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European Journal of Pharmacology 364 (1999) 43–48

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Effects of HMG-CoA reductase inhibitors on excitation–contraction coupling of rat skeletal muscle

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Received 23 September 1998; accepted 3 November 1998

Abstract

3-Hydroxy-3-methyl glutaryl coenzyme A (HMG-CoA) reductase inhibitors currently used as cholesterol-lowering drugs produce side effects in patients, one of which is myopathy. In the present study we compared the effect of a 3-month chronic treatment with two different compounds, simvastatin and pravastatin, on the excitation–contraction coupling of rat skeletal muscle fibers, the mechanism which links membrane depolarization to the movements of cytosolic Ca^{2+} from intracellular stores. The voltage threshold for mechanical activation of extensor digitorum longus muscle fibers in response to depolarizing pulses of various durations was studied in vitro by the two intracellular microelectrode method in 'point' voltage clamp mode. Simvastatin (5–50 mg/kg) modified the mechanical threshold of striated fibers in a dose-dependent manner. The muscle fibers of rats treated with 10 mg/kg and 50 mg/kg of simvastatin needed significantly less depolarization to contract than did untreated fibers at each pulse duration, suggesting that levels of cytosolic Ca^{2+} were higher. Consequently, the rheobase voltage for fiber contraction was significantly shifted toward more negative potentials with respect to controls by 2.4 mV and 7.1 mV in the 10 mg/kg and 50 mg/kg simvastatin-treated animals, respectively. Pravastatin treatment at 100 mg/kg did not produce any alteration of excitation–contraction coupling since the rheobase voltage was similar to that of controls. The different physicochemical properties of the two drugs may underlie the different effect observed because lipophilic agents, such as simvastatin, have been shown to affect sterol biosynthesis in many tissues, whereas the hydrophilic pravastatin is hepato-selective.

Keywords: Simvastatin; Pravastatin; Skeletal muscle; Excitation–contraction coupling

1. Introduction

Hypercholesterolemia is the major risk factor for atherosclerosis and coronary heart disease (Sirtori, 1993; Brown et al., 1993). Therapeutic strategies involving the use of lipid-lowering drugs, such as 3-hydroxy-3-methyl glutaryl coenzyme A (HMG-CoA) reductase inhibitors (i.e., simvastatin, pravastatin, lovastatin), are useful in the prevention of dyslipidemias because they cause a large reduction in total and low-density lipoprotein (LDL) cholesterol levels (Grundy, 1988; Blum, 1994). One of the most feared side effects of all such drugs in clinical use is myopathy, characterized by rhabdomyolysis due to extensive muscle fiber necrosis, with clinical signs of weakness and pain (Sirtori, 1993). Although controversial data exist, there are reports showing that hepatoselective drugs, such as pravastatin and fluvastatin, have fewer myopathic effects (Reijneveld et al., 1996; Hamelin and Turgeon, 1998), unless they are used in combination with fibric acid and/or immunosuppressive agents (Pierce et al., 1990; Smith et al., 1991; Kobashigawa et al., 1995). The pharmacological basis for the muscle toxicity of HMG-CoA reductase inhibitors is however largely unknown. Myopathic reactions have been previously observed with other hypocholesterolemic drugs such as clofibrate. This drug and its in vivo metabolite, clofibric acid, may indeed produce myotonia in the rat by lowering muscle membrane Cl^- conductance (GCl) (Conte Camerino et al., 1984; De Luca et al., 1992). This conductance maintains the electrical stability of the sarcolemma (Cannon, 1996; Lehmann-Horn and Rüdel, 1996). It is known that genetic mutations of the main skeletal muscle Cl^- channel, which sustains the macroscopic GCl, are responsible for the hyperexcitability

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0014-2999/99/$ - see front matter
PII: S0014-2999(98)00817-6

characteristic of myotonic muscles (George, 1995). Prolonged chronic treatment of rats with high doses of different HMG-CoA reductase inhibitors has shown that simvastatin, a lipophilic compound, unlike the hydrophilic pravastatin, reduces GCl and can give rise to myotonic symptoms; however, the drug was tested at doses far exceeding those used clinically (Pierno et al., 1995). We ascribed the different result to the diversity in the physicochemical characteristics of the two drugs, characteristics that regulate their tissue distribution and consequently the target of action and their potential toxicity. Both drugs show similar strong inhibition of cholesterol biosynthesis in the liver, but pravastatin does not affect this biosynthesis in extrahepatic tissues (Komai and Tsujita, 1994). In contrast, simvastatin reduces cholesterol biosynthesis by over 40% in almost all tissues (Koga et al., 1990; Sirtori, 1993), including skeletal muscle, where the drug may interfere with the structural and functional integrity of the sarcolemma and other intracellular membranes. In this regard it has recently been proposed that simvastatin, but not pravastatin, may be cytotoxic for muscle function since it increases cytosolic Ca^{2+} in cultured rat myoblasts (Nakahara et al., 1994). It is known that in skeletal muscle the Ca^{2+} necessary for contraction is released from the sarcoplasmic reticulum in response to electrical signals from the sarcolemma triggered by the voltage sensor of dihydropyridine receptors (Rios and Pizarro, 1991). The decrease in the membrane cholesterol content induced by lipophilic HMG-CoA reductase inhibitors may influence the function of cellular membranes, including that of intracellular organelles, such as the sarcoplasmic reticulum, thereby altering the mechanisms involved in Ca^{2+} movements. On the basis of the above findings, the aim of the present study was to evaluate the effects of pravastatin and simvastatin on the excitation–contraction coupling mechanism, by measuring the voltage threshold for contraction of rat skeletal muscle fibers.

2. Materials and methods

2.1. In vivo studies

Adult male Wistar rats (Charles River Laboratories, Calco, Italy), weighing 200–250 g at the beginning of the treatment, were used. Three different groups of animals were treated with simvastatin at doses of 5–10–50 mg/kg, respectively. A 4th group of animals was treated with 100 mg/kg of pravastatin and a 5th group was used as control. Appropriate suspensions of simvastatin (kindly provided by Merck Sharp and Dohme, West Point, PA, USA) and pravastatin (kindly provided by Bristol-Myers Squibb, Rome, Italy) were prepared in 0.5% aqueous methylcellulose (1500 mg/20 ml for pravastatin and 350 mg/12 ml for simvastatin) and administered to animals orally via an esophageal cannula, once a day, for 3 months. For each rat, the weight-related dose was formulated so that the volume of pravastatin- or simvastatin-containing solutions was 0.3–1 ml. Control animals were dosed in a similar fashion with the vehicle (aqueous methylcellulose) only (Pierno et al., 1995).

To evaluate skeletal muscle performance the righting response (the ability of the rat to straighten itself on four legs when turned on its back) was assessed daily in each rat throughout the treatment period (Pierno et al., 1995).

2.2. In vitro electrophysiological measurements

The electrophysiological experiments were performed in vitro with extensor digitorum longus muscle at the end of the treatment period. Muscles were removed under urethane anesthesia (1.2 g/kg, i.p.) from both treated and control rats. Soon after dissection, while they were still anesthetized, the rats were euthanized by a urethane overdose. Muscle preparations were immediately placed in a temperature-controlled muscle chamber at 30°C and perfused with normal physiological solution of the following composition (mM): NaCl 148; KCl 4.5; $CaCl_2$ 2.0; $MgCl_2$ 1.0; NaH_2PO_4 0.44; $NaHCO_3$ 12 and glucose 5.55. The physiological solution was continuously bubbled with 95% O_2 and 5% CO_2 and the pH was carefully maintained at 7.2–7.3 (Pierno et al., 1995).

The mechanical threshold of the fibers was determined by using the two microelectrode 'point' voltage-clamp method as previously described (Dulhunty, 1988; Heiny et al., 1990; De Luca et al., 1996). A voltage-sensing electrode (3 M KCl) and a current-passing electrode (2 M potassium citrate) were inserted within 50 μm of each other into a randomly selected superficial fiber which was continuously viewed under a stereomicroscope (100× magnification). The holding potential was set at −90 mV and depolarizing command pulses of variable duration were given at a rate of about 0.3 Hz. During recordings tetrodotoxin (3 μM) was added to prevent action potential generation (Dulhunty, 1988; Heiny et al., 1990; De Luca et al., 1996). As a standard protocol, the command pulse duration was set sequentially to each of the following values: 500, 50, 5, 200, 20, 100 and 10 ms. At each duration, the command voltage was increased by using an analogue control until contraction was just visible and then backed down until the contraction disappeared. A digital sample-and-hold millivoltmeter stored the value of the threshold membrane potential at this point. We estimated the uncertainty of any single measurement for a given fiber to be 1–2 mV. The measurements were performed for all experimental conditions in an identical manner, with about the same length of time involved in each determination so as to exclude any effect on the mechanical threshold of intracellular citrate ions from the electrodes (Dulhunty, 1988). The threshold membrane potential V (mV) for each fiber was averaged at each pulse duration t (ms) and then the mean values were plotted against duration, giving a

'strength–duration' relationship. A fit estimate of the rheobase voltage (R) and of the time constant to reach the rheobase was obtained by using a nonlinear least-squares algorithm and the following equation: $V = [H - R \exp(t/\tau)]/[1 - \exp(t/\tau)]$; where H is the holding potential (mV), R is the rheobase (mV) and τ is the time constant (Miledi et al., 1983; De Luca and Conte Camerino, 1992). In the fitting algorithm, each point was weighted by the reciprocal of the variance of that mean V, and the best fit of the parameters R and τ were estimated. We used this procedure in order to be able to incorporate all of our determination points and their associated errors into our estimate of R under each condition. The standard error (S.E.) of the fitted rheobase values were determined from the variance–covariance matrix in the nonlinear least-squares fitting algorithm.

The statistical significance of the fitted rheobase values and their differences from each other were estimated by a Student's t-distribution, using a number of degrees of freedom equal to the total number of threshold values minus the number of means minus two for the free parameters. One-way analysis of variance (ANOVA) was used to compare the threshold membrane potentials at each pulse duration between each experimental group and the control group (Tallarida and Murray, 1987).

3. Results

3.1. In vivo studies

As previously reported (Pierno et al., 1995), the righting reflex was normal in all rats treated with either pravastatin or simvastatin at all the doses tested, during the whole period of treatment. Moreover, none of the animals showed any adverse physical sign or mortality, even after administration of the higher doses of the two HMG-CoA reductase inhibitors.

3.2. In vitro studies: effects of simvastatin and pravastatin treatment on the mechanical threshold of rat skeletal muscle fibers

The threshold potential for contraction of extensor digitorum longus muscle fibers from both control and HMG-CoA reductase inhibitor-treated rats showed the typical dependence on command pulse duration, in that the threshold potential was more negative the longer the duration of the pulse. Under the experimental conditions used in the present study ($t = 30$°C and rate of about 0.3 Hz), a constant rheobase value was almost always reached with the longest pulse duration used, as commonly seen with mammalian muscle fibers (De Luca and Conte Camerino, 1992). However, simvastatin treatment affected, in a dose-dependent manner, the mechanical threshold of the fibers. Indeed, at each pulse duration, muscle fibers of rats treated with the highest dose of simvastatin (50 mg/kg) needed a significantly smaller depolarization to contract with respect to that of controls (Table 1). Consequently, the strength–duration curve constructed as described in Section 2 was shifted toward more negative potentials in the 50 mg/kg simvastatin-treated rats with respect to that of untreated controls (Fig. 1). This curve was based on the mean results from five animals, all of which were significantly affected

Table 1
Effects of chronic treatment with pravastatin and simvastatin on the mechanical threshold of rat extensor digitorum longus muscle fibers

Experimental condition	Dose	Duration (ms)						
		5	10	20	50	100	200	500
Mean 4 Control rats		−43.5 ± 1.2 (20)	−55.1 ± 1.5 (17)	−58.0 ± 1.6 (20)	−59.0 ± 1.2 (24)	−60.0 ± 1.4 (17)	−62.0 ± 0.8 (20)	−63.1 ± 0.6 (26)
Mean 5 Pravastatin-treated rats	100 mg/kg	−43.3 ± 0.8 (45)	−55.6 ± 0.9 (40)	−58.7 ± 0.8 (43)	−59.1 ± 0.6 (48)	−61.2 ± 0.6 (41)	−62.8 ± 0.5 (45)	−63.3 ± 0.4 (48)
Mean 3 Simvastatin-treated rats	5 mg/kg	−44.6 ± 1.6 (18)	−54.2 ± 1.5 (16)	−60.5 ± 1.4 (18)	−60.9 ± 1.3 (22)	−61.1 ± 1.4 (21)	−62.0 ± 1.6 (25)	−62.0 ± 1.1 (32)
Mean 3 Simvastatin-treated rats	10 mg/kg	−45.6 ± 1.3 (19)	−56.0 ± 1.8 (16)	−59.9 ± 1.7 (17)	−63.9 ± 1.6 (22)	−64.0 ± 2.1 (19)	−65.3 ± 1.5 (19)	−66.0 ± 1.2 (26)
Mean 5 Simvastatin-treated rats	50 mg/kg	−48.0 ± 1.1 (33)	−60.0 ± 1.0 (29)	−66.0 ± 0.9 (32)	−68.0 ± 1.2 (37)	−68.0 ± 1.1 (29)	−70.0 ± 0.8 (31)	−70.0 ± 0.6 (43)

The columns from left to right are as follows: Experimental conditions: fibers sampled from extensor digitorum longus muscles of control, pravastatin- and simvastatin-treated rats. For each experimental condition are shown the threshold membrane potential values obtained with a depolarizing command pulse of duration ranging from 5 ms up to 500 ms. The values are expressed as means ± S.E.M. from the number of fibers shown in brackets below each value. Statistical differences between treated and control rats were evaluated by one-way ANOVA. Rats treated with 10 mg/kg simvastatin showed significant differences vs. untreated controls at 50 ms ($F(1,44) = 8.74$, $P < 0.01$), 200 ms ($F(1,37) = 5.03$, $P < 0.05$) and 500 ms ($F(1,50) = 5.39$, $P < 0.05$). Rats treated with 50 mg/kg simvastatin showed significant differences vs. untreated control rats at all the duration tested: 5 ms ($F(1,51) = 5.51$, $P < 0.025$), 10 ms ($F(1,44) = 5.00$, $P < 0.05$), 20 ms ($F(1,50) = 15.89$, $P < 0.005$), 50 ms ($F(1,59) = 195.6$, $P < 0.005$), 100 ms ($F(1,44) = 12.9$, $P < 0.005$), 200 ms ($F(1,49) = 16.22$, $P < 0.005$) and 500 ms ($F(1,67) = 5.09$, $P < 0.05$). No significant differences were observed between the rats treated with 5 mg/kg simvastatin or 100 mg/kg pravastatin vs. control rats.

by the treatment. In rats treated with 10 mg/kg of simvastatin the threshold membrane potentials at all the pulse durations tested were more negative with respect to the control values and at pulse durations of 50, 200 and 500 ms the differences were statistically significant (Table 1). The voltage at rheobase estimated from the fit of the available experimental points was -69.0 ± 0.39 mV and -64.3 ± 0.7 mV for 50 mg/kg and 10 mg/kg simvastatin-treated rats, respectively, and both values were significantly different with respect to the control value, which was -61.86 ± 0.41 mV. The time constant (τ) to reach the rheobase, obtained from the fit, was also significantly increased from 5.50 ± 0.26 ms in controls to 7.53 ± 0.33 ms in 50 mg/kg simvastatin-treated rats. However the time constant recorded for the 10 mg/kg simvastatin-treated rats was not significantly different with respect to the control value, being 5.94 ± 0.38 ms (Fig. 2).

Lower doses of simvastatin (5 mg/kg) did not affect the mechanical threshold for contraction (Table 1). As a consequence, the rheobase voltage and the time constant did not differ with respect to control values, being -61.27 ± 0.6 mV and 5.2 ± 0.35 ms, respectively (Fig. 2).

Pravastatin treatment at 100 mg/kg did not alter the mechanical threshold of any of the 5 treated rats and the mean values were similar to those of controls (Table 1; Fig. 1). As expected, neither the rheobase voltage (-61.84 ± 0.24 mV) nor the time constant (5.5 ± 0.17 ms) differed from that of controls (Fig. 2).

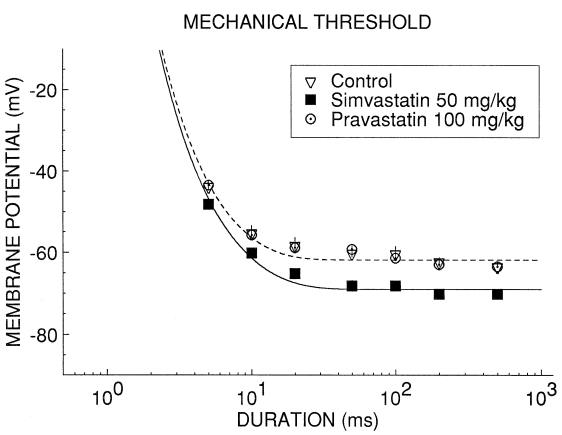

Fig. 1. Strength-duration curves for threshold of mechanical activation of extensor digitorum longus muscle fibers from control (4 muscle preparations), 50 mg/kg simvastatin-treated (5 muscle preparations) and 100 mg/kg pravastatin-treated (5 muscle preparations) rats. At each pulse duration the threshold voltage was determined in 17–48 fibers and the mean ± S.E.M. values were plotted against duration. Smooth curves were fitted to the points by a nonlinear least-squares algorithm, as described in Section 2. The voltage at rheobase estimated from the fit was -61.86 ± 0.41 mV for control rats, -61.84 ± 0.24 mV for pravastatin-treated rats and -68.99 ± 0.39 mV for simvastatin-treated rats. The difference between control and simvastatin-treated rats, determined by one-way analysis of variance (ANOVA), was statistically significant at each duration ($P < 0.05$ or less).

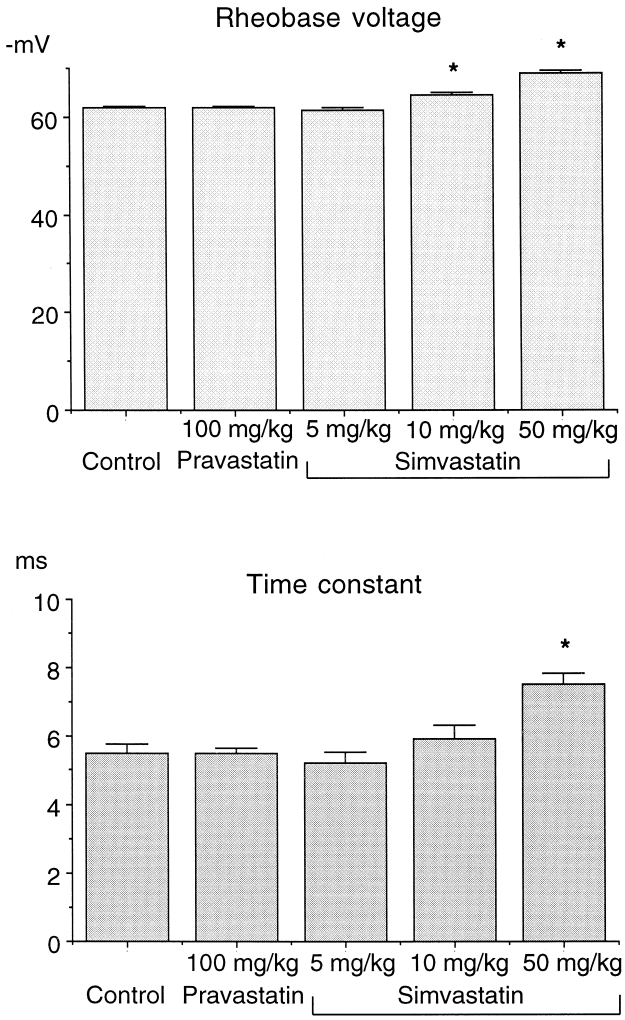

Fig. 2. Effects of chronic treatment with pravastatin (100 mg/kg) and simvastatin (5–50 mg/kg) on the rheobase voltage and the time constant of rat extensor digitorum longus muscle fibers. The values ± S.E. were obtained from the fit of the experimental points. The number of fibers was calculated as described in Section 2. * Significantly different from untreated control ($P < 0.005$ or less) by Student's *t*-test.

4. Discussion

The present results showed that simvastatin administration affects dose dependently the mechanical threshold of rat skeletal muscle fibers. The rheobase voltage for contraction was significantly more negative in the rats treated with simvastatin than it was in untreated controls, especially in those rats treated with the highest doses tested. It should be underlined that the doses of the drugs used in the present study were far higher than those used clinically to lower cholesterol levels. However pravastatin, even at a dose exceeding the therapeutic dose (100 mg/kg), did not modify this parameter; indeed the rheobase voltage was not different from that of control fibers. The changes in the

rheobase voltage for contraction observed in the rats treated with simvastatin are consistent with a modification of the excitation–contraction coupling and then of muscle contractility. In fact, the rheobase, the lowest voltage threshold needed to elicit contraction, is shifted in a negative direction by any pathological or pharmacological intervention that either increases the release of Ca^{2+} from the sarcoplasmic reticulum to a greater extent than it affects uptake, or reduces the uptake of Ca^{2+} (Dulhunty, 1988). One such pathophysiological situation is aging. In fact, the shift in the mechanical threshold toward negative potentials observed by us in extensor digitorum longus muscle from aged rats is correlated to the aging-induced decrease in the Ca^{2+} transport activity of sarcoplasmic reticulum described by others (Larsson and Salviati, 1989; Lompré et al., 1991; De Luca and Conte Camerino, 1992). Moreover, drugs able to increase the cytosolic Ca^{2+} levels, such as the Ca^{2+} ionophore A23187, also shift the mechanical threshold toward more negative potentials (Morgan and Bryant, 1977).

With regard to the mechanism through which simvastatin exerted the observed effect on the mechanical threshold, it is likely that the lipophilic nature of simvastatin vs. the hydrophilic nature of pravastatin plays a role. Lipophilic drugs, such as simvastatin, have easy access to the intracellular space of skeletal muscle fibers and can directly inhibit tissue sterol synthesis (Komai and Tsujita, 1994). Cholesterol has an essential role in modulating membrane fluidity in mammalian cells, and small changes in the ratio between cholesterol and phospholipids may be sufficient to induce significant perturbations in membrane properties (Goldstein and Brown, 1990; Ng et al., 1994). Such a mechanism has been already proposed to account for the different effect of the two compounds on the electrical properties of rat skeletal muscle fibers and in particular on GCl, were simvastatin produces a significant lowering of this parameter compared with the effect of pravastatin (Conte Camerino et al., 1984; Pierno et al., 1995). In a previous study we ruled out the possibility that a decrease in GCl, by modulating the voltage sensitivity in the transverse tubular membranes, could directly affect the threshold membrane potential for contraction (De Luca et al., 1996). Thus, simvastatin may independently affect the two functions, probably through a common mechanism, i.e., the modification of the membrane composition and microviscosity. Such a modification at the level of the intracellular stores may lead to an increased membrane permeability to Ca^{2+}, thereby facilitating Ca^{2+} release and affecting mechanical threshold. The few and controversial data available in the literature about the role of membrane cholesterol in the function of cation pumps (Lijnen and Petrov, 1995, Lijnen et al., 1996; Makarov and Kuznetson, 1995) do not allow us to exclude the possibility that the change in microviscosity could also affect the activity of the Ca^{2+}-ATPase, thus reducing the calcium reuptake mechanism.

The hypothesis that simvastatin may directly affect the intracellular sites responsible for Ca^{2+} homeostasis involved in excitation–contraction coupling is supported by the finding that the acute application of simvastatin to cultured rat myoblasts increased cytosolic free Ca^{2+} both by enhancing Ca^{2+} release from intracellular stores and Ca^{2+} influx from the extracellular solution, thus accounting for muscle cell damage and necrosis (Nakahara et al., 1994). In contrast, pravastatin did not induce an increase in intracellular Ca^{2+} or cell damage (Nakahara et al., 1994). Other studies of vascular smooth muscle cells showed that simvastatin promotes the release of calcium from a thapsigargin-sensitive pool (Escobales et al., 1996). Thus the proposed ability of simvastatin to alter the composition of membranes and the movement of Ca^{2+} can explain the signs of myopathy and muscle pain reported by various patients during therapy. Although statins are generally well tolerated, the results of the present study suggest that the tissue-selectivity of pravastatin may be clinically advantageous since the inhibitory effect on cholesterol biosynthesis is maintained in the liver, thus reducing the potential risk of side effects in peripheral organs such as skeletal muscle. This is of clinical interest for subjects affected by severe hyperlipidemia, who often require combined therapy with HMG-CoA reductase inhibitors and other lipid-lowering drugs and also for transplanted patients cotreated with cyclosporine A (Smith et al., 1991), drug combinations that are known to increase the incidence of myopathy.

Acknowledgements

The authors are grateful to Prof. Cesare Sirtori for his generous support and helpful discussions and to Dr. Fedele Natuzzi for his collaboration during the experiments.

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