THE INFLUENCE OF SIMVASTATIN AT HIGH DOSE AND DILTIAZEM ON MYOCARDIUM IN RABBITS, THE BIOCHEMICAL STUDY

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Abstract : 3-Hydroxy-3-methyl-glytaryl coenzyme A (HMG-CoA) reductase inhibitors ("statins") have been proved to be extremely useful in the management of hypercholesterolemia, as well as in prevention of primary and secondary coronary heart disease. However, they may produce rare but severe muscle-related symptoms such as myopathy and rhabdomyolysis. Recent findings *in vitro* have shown that statins can reduce cardiomyocyte viability. The exact mechanism of statin myotoxicity still remains unclear. Diltiazem as CYP3A4 inhibitor, is a well recognized risk factor of skeletal muscles myopathy, if co-administered with simvastatin. It is not known whether such interaction affects myocardial efficiency causing biochemical changes. The experiments were performed on thirty six New Zealand white rabbits. The animals were divided into four groups receiving: 0.2% MC (control group); diltiazem (5 mg/kg); simvastatin (50 mg/kg) or diltiazem + simvastatin, daily for 14 days (*po*). The following biochemical parameters were estimated: creatine kinase (CK), serum transaminases (ALT and AST), as well as myocardial injury markers: troponin I (TnI) and creatine kinase MB (CK-MB). Simultaneous administration of simvastatin and diltiazem caused 23-fold increase ($p < 0.01$), in rabbit serum CK levels and 20-fold increase ($p = 0.056$) in TnI levels, as compared to the initial values. Also in these rabbits significant increase in CK (12411,60 *vs* 839,87 IU/L) and TnI (0,26 vs 0,014 ng/mL), as compared to control group were observed. Significant increase in CK (12411,60 *vs* 1100,92 IU/L) and TnI (0,26 vs 0,012 ng/mL), as compared to diltiazem alone were noted, too. This may suggest another mechanism of drug-drug interaction than the one based on CYP3A4 inhibition if the impact on cardiac or skeletal muscle is considered.

Keywords: simvastatin, diltiazem, biochemical parameters, rabbits

3-Hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase inhibitors ("statins", HMGRI) have been proved to be extremely useful in the management of hypercholesterolemia, especially in cases of elevated concentrations of low-density lipoprotein cholesterol (LDL-C). Treatment with HMGRIs has a major impact on the prevention of primary and secondary coronary heart disease (1-5).

Statins are well-tolerated by most patients, but may produce a variety of muscle-related symptoms such as myopathy and rhabdomyolysis, accounting for 2 to 5% of all musculoskeletal effects (6, 7). However, the exact mechanism of statin myotoxicity still remains unclear. It is known, that the risk of statin-induced myopathy rises with plasma drug concentration. Thus, it may increase significantly, when HMGRIs, metabolized by the CYP3A4 izoenzyme, are prescribed concomitantly with other drugs inhibiting their metabolism *via* CYP3A4 pathway. The above mentioned interactions were shown for antifungal and immunosuppressive drugs or macrolides (8, 9). Co-administration of simvastatin and diltiazem or mibefradil has also been proved to increase the circulating simvastatin concentration (10-12). Moreover, case reports indicate the potentially fatal interaction with rhabdomyolysis of diltiazem and statins (11).

Recent findings *in vitro* show that statins can also damage myocardium. It has been demonstrated that HMGRIs, including simvastatin produced a dose-dependent reduction of cardiomyocyte viability with oncotic and apoptotic cell death, indicating the possibility that part of the mortality in rhabdomyolysis might be due to cardiac toxicity of statins (13-15). However, the correlation between possible statin cardiotoxicity assessed *in vitro* and cardiac efficiency with biochemical changes investigated *in vivo* has not been estimated so far. On the other hand a much bigger role has been assigned to the aggressive lowering of LDL-C, which is connected with higher doses of hypolipemic drugs, especially statins. Moreover, quite frequently, there is a need for combined administration of statins and diltiazem. Thus, the evaluation of the combined administration of these drugs is of high clinical importance for the myocardium efficiency, especially in the case of high dosage.

It has been suggested that changes of biochemical parameters might be the consequence of interaction on the izoenzyme CYP3A level. Previous findings have confirmed the functional similarity of intestinal CYP3A forms in rabbits and humans, suggesting that the rabbit is a beneficial *in vivo* model for the assessment of drug interaction occurring at the first pass of drugs ingested (16). Moreover, studies performed on rabbits, evaluating the pharmacokinetics of other drugs metabolized in humans *via* CYP3A4 pathway (i.e. diltiazem, saquinavir) (17, 18), have confirmed the usefulness of the rabbit model for such investigations.

The aim of the present experiment was to establish the influence of high dose simvastatin administered concomitantly with diltiazem on the myocardium of rabbits with biochemical examination.

MATERIALS AND METHODS

Drugs: simvastatin (series no. KY-SI-M20030102), diltiazem (Diltiazemi hydrochloridum,

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series no. 010203) both from "Polfarmex", Poland, methylcellulose (MC) (Fluka, Switzerland, series no. RB 13425).

The experiments were performed on thirty six outbred New Zealand white rabbits, both sexes, body weight 2.5-5 kg, fed on granulated mix "LSK" with free access to water. The animals were housed in standard cages, one animal per cage. The experimental procedures were carried out in accordance with international guidelines for care and use of laboratory animals. All efforts were made to minimize animal suffering and reduce the number of animals used in the experiments. All the procedures in these experiments were approved by the Ethics Committee of the Medical University of Łódź (Poland).

The animals were divided into four groups of rabbits, randomly allocated to every group: Group I: 0.2% MC, 1 mL/kg, *po* daily for 14 days (n = 9); Group II:: Diltiazem 5 mg/kg, *po* daily for 14 days (n = 6); Group III: Simvastatin 50 mg/kg, *po* daily for 14 days (n = 10); Group IV: Simvastatin 50 mg/kg + diltiazem 5 mg/kg, *po* daily for 14 days (n = 11).

All drugs, in doses used in the previous experiments in rabbits (19-22), were administered suspended in 0.2% MC, by oral gavage,.

Biochemical studies

Blood samples were obtained from a marginal ear vein on 1 and 14 day of drug administration in the volume of 1.5 mL. 1.5 mL of rabbit plasma was centrifuged for 5 min at 3000 rpm to obtain 700 μL of heparinized plasma.

Quantitative determination of the activity of serum transaminases (ALT and AST) and serum creatine kinase (CK), was performed using commercial spectrophotometric method, recommended by International Federation of Clinical Chemistry (IFCC) (23-24), using BIOLABO reagents. The change in absorbance resulting from the formation of NADH or NAD^+ in the following reactions, catalyzed by ALT, AST or CK enzymes was proportional to their activity in rabbit serum. The measurement of absorbance was performed at 37°C and $\lambda = 340$ nm.

Quantitative determination of serum cardiac troponin I and CK-MB concentration was performed using colorimetric immunoassay. As polyclonal antibodies specifically prepared against human cardiac troponin I have been shown to react with troponin I in the supernatants of heart homogenates of rabbits, cardiac troponin I was evaluated using a commercially available DADE-BEHRING reagent for human c-troponin I, in which monoclonal antibodies are conjugated with alkaline phoshatase acting on the fluorogenic substrate 4-methyl-umbelliferil-phosphate (25). The released chromogen was proportional to the amount of TnI. The measurement was performed at 37°C and $\lambda = 510$ nm.

As the sequences of human and rabbit CK-MB are very similar, CK-MB was measured in heparin plasma using a commercially available DADE-BEHRING reagent for human CK-MB, in which monoclonal antibodies specific for the CK-MB subunit and for the CK-MB izoenzyme are conjugated with a chlorophenol red β-D-galactopyranoside chromogenic substrate (26). The released chromogen was proportional to the amount of CK-MB. The measurement was performed at 37°C and $\lambda = 550$ nm.

Statistics

The statistical analysis of haemodynamic parameters was performed using the Statistica version 5.0 Statsoft program. The statistical evaluation was performed using analysis of variance (ANOVA) and *post-hoc* comparisons were performed using Duncan test. Normal distribution of a parameter was checked by means of Kolmogorov-Smirnov test with Lillieforce correction. The homogeneity of variance was tested by Levene'a test. If data were not normally distributed or the values of variance were different, ANOVA test Kruskal-Wallis and U Mann-Whitney's test were used. All parameters were considered statistically significantly different if $p < 0.05$.

RESULTS

Alanine aminotransferase (ALT)

Simultaneous administration of simvastatin and diltiazem caused increase in rabbit serum ALT activity, average by 86% as compared to the initial values. However, the observed changes were statistically insignificant.

These rabbits also showed significant ($p < 0.05$) increase in rabbit serum ALT activity as compared to rabbits receiving diltiazem alone. Some changes, bordering the statistical significance ($p = 0.053$), of serum ALT activity in rabbits receiving simvastatin with diltiazem as compared to diltiazem alone were observed, as well (Table 1).

Asparagine aminotransferase (AST)

After 14 days of simvastatin administration AST activity increased, on average by 30%, as compared to the initial values (day 1). The observed changes were statistically insignificant. These rabbits also showed a slight ($p = 0.072$) elevation of transaminase as compared to the control group.

Simultaneous administration of simvastatin and diltiazem caused insignificant ($p > 0.05$) increase in rabbit AST activity as compared to the initial values. No significant changes in serum AST levels were observed in rabbits receiving simvastatin with diltiazem as compared to placebo, simvastatin or diltiazem alone (Table 1).

Creatine kinase (CK)

After 14 days of simvastatin administration CK serum levels were markedly ($p < 0.05$) increased as compared to the initial values (day 1). These rabbits also showed a slight, but insignificant elevation of CK as compared to the control group.

Simultaneous administration of simvastatin and diltiazem caused significant, average 23-fold ($p < 0.01$) increase in rabbit CK activity as compared to the initial values.

In these rabbits CK activity levels were markedly ($p < 0.01$) increased as compared to rabbits receiving placebo. The significant ($p < 0.05$) increase in CK levels as compared to rabbits receiving diltiazem alone was observed, as well. Simultaneous administration of sim-

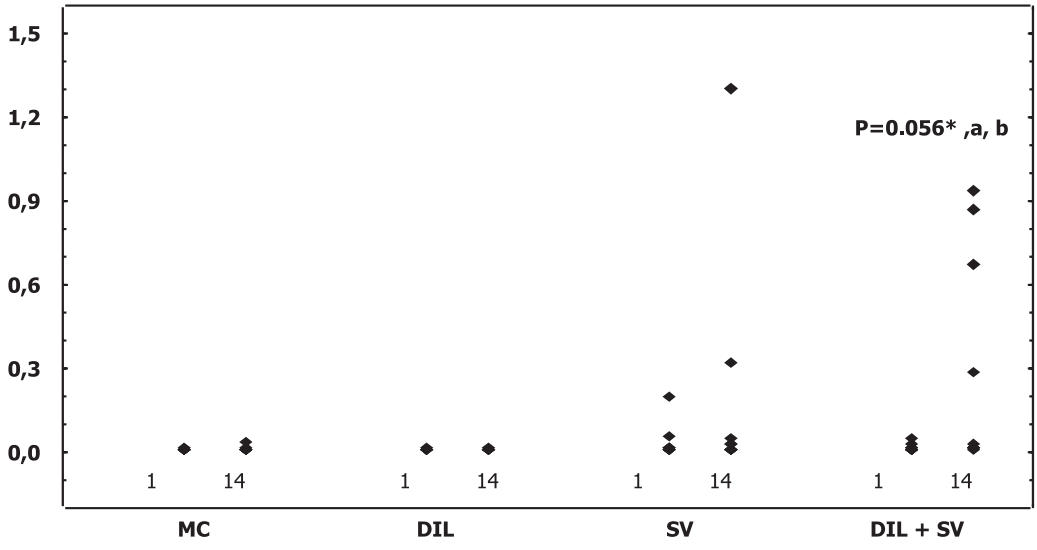

Figure 1. The influence of simultaneous administration of simvastatin and diltiazem on serum TnI concentration. (*) in comparison to the initial values (day 1); (a) $p < 0.01$, in comparison to the control group. MC – control group receiving methylcellulose; DIL – diltiazem, SV – simvastatin; DIL + SV – diltiazem + simvastatin.

vastatin and diltiazem caused increase in CK activity ($p = 0.07$) as compared to rabbits receiving simvastatin alone (Table 1).

Troponin I (TnI)

14–Day simvastatin administration resulted in slight elevation of troponin I as compared to the initial values (day 1). However, the observed changes were statistically insignificant. These rabbits also showed an insignificant changes of TnI levels as compared to the control group.

Simultaneous administration of simvastatin and diltiazem caused 20-fold increase ($p = 0.056$) in TnI activity as compared to the initial values. These rabbits also showed marked ($p < 0.01$) elevation of troponin I as compared to rabbits receiving diltiazem alone or placebo (Figure 1; Table 1).

Creatine kinase MB (CK-MB)

14–Day administration of simvastatin alone or with diltiazem did not result in significant elevation of CK-MB as compared to the initial values (day 1). Simultaneous administration of simvastatin and diltiazem caused insignificant increase in CK-MB level as compared to simvastatin alone. No other marked changes of CK-MB level were noted (Table 1).

DISCUSSION

The aim of the present study was to establish the influence of high dose simvastatin on cardiac efficiency evaluating the biochemical results of interaction with diltiazem.

In our study, simvastatin administration markedly increased CK activity. This confirmed previous findings (22) describing simvastatin-induced myopathy on rabbit model. Moreover, simultaneous administration of simvastatin and diltiazem resulted in marked elevation of serum CK levels as compared to diltiazem alone. CK is non-specific marker of skeletal muscle injury. Thus, the increased CK activity in rabbit serum may suggest the possibility of further myopathy development. 14-day simvastatin administration caused significant increase in ALT, but not AST levels as compared to the control group. Similar results were obtained by others (22).

Combined administration of HMGRI and CCB caused a significant increase in TnI level. Moreover, three rabbits showed over 1 ng/mL in the TnI level, 100 times over the normal limit. The obtained results were similar to the values noted by others (27) in rabbits with cardiac necrosis. Troponin I is specific marker of cardiac injury, in ischaemic coronary syndromes or myocarditis. It is not expressed in regenerating human skeletal muscles and is not found in the serum of subjects with chronic skeletal muscle damage unless concurrent heart muscle injury was also present Also, TnI levels remain unchanged during renal insufficiency (28-30). Thus, the obtained results could suggest myocardial injury induced by simultaneous simvastatin and nifedipine administration. On the other hand, the markedly unchanged CK-MB levels in terms of combined simvastatin and diltiazem administration as well as pathogenic background of the observed changes remain questionable.

Diltiazem is a recognized risk factor of statin-induced myopathy of skeletal muscles. As an inhibitor of the human liver microsomal enzyme CYP3A4, by which simvastatin is metabolized as well, diltiazem significantly increases the mean peak serum concentration of simvastatin (by 3,6-fold) as well as it increases the area under the serum concentration-time curve (by 5-fold) (10). Pharmacokinetic interaction between statin and diltiazem is known to enhance the pharmacodynamic effect of simvastatin (31) and may lead to increased risk of statin side effects, including myopathic symptoms. On the other hand, the magnitude of this

Table 1. The influence of simultaneous administration of simvastatin and diltiazem on serum ALT, AST, CK activity and serum TnI, CK-MB concentration. Each value represents the mean ± SEM.

Group	No. of rabbits	ALT (IU/L)		AST (IU/L)		CK (IU/L)		TnI (ng/mL)		CK-MB (ng/mL)	
		Day-1	Day-14	Day-1	Day-14	Day-1	Day-14	Day-1	Day-14	Day-1	Day-14
MC (0.2%)l	9	48.42 ±6.86	56.20± 6.84	38.59 ± 6.67	51.44± 4.04	820.61 ± 73.03	839.87 ± 145.85	0.011± 0.001	0.014±0.003	0,74 ± 0,551	1,80 ± 0,58
DIL	6	56.40 ± 17.50	41.67±4.86	59.1 ± 5.34	74.78± 9,53	853.75 ± 161.46	1100.92 ± 136.59	0.012± 0.002	0.012±0.002	2,40 ± 0,98	1,38 ± 0,39
SV	10	68.56 ± 12.90	99.47± 15.07 *, *a*	71.49 ± 25.72	92.89 ± 21.61 *p = 0.072*^	276.76 ± 75.09	2975.69 ± 1484.40*	0.037 ±0.019	0.180±0.130	0,42 ± 0,13	2,23 ± 1,38
DIL + SV	11	58.75 ± 9.85	109.17 ± 31.15 *b, p = 0.053*^	58.3 ± 14.55	78.99± 18,77	537.79 ± 53.35	12411.60± 6553.65* *a, b, p = 0.067***	0.012 ±0.004	0.26 ± 0.01 *p = 0.056*, a, b*	0,84 ± 0.20	5,40 ± 1,84

(*) $p < 0.05$ in comparison to the initial values (day 1); (a) $p < 0.05$, (^) in comparison to the control group; (b) $p < 0.05$ in comparison to nifedipine alone; (c) $p < 0.05$ in comparison to nifedipine alone, (**) in comparison to simvastatin.

MC – control group receiving methylcellulose; DIL – diltiazem, SV – simvastatin; DIL+SV – diltiazem + simvastatin

interaction is smaller than observed with the CYP3A4 inhibitors such as itraconazole (9) or mibefradil, a selective T-type calcium channel blocker inhibitor which has been removed from the market because of adverse interactions with co-administered CYP3A4 substrates (32).

Another point is that recent findings *in vitro* show that statins can damage myocardium. It has been demonstrated that HMGRIs, including simvastatin produced a dose-dependent reduction of cardiomyocyte viability with oncotic and apoptotic cell death (13-15). Moreover, the authors indicated the possibility that part of the mortality in rhabdomyolysis might be due to cardiac toxicity of statins. One of the postulated mechanisms of statin-induced myopathy involves intracellular calcium overload. Nakahara et al. (33) revealed that simvastatin and simvastatin acid elevated [Ca^{2+}] in L6 rat myoblasts. The authors suggested that it may lead to skeletal muscle cell damage and myopathy. Similarly, intracellular [Ca^{2+}] increase in cardiomyocytes was reported as well (7). Chronic [Ca^{2+}] elevation may contribute to apoptosis induction by cellular proteases (caspases) activation (34). It has been demonstrated that HMGRIs may induce cardiac toxicity via activation of proapoptotic factors: caspase-2 and caspase-3 (35). The exact relationship between [Ca^{2+}] elevation and caspase activation has not been elucidated, however, caspase-3 activation was demonstrated in myocardial ischaemic injury and cardiomyopathy (36, 37).

Diltiazem, by inhibition of smooth muscle L-type calcium channels, decreases mainly intracellular calcium concentration in smooth muscle cell, having also some impact on those of the heart [38-39]. However, if Ca^{2+} elevation induced by statins plays a role in myotoxity development, the cause of serum TnI level elevation, suggesting the worsening of myocardium efficiency, remains questionable. Especially, that rather the positive impact on the damaged myocardium performance, resulting from inhibition of Ca^{2+} inflow to the ischaemic myocardium and mitochondrial damage prevention, is already known.

We suggest that the influence of diltiazem, co-administered with simvastatin, on myocardium might be rather the negative consequence of CYP3A4 inhibition, than the impact on calcium signaling. On the other hand, we must note that the further development of skeletal muscle injury as compared to rabbits receiving simvastatin alone and expressed by increase in CK activity, was accompanied by quite small elevation of TnI – cardiac injury indicator.

The limitation of the extrapolation of our data to humans is the relatively high dose of simvastatin used in our experiments. However, the aim of the present study was to assess whether simvastatin co-administered with CYP3A4 inhibitor, in the dose provoking myopathy of skeletal muscles, influence on biochemical markers, especially cardiac injury ones. Thus, dose and dosage of simvastatin (50 mg/kg per day, 14 d) were established based on the previous experimental simvastatin-induced myopathy rabbit model (20-22).

The role of HMGRIs in the primary and secondary prevention of ischaemic heart disease is well established. However, the mechanism of the possible highest statin affinity to skeletal muscle in comparison to cardiac muscle in the development of HMGRI myotoxicity has not

been determined. In present study, performed on rabbit myopathy model, diltiazem, CYP3A4 inhibitor and calcium channel blocker, co-administered with simvastatin given in myopathy-induced dose, increased CK and TnI levels as compared to simvastatin alone. It indicates the possibility of further development of skeletal muscle injury accompanied by rather less significant negative changes of myocardium. However, it remains questionable, why concomitant administration of simvastatin and diltiazem showed any changes in TnI level. Further studies involving smaller doses of simvastatin are needed.

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