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To cite this article: Seyed Shahabeddin Mortazavi-Jahromi, Shahab Alizadeh, Mohammad Hassan Javanbakht & Abbas Mirshafiey (2018): Cardioprotective effect of β-d-mannuronic acid (M2000) as a novel NSAID on gene expression of oxLDL scavenger receptors in the experimental diabetic model, Immunopharmacology and Immunotoxicology, DOI: 10.1080/08923973.2018.1455209

To link to this article: https://doi.org/10.1080/08923973.2018.1455209

Published online: 05 Apr 2018.
Cardioprotective effect of β-D-mannuronic acid (M2000) as a novel NSAID on gene expression of oxLDL scavenger receptors in the experimental diabetic model

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ABSTRACT

Context: The investigations have shown that patients with diabetes have the elevated levels of glucose and oxLDL. These two play an important role in increased expression levels of oxLDL scavenger receptors on the surface of macrophages and endothelial cells that leads to deposition of oxLDL and macrophages in vascular walls.

Objective: The present study intends to show the effects of β-D-mannuronic acid (M2000) on the expression profile of ox-LDL scavenger receptors (including SR-A, LOX-1, CD36, and CD68) in an experimental model of diabetes.

Materials and methods: Eighteen Sprague-Dawley rats were randomly divided into three 6-member groups of the healthy control, diabetic control, and treated rats by M2000. Diabetes was induced in rats by intraperitoneal (IP) administration of 60 mg/kg streptozotocin. The treated rats were given daily intraperitoneal injections of M2000 with a dose of 25 mg/kg for 28 days and at the end of the 28th day, their aortas were removed. The qRT-PCR technique was then used to evaluate the expression levels of the proposed gene.

Results: The gene expression levels of the SR-A, LOX-1, CD36, and CD68 significantly declined in the diabetic group that received M2000 compared with untreated diabetic rats.

Conclusions: The M2000, as a novel NSAID is able to modify by lowering the gene expression levels of SR-A, LOX-1, CD36, and CD68 in treated rats compared to the untreated diabetic group, which may play an important role in preventing the complications that could lead to a cardioprotective efficacy.

ARTICLE HISTORY

Received 10 November 2017
Accepted 1 March 2018

KEYWORDS

M2000; mannuronic acid; scavenger receptors; ox-LDL; cardioprotective

Introduction

Cardiovascular diseases (CVDs) have been one of the leading causes of disability and mortality during the past five decades and it has been reported that around one cause of death in the year 2015 [1,2]. Atherosclerosis is the main origin of most CVDs, including coronary artery disease (CAD), heart failure, and stroke [3]. Patients with type 1 and 2 diabetes are at risk for developing CVD due to the acceleration of processes that lead to atherosclerosis [4]. In fact, diabetic patients have a 2–3.5 time higher risk of death from heart disease than healthy control [5]. The exact mechanism of accelerated atherosclerosis in diabetic patients is not clear yet, although the various mechanisms have been suggested. The resistance to insulin is often accompanied by the metabolic disorders such as dyslipidemia, hypertension, and obesity. These risk factors can explain a part of the reason for the high prevalence rates of atherosclerosis in diabetic patients [6].

Dyslipidemia is a prevalent metabolic disorder in diabetic patients, and it has been suggested as one of the factors that lead to the development of atherosclerosis in these patients. The increased levels of low-density lipoproteins (LDLs) are a major risk factor for atherosclerosis [7]. At present, we know that the oxidized low-density lipoproteins (oxLDLs) play a more vital role in the development and progression of atherosclerosis than intact LDL [8,9]. Increasing evidence has shown that the uptake of oxLDL by monocytes deposited below the vascular endothelium is one of the potential factors in the differentiation of monocytes into macrophages and foam cells and, eventually, in the formation of atheromatous plaques [10]. About 75–90% of oxLDL uptake takes place in macrophages via class A scavenger receptors (SR-A) and a cluster of differentiation (CD) 36 receptors [11]. In addition to these receptors, it has been recently suggested that lectin-like oxLDL receptor-1 (LOX-1) [12] and CD68 [13] also act as scavenger receptors of oxLDL in macrophages and vascular endothelial cells. In diabetic patients, the glucose and oxLDL levels are elevated, and these two play a role in the increased expression of oxLDL...
scavenger receptors on the surface of macrophages and endothelial cells. This increased expression leads to the depositing of oxLDL and macrophage in vascular walls, the start of inflammatory response cascade and, finally, in forming the atheromatous plaque in patients with diabetes [14–16].

The release of some factors that regulate inflammatory response, such as interleukin-1 (IL-1), CD14, and tumor necrosis factor-α (TNF-α) shows that atherosclerosis has an inflammatory origin and these factors play a role in regulating the inflammation process [17]. The use of non-steroidal anti-inflammatory drugs (NSAIDs) may be useful in preventing or reducing atherosclerosis complications. Although coxibs, including rofecoxib, celecoxib, and valdecoxib, are effective in inflammatory reactions, but they have many side effects on various systems of the body, especially the heart and the blood vessels [18,19]. Therefore, in recent years, the researchers have tried to identify the safer and more effective anti-inflammatory drugs. The small molecule β-D-mannuronic acid (M2000) (patented, DEU: 102016113018.4) is an anti-inflammatory drug with very low molecular weight and without any side effect for the digestive system and kidney function. It has exhibited the therapeutic effects with the highest levels of tolerability and efficiency in various experimental models, such as experimental autoimmune encephalomyelitis, adjuvant-induced arthritis, nephritic syndrome, and acute glomerulonephritis [20–24].

The present research intends to assay the effects of M2000 on gene expression level of the oxLDL scavenger receptors (including SR-A, LOX-1, CD36, and CD68) in the experimental diabetes model.

**Methods**

**Preparation of M2000**

The small molecule (C₁₀H₁₆O₇), M2000, with very low molecular weight (194.139 Da) patented (DE-102016113018.4) was synthesized by the acid hydrolysis from sodium alginate (Sigma-Aldrich, St Louis, MO, USA) based on the Mirshafiey et al. method [25]. Thereafter, the purity of M2000 was verified and confirmed using carbon-13 nuclear magnetic resonance (¹³C NMR) and Fourier-transform infrared (FTIR) spectroscopy.

**Animals**

A total of 18 adult male Sprague-Dawley rats, weighing 180–250 g, were included in the present experimental study. They were purchased from the central animal house of Pharmacology College at Tehran University of Medical Sciences. The animals were housed in clean capacious cages under normal light period (12 h dark and 12 h light), appropriate temperature (25±2°C), and controlled relative humidity (50±15%) conditions. 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**

The rats were divided into three 6-member groups. To induce diabetes, after 14-h fasting, rats of two groups received a single intraperitoneal (IP) injection of 60 mg/kg streptozotocin (STZ; Sigma Chemicals Co., St Louis, MO, USA) freshly dissolved in phosphate-buffered saline (PBS). After 48 h, blood was collected from the tail veins and the fasting blood glucose (FBG) concentration was determined using a glucometer (Bionime GM300, SwissDesign, Berneck, Switzerland). The glucose concentrations in all STZ-injected rats were greater than 220 mg/dl. The M2000/diabetic rats then received daily injections of 25 mg/kg of M2000 for four weeks, the diabetic control (patient) rats and the normal control (healthy) rats received 0.5 ml sterile saline 0.9% daily for four weeks. All injections were IP.

**Tissue preparation**

At the end of the four-week study, the animals were anesthetized with ketamine and xylazine after 12 h fasting. The aortas were excised, weighted, rapidly frozen in liquid nitrogen and stored at −80°C until analysis.

**RNA extraction and cDNA synthesis**

The 50 mg of aortic tissue samples were homogenized in liquid nitrogen and total RNA was extracted from the control groups and treated group using Hybrid-R™ Mini kit (GeneAll, Songpa-gu, Republic of Korea) according to the manufacturer’s guidelines. The quality of isolated RNA was determined by agarose gel electrophoresis on the GelRed™ (Biotin, Fremont, CA), and total RNA concentration was assessed by NanoDrop 2000 UV spectrophotometer (Thermo Scientific, Salt Lake City, UT USA) and the samples with A260/280 ratio in the range 1.7–2.0 were selected for cDNA synthesis. Equal amounts of cDNA were synthesized using RNA (1 μg) via oligo (dT) and random hexamer primers in the presence of cDNA reverse transcriptase by the use of PrimeScript™ RT reagent kit (Takara, Shiga, Japan). A list of used primer sequences in the current study has been represented in Table 1.

**Quantitative real-time PCR (qRT-PCR)**

PCR amplifications were performed using Syber premix Ex Taq™ II (Takara, Shiga, Japan) in ABI Step One Plus™ Real-time PCR system (Thermo Scientific, Salt Lake City, UT, USA). Briefly, the reaction conditions included 2 μl cDNA, 12.5 μl SYBR Green Master Mix, 8.5 μl RNase Free-Water, 1 μl forward primer, 1 μl reverse primer. The amplification was carried out as follows: an initial denaturation at 95°C for 30 s, followed by 40 cycles of denaturation for 5 s at 95°C and annealing for 30 s at 60°C. The gene levels were normalized to the housekeeping gene (β-actin) as an internal control. The relative changes in gene expression were calculated using the 2⁻ΔΔCt method (Ct refers to the threshold value).
Statistical analysis was performed using GraphPad Prism Software Version 6.02 (GraphPad Software, San Diego, CA, USA; www.graphpad.com). Repeated measures one-way analysis of variance (ANOVA) with Tukey’s multiple comparisons post hoc tests were utilized to analyze the expression of the gene between treated and untreated (control) group. The results were expressed as the mean value±standard deviation (SD). A p value <.05 was considered to be a statistically significant difference.

**Results**

In this study, the FBG concentration which was determined using glucometer in diabetic rats (non-treated and treated by M2000) as the most important parameter of diabetes was greater than 220 mg/dl, as baseline determinants.

**Effects of M2000 on SR-A gene expression**

Our data demonstrated that the gene expression of SR-A in the aorta of STZ-induced diabetic rats was significantly decreased 39.6%±4 by M2000, in comparison to diabetic control group (144.7%±9.7) with p<.01 vs. diabetic control group and ###p<.001 vs. healthy control group. (Figure 1).

**Effects of M2000 on LOX-1 gene expression**

The results illustrated that the gene expression of LOX-1 in the aorta of STZ-induced diabetic rats was significantly decreased 47.9%±2.4 by M2000, in comparison to diabetic control group (154.5%±7.4) with *p<.05 vs. diabetic control group and ##p<.01 vs. healthy control group. (Figure 2).

**Effects of M2000 on CD36 gene expression**

Our findings indicated that the gene expression of CD36 in the aorta of STZ-induced diabetic rats was significantly decreased 39.1%±5.9 by M2000, in comparison to diabetic control group (160.9%±9) with p<.05 (Figure 3).

**Effects of M2000 on CD68 gene expression**

The results showed that the gene expression of CD68 in the aorta of STZ-induced diabetic rats was significantly decreased
Diabetic patients are at the risk of developing atherosclerotic CVDs including stroke, CAD, and peripheral vascular disease and finally a macrovascular complication of diabetes. The patients suffering from the two main types of diabetes, i.e. type 1 and type 2, are at the greater risk of developing such diseases even at an early age. The investigations show that the oxLDL could be the most important factor involved in the pathogenesis of cardiopathy and atherosclerotic diseases [26,27]. The oxLDL releases from the sedimented monocytes in the vascular subendothelial space potentially contribute to monocyte differentiation into macrophages and foam cells and, consequently, atheromatous plaques formation [28]. This is done through the ‘scavenger receptors’ such as SR-A, LOX-1, CD36 and CD68, expressed in macrophages and vascular endothelial cells.

Ganjifrockwala et al. reported a higher level of oxLDL in diabetic patients compared with non-diabetics [29]. After activating the NF-κB signaling pathway, the oxLDL increases the production of proinflammatory cytokines in human dendritic cells, contributing to their maturation and differentiation [30]. Lu et al. indicated that diabetes-induced hyperglycemia increases oxLDL uptake by dendritic cells and thus potentially contributes to the development and exacerbation of atherosclerosis [31]. Ye et al. showed that SR-A is a major receptor for oxLDL uptake by mesenchymal stem cells whose expression is increased through exposure to oxLDL [32]. Handberg et al. for the first time illustrated that the soluble CD36 levels are increased in diabetic samples. The soluble CD36 is strongly associated with atherosclerosis risk factors such as insulin resistance and glycemic control. It has been reported that the CD36 is able to predict the atherosclerosis process in diabetic patients [33]. It has been revealed in two separate studies that diabetes-induced hyperglycemia increases LOX-1 gene expression and differentiates foam cells into macrophages, leading to diabetes-induced atherosclerosis [14,34]. Yoshida et al. reported that oxLDL could increase scavenger receptor expressions (SR-A, CD36, CD68) in rat peritoneal macrophages [35].

The potential therapeutic effect of M2000, as a novel NSAID has been examined in various experimental models such as glomerulonephritis, nephrotic syndrome, rheumatoid arthritis and multiple sclerosis [20–24]. The administration of M2000 in immune complex glomerulonephritis and experimental nephrotic syndrome has been demonstrated a significantly reduced blood urea nitrogen (BUN), proteinuria, serum creatinine, and cholesterol levels as well as glomerular lesions in rats [20,21]. The results of another study conducted on Wistar rats and the naval medical research institute (NMRI) mice for assessment of acute and subacute pharmacotoxicity revealed no significant clinical or histopathological side effects. The evaluation of hematological and biochemical indices point to no systemic side effects of this novel drug. In addition, the results in this research indicate that the oral administration of M2000 causes to the lack of side effects in animals even up to 1250 mg/kg body weight dose levels, i.e. the highest tested dose [25]. The pharmacotoxicological studies show M2000 to be a safe anti-inflammatory drug compared with dexamethasone and commonly tested NSAIDs [22]. This novel NSAID is able to modify TLR signaling pathways by suppressing the adaptor molecules IRAK1 and TRAF6, the transcription factor NF-κB and miR-146a as a new therapeutic approach [36]. At present, this anti-inflammatory drug has been tested in phase 1/2 clinical trial with the registered no. IRCT2013062213739N1 in ankylosing spondylitis.
patients and phase 1/2 clinical trial with the registered no. IRCT2014011213739N2 in rheumatoid arthritis patients without any side effects, even without cardiovascular problems [37–40], whereas cardiopathy could be one of the NSAIDs side effects.

Based on the significant role of scavenger receptors in inflammatory diseases such as diabetes-induced atherosclerosis, demonstrated in previous studies, the present research aimed to study the effects of M2000 on the expression level of the oxLDL scavenger receptors’ profile (including SR-A, LOX-1, CD36, and CD68) in the experimental diabetes model. The results revealed that the IP injection of M2000 to diabetic adult male rats for four weeks and at a dosage of 25 mg/kg/day could significantly reduce SR-A, LOX-1, CD36, and CD68 gene expressions compared with the diabetic control group (Figures 1–4).

Collectively, considering scavenger receptors as drug targets, numerous researchers have attempted to alleviate atherosclerotic CVDs and control the development of inflammatory diseases using NSAIDs with lowest toxicity levels and side effects. The present research showed that the administration of M2000, as a novel NSAID might modify the SR-A, LOX-1, CD36, and CD68 gene expressions in diabetic rats which it could be probably recommended as a preventive agent in the angiocardioopathy process.

Acknowledgements
We would like to thank all the members that have participated in the research.

Disclosure statement
The authors report that they have no conflicts of interest.

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