Measurement of malondialdehyde (MDA) level in rat plasma after simvastatin treatment using two different analytical methods

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Abstract

Background and Purpose: *The aim of this study was to investigate the effect of chronic administration of simvastatin (SIMV) on plasma malondialdehyde (MDA) level using two different methods. We also wanted to examine the plasma MDA level 10 days after the last administration of SIMV.*

Materials and Methods: *The first two groups of Wistar rats were given 10 mg/kg/day of SIMV and the third and fourth groups 50 mg/kg/day of SIMV for 21 days. Two control groups were on saline for the same period. Plasma MDA level was measured after the end of treatment and 10 days after the last dose. Two methods were used: UV-VIS spectrophotometric method and HPLC-MS method. Statistics: Kruskal-Wallis test and Steel test for post-hoc comparison with the control group. P values less or equal to 0.05 were considered as statistically significant.*

Results: *MDA levels in all groups, measured by both techniques, showed that SIMV treatment caused a dose-dependent decrease (significant in high dose) in plasma MDA level. The decrease in MDA level was also well-maintained for 10 days after the last administration of SIMV in both doses (significantly in high dose).*

Conclusion: *Both doses of SIMV decreased plasma MDA level after 21 day treatment and it remained decreased 10 days after the last dose, regardless of the measurement method used. These results showed that SIMV has antioxidant activity that persists after discontinuation of therapy.*

Abbreviations:

BHT	Butylated hydroxytoluene
DAD	Diode Array Detector
HPLC-MS	High Performance Liquid Chromatography – Mass Detection
LDL	Low-density lipoprotein
MDA	Malondialdehyde
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SIMV	Simvastatin
TBA	2-thiobarbituric acid
TCA	Trichloroacetic acid
WA	Wash-out period

INTRODUCTION

Oxidative stress is a term denoting an imbalance between the production of oxidants and respective defense systems of an organism *(1)*. Oxidants encompass reactive oxygen species (ROS), reactive nitrogen species (RNS), sulfur-centered radicals and others. Not all of these reactive species are arene-radicals, i.e. molecules with one (or more) unpaired electrons, but in many cases the reactive non-radical species will end up as radicals, damaging biomolecules by (one-electron) oxidation. The danger of this type of reaction is that the oxidation products formed are radicals themselves, which are in many cases able to propagate the reaction, leading to extensive damage. Oxidants can be generated in numerous ways, such as by ionizing radiation, by chemical reactions, enzymatically, through redox-catalysis involving free transition metal ions, or metal ions bound to enzymes. Important cellular sources of oxidative stress are: (a) the formation of reactive oxygen species by incomplete reduction of oxygen in the respiratory chain of mitochondria, and (b) host defense systems, which include the »*oxidative burst*« mediated by NADPH oxidase, producing superoxide radical, and myeloperoxidase, leading to the formation of hypochlorous acid *(2)*.

There is extensive evidence that links hypercholesterolemia with increased lipid peroxidation and increased oxidative stress *(3, 4)*. The oxidative modification of lipoproteins – particularly low-density lipoproteins (LDL) – has emerged as a fundamental process in the development of atherosclerosis *(5)*. Oxidatively modified LDLs, that have been heralded as an initiating factor in atherogenesis, possess numerous unfavorable biological effects, including the induction of endothelial dysfunction, activation of endothelial adhesiveness, monocyte differentiation and adhesion, and smooth muscle cell proliferation *(6–9)*. Several studies suggest the relationship between oxidized LDL and severity of atherosclerosis in coronary *(10)*, carotid or brachial arteries *(11, 12)*. Quantification of primary lipid peroxidation products (hydroperoxides) is difficult due to the unstable and reactive nature of these compounds *(13)*. Thus, the assessment of lipid peroxidation is usually performed by analyzing secondary oxidation products such as malondialdehyde (MDA). This distinctive compound has long been employed as a model compound for studying secondary degradation products of lipid peroxidation. The condensation of MDA with two molecules of 2-thiobarbituric acid (TBA) has been widely used to measure the extent of oxidative deterioration of lipids in biological and food systems *(14–16)*. The absorbance of the complex is usually measured by spectrophotometry or spectrofluorometry *(17)*, or by specific techniques, also based on TBA-adduct, where HPLC separation with spectrophotometric or spectrofluorometric or MS detection is employed. The level of oxidative stress in plasma and tissue usually correlates with MDA concentration (Scheme 1).

Statins have been used for treatment of hypercholesterolemia for a very long period. They lower the level of LDL due to specific, competitive, reversible inhibition of
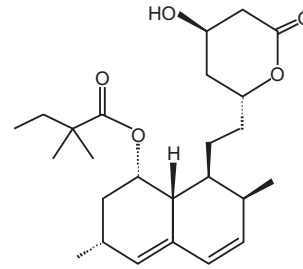

Figure 1. *Structures of the Simvastatin.*

3-hydroxyl 3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase). Some studies have shown the ability of statins to reduce the levels of circulating oxidized LDL or of circulating MDA as well *(18–20)*. The known member of statins is simvastatin (Figure 1), which has also shown antioxidant, anti-inflammatory and protective cardiovascular effects.

The aim of this study was to investigate the effect of simvastatin (SIMV) on plasma MDA level using two different analytical techniques, and to compare the results of these two analytical approaches in order to decide which technique is more reliable and accurate for further investigation of antioxidant action of statins. Because the plasma level of MDA indirectly indicates the degree of oxidative stress, it was of interest to measure the MDA level immediately after the discontinuation of SIMV treatment, and 10 days after the last dose (wash-out period). Additionally, the effect of SIMV on plasma MDA could indirectly show its protective effect on LDL oxidation.

MATERIALS AND METHODS

Test Substance. Simvastatin (CAS-*79902-63-9*) was obtained from Pliva Hrvatska d.o.o., Zagreb (Croatia) (Stateks® 20) and administered daily into stomach by oral gavage in two different doses (10 and 50 mg/kg body weight/day) in saline.

Treatment of animals

Forty-six male Wistar rats (Department of Pharmacology, School of Medicine, University of Zagreb, Zagreb, Croatia) weighing 170–200 g, were randomized in two control groups (n=7 each) treated with saline, and four experimental groups (n=8 each). The first two experimental groups were given a small dose of SIMV (10 mg/kg/day), and the third and fourth experimental groups were given a high dose of SIMV (50 mg/kg/day). The drug treatment period for all experimental groups was 3 weeks. On 21st day of treatment and following an overnight fast, the rats from one control group and from the first and third treatment groups were sacrificed under diethyl ether anesthesia, and blood sera were obtained directly from the heart. Plasma samples for measuring MDA were frozen immediately after sampling at –70 °C until further processing. Other groups of rats were sacrificed 10 days after the last administration of saline

(control) or SIMV (two experimental groups on small and high doses). The rats were housed in an air-conditioned room (20–22 °C) at 12 h light/12 h dark cycles, and were on standard diet (food for laboratory rats and mice manufactured by Mucedola s.r.l, Italia) and water *ad libitum*. Handling and treatment of the animals were conducted following the international guidelines regarding the use of laboratory animals. The experiments had been approved by the local ethics committee.

MDA

The level of MDA was measured spectrophotometrically on UV-VIS spectrophotometer (Hitachi, Japan) at 535 nm or by reverse phase HPLC-MS method with single ion monitoring technique. MDA levels are expressed as µmol/L concentrations using calibrating curves and expressed as percentage of decrease in MDA level compared to negative control.

Chemicals

The chemicals used for spectrophotometric and HPLC-MS determination of MDA-TBA: ammonium acetate, potassium dihydrogen phosphate and trichloroacetic acid (TCA) were obtained from Merck. Thiobarbituric acid (TBA) and butylated hydroxytoluene (BHT) were purchased from Aldrich. The solvents used in experiments and analysis were of analytical purity (p.a.) or HPLC grade purity purchased from Merck.

Spectrophotometric determination of MDA-TBA

The TBA assay was adopted from Angulo *et al.* (17) and Botsoglou *et al.* (21) and adapted for our purposes.

In the first step, an aliquot of plasma (250 mL) was transferred into a 5 mL Nunc CryotubeE (Polylabo, Geneva, Switzerland), followed by successive additions of 25 µL 0.2% BHT (dissolved in ethanol) and 1 mL 15% aqueous TCA. The mixture was then centrifuged (Universal 32 R, Hettich Zentrifugen) at 4000*g* for 15 minutes at 4 °C. The deproteinized supernatant (stock) was stored at –70 °C.

From that stock, an aliquot of 500 µL was transferred into a 5 mL Nunc CryotubeE and 1 mL TBA (0.375% in 0.25 M HCl) was added. It was then heated at 100 °C for 15 minutes. After cooling, the solution was analyzed by spectrophotometry using 1 cm absorption cell (Hitachi, Japan) at 535 nm.

HPLC-MS determination of MDA-TBA

A portion of the deproteinized stock was also used for HPLC-MS measurements.

A Waters HPLC-MS Quatro-Micro instrument equipped with a diode-array detector (DAD) was used for quantification. The analytical reverse phase (RPC-18) column (X-Terra) from Waters, 100 mm long and 4.6 mm wide with 5 µ particle size was used and the solvent was mixture of acetonitrile / water acidified by 0.01% formic acid. The analyses were conducted by injecting 1 µl of

sample on the column by autoinjector using programmed gradient mode which started from 5% water / 95% acetonitrile at the beginning of chromatography to 25% water / 75% of acetonitrile over 15 min period at the flow-rate of 1 mL/min. During the chromatography molecular ion for MDA-TBA adduct was simultaneously monitored, as well as UV-VIS absorbance at 536 nm. Data acquisition and processing were carried out with MsLynx software. The MDA derivative was quantitated based on the calibration curve previously measured for different MDA-TBA concentrations.

Statistical analysis

Data are shown as mean ± standard error of the mean. Data analysis was made using Kruskal-Wallis test and Steel test for *post-hoc* comparison with the control group. P values less or equal to 0.05 were considered statistically significant. All applied tests were two-tailed.

RESULTS AND DISCUSSION

The levels of MDA in all experimental groups (measured by both techniques) in comparison with their corresponding control show that SIMV treatment caused a dose-dependent decrease in plasma MDA level. Only the treatment with high dose of SIMV resulted in the significantly greater decrease of MDA level, which was the same regardless of the measurement method (Figures 2 and 3).

Spectrophotometric analysis of MDA level showed a decrease in plasma concentration by 9.0% and 39.0% (p<0.05) after the administration of SIMV in doses of 10 and 50 mg/kg/day during 3 weeks. According to our results, the decrease in MDA level was well-maintained for 10 days after the last administration of SIMV in both doses (Figure 2) (wash-out period). As shown in Figure 2, 10 days after the last small dose of SIMV, the decrease in MDA was greater (30%) in comparison with the MDA level measured after the last administration (after 3 weeks),
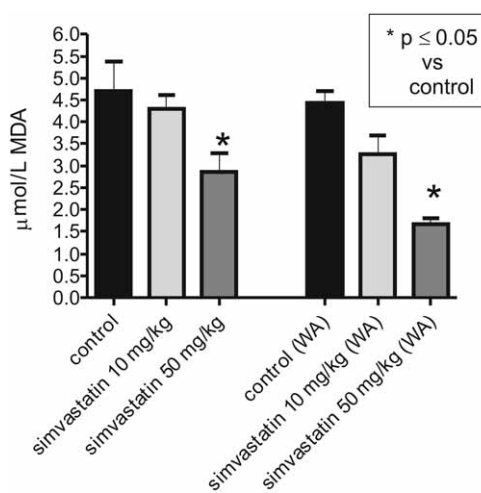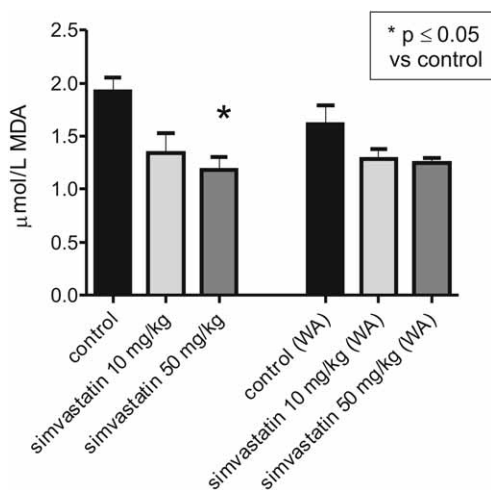

Figure 2. *Effect of Simvastatin treatment on MDA level in rat plasma measured by UV-VIS*

but insignificant. The decrease in the MDA level measured 10 days after the last administration of high dose was greater in comparison with the small dose, and significant (63%).

HPLC-MS analysis showed decrease in MDA level by 30.0% (small dose) and 38.3% (p<0.05) (high dose) after the 3 week SIMV treatment (Figure 3). It showed that the decrease in MDA level was also well-maintained for 10 days after the last administration of both doses, but insignificant (Figure 3, wash-out period).

Based on our results, we suggested that both methods, spectrophotometric method and HPLC-MS, give results that have similar direction, but with difference in magnitude and sensitivity. We suppose that this difference is due to the high specific quality of HPLC-MS which measures only MDA-TBA adduct, while UV-VIS method lacks that specificity and absorption and at wavelength of 536 nm

Figure 3. *Effect of Simvastatin treatment on MDA level in rat plasma measured by HPLC-MS.*
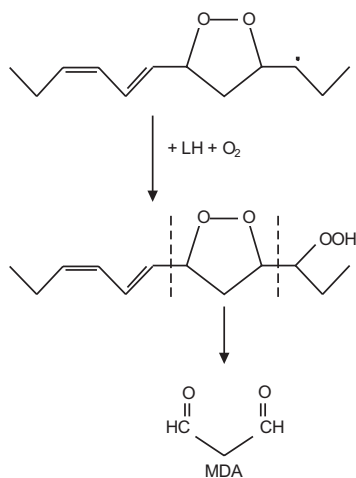
Scheme 1. *Mechanism of lipoxigenation of polyunsaturated fatty acids (LH).*

was affected by other UV-VIS active species present in plasma.

According to our data (Figure 2 and 3), SIMV decreases the plasma level of MDA. Because MDA is described as an oxidative marker, the results of this study indicate that the SIMV administered at high dose (50 mg/kg/day) significantly decreases the level of oxidative stress in the healthy rat.

Our results obtained by spectrophotometric measurement seem to agree with the results of Beltowski *et al.* *(22a)*, and Beltowski *et al.* *(22b)* who, using the same method, found that cerivastatin and fluvastatin caused a significant reduction in plasma concentration of MDA+ hydroxydialkenals (HAD) and lipid hydroperoxides. Plasma concentration of MDA+4-HAD in a group of rats on a low dose of cerivastatin (0.03 mg/kg/day for 21 days) tended to be lower than in the control, but the difference was not significant. In the high dose group (0.3 mg/kg/day for 21 days), MDA+4-HAD level decreased by 46.6% in comparison with the control *(22a)*. The same results were achieved with fluvastatin, which caused the plasma decrease of MDA+4-HAD level by 19.8% and 30.9% after administration of fluvastatin in doses of 2 and 20 mg/kg/day for 21 days *(22b)*, perspectively. Our results are also congruent with the results mentioned above in the fact that the action of SIMV, cerivastatin and fluvastatin on MDA is dose-dependent. In the *in vitro* experiments, Nakashima A *et al.* *(23)* found that fluvastatin and its metabolites had the potential to protect against oxidative stress mediated by several ROS, such as singlet oxygen, superoxide anion, hydroxyl radical, hypochlorite ion, and linoleic acid peroxide. These findings also support our results on the antioxidant action of simvastatin. We showed that the well-maintained decrease in plasma MDA level persisted 10 days after the last administration of SIMV (Figure 2 and 3). Due to this reason, we suggest that this extended action of SIMV on plasma MDA level is the new evidence about its antioxidant action. The effect of simvastatin on plasma MDA level which we showed in our experiments on rats is also coherent with a clinical report *of* Skrha *et al.* who observed a significant decrease in MDA after 3-month treatment of diabetic patients with combined hyperlipidemia *(24)*.

In conclusion, our results show that spectrophotometric method and HPLC-MS analysis give results that have similar direction. We showed a decrease in the plasma level of MDA immediately after the end of SIMV treatment in small and high doses for 21 days, regardless of the method used. Also the decrease in plasma MDA level was verified 10 days after the last dose. The only significant results were those obtained after the high dose treatment. Our results showed that SIMV has antioxidant activity that persists after therapy discontinuation.

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