Reduction of Rage Expression by Vitamin D in Isolated Diabetic Rat Aortas

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High blood glucose level and advanced glycation end-products (AGE) are the major contributors to the development of vascular complications in diabetes mellitus. The receptors for advanced glycation end-products (RAGE) are upregulated in response to high blood glucose level and may play a role in the occurrence of diabetic-related vascular disease. Vitamin D is widely reported as a cardiovascular protective agent. The present study assessed the effects of 1,25-dihydroxycholecalciferol (1,25-VitD$_3$) on the endothelial dependent relaxation (EDR) in organ bath studies and on vascular expression of RAGE by immunohistochemistry studies in aortic samples isolated from rats with experimental diabetes (streptozotocin, 50 mg/kg, single dose). Our findings indicate that: i) in diabetic aortas the EDR was significantly impaired (vs. control) together with a high expression of RAGE in the entire structure of the aortic wall and, ii) ex vivo treatment with 1,25-VitD$_3$ (0.1 μM) significantly improved the relaxation response and reduced the aortic expression of RAGE in diabetic vascular samples. In conclusion, low amounts of vitamin D exerts beneficial effects on endothelial function in vitro; in particular, we firstly demonstrated the modulation of RAGE expression in the settings of experimental diabetes. Investigations aimed at elucidating the underlying signal transduction pathways are clearly warranted view the potential therapeutic effect.

Keywords: vitamin D, diabetes mellitus, receptor for advanced glycation products

Atherosclerosis, the most important cause of cardiovascular diseases, has a complex pathogenesis that comprises the activation/recruitment of numerous cells (endothelial, vascular smooth muscle and immune cells) as well as inflammatory mediators regulating the vascular tone that are collectively responsible for the development and progression of endothelial dysfunction [1]. The process of atherosclerosis occurs prematurely and is accelerated in the presence of diabetes mellitus, the most severe metabolic disease. Increased generation of advanced glycation end-products (AGEs) from the non-enzymatic reaction of reducing blood glucose with proteins, lipids or nucleic acids is a key event in the pathogenesis micro- and macrovascular complications in diabetes [2]. AGEs activate their specific surface receptors (RAGE) located on the above mentioned cells [3,4]. The deleterious effects associated with RAGE activation are related to its pro-inflammatory, pro-apoptotic, pro-thrombotic, and pro-oxidant effects [7]. These effects are responsible for the severe local inflammation and endothelial dysfunction elicited by RAGE in the settings of chronic metabolic and inflammatory diseases leading to their vascular complications [5,6].

An increasing body of evidence links vitamin D deficiency with atherosclerosis and cardiovascular disease (reviewed in [8]). Indeed, in addition to its well-known role in phosphate-calcium metabolism, vitamin D has been found to be an important factor in the cardiovascular system. Calcitriol (1,25-dihydroxicholecalciferol), the active metabolite of vitamin D, exerts protective effects against atherosclerosis via several mechanisms: inhibits platelets' adhesion and aggregation, inhibits the release of pro-inflammatory cytokines , and the expression of adhesion molecules such as the intercellular adhesion molecule (ICAM-1), vascular cell adhesion molecule (VCAM-1) and E-selectin through a nuclear factor- κB (NFκB)-mediated mechanism [9-11]. Moreover, it also modulates the vascular tone via reducing calcium influx into endothelial cells, decreasing the production of endothelium-derived contracting factors and improving the endothelial nitric oxide (NO) synthase (eNOS) coupling that will further promote NO synthesis while decreasing reactive oxygen species (ROS) generation [8].

The aim of the present study was to assess the effects of 1,25-dihydroxi-cholecalciferol on vascular function and RAGE expression in aortic segments isolated from streptozotocin-induced diabetic rats.

Experimental part

Material and methods

Animal model

Wistar male rats were purchased from Cantacuzino Institute (Bucharest, Romania) and were acclimated for 2 weeks prior to the study. At the age of 8 weeks, diabetes

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was induced by a single injection of streptozotocin (50 mg/kg STZ, IP). Age-matched control rats received an equal volume of vehicle (0.01 M citrate buffer, *p*H 4.5). Two days after the injection, a blood sample was collected from the tail vein to measure the blood glucose. Rats with a glycemia over 200 mg/dl were considered diabetic. Animals were housed under standard conditions (constant temperature and humidity of 22.5 ± 2° C and 55 + 5%, 12-h light/dark cycle). The duration of the diabetes evolution was 1 month and glycemia and body weight were systematically monitored. Twenty-four hours prior to the experiment solid food was withdrawn with no limitation in water supply.

All experimental procedures used in this study were conducted in accordance with the Directive 2010/63/EU and the Romanian Law nr. 43/May 2014 concerning the protection of animals used for scientific purposes. The experimental protocol was approved by the Committee for Research Ethics of "Victor Babeş" University for Medicine and Pharmacy of Timişoara, Romania.

All reagents used were of the highest quality available and were purchased from Sigma Aldrich, Invitrogen, Applichem and Abcam.

Organ culture

Rat aortic segments were dissected under sterile conditions, cleaned, and incubated for 12 h at 37°C in EBM culture medium containing 0.1% BSA, in the presence or absence of 1,25-cholecalciferol (0.1μM, Sigma-Aldrich). Subsequently, the vascular tissue was studied in organ bath or frozen and used for immune histology.

Immune histology

Tissue expresion of the RAGE was determined on frozen sections of rat aortas using the RAGE primary antibodies (Abcam, ab3611, 1:50) and Texas-Red labeled secondary goat anti-rabbit antibody (Santa Cruz, SC2780, 1:200), respectively. Nuclear staining was obtained with DAPI (Santa Cruz, SC3598). Slides were examined on an Olympus Fluoview FV1000 confocal microscope (DAPI ex/em 405/461nm, Texas-Red ex/em 543/612nm). Images were analyzed with Icy, a free open source image analysis software developed by the Quantitative Image Analysis Unit at Institut Pasteur Paris according to de Chaumont [12].

Results and discussions

Experimental diabetes

Four weeks after the induction of diabetes with STZ, body weight was significantly decreased in diabetic rats *vs*. controls and blood glucose level was significantly higher in diabetic rats *vs.* controls (fig. 1).

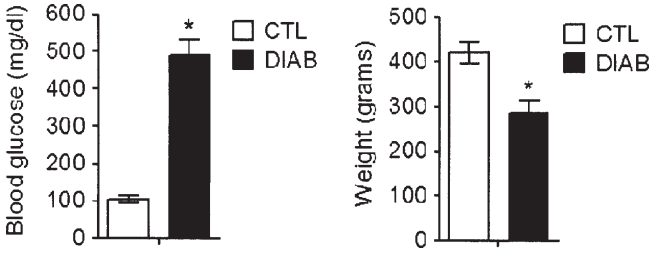

Fig. 1. Body weight and blood glucose at 4 weeks after the induction of diabetes

Vitamin D Improves the Endothelial-Dependent Relaxation of Diabetic Rat Aortas

The effect of vitamin D3 on vascular function was studied in aortic segments isolated from diabetic rats and the corresponding controls. After 1 month of constant hyperglycemia, thoracic aortas were isolated, incubated for 24 h with 1,25-dihydroxicholecalciferol (0.1 μM) and used for organ bath experiments according to a previously described protocol [13-15]. In brief, the endothelium-dependent relaxation (EDR) of preconstricted aortic rings to increasing concentrations of acetylcholine (ACh) was recorded. The concentration of phenylephrine (Phe) used for preconstriction, was adjusted to obtain an identical preconstriction level of 80% of the contraction elicited by KCl (80 mmol/L).

At the end of 4 weeks of diabetes evolution, hyperglycemia significantly attenuated the endothelium-dependent relaxation, whereas 12 h incubation with 1,25-VitD_3 partially restored the relaxation response (table 1).

Vitamin D exerts protective effects on endothelium through several mechanisms such as: suppression of NADPH oxidase, inhibition of the expression of interleukin IL-6 and IL-8 and of several adhesion molecules [9, 10, 16], reduction of the expression of the inducible cyclooxygenase-2 with the subsequent decrease of vasoconstrictor metabolites as well as the modulation of the immune response [10, 11]. In chronic administration it also reduces calcium influx into the endothelial cells thus preventing the activation of phospholipase A2 and reducing the endothelium-dependent contractions [8, 17]. Nevertheless, the most important effect of vitamin D is related to the enhancement of the endothelial nitric oxide (NO) synthase coupling with the increase in bioavailability of nitric oxide [17-19].

RAGE Expression is Increased in Diabetic Rat Aortas

AGEs-induced deleterious effects in the vasculature are central in the pathogenesis of diabetes complications and are partly mediated by their receptors (RAGE) that are present on the surface of both endothelial and vascular

Parameters	CTL (n = 10)	CTL+VIT D (n = 10)	DIAB (n = 10)	DIAB+VIT D (n = 10)
Maximal relaxation (%)	89.89±4.50	86.03±3.758	72.37±4.03	81.6±3,46
EC_{50} (-Log[M])	7.482±0.15	7.403±0.08	6.674±0.09	6.961±0.12
Hill slope	-0.7344	-0.7442	-0.8905	-0.8005

Table 1
PARAMETERS OF ENDOTHELIAL-DEPENDENT RELAXATION TO CUMULATIVE CONCENTRATIONS OF ACh IN AORTIC SEGMENTS ISOLATED FROM DIABETIC AND CONTROL RATS INCUBATED OR NOT WITH 1,25-DIHYDROXYCHOLECALCIFEROL (0,1 μM).

$p^1 < 0.05$ CTL vs. DIAB , $p^2 < 0.05$ DIAB vs. DIAB+VIT D

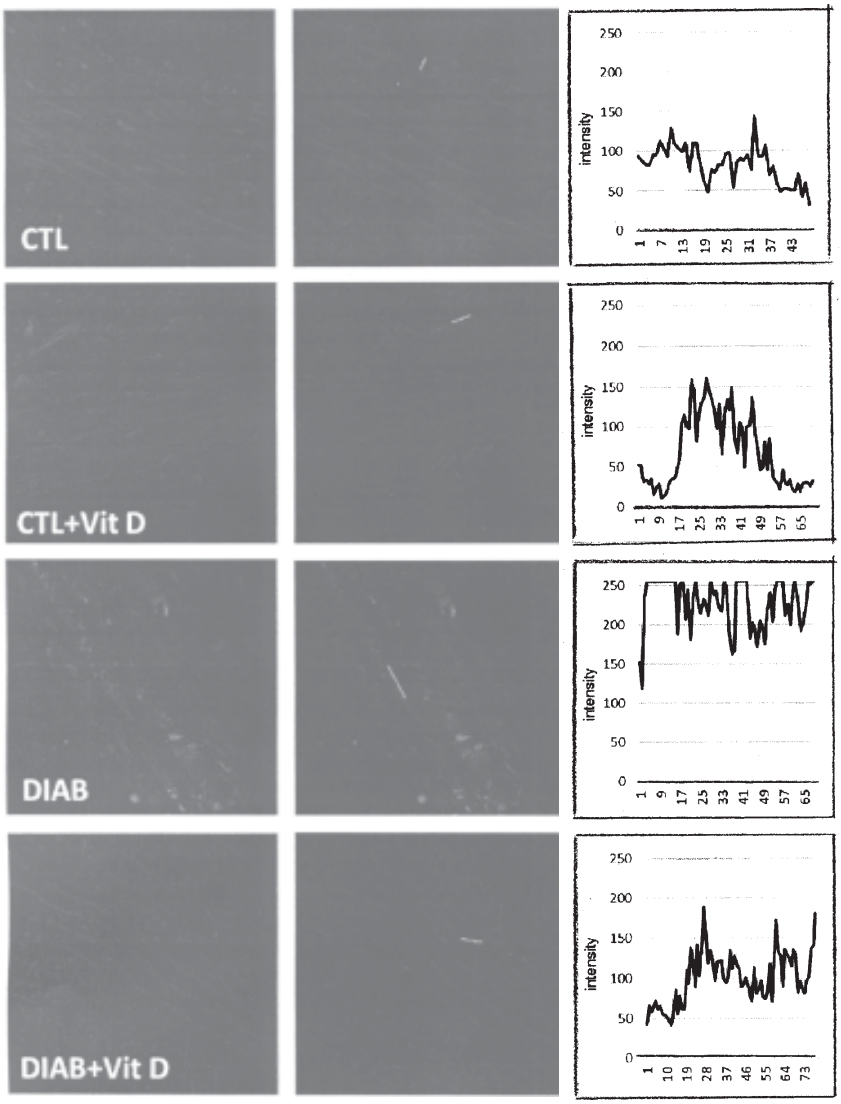

Fig. 2. RAGE expression in vascular preparations from diabetic and non-diabetic rats incubated with 1,25-VitD_3 (0.1 μM)

smooth muscle cells. RAGE expression was found to be upregulated in both human atherosclerotic lesions and diabetes [20, 21] but the relation with vitamin D was not investigated so far. In this regard, we firstly assessed the expression of RAGE in aortas of rats with STZ-induced diabetes *vs.* control animals. Experiments were performed 1 month after the diabetes induction. As demonstrated by the immune-fluorescence staining, RAGE was more abundant in samples harvested from diabetic rats as compared to controls (fig. 2).

Vitamin D Treatment Decreases RAGE Expression in Aortic Segments From Diabetic Rats

In order to investigate whether vitamin D can modulate RAGE expression, we incubated for 12 h aortic samples from diabetic and control rats with its active form, 1,25-dihydroxi-cholecalciferol and evaluated RAGE expression by immune histology. As revealed by the fluorescence staining, *in vitro* treatment with 1,25-dihydroxi-cholecalciferol decreased RAGE expression in the diabetic vascular samples and had no effect on controls (fig. 2).

Atherogenesis is particularly severe in patients with diabetes and the accumulation of AGEs with the subsequent activation of RAGE is an important pathomechanism that leads to endothelial activation, local inflammation, oxidative stress and acceleration of the development of atherosclerotic plaques [7, 22, 23].

RAGE is a member of the immunoglobulin receptor family that is present on the surface of all cells relevant to the atherosclerotic process being involved in intracellular signal transduction [24]. In diabetic patients its activation has been reported to promote inflammation, thrombosis, and oxidative stress [25]. Also, RAGE is upregulated in the atheromatous lesions pointing to the contribution of AGEs-related signal transduction to the progression of the disease [26].

Recent reports have shown beneficial effects of calcitriol on cardiovascular functions especially in conditions associated with AGE accumulation [27-29]. In line with these observations we decided to study the effect of calcitriol on endothelial function and RAGE expression in rat vasculature in the settings of experimental diabetes. Regarding the effect of vitamin D metabolites on RAGE expression, it was showed that calcitriol can modulate RAGE expression in diabetic hearts [9], in cultured HUVEC exposed to diabetic-like environment [30] or to inflammatory conditions (induced by lipopolysaccharide) [31], and in HUVEC stimulated with AGEs [32]. In our experiments we selected a very low concentration of 1,25-dyhidroxycolecalciferol to be tested *in vitro*. Our findings support the results of the increasing number of studies suggesting the beneficial effects of vitamin D_3 in the cardiovascular system and also, the fact that these effects occur at very low concentrations [17]. Elucidation of the mechanisms of vitamin D-related beneficial effects and their demonstration *in vivo* are paramount view its potential therapeutic use.

Conclusions

In summary, we showed for the first time that low amounts of calcitriol counteracts the RAGE overexpression found in rat diabetic aortic segments. The effect of calcitriol on RAGE expression may explain the improvement of endothelial-dependent relaxation. Whether the positive effects of vitamin D on vasculature can be recapitulated *in vivo* remains to be demonstrated.

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