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Atorvastatin and rosuvastatin improve physiological parameters and alleviate immune dysfunction in metabolic disorders

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ABSTRACT

This study was designed to characterize the potential therapeutic effects of two statin drugs commonly used to treat dyslipidemia in inflammation-linked metabolic disorders related to type 2 diabetes.

Atorvastatin (10 mg/kg/day) and rosuvastatin (3 mg/kg/day) were administered to mice with diet-induced obesity (DIO). The statins lowered serum total and LDL cholesterol levels, and improved the atherogenic index and cardiac risk index. Furthermore, the drugs decreased fasting glucose levels, improved glucose tolerance, and decreased fat tissue weight and adipocyte size; this was accompanied by an overall body weight loss tendency. The statins also improved antigen-specific immunity. The killing activity of cytotoxic T cells and exacerbation of IgG secretion levels were considerably normalized. Most importantly, serum tumor necrosis factor-α and interleukin 6 levels decreased, while their RNA expression levels in fat tissue were regulated by the statins as well.

This study is the first to indicate that low doses of atorvastatin and rosuvastatin, the dosing regimen for which has been controversial, could significantly improve diabetes-related metabolic disorders, and could modulate pro-inflammatory cytokines, alleviating inflammation and simultaneously restoring overall humoral and cell-mediated immunity.

1. Introduction

Dyslipidemia is a condition featuring pathologically high blood cholesterol levels, and is considered a typical metabolic disorder. Statin drugs are generally prescribed for dyslipidemia to regulate cholesterol biosynthesis [1,2]. However, studies have confirmed various pleiotropic effects of statins, including improving epithelial cell function, reducing oxidative stress, and alleviating arteriosclerosis and inflammation [3–5]. Since fatty acid and lipid metabolites are closely related to inflammation, lipid regulation by statins can contribute to alleviating inflammation. In this regard, numerous attempts have been made to evaluate whether statins could exert beneficial effects on other disorders where inflammation acts as a major factor, such as cardiovascular diseases, stroke, and insulin resistance [6–9].

There has been much controversy on the topic of treating diabetes with statins, especially regarding their dose regimen [10,11]. Studies asserting that an inflammatory reaction between hypertrophic adipocytes and macrophages is the main pathologic mechanism underlying type 2 diabetes [12–14] argue that statins' anti-inflammatory effect could lead to an anti-diabetic effect [15–17]. Other studies, however, found conflicting results indicating that high doses of statins (atorvastatin ≥80 mg and rosuvastatin ≥40 mg) aggravated diabetic symptoms [18,19].

The objective of this study was to examine whether low doses of statins improve diabetic symptoms or other metabolic phenotypes, and further identify statins' anti-inflammatory effects and immunologic improvements. Atorvastatin and rosuvastatin, the most-prescribed statins in Korea, were administered to mice with diet-induced obesity (DIO), an animal model for obesity and diabetes, at low doses (10 mg/kg/day and 3 mg/kg/day respectively) [12,20,21].

2. Materials and methods

2.1. Animals and experimental protocol

Male C57BL/6 mice (specifically pathogen-free, 4 weeks of age)

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were purchased from Samtako (Osan, Korea) and acclimated for 2 weeks to laboratory conditions at temperature and humidity ranges of approximately 20–24 °C and 30–70% respectively, with a 12-h light-dark cycle and free access to commercial rodent feed and sterile water. The normal mice were fed irradiated rodent diet (Purina Rodent Chow 38057), while the DIO mice were fed 60% high fat diets (D12492, Research Diets, Inc.) for 27 weeks. All experimental procedures were performed in strict compliance with the Guidelines for the Care and Use of Laboratory Animals issued by Sahmyook University (IACUC number: SYUIACUC 2014039). The regular diet (RD) group and the high-fat diet (HFD) group served as controls. The atorvastatin and rosuvastatin groups, which consisted of DIO mice alone, were administered 10 mg/kg of atorvastatin or 3 mg/kg of rosuvastatin daily for 48 consecutive days. The dosing volume was 5 mL/kg.

Immunological analysis was adapted from an existing protocol [22]. Male 4-week-old C57BL/6 mice were purchased from Samtako (Osan, Korea) and allowed to acclimatize for 1 week. The mice were housed in animal facility with the conditions as described above. All experimental procedures were performed in strict compliance with the Guidelines for the Care and Use of Laboratory Animals issued by Sahmyook University (IACUC number: SYUIACUC 2015001). To induce metabolic diseases such as obesity, hyperglycemia, and hyperlipidemia, the mice were fed a 45 kcal HFD for 23 weeks; disease progression was confirmed by monitoring body weight, blood glucose, and cholesterol levels. Atorvastatin and rosuvastatin were then administered orally for 16 weeks at the same doses described above.

2.2. Fasting blood glucose and intraperitoneal glucose tolerance test (IPGTT)

Fasting blood glucose levels were measured once a week during the study after week 3, using Accu-chek® Performa (Roche Diagnostics, Risch-Rotkreuz, Switzerland) after 16 h of fasting by making a cut in the tail using a blade. IPGTTs were conducted on study day 45 in all study groups. Animals were fasted overnight (16 h) prior to IPGTT, and a dose of 2 g/kg glucose was injected into the intraperitoneal cavity. Blood was sampled at 0 (pre-dose), 15, 30, 60, and 120 min.

2.3. Serum collection and analysis

After final dosing, Animals were fasted overnight (16 h) prior to scheduled necropsy. Blood was sampled by cardiac puncture under ether anesthesia. Collected blood was transferred into 1.5-mL microtubes and left for 30 min at room temperature to facilitate clotting; the blood was then centrifuged at 10,000 rpm for 2 min to separate the serum. Serum was stored below −70 °C prior to analysis. Blood chemistry analysis was performed at the Korea Animal Clinical Medical Center (Guri, Korea) using a blood chemistry analyzer (AU400, Olympus, Tokyo, Japan).

2.4. Histological analysis

Gonadal fat tissue was isolated from mice, fixed in 10% formalin, and embedded in paraffin. Four-micrometer-thick sections were obtained, mounted on glass slides, and stained with hematoxylin and eosin (H&E). Sections were imaged with a microscope (Nikon, Tokyo, Japan) at ×200 magnification.

2.5. In vivo cytotoxic T lymphocyte (CTL) assay

Four mice per group were immunized with soluble ovalbumin (OVA, 100 μg/mouse) intravenously [23]. After 7 days, splenocytes from naive syngeneic mice were prepared as target cells, and pulsed with OVA peptide for 1 h at 37 °C, followed by labeling with a high concentration of carboxyfluorescein succinimidyl ester (CFSE, 5 μM, $CFSE_{high}$) for 10 min at room temperature. The same number of unpulsed syngeneic cells was prepared and labeled with a low concentration of CFSE (1 μM, $CFSE_{low}$). A 1:1 mixture of each target cell was injected via the tail vein of the OVA-immunized recipient mice, which were sacrificed 18 h later. Splenocytes were harvested and analyzed using fluorescence-activated cell sorting (FACS).

2.6. Immunoglobulin G (IgG) assay

The mice in each group were injected subcutaneously with OVA peptide suspended in complete Freund's adjuvant (CFA, Sigma-Aldrich, St. Louis, MO, USA), followed by a secondary injection of OVA peptide in incomplete Freund's adjuvant (IFA, Sigma-Aldrich). After the secondary immunization, the mice were euthanized, whole blood samples were collected, the serum was separated, and IgG levels were measured using in an enzyme-linked immunosorbent assay (ELISA) as previously described with slight modification [24].

2.7. Pro-inflammatory cytokine assay

The levels of pro-inflammatory cytokines in mouse serum were measured using an ELISA kit (eBioscience Inc., San Diego, CA, USA) according to the manufacturer's instructions. Absorbance values were measured at 450 and 570 nm using a microplate reader (Molecular Devices). The values obtained at 570 nm were subtracted from those at 450 nm, and the concentration of each cytokine was calculated according to a standard curve constructed by using each of the recombinant cytokines in the ELISA kit.

2.8. RNA expression

Total RNA was extracted from adipose tissue using a commercial purification kit (GeneAll Hybrid-R™, GeneAll Biotechnology, Seoul, Korea). RNA concentrations were equalized and converted to cDNA using a RT-premix (GeneAll HyperScript™). Gene expression levels were measured by qRT-PCR (StepOnePlus™ System, Applied Biosystems, Foster City, CA, USA) using PCR Master Mix (AB Power SYBR® Green). The cDNA template was denatured at 95 °C for 10 min followed by 40 cycles of PCR (PCR conditions varied depending on the primer). The primers used in the experiments are listed in Table 1 β-Actin was used as an internal control for all mRNA levels.

2.9. Statistical analysis

All results are presented as mean ± standard error of the mean (SEM), and the differences between the control and treated groups were statistically analyzed using one-way analysis of variance (ANOVA) followed by a Dunnett's multiple comparisons test for each parameter of interest. $p < 0.05$ was considered statistically significant.

3. Results

Fasting glucose tests and IPGTTs were conducted to study the influence of atorvastatin and rosuvastatin on non-insulin-dependent diabetes mellitus (NIDDM)-related pathological symptoms. During the experimental period, the HFD-fed C57BL/6 mice showed significantly higher glucose levels than the RD group ($p < 0.001$). This elevation decreased when atorvastatin and

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