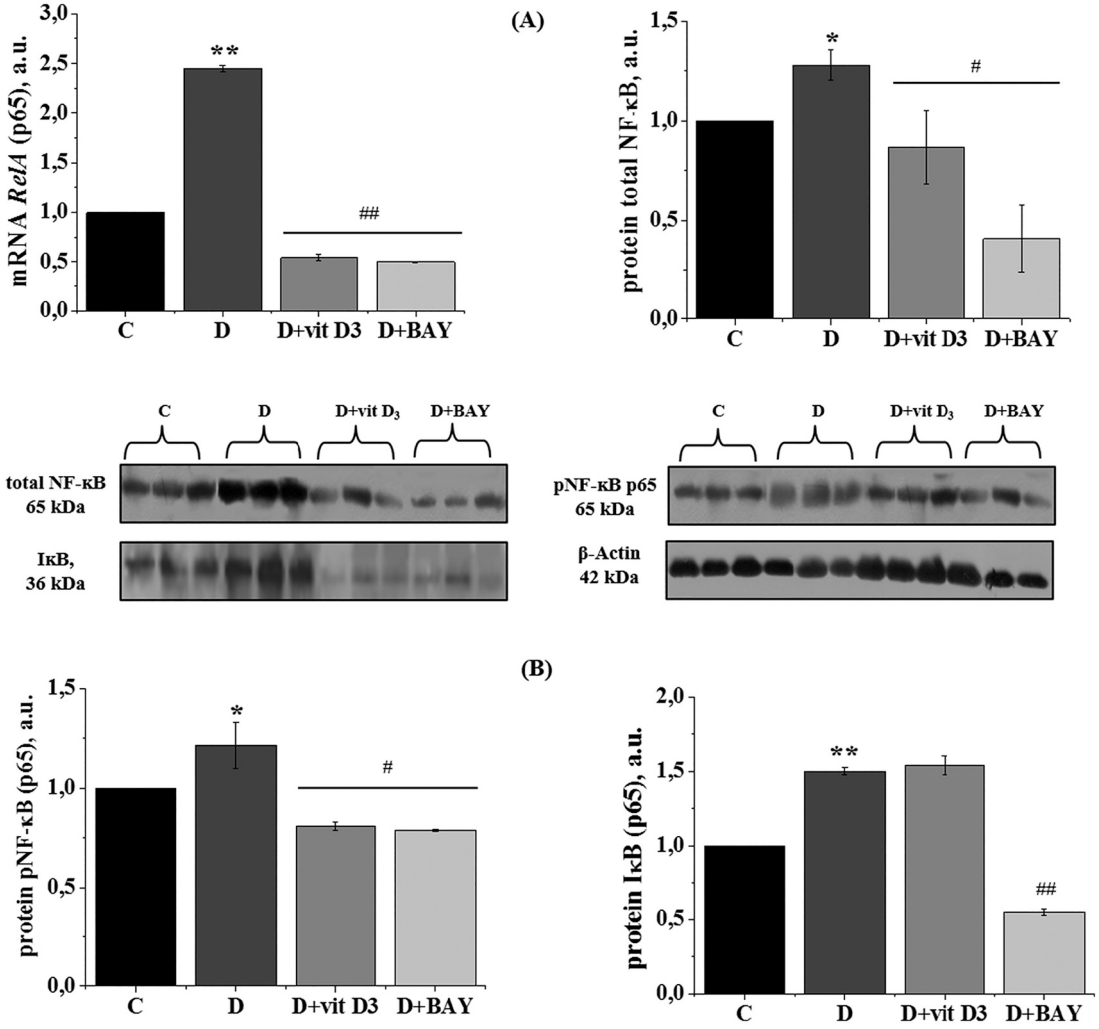

Fig. 4. Kidney NF-κB/IκB system in diabetes and after vitamin D_3/BAY 11–7082 supplementation. Transcript level (mRNA) and protein abundance of *RelA (p65)*/ total NF-κB (A), protein abundance of Ser 311 pNF-κB (B) and IκB-α (C). ANOVA with Tukey post hot test was used to analyze differences between groups. Statistically significant differences are expressed as mean ± SEM of three experiments (n = 3) in duplicate. *p ≤ 0.002 vs. control; $^{\#}$p ≤ 0.002 vs. diabetes, **p ≤ 0.001 vs. control; $^{\#\#}$p ≤ 0.001 vs. diabetes.

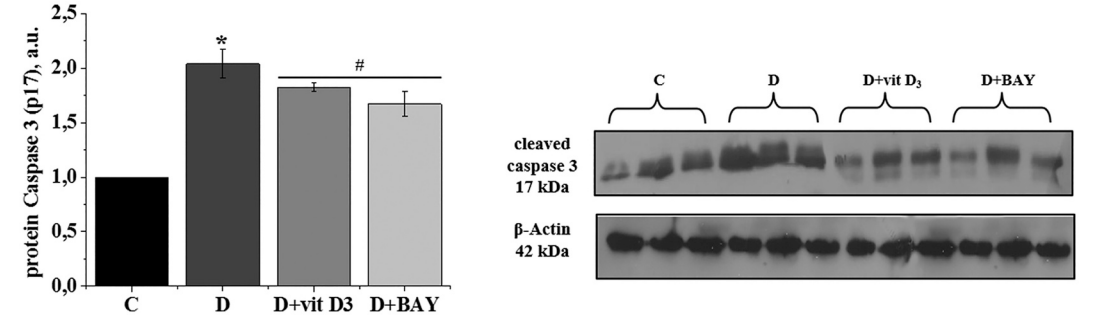

Fig. 5. Caspase-3 p17 protein levels in the kidneys of diabetic animals and the effect of vitamin D_3/BAY 11–7082 supplementation. ANOVA with Tukey post hot test was used to analyze differences between groups. Statistically significant differences are expressed as mean ± SEM of three experiments (n = 3) in duplicate. *$p \leq$ 0.003 vs. control; #p ≤ 0.003 vs. diabetes.

given that NF-κB can be effectively suppressed by liganded VDR, we can nonetheless argue based on the findings of other authors that cholecalciferol probably contributes significantly to more diverse regulatory effects in maintaining the endocrine system of vitamin D. It could act not only directly on the promoter regions of the *Vdr* and *Cyps* genes or inhibit transcriptional activation of NF-κB, but, probably, by attracting a number of other factors – the fibroblast growth factor-23 (FGF-23), parathyroid hormone and Ca^{2+} etc. [15,17,41].

Since its discovery in 1986, NF-κB has been considered a key factor in the pathogenesis of a number of human diseases, including type 1 diabetes and its complications [42]. In particular, one of the pathogenic mechanisms in the development of renal dysfunction associated with

diabetes is the activation of NF-κB under the action of angiotensin II followed by inflammation, which leads to apoptosis of kidney cells and the formation of fibrous tissue. In this study, we found an increase in the level of both total NF-κB and its active form phosphorylated at Ser 311 in diabetic kidneys. It is well known that tumor necrosis factor α (TNF-α) is a classical inducer of p65/NF-κB phosphorylation at Ser 311, which acts through PKCζ activation [43]. Currently, the issue of whether hyperglycemia can promote the activation of NF-κB by phosphorylation directly at Ser 311 or additional factors (e.g., IL-1β or RANKL) are involved remains largely open and requires further comprehensive study [42,44].

One of the principal mechanisms for regulating the activity of NF-κB is its reversible inhibition by binding to its natural regulatory protein – IκB (inhibitor of nuclear factor kappa B). Phosphorylation of IκB-α (major isoform) by the IκB kinase (IKK) enzyme complex, degrades IκB-α and liberates NF-κB from its inhibitory control. The latter translocates to the nucleus and is involved in transcriptional activation or inhibition of target genes. A significant elevation of the IκB-α protein level in diabetic kidneys, demonstrated in our study, may be a compensatory response to a profound increase in the expression and activation of NF-κB. Thus, the development of experimental type 1 diabetes is accompanied by an up-regulation of NF-κB synthesis and its activation (assessed by phosphorylation of the p65 subunit at Ser 311), the most likely compensatory response to which is a rise in IκB-α production. The suppressive role of BAY 11–7082 on NF-κB signaling observed in the present study is realized through the inhibition of IκB-kinase β (IKKβ), which is necessary for phosphorylation of IκB and its subsequent dissociation from the RelA (p65)/p50 complex (canonical pathway of NF-κB activation) [45].

Mounting evidence suggests that the non-canonical NF-κB pathway is also involved in the development of a number of inflammatory diseases, in particular kidney inflammation and injury, which cannot be regulated by BAY 11–7082 [46]. In addition to TNF-α and IL-1β, common activators of the canonical and non-canonical pathways, the non-canonical pathway of NF-κB activation can be regulated by a number of specific factors, such as the receptor activator of nuclear factor κB ligand (RANKL) [47]. Notably, in our previous study we showed that vitamin D_3 can modulate NF-κB activation and its translocation to the nucleus via the RANKL/RANK (receptor activator of nuclear factor κB)/OPG (osteoprotegerin) cytokine system [48]. Based on our findings, we can speculate that the use of vitamin D_3 in the treatment of diabetes-induced kidney failure allows modulating the activity of NF-κB not only through the canonical pathway via IκB and IKKβ, but also very likely by non-canonical activation via the RANKL/RANK signaling pathway.

Activation of cellular signaling downstream NF-κB can lead to apoptotic/necrotic cell death [47]. One of the most likely arguments in favor of diabetic-induced renal cell apoptosis is the sequential activation of caspases (cysteine-dependent specific aspartate proteases) under the influence of extrinsic (via TNF-α or Fas-ligand) or intrinsic (due to a pro-apoptotic Bax/Bcl balance) pathways [49]. Of notice, indirect NF-κB stimulation, in particular through IL-6, has been shown to activate caspase-3 and, ultimately, induce apoptotic cell death [50]. In light of this, our results suggest a possible link between diabetes-related NF-κB activation and caspase-3 mediated apoptotic cell death in renal tissue, which is largely consistent with previously published data. The supplementation of vitamin D_3 to diabetic animals or using BAY 11–7082 ensued to lower levels of p17 cleaved caspase-3 protein in the kidney compared to the diabetes group. This finding indicates that inhibition of the canonical or both canonical and non-canonical pathways of NF-κB activation can prevent the inflammatory response and correlates with a lower level of pro-apoptotic marker – cleaved caspase-3. However, it is obvious that one change in the renal level of caspase-3 is clearly insufficient for a comprehensive assessment of cell death pathways caused by diabetes, which requires a more detailed study of the intensity and mechanisms of cell apoptosis in our future trials.

Finally, it is worth noting that one of the significant aspects of the alteration in renal vitamin D metabolism that we have demonstrated in the present study may be closely related to the development of oxidative stress due to disturbances in the enzymatic system for the synthesis of glutathione and, as a result, a decrease in the level of glutathione in kidney tissue. Moreover, a negative effect of oxidative stress on vitamin D status in mice treated with HFD has been previously demonstrated [51]. The data available in the scientific literature fully correlate with ours. Potential molecular mechanisms that can cause an elevation of ROS generation in peripheral blood mononuclear cells, identified here, can be attributed to the activation of protein kinase C (PKC) under conditions of hyperglycemia, the diacylglycerol (DAG) pathway, mitochondrial dysfunction, etc. [52]. Some of NF-κB-dependent signaling pathways are known to be ROS-sensitive and strongly correlate with systemic inflammation that affects various tissues, in particular the kidneys, in patients with diabetes mellitus [53]. It was found that the hormonally active form of vitamin D_3 is able to suppress oxidative stress in the U937 monocyte cell line by inhibiting the production of pro-inflammatory cytokines (IL-8 and monocyte chemoattractant protein (MCP)-1) [54]. Such effects of cholecalciferol can be explained by the inhibition of NF-κB, since the expression of the aforementioned pro-inflammatory factors is under its transcriptional control [55]. Another mechanism of the corrective action of vitamin D_3 in diabetes is the attenuation of hyperglycemia due to the activation of the GLUT transporter expression, which, together with a decrease in NF-κB phosphorylation, leads to the suppression of generalized oxidative damage to cellular structures and stimulation of excessive glucose uptake by peripheral tissues [56].

We can summarize that the development of experimental STZ-induced type 1 diabetes is associated with vitamin D insufficiency/deficiency, as evidenced by a significant decline in the level of serum 25 (OH)D. Diabetes-related impairment of vitamin D bioavailability can occur due to disturbances of the kidney tissue histological structure, and impaired synthesis of kidneys' endocytotic receptor system, as well as key components of vitamin D-auto/para/endocrine system. The use of the NF-κB inhibitor, BAY 11–7082, further confirmed that the nuclear factor κB probably plays one of the leading roles in the development of diabetes-induced kidney dysfunction. Vitamin D_3 administration helped to restore the vitamin D status and normalize the biosynthesis of VDR, as well as the main hydroxylases involved in the metabolism of vitamin D – CYP27B1 and CYP24A1 – in the kidneys. The restoration of vitamin D bioavailability in diabetes led to a decrease in the biosynthesis and activation of NF-κB, which correlated with a lower level of cleaved (p17) caspase-3 apoptosis marker. Thus, we can speculate that the normal vitamin D status of diabetic animals can prevent the development of diabetic-induced kidney damage by reducing oxidative stress and NF-κB-mediated inflammation.

Author contributions

All authors have read and approved the final version of the manuscript.

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CRediT authorship contribution statement

Anna Mazanova: Conceptualization, Methodology, Investigation, Writing – original draft. **Ihor Shymanskyi:** Conceptualization, Project administration, Data curation, Writing – original draft. **Olha Lisakovska:** Investigation, Writing – review & editing. **Dmytro**

Labudzynskyi: Investigation, Methodology. **Anna Khomenko:** Investigation, Methodology. **Mykola Veliky:** Supervision, Conceptualization, Data curation, Methodology, Writing – original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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