ASSESSMENT OF ANTI-INFLAMATORY ACTIVITY OF STATIN

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ABSTRACT

Objective: Previous studies have reported inhibition of 3-hydroxy-3-methyl-glutaryl CoA reductase (HMG-CoA reductase) as the mechanism by which statin drugs reduce excessive endogenous cholesterol biosynthesis and thereby prevent cardiovascular diseases. However, there has been little or no report on its possible anti-inflammatory actions which may be another mechanism by which statin protects against coronary heart disease (CHD). This study investigated anti-inflammatory tendencies of simvastatin in albino rats.

Method: Simvastatin was administered for a period of 28 days. Serum concentrations of interleukine-2 (IL-2) and tumour necrosis factor-alpha (TNF-α) were determined using ELISA method while serum lipids were measured spectrophotometrically.

Results: Administration of statin caused a decrease in TNF-α, total cholesterol, triglyceride and low density lipoprotein cholesterol while a mild increase was observed for IL-2 and high density lipoprotein cholesterol.

Conclusion: The reduction in serum concentration of TNF-α in rats given statin may be an indication that statin prevents CHD by way of repressing expression of proteins involved in inflammatory processes.

Keywords: Simvastatin, Inflammation, Cytokines, Lipids, Cardiovascular

INTRODUCTION

Cardiovascular disease is a class of disease that involves the heart or blood vessels (arteries and veins), [1] while the term technically refers to any disease that affects the cardiovascular system. It is usually used to refer to those related to atherosclerosis (arterial disease). These conditions usually have similar causes, mechanisms, and treatments [1].

Cardiovascular disease remains the biggest cause of deaths worldwide, though over the last two decades, cardiovascular mortality rate have declined in many high-income countries. The percentage of premature death from cardiovascular disease ranges from 4% in high-income countries to 42% in low-income countries [2].

Research on disease dimorphism suggests that women who suffer cardiovascular disease usually suffer from forms that affect the blood vessels while men usually suffer from forms that affect the blood vessels while men usually suffer from forms that affect the heart muscles [1]. Each year, cardiovascular disease kills more Americans than cancer. In recent year, cardiovascular risks in women have been increasing and have killed more women than breast cancer.

Inflammation had been implicated in atherogenesis. This may be because inflammation induces generation of free radicals some of which may oxidize intact low density lipoprotein cholesterol (LDL) forming oxidized LDL (OX-LDL). Oxidation of LDL leads to increase influx of cholesterol into the artery forming plaque on the intima of the artery. However, markers of inflammation include synthesis of interleukin-2 and tumor necrosis factor-alpha. Elevated serum interleukin-2 and tumor necrosis factor alpha have been associated with increased progression to occurrence of cardiovascular disease. There is therefore increased emphasis in preventing cardiovascular disorder by modifying risk factor. Researches have shown anti-inflammatory activities of both natural products and synthetic drugs [3,4,5].Although statin, have been applied in many instances for the treatment of hyperlipidemia due to its ability to reduce synthesis of cholesterol, however, there has been no information of its application in an attempt to repress synthesis of interleukin-2 and tumor necrosis factor alpha. Therefore this study is designed to assess ability of statin to repress synthesis of interleukin-2 and tumor necrosis factor.

METHODOLOGY

Simvastatin drug was purchased from a local pharmacy in Ogbomoso. The ELISA kits for the determination of rat Interleukin-2 (IL-2) and Tumor Necrosis Factor-alpha (TNF-α) were products of RayBiotech, Inc. USA, while those for Total Cholesterol, Triacylglyceride, and High Density Lipoprotein Cholesterol (HDL-C) were products of LABKIT, CHEMELEX, S.A. Pol. Canovelles-Barcelona, Spain. All other chemicals used including solvents are of analytical grade.

Study design

Thirty adult Wistar rats with average weight of 190 grammes were purchased and housed in well ventilated wire cages to acclimatize for few days. They were fed with normal pelleted animal feed and were given unrestricted access to clean water. The protocol conforms to the guidelines of the National Institute of Health (NIH) (NIH publication 85–23, 1985) for laboratory animal care and use. Animals were randomly distributed into two groups of fifteen animals each. Rats in group 1 were administered simvastatin (0.05mg/g) per rat per day for 28 consecutive days. Group 2 served as control (received drug-vehicle, water).On day 29, the rats were sacrificed by cervical dislocation. Fasting blood samples were collected through cardiac puncture and were centrifuged at 1,500 g/min at 4 °C for 10 min to obtain serum and stored at -20°c till further analysis was done.

Determination of biochemical parameters

Analyses of lipids, lipoproteins, total proteins and albumin

Total cholesterol, HDL-C, LDL-C, triglyceride, total proteins and albumin were analyzed using spectrophotometric methods. Globulin concentration was calculated from the difference between total protein and albumin concentrations. Total cholesterol concentration in the serum was determined spectrophotometrically using the cholesterol oxidase method at 546 nm, 37[0]C [6]. HDL-C was determined by spectrophotometric method of [7] at 500 nm, 37[0]C. Triglycerides were determined using spectrophotometric method [8]. Low density lipoprotein cholesterol was calculated using Friedwald formular[9].

Principle of the determination of cytokines using RayBio elisa kit

The RayBio® Rat ELISA (Enzyme – Linked Immunosorbent Assay) kit is an in vitro enzyme-linked immunosorbent assay for the quantitative measurement of rat cytokines in serum (rat IL-2 concentration is pretty low in normal serum/plasma, it may not be detected in this assay), plasma and cell culture supernatants. This assay employs an antibody specific for the cytokine coated on a 96 – well plate. Standards and samples are pipetted into the wells and cytokine present in a sample is bound to the wells by

the immobilized antibody. The wells are washed and biotinylated anti-Rat cytokine antibody is added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin is pipetted to the wells. The wells are again washed, a TMB substrate solution is added to the wells and color develops in proportion to the amount of cytokine bound. The stop solution changes the color from blue to yellow, and the intensity of the color is measured at 450nm.

Assay Procedure for the cytokines

All reagents and samples were brought to room temperature (18-25⁰C) before use. According to the recommendation of the manufacturer, all standards and samples were run at least in duplicate. Hundred microlitres (100 μ L) of each standard and sample were added into appropriate wells. These wells were covered and incubated for 2.5hours at room temperature with gentle shaking. The solution was discarded and the well washed 4 times with 1x wash solution. The wells were washed by filling each well with wash buffer (300 μ L) using a multi-channel pipette or auto washer. The liquid was completely removed at each step as this is essential for good performance. After the last wash, remaining wash buffer was removed by aspirating. The plates were inverted and blotted against clean paper towels. One hundred microlitres (100 μ L) of 1x prepared biotinylated antibody was added to each well. This was incubated for 1 hour at room temperature with gentle shaking. The solution was discarded and washed 4 times with 1x wash solution.

One hundred microlitres (100 μ L) of prepared streptavidin solution was then added to each well. This was incubated for 45 minutes at room temperature with gentle shaking. The solution was discarded and washed 4 times with 1x wash solution. One hundred microlitres (100 μ L) of TMB one-step substrate reagent was added to each well and incubated for 30minutes at room temperature in the dark with gentle shaking. Finally, fifty microlitres (50 μ L) of stop solution was added to each well read at 450nm immediately.

RESULTS

Results showed that administration of statin caused reduction in the mean serum concentrations of total cholesterol, triglyceride, high density lipoprotein cholesterol and low density lipoprotein cholesterol (Table 1). Administration of statin caused increased mean serum concentration of interleukin-2 and reduced mean serum concentration of tumour necrosis factor alpha (Table 2). There were negative correlations between serum concentrations of interleukin-2 and those of total cholesterol, triglyceride, high density lipoprotein cholesterol and low density lipoprotein cholesterol (Table 3). There was negative correlation between mean serum concentrations of triglyceride and tumour necrosis factor alpha (Table 3). There were positive correlations between mean serum concentrations of tumour necrosis factor alpha and those of total cholesterol, high density lipoprotein cholesterol and low density lipoprotein cholesterol (Table 3). The histogram compared the mean serum concentrations of the lipids and lipoproteins between rats given statin and the control (fig. 1). In figure 2, the mean serum concentrations of cytokines between control and rats given statin.

Table 1: Effects of administration of statin on serum concentrations of lipids and lipoproteins.

	Total cholesterol	Triglyceride	HDL-C	LDL-C
Control	41.98± 0.48	50.96 ± 0.4	12.34 ± 0.45	19.45 ± 0.29
Test	33.44 ± 1.36	50.76 ± 0.18	12.24 ± 0.37	17.05 ± 1.39

Table 2: Effects of administration of statin on serum concentrations of interleukin-2 and tumour necrosis factor alpha.

Groups	IL-2	TNF-α
Control	23.03 ± 4.82	3414 ± 252
Test	24.21 ± 3.72	1543 ± 944

Table 3: Correlation between serum lipids concentrations and cytokines.

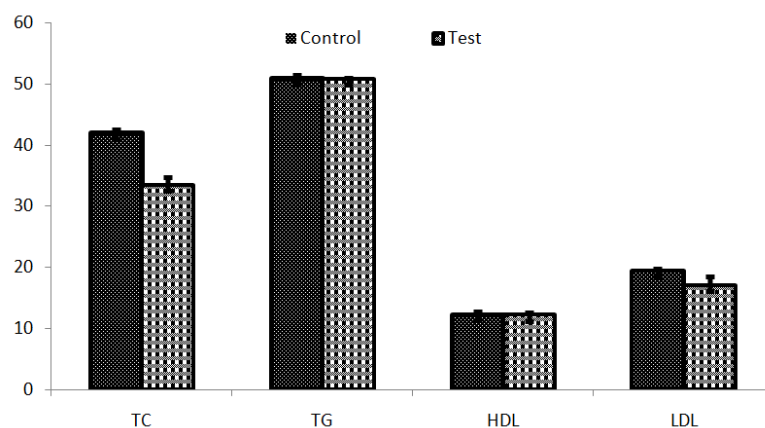|	IL-2	TNF-α
T.chol	-0.087	0.444
Triglyceride	-0.277	-0.003
HDL-C	-0.236	0.475
LDL-C	-0.014	0.307

Fig. 1: Comparisons of mean serum concentrations of lipids in both control and rats given statin.
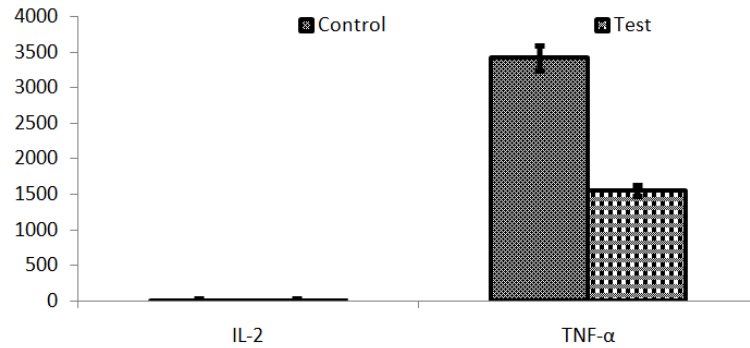
Fig. 2: Comparisons of mean serum concentrations of cytokines in both control and rats given statin.

DISCUSSION

Cholesterol is an important part of the human cells by serving as the building block of some hormones, membrane transport, fat metabolism etc [10]. The liver biosyntheses virtually all the cholesterol the body needs. However, a significant amount of cholesterol is introduced to the human body from dietary sources, such as animal-based foods like milk, eggs, and meat. Increase in blood cholesterol levels has been linked with clinical conditions, such as an increase in the risk of coronary artery disease, stroke and death [11]. Sequel to the clinical conditions that require the reduction of blood cholesterol levels, statin drugs are prescribed with the view of preventing heart related problems and stroke. Recent studies show that, in certain people, statin drugs reduce the risk of heart attack, stroke, and even death from heart disease by about 25% to 35% and/ reduce the chances of recurrent strokes or heart attacks by about 40% [12,13]. These statin drugs work by blocking the action of 3-hydroxy-3-methyl-glutarylCoA reductase (HMG-CoA reductase) in the liver, which is responsible for cholesterol biosynthesis *in vivo* [13].

The result obtained in the serum total cholesterol (TC), triacylglycerol (TG), low density lipoprotein cholesterol (LDL-C) and high density lipoprotein cholesterol (HDL-C) concentrations in the rats administered with statin drug (Table 1) is due to the statin drug. This result supported the previous reports of [14] and [12] that the administration of statin drugs reduced serum TC, LDL-C, the "bad" cholesterol, and triglycerides, while they had a mild effect in increasing HDL-C, the "good" cholesterol. Marked increases in the serum TC and LDL-C have been correlated with a buildup of plaque on the walls of the arteries that could eventually cause the arteries to narrow or harden, thereby, leading to the formation of blood clots in these narrowed arteries, which is the basis for a heart attack or stroke [11,12]. Recently, however, about half of all reported cases of heart attacks and strokes happen in apparently healthy people with LDL-C levels below the current level of concern. Therefore, the role of inflammation in the incidence of heart diseases and related clinical manifestations can't be overemphasized.

In this study, the administration of the statin drug to the rats recorded an increase in the serum interleukin 2 (IL-2) concentration (Table 2), which is an inflammatory marker. Increased serum IL-2 concentration is implicated in the induction of severe chronic hypocholesterolemia that is mediated by the inhibition of lecithin: cholesteryl acyltransferase (LCAT) activity [15,16]. Thus, the increased serum (IL-2) concentration could be responsible for the trends obtained in the serum TC and LDL-C (Table 1) concentrations in the rats administered with the statin drug. However, marked increases in serum IL-2 levels were reported to induce adverse cardiovascular and hemodynamic effects similar to septic shock, which can lead to hypotension, vascular leak syndrome, and myocardial infaction, cardiomyopathy, and thrombotic events such as deep vein thrombosis, pulmonary embolism and arterial thrombosis [17,18].

In addition, the induction of the release of IL-2 in the serum is always due to immunological responses triggered by inflammation in cells for the increased activities of T lymphocytes in containing the antigen (which could be oxidized LDL-C forming foam cells in the arteries or infected and dead cell as the case might be) [19]. However, the recorded increase in the serum IL-2 concentration in this study did not agree with the previous finding of [14] that the administration of Simvastatin decreased the concentration of IL-2 in hypercholesterolemic patients. This variation could be accounted for since the rats administered with Simvastatin in this study were not induced with hypercholesterolemia and markedly increased IL-2 levels are also known to induce hypocholesterolemia, as recorded in the study.

The marked reduction the serum tumour necrosis factor α (TNF-α) in rats administered with Simvastatin (Table 2) indicated that there was no induction of chronic inflammatory responses in the rats. This is because TNF-α modulates both cardiac contractility and peripheral resistance, which are the two most important haemodynamic determinants of cardiac function. Thus, increased levels of TNF-α or of its soluble receptors have been implicated in the pathophysiology of ischaemia-reperfusion injury, myocarditis, cardiac allograft and, more recently, also in the progression of congestive heart failure [20]. Therefore, the trend obtained in the serum IL-2 concentration could have been an immunological response (immunomodulation) to further enhance the activities of T lymphocytes in warding off infected or dead cells [21]. That is, the increased IL-2 concentration might be a boost in the immunological status of the rats and not probably of any physiological detriment.Conclusively, administration of statin drug, Simvastatin modulates inflammatory activities.

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