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Vitamin D3 Induces Gene Expression of Ox-LDL Scavenger Receptors in Streptozotocin-Induced Diabetic Rat Aortas: New Insight into the Role of Vitamin D in Diabetic Atherosclerosis

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Abstract

Background: Several lines of evidence suggest that oxidized LDL (Ox-LDL) scavenger receptors play a crucial role in the genesis and progression of diabetic atherosclerosis. This study aimed to elucidate the effect of vitamin D3 on gene expression of lectin-like oxidized LDL receptor-1 (LOX-1), scavenger receptor-A (SR-A), Cluster of Differentiation 36 (CD36), and Cluster of Differentiation 68 (CD68) as the main Ox-LDL receptors in streptozotocin (STZ)-induced diabetic rat aortas.

Methods: Eighteen Sprague-Dawley rats were randomly divided into three groups of six rats each. Two rats died during the study so five rats from each group were analyzed at the study's end. Diabetes was induced in overnight starved rats in two of the groups by intraperitoneal injections of 60 mg/kg of STZ. The vitamin D3/diabetic group then received weekly intraperitoneal injections of 5000 IU/kg of vitamin D3 dissolved in cottonseed oil for four weeks, diabetic controls received cottonseed oil, and healthy controls received sterile saline weekly for the same period. At the end of the four-week study period the animals were killed and the aortas were collected to examine the mRNA expression using real-time polymerase chain reaction (RT-PCR).

Results: SR-A and CD36 mRNA expression were significantly greater in the vitamin D3/diabetic rats than in both the diabetic control and healthy control rats. CD68 and LOX-1 expression were greater in the vitamin D3/diabetic rats than in the diabetic control and healthy control rats, respectively.

Conclusions: Vitamin D3 may increase the risk of diabetic atherosclerosis by inducing scavenger receptors expression.

Keywords: Atherosclerosis, Diabetes, Ox-LDL, Scavenger receptor.

Introduction

Cardiovascular disease (CVD) is a major cause of disability and death globally (1). Atherosclerosis is the main contributor to most CVD (2). Individuals with diabetes mellitus (DM) are at increased risk for CVD, due to accelerated vascular disease and atherosclerosis (3). Several conditions contribute to the increased risk of atherosclerosis in diabetic patients; these include hyperglycemia, insulin resistance, hypertension, and high serum levels of low-density lipoprotein–cholesterol (LDL) (4, 5). Of these, the high LDL serum level has been proposed as one of the most important risk factors for atherosclerosis (5). Several lines of evidence suggest that oxidized LDL (Ox-LDL) plays a more important role in the genesis and progression of atherosclerosis than native LDL (5). Increased

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oxidant species in diabetic patients modify LDL and produce Ox-LDL (6). The internalization of Ox-LDL into macrophages and activated smooth muscle cells leads to the differentiation of these cells to foam cells and cholesterol-engorged cells, which are the hallmark of early atherosclerotic lesions (7). Ox-LDL is taken up by macrophages and aortic endothelial cells via scavenger receptors, which mediate the cellular function of Ox-LDL (7, 8). Lectin-like oxidized LDL receptor-1 (LOX-1) (9), class A scavenger receptor (SR-A) (10), class B scavenger receptor (CD36) (8), and macrosialin (CD68) (11) are four main receptors for ox-LDL on endothelial cell and macrophage membranes. Although the pathophysiologic roles played by these receptors in human disease are not yet known, animal models of atherosclerosis have revealed a crucial role for these receptors in atherosclerotic foam cell development and plaque formation (12, 13). It has been shown that elevated glucose and ox-LDL in diabetic patients can increase the expression of scavenger receptors CD36 (14), SR-A (15), CD68, and LOX-1 (16, 17) in endothelial cells and macrophages, thereby contributing to diabetic atherosclerosis (18).

There is growing interest in identifying the effects of vitamin D on human health. Beyond its well-defined role in osteoporosis, vitamin D has recently been reported as an important factor in cardiovascular health (19). *In vitro* studies using animal models showed that the vitamin D receptor is expressed in cardiomyocytes, endothelial cells, and vascular smooth muscle (20), supporting the hypothesis that vitamin D plays a role in the regulation of vascular cell functions. In the past decades, many studies have attempted to reveal an association between vitamin D and cardiovascular disease. Nevertheless, the results have been inconclusive, with most studies reporting neither beneficial nor adverse effects from vitamin D (21). The role of vitamin D in the management and prevention of, as well as its possible contribution to, CVD remains to be demonstrated (22). In the present study, to further elucidate the role of vitamin D in CVD in subjects with diabetes, we examined the effect of vitamin D_3 on gene expression of ox-LDL scavenger receptors in aortic cells of streptozotocin-induced diabetic and normal rats.

Materials and Methods

Animals

Eighteen adult male Sprague-Dawley rats weighing 180 – 250 g were used in the study. They were purchased from the central animal house of Pharmacology College at Tehran University of Medical Sciences, Tehran, Iran. The animals were housed in clean capacious cages with 12 hr dark and 12 hr light photoperiods, temperature of 25 ± 2 °C, and controlled relative humidity of 50 ± 15%. The rats were fed regular chow diet and water ad libitum. All animal procedures were approved by the Institutional Animal Care and Use Committee of Tehran University of Medical Sciences.

Diabetes induction and treatment regimen

Eighteen rats began the study. The rats were divided into three groups of six rats each. To induce diabetes, after a 14-hour fast, rats from two of the groups received a single intraperitoneal (IP) injection of 60 mg/Kg streptozotocin (STZ; Sigma Chemicals Co. L. St Louis, Mo) freshly dissolved in phosphate buffered saline (PBS). After 48 hours, blood was collected from the tail veins and the fasting blood glucose (FBG) concentrations were determined using a glucometer (Bionime GM300, SwissDesign, Berneck, Switzerland). Glucose concentrations in all the STZ-injected rats were greater than 220 mg/dL. The vitamin D3/diabetic rats then received weekly injections of 5000 IU/kg of vitamin D3 dissolved in 0.5 ml cottonseed oil for four weeks, the diabetic control rats received 0.5 ml cottonseed oil, and the normal control rats, who had not received STZ, received 0.5 ml sterile saline 0.9% weekly for four weeks. All injections were IP. One rat from the vitamin D3/diabetic group and one from the diabetic control group died during the study so 15 rats were analyzed at the study's end.

Tissue preparation

At the end of the four-week study, the animals were euthanized with ketamine and xylazine after a 12-hr fast. The aortas were excised, weighed, rapidly frozen in liquid nitrogen, and stored at −80 °C until analysis.

RNA extraction and quantitative real-time polymerase chain reaction (Q-PCR)

Fifty mg of aortic tissue samples were homogenized in liquid nitrogen and total RNA was isolated using a Hybrid-RTM miRNA kit (Geneall, Cat# 325-150) according to the manufacturer's protocol. RNA concentration was determined with a spectrophotometer. Total RNA from each sample was reverse-transcribed using a PrimeScript™ First Strand cDNA Synthesis Kit (Takara Biotechnology Co., cat# 6110A) according to the manufacturer's instructions. The transcribed cDNA (0.8 μl) was used for *Q-PCR* amplification with specific primers for rat LOX-1, SR-A, CD36, and CD68 (Table 1). All amplifications were performed in duplicate. Quantitative PCR using SYBR Green reagent was performed on the ABI 7500 Real-time PCR System (Applied Biosystems, United States). The threshold cycle (CT) values were determined and normalized to the housekeeping gene B-actin. The relative amount of each target gene was calculated using the $2^{-\Delta\Delta Ct}$ method (23).

Table 1. PCR primers design.

Gene	Primer sequences (5′-3′)	Length (bp)	TM	CG (%)
CD36	F: GGTCCTTACACATACAGAGTTCG	23	58.58	47.83
	R: TCCAACAGACAGTGAAGGCTC	21	59.93	52.38
CD68	F: TCTGTTGCGGAAATACAAGC	20	56.73	45.00
	R: GCAGCAAGAGAGATTGGTC	19	56.01	52.63
LOX-1	F: GCTATCCTTTCTTGGGTGTAAAAC	24	58.05	41.67
	R: TTGCTTCTGGTCTTTGTCTCTG	22	58.53	45.45
SR-A	F: ACTTCAGCATGGCAACCGAC	20	61.24	55.00
	R: GAAATTGCATCCAGGGACTCC	21	58.97	52.38
B-actin	F: CCTCTATGCCAACACAGTGC	20	58.91	55.00
	R: GTACTCCTGCTTGCTGATCC	20	58.05	55.00

CD36, Cluster of Differentiation 36; CD68, Cluster of Differentiation 68; -1, Lectin-like oxLDL receptor 1; SR-A, Scavenger receptor A.

Statistical analysis

All data are expressed as the mean ± standard deviation (SD). Normality of parameters distribution was assessed with the Kolmogorov–Smirnov test. The variables did not follow a normal distribution ($p < 0.05$); thus, data were analyzed by non-parametric tests. Differences between experimental groups were statistically analyzed using a Kruskal-Wallis H test. Statistical significances between two groups were determined using the Mann-Whitney U test, and paired data (weight and food intake) were analyzed by Wilcoxon's paired rank-sum test. Differences were considered to be statistically significant at $p < 0.05$. Statistics were analyzed using SPSS software (version 23.0; SPSS, Inc., Chicago, IL).

Results

Body weight and food intake

Of 18 rats, 16 survived until the end of the study. One rat in the vitamin D/diabetic group and one in the diabetic control group died during the study period. Table 2 reports the body weight and food intake of different groups of the rats at baseline, On the day of diabetes induction, which was two days before treatment with vitamin D, and the end of the study, the 28th day of treatment with vitamin D. No significant differences were found in the initial and final body weights between the groups. Weights in all groups were significantly greater at the end of the study than at baseline. Regarding food intake, before the experiment (On the day of diabetes induction, which was two days before treatment with vitamin D), the healthy control group consumed significantly more food than either the vitamin D/diabetic or diabetic control group, while no significant difference in food intake was found between the groups at the end of the study (the 28th day of treatment with vitamin D). Furthermore, there were significant within-group changes in food intake in vitamin D/diabetic and diabetic control rats.

Table 2. Weight and food intake of rats among the different groups at baseline and end of the experiment.

	Weight (g)			Food intake (g)		
Group	**Before[a]**	**After[b]**	**p-value***	**Before[a]**	**After[b]**	**p-value***
Vitamin D	204.20 ± 46.68	282.20 ± 29.51	0.04	16.40 ± 0.54	23.60 ± 0.54	**0.03**
Healthy control	231.50 ± 45.52	273.17 ± 43.03	0.02	25.00 ± 1.09[a]	24.00 ± 5.47	**0.33**
Diabetic control	223.80 ± 18.44	306.20 ± 26.98	0.04	17.40 ± 0.54	23.80 ± 3.83	**0.03**

P-value*, p-value for within group; a, p-value < 0.5 compared to vitamin D and diabetic control groups.
[a] On the day of diabetes induction, which was two days before treatment with vitamin D
[b] the 28th day of treatment with vitamin D

mRNA expression of SR-A, CD68, CD36, and LOX-1 in aortas

The SR-A, CD68, CD36, and LOX-1 expression levels in the rat aortas are shown in Figure 1. SR-A mRNA expression was greater in the vitamin D/diabetic rats than in either the diabetic (2.1-fold, p = 0.03) or healthy controls (2.74-fold, p = 0.009) (Fig. 1A). CD68 mRNA expression was significantly greater in the vitamin D3/diabetic rats than in the diabetic controls (2.44-fold, p = 0.008), but not significantly different from that of the healthy control rats (Fig. 1B). CD36 mRNA expression was significantly greater in the vitamin D3/diabetic rats than in either the diabetic (2-fold, p = 0.04) or healthy controls (3.14-fold, p = 0.004) (Fig. 1C). LOX-1 mRNA expression was significantly greater in the vitamin D3/diabetic than in the healthy control rats, (3.89-fold, p = 0.04) but not significantly different from the diabetic control rats (Fig. 1D). No significant differences between healthy control and the diabetic control rats were seen for SR-A, LOX-1, CD68, or CD36.

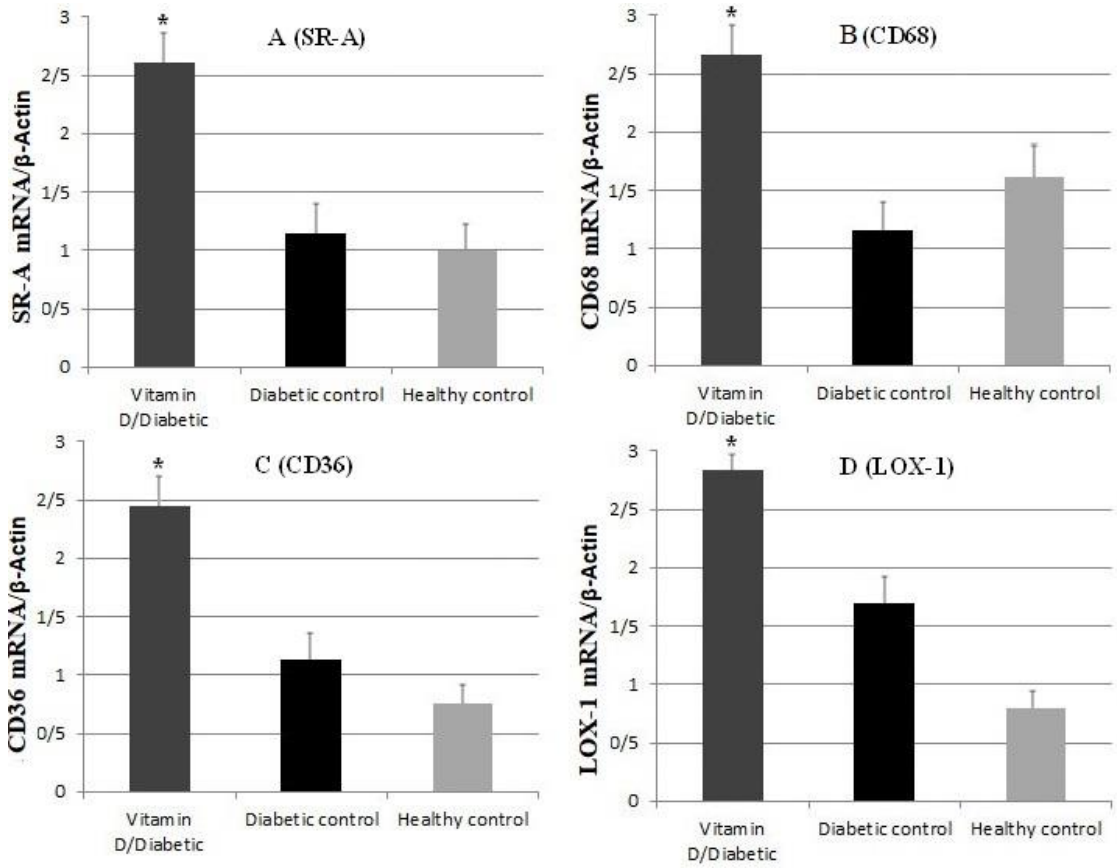

Fig. 1. Effect of treatment with vitamin D3 on gene expression of ox-LDL scavenger receptors in STZ-induced diabetic rats. Quantitative RT-PCR was performed to assess the changes in mRNA levels in the rat aorta tissues for SR-A (A), CD68 (B), CD36 (C), and LOX-1 (D). The levels of mRNA are represented as the ratio to B-actin. The results are reported as the mean ± SD. The rats were divided into three groups and six rats were included in each group.

Discussion

In vitro studies have provided important insights into potential mechanisms by which diabetes might damage arterial cells or play a role in atherogenesis (3). In this regard, ox-LDL and its receptors, which are increased in chronic hyperglycemia, have been of great interest in the study of diabetic atherosclerosis over the past decade (24). Because endothelial cells express the vitamin D receptor and 1α-hydroxylase (25), which is required for the conversion of 25(OH)D to calcitriol, studies have proposed that vitamin D may act as a modulator of endothelial function (19). Accordingly, this study was performed to investigate the effect of vitamin D3 on gene expression of Ox-LDL scavenger receptors in diabetic rat aortas. In this study, SR-A, CD36, and LOX-1 expression were greater in diabetic than in healthy control rats, but these differences were not significant. However, expression of SR-A, CD36, and CD68 was significantly greater in the vitamin D/diabetic rats than in control diabetic rats, indicating that vitamin D3 might play a role in atherogenesis.

Some reports suggested that hypovitaminosis D induces foam cell formation (26, 27) and that supplementation with vitamin D could reduce the risk of vascular disease (28). In recent randomized clinical trials, vitamin D supplementation failed to reverse the deleterious effects of cardiovascular diseases (28, 29). A comprehensive study, after the re-analysis of the data of the RECORD trial, other RCTs, meta-analyses and systematic reviews found that, except for possible protective effects against heart failure, vitamin D monotherapy has neutral effects of on cardiovascular events (30). Moreover, some evidence from experimental, epidemiological, and clinical studies propose that vitamin D might initiate cell degeneration, calcification, lipid particle accumulation in aortic media, and plaque formation, and eventually could accelerate the progression of atherosclerotic disease (31). The assumption of the biphasic effect of vitamin D on CVD is supported by prospective studies. These studies found a U-shaped association between circulating 25OHD and CVD outcomes and showed that both vitamin D deficiency and hypervitaminosis D are independently associated with increased CVD morbidity and mortality (22, 32). Nevertheless, the role of vitamin D in cardiovascular health and disease remains controversial.

The results of our study to some extent shed light on the potential mechanism by which vitamin D leads to increased atherosclerosis risk. Our findings showed that vitamin D3 increases common Ox-LDL receptors in diabetic rat aortas. In the study by Suzuki et al. (33), atherosclerotic lesions were reduced by 60% in apolipoprotein E (apo E) -knockout mice with macrophage SR-A deficiency. Kunjathoor et al. (34) also reported that macrophages lacking both SR-A and CD36 exhibited a 75–90% decrease in the internalization and degradation of modified lipoproteins. In agreement with these studies, many reports confirm that targeted silencing of scavenger receptors attenuates lipid accumulation and atherosclerosis formation (13, 35, 36). Thus, up-regulation of scavenger receptors by vitamin D could, at least in part, explain the possible direct association between vitamin D and atherosclerosis. To the best of our knowledge, until now, only three original studies (26, 37, 38) explored the effect of vitamin D on protein or mRNA expression of SRA and CD36. These studies were conducted on macrophage cell lines. In contrast to our results, in these studies vitamin D downregulated SR-A and CD36. Studies on cultured cells provide only hypothesis-generating findings that then should be investigated *in vivo*. There are many reasons why studies on cultured vascular cells might not mimic the responses of these cells *in vivo*. For instance, cultured cells are often phenotypically different from those *in vivo*, and *in vivo* responses are a complex result of interactions between many factors (3). Thus, evidence regarding the effect of vitamin D on scavenger receptors is limited, and to achieve a conclusive result, further *in vivo* and *in vitro* studies are required to assess the effect of vitamin D on the function of scavenger receptors and atherosclerosis.

This is the first *in vivo* study exploring the effect of vitamin D3 on scavenger receptor expression. Further, our study is the first to assess the effect of vitamin D3 on LOX-1 and CD68 expression. Nevertheless, some limitations of the present study require discussion. First, this study measured only

mRNA expression; however, mRNA expression changes do not always result in protein content changes (39). Determining protein expression by western blot analysis is recommended for future experiments. Second, atherosclerosis is a chronic complication of diabetes; yet no atherosclerosis was observed in the streptozotocin-induced diabetic rats during the four-week study. Thus, investigating the effect of vitamin D3 on both gene and protein expression of ox-LDL scavenger receptors in animal models of atherosclerosis is needed to confirm our findings. Third, many studies show that scavenger receptor expression is up-regulated in hyperglycemia and diabetes (3). In our study, diabetic control rats exhibited a non-significant increase in SR-A, CD36, and LOX-1 expression compared with non-diabetic mice, which suggest that diabetes might influence expression of these receptors over the long term.

In conclusion, the current study showed for the first time that treatment with vitamin D3 resulted in increased expression of SR-A, CD36, CD68, and LOX-1in diabetic rats; this might be a novel mechanism for the atherogenic effects of vitamin D3. Hence, it seems that vitamin D3 may contribute to diabetic atherosclerosis. Additional large, well-designed studies are required to examine the potential effects of vitamin D on cardiovascular disease.

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