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Hydroxymethylglutaryl-coenzyme A reductase inhibitors induce apoptosis in human cardiac myocytes in vitro

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ABSTRACT

Recent findings have implicated hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors or statins, an established class of drugs for the treatment of hypercholesterolemia, in tissue remodeling in the heart. Statins induce apoptosis in different cell culture systems including rat neonatal cardiomyocytes. We investigated possible effects of different statins in vitro in human adult cardiac myocytes on the expression of proteins thought to be involved in the regulation of apoptosis such as Mcl-1, an inhibitor of apoptosis, Bax, an inducer of apoptosis, as well as on cytoplasmic histone-associated-DNA-fragments. Human adult cardiac myocytes (HACM) were treated with different statins at concentrations from 0.01 to 5 μM for up to 96 h. Whereas the lipophilic statin simvastatin at a concentration of 5 μM downregulated Mcl-1 mRNA by 49%, the hydrophilic pravastatin had no effect. Bax mRNA levels were not affected by neither of the statins. Simvastatin but not pravastatin reduced Mcl-1 protein expression whereas Bax protein was not detectable in HACM as determined by Western blotting. Simvastatin, atorvastatin and fluvastatin induced an up to seven-fold increase in histone-associated-DNA-fragments whereas pravastatin did not. Simvastatin up regulated histone-associated-DNA-fragments dose-dependently, and mevalonate and geranylgeranyl pyrophosphate reversed this effect to control levels. Our results show that lipophilic statins can induce a pro-apoptotic state in human adult cardiac myocytes in vitro. We speculate that, similar to findings in animal models, statins might be involved in the attenuation of cardiac hypertrophy and remodeling in humans by modulating the balance between cell survival and apoptosis.

1. Introduction

Hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors or statins represent an established class of drugs for the treatment of hypercholesterolemia and large-scale clinical trials have emphasized their benefits in the primary and secondary prevention of atherosclerosis and its complications [1–4]. The beneficial effects of statins are believed to result from their ability to reduce cholesterol biosynthesis and in addition from pleiotropic actions such as anti-inflamma-

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tory, anti-thrombotic and antioxidant effects, mobilization of progenitor cells, and their ability to improve impaired endothelial function [5].

Cardiac hypertrophy is a major determinant of morbidity in all forms of cardiovascular diseases [6]. Recent findings that statins attenuate myocardial hypertrophy in vitro and in vivo provide evidence that these drugs might also be involved in tissue remodeling in the heart [7–10]. This view was further supported by studies showing that statins positively affect left ventricular (LV) remodeling in animal models of myocardial infarction and heart failure [11–13]. Simvastatin prevented progression and reversed established right ventricular hypertrophy in rats with pulmonary hypertension and significantly decreased LV weight and LV myocyte size in hypercholesterolemic rabbits [14,15]. Treatment with quinapril and atorvastatin reduced ventricular weight in spontaneously hypertensive rats (SHR) and increased the number of apoptotic smooth muscle cells in the arteries from both SHR and normotensive rats [16].

The exact role of apoptosis, the programmed cell death, in the heart is unknown and remains speculative. Apoptosis has been detected in the myocardium in cardiac pathologies such as myocardial infarction, cardiomyopathy, arrhythmogenic right ventricular dysplasia and heart failure [17,18]. In the heart apoptosis is involved in postnatal shaping of the right ventricle by eliminating unnecessary cells [19] and plays an important role in the disappearance of both the infiltrated leukocytes and the proliferated interstitial cells after myocardial infarction [20]. Apoptosis mediates the controlled deletion of so-called ''unwanted'' cells and can be a potential mechanism contributing to the suppression of cardiac hypertrophy [21,22]. An association between apoptosis and regression of cardiac hypertrophy in SHR has been reported [23]. It was shown that statins induce apoptosis in rat neonatal cardiomyocytes and thus may attenuate cardiac hypertrophy and remodeling [24,25]. In this study we investigated the effect of different statins on apoptosis in human adult cardiac myocytes in vitro.

2. Materials and methods

2.1. Materials

Stock solutions of atorvastatin (kindly provided by Pfizer, Sandwich, UK) and fluvastatin (kindly provided by Novartis, Basel, Switzerland) were prepared at a concentration of 10 mM in ethanol. A stock solution of pravastatin (kindly provided by Bristol-Myers-Squibb, Paris, France) at the same concentration was prepared in distilled water. A stock solution (10 mM) of simvastatin (kindly provided by Merck Sharpe and Dome, Ballydine, Ireland) was prepared after activation of the molecule as described [26]. Such stocks were aliquoted and stored at −80 °C. Mevalonate and geranylgeranyl pyrophosphate (GGPP) were purchased from Sigma (St. Louis, MO, USA).

2.2. Cell culture

Primary human adult cardiac myocytes (HACM) were cultured from ventricular tissue obtained from explanted recipients' hearts after heart transplantation by mechanical dispersion of the tissue and separated from fibroblasts by preplating as described previously [27]. This procedure resulted in cell populations of >95% rod-shaped cells with a viability of >90%. HACM were cultivated in culture flasks that had been coated with fibronectin (Roche, Basel, Switzerland) in minimum essential medium (M199; Sigma) containing 10% FCS as well as 100 U/mL penicillin, 100 μg/mL streptomycin, 10 μg/mL transferrin (Sigma) and 10 μg/mL insulin (Sigma) at 37 °C in a humidified atmosphere of 5% CO_2:95% air. These cells were transferred into 24-well plates and were incubated in M199 containing 0.1% bovine serum albumin (BSA; Sigma) for 24 h prior to treatment with statins. All human material was obtained and processed according to the recommendations of the Hospital's Ethics Committee and Security Board.

2.3. Treatment of cells with statins

HACM were treated with different statins at concentrations from 0.01 to 5 μM for up to 96 h. In additional experiments HACM were incubated with simvastatin at a concentration of 5 μM in the absence or presence of 100 μM mevalonate or 10 μM GGPP for 48 h. The culture supernatants were then collected followed by removal of cell debris by centrifugation and stored at −80 °C until used. The total cell number of the respective cultures after trypsinisation was counted with a haemocytometer.

2.4. mRNA purification

Cells were treated as described, supernatants were removed and mRNA were isolated using QuickPrep™ Micro mRNA Purification Kit (Amersham Biosciences, Buckinghamshire, UK) according to the manufacturer's instructions.

2.5. RealTime-polymerase chain reaction

Specific mRNA levels for Mcl-1 and Bax were determined by RealTime-polymerase chain reaction (RealTime-PCR) using LightCycler-RNA Master SYBR Green I (Roche) according to the manufacturer's instructions. Primers were designed with LightCycler Probe Design Software Version 1.0 (Roche) and Primer3 Software (http://frodo.wi.mit.edu/), as follows (annealing temperature in parentheses and corresponding position in brackets): Mcl-1 (67 °C), forward primer: 5′-tgc tgg agt agg agc tgg tt-3′ [1077–1096], reverse primer: 5′-ttc tgg cta ggt tgc tag gg-3′ [1235–1016]; Bax (65 °C), forward primer: 5′-tgc ttc agg gtt tca tcc a-3′ [129–147], reverse primer: 5′-tga gac act cgc tca gct tc-3′ [242–223]; GAPDH (65 °C), forward primer: 5′-aca gtc cat gcc atc act gcc-3′ [604–625], reverse primer: 5′-gcc tgc ttc acc acc ttc ttg-3′ [890–869]. The amplification conditions consisted of an initial incubation at 61 °C for 20 min, followed by incubation at 95 °C for 30 s, 50 cycles of 95 °C for 1 s, the respective annealing temperature for 10 s and 72 °C for 10 s, a melting step from 45 to 95 °C with increases of 0.1 °C/s, and a final cooling to 40 °C. Data was analyzed using LightCycler Software Version 3.5 (Roche).

2.6. Western blotting

Confluent monolayers of HACM (10^6 cells) were incubated in the absence or presence of simvastatin or pravastatin at a

concentration of 5 μM for 24 h, at 1.25 μM for 48 h or at 0.1 μM for 96 h. At the end of the incubation, cells were harvested and lysed in NP-40 buffer (1% NP-40 in PBS) containing 1 mM PMSF and 100 μM sodium orthovanadate (Sigma). Protein concentrations were measured by a Micro BCA Protein Assay Reagent (Pierce, Rockford, IL, USA). Lysates were subjected to SDS-PAGE in 12% gels. Prior to SDS-PAGE, lysates were mixed with reducing SDS-loading buffer (7.2% SDS, 9 mmol/L EDTA, 20% glycerol, 10 mmol/L dithiothreitol, 13 mmol/L Tris–phosphate buffer, pH 6.8) and boiled for 5 min. Separated proteins were then electroblotted onto a nitrocellulose membrane (Schleicher & Schüll, Dassel, Germany) at 0.8 mA/cm^2 for 2 h. The membranes containing the complete set of samples were blocked in blocking buffer (5% non-fat dry milk in PBS) followed by immune overlay with the following antibodies: Mcl-1 (Dako, Glostrup, Denmark) and Bax (Ab-1, Calbiochem, Darmstadt, Germany). After washing, bound antibodies were detected with HRP-labelled sheep anti-mouse IgG (Amersham) or HRP-labelled goat anti-rabbit IgG (Pierce), respectively. After rinsing in Western blotting detection reagent ECL (Amersham), membranes were exposed to a X-OMAT-AR film (Eastman Kodak, Rochester, NY, USA) for 5, 30 and 60 min each. Western blot bands were quantified by GelPro Analyzer 4.0 (Media Cybernetics, Silver Spring, MD, USA). Membranes were also stained with Coomassie blue and quantified to account for uneven loading.

2.7. Detection of histone-associated-DNA-fragments by ELISA

Histone-associated-DNA-fragments were quantitatively determined by Cell Death Detection ELISA^{PLUS} (Roche Diagnostics, Mannheim, Germany). Briefly, 10^4 cells cultured in 24-well plates were treated with atorvastatin, fluvastatin, simvastatin or pravastatin at the indicated concentrations for 48 h. At the end of the incubation, culture medium was removed to an Eppendorf tube and centrifuged at 16,000 × *g*. The resulting pellets contained oligonucleosomal fragments. Adherent HACM were permeabilized in lysis buffer provided with the assay kit. The supernatants containing the cytoplasmic oligonucleosomes released from HACM nuclei were combined with the oligonucleosomal fragments in medium. The combined supernatants were transferred into a streptavidin-coated 96-well microplate. A mixture of anti-histone-biotin antibodies and anti-DNA-peroxidase (POD) antibodies were added and incubated. During the incubation period, the anti-histone antibodies bind to the histone-component of the nucleosomes and simultaneously capture the immunocomplex to the streptavidin-coated microplate via its biotinylation. Additionally, the anti-DNA-POD antibodies react with the DNA-component of the nucleosomes. Unbound components (antibodies) were removed by a washing step. Quantitative determination of the amount of nucleosomes by the POD retained in the immunocoplex was performed photometrically with ABTS as substrate. All measurements were performed in triplicates.

2.8. Statistical analysis

Values are expressed as mean ± S.D. Data were compared by ANOVA. Values of $p < 0.05$ were considered significant.

3. Results

3.1. Effect of statins on Mcl-1 and Bax in HACM

Simvastatin decreased Mcl-1 protein level in human cardiac myocytes as shown by Western blotting to 65% at 5 μM after 24 h, to 34% at 1.25 μM after 48 h and to 62% at 0.1 μM after 96 h, respectively, when compared to the respective control. Interestingly, pravastatin at 5 and 1.25 μM slightly increased Mcl-1 protein to 126% after 24 h and 123% after 48 h when compared to the respective control (Fig. 1). In contrast to Mcl-1, Bax could not be detected by Western blotting in HACM.

As demonstrated by RealTime-PCR, treatment of HACM with simvastatin at a concentration of 5 μM for 24 h also resulted in a robust decrease in mRNA specific for Mcl-1 (51% of respective untreated control) whereas no changes in mRNA specific for Bax (106% of respective untreated control) were observed. Consequently the Mcl-1/Bax ratio decreased to 0.48. In contrast pravastatin had no effect on Mcl-1 and Bax mRNA expression, probably due to its hydrophilic properties (data not shown).

3.2. Effect of statins on release of histone-associated-DNA-fragments from HACM

Atorvastatin, fluvastatin, simvastatin, but not pravastatin at a concentration of 5 μM increased the release of histone-associated-DNA-fragments from HACM up to seven-fold. The results for atorvastatin, fluvastatin and simvastatin were confirmed with HACM isolated from hearts from four different donors (Table 1). Atorvastatin, fluvastatin and simvastatin also at a concentration of 0.5 μM slightly, but not always significantly increased the release of histone-associated-DNA-fragments from HACM. The effect of simvastatin on histone-associated-DNA-fragments release was dose-dependent as

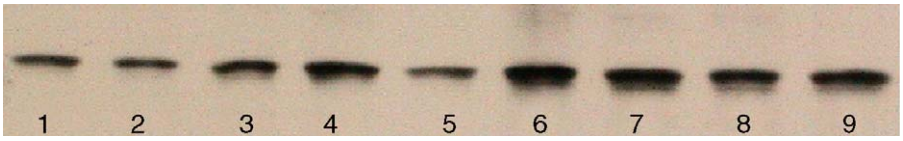

Fig. 1 – Effect of statins on Mcl-1 protein expression in HACM. Confluent monolayers of HACM (10^6 cells) were incubated in the absence or presence of simvastatin or pravastatin at a concentration of 5 μM for 24 h, 1.25 μM for 48 h or 0.1 μM for 96 h. The cells were lysed and Western blot was performed as described in Section 2. Lane 1: control, 24 h; lane 2: simvastatin 5 μM, 24 h; lane 3: pravastatin 5 μM, 24 h; lane 4: control, 48 h; lane 5: simvastatin 1.25 μM, 48 h; lane 6: pravastatin 1.25 μM, 48 h; lane 7: control, 96 h; lane 8: simvastatin 0.1 μM, 96 h; lane 9: pravastatin 0.1 μM, 96 h.

Table 1 – Lipophilic atorvastatin, fluvastatin and simvastatin but not hydrophilic pravastatin induce release of histone-associated-DNA-fragments from the human adult cardiac myocytes (HACM)

	Donor 1	Donor 2	Donor 3	Donor 4
Control	0.143 ± 0.002	0.102 ± 0.013	0.206 ± 0.003	0.297 ± 0.028
Atorva 0.5 μM	n.d.	n.d.	0.352 ± 0.056*	0.360 ± 0.045
Atorva 5 μM	0.550 ± 0.048*	n.d.	1.331 ± 0.059*	0.633 ± 0.001*
Fluva 0.5 μM	n.d.	n.d.	0.446 ± 0.051*	0.385 ± 0.025*
Fluva 5 μM	0.535 ± 0.03*	n.d.	1.527 ± 0.087*	0.624 ± 0.109*
Simva 0.5 μM	n.d.	0.116 ± 0.013	0.410 ± 0.05*	n.d.
Simva 5 μM	n.d.	0.226 ± 0.022*	1.433 ± 0.098*	0.713 ± 0.020*
Prava 5 μM	0.151 ± 0.014	n.d.	n.d.	n.d.

Confluent monolayers of HACM, isolated from four different donors, were incubated for 48 h in the absence or presence of atorvastatin, fluvastatin and simvastatin at the concentrations of 0.5 or 5 μM or pravastatin at a concentration of 5 μM. The cells were collected and histone-associated-DNA-fragments were determined by ELISA as described in Section 2. Values are given in absorbance (405 nm)/10^4 cells/48 h and represent mean values ± S.D. of three independent experiments ($n = 3$). n.d., Not determined.

* $p < 0.05$.

demonstrated in Fig. 2. This effect was already observed at a concentration of 0.5 μM of simvastatin and reached a maximum between 2.5 and 5 μM of simvastatin after incubation for 48 h.

3.3. *Mevalonate and geranylgeranyl pyrophosphate reversed simvastatin-induced release of histone-associated-DNA-fragments in HACM*

To determine whether the effects of the statins on histone-associated-DNA-fragments release depend on their capacity to inhibit mevalonate synthesis by HMG-CoA reductase, cardiac myocytes were treated with simvastatin in the absence or presence of 100 μM mevalonate or 10 μM GGPP. When mevalonate or GGPP were present during the incubation period for 48 h together with simvastatin, histone-associated-DNA-fragments levels were similar to the level seen in untreated cells (Fig. 3).

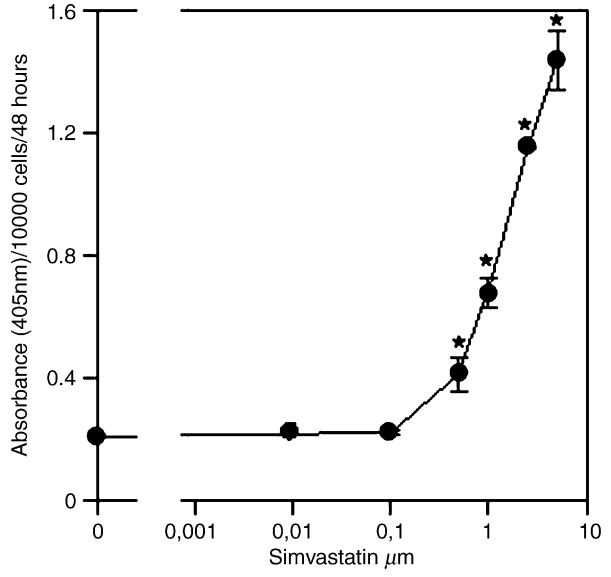

Fig. 2 – Effect of simvastatin on histone-associated-DNA-fragments release from HACM. Confluent monolayers of HACM were incubated for 48 h in the absence or presence of simvastatin at a concentration of 0.01, 0.1, 0.5, 1, 2.5 and 5 μM. Samples were collected and histone-associated-DNA-fragments were determined by ELISA as described in Section 2. Values are given as absorbance (405 nm)/10^4 cells/48 h and represent mean values ± S.D. of three independent experiments ($n = 3$) with cells isolated from one donor. Experiments were performed three times with HACM obtained from three different donors with similar results. A representative experiment is shown. * $p < 0.05$.

4. Discussion

Multicellular organisms are dependent on an ordered elimination of unwanted cells for their normal development as well as for the maintenance of balanced tissue homeostasis. An essential mechanism for elimination of unwanted cells is apoptosis or programmed cell death [28]. Besides other pleiotropic effects, statins have been recently also implicated in the regulation of apoptosis. Through the induction of apoptosis in endothelial cells and smooth muscle cells statins are thought to have beneficial effects such as improving endothelial dysfunction or preventing restenosis after angioplasty [29–34]. Statins also arrest the functional differentiation of monocytes into macrophages and steer these cells into apoptosis, suggesting a mechanism for the vasculoprotective properties of statins [35]. Induction of apoptosis by fluvastatin in rat neonatal cardiac myocytes was proposed as a possible mechanism of statin-attenuated cardiac hypertrophy [24]. By inducing apoptosis simvastatin has been shown to rescue rats from fatal pulmonary hypertension, and to prevent progression and even to reverse established right ventricular hypertrophy [14].

Here we present for the first time evidence for the involvement of statins in inducing apoptosis in human cardiac myocytes. We could show that the lipophilic statin simvastatin reduced the levels of the anti-apoptotic protein Mcl-1 in human primary cardiac myocytes down to 34% of control whereas the hydrophilic statin pravastatin, probably due to its physico-chemical properties showed only a minor effect [8,24,30,33,36,37]. Mcl-1, a B cell leukemia-2 (Bcl-2) homologue [38], is a member of the Bcl-2 family of proteins, which have emerged as key regulatory component of the cell death process, with particular members, e.g. Bcl-2, Bcl-xL or Mcl-1, providing an anti-apoptotic signal and others, e.g. Bcl-2-

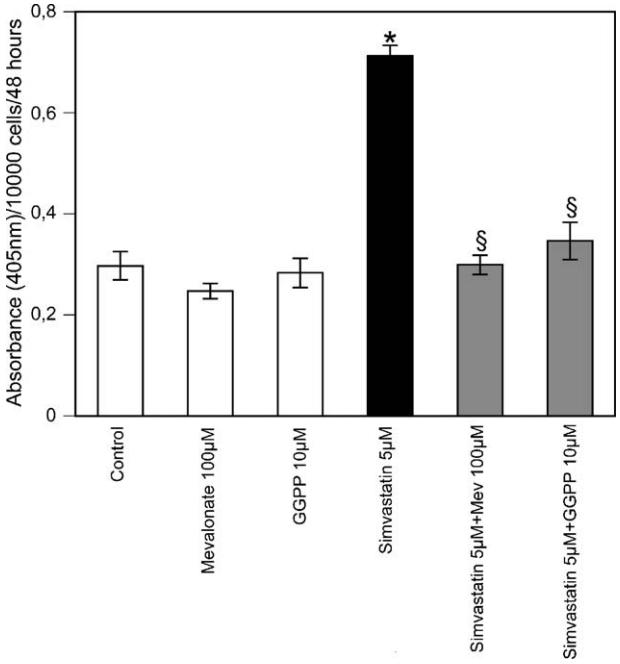

Fig. 3 – Effect of mevalonate and geranylgeranyl pyrophosphate (GGPP) on simvastatin-induced histone-associated-DNA-fragments release from HACM. Confluent monolayers of HACM were incubated for 48 h in the absence or presence of 100 μM mevalonate, 10 μM GGPP or 5 μM simvastatin alone or in combination. Samples were collected and histone-associated-DNA-fragments were determined by ELISA as described in Section 2. Values are given as absorbance (405 nm)/10^4 cells/48 h and represent mean values ± S.D. of three independent experiments ($n = 3$) with cells isolated from one donor. Experiments were performed three times with HACM obtained from three different donors with similar results. A representative experiment is shown. $^{*}p < 0.05$ as compared to control, $^{\S}p < 0.05$ as compared to simvastatin alone; Mev, mevalonate.

associated protein X (Bax), enhancing the cell death process [39]. By using RealTime-PCR we could confirm our protein data on the level of specific mRNA. Statins decreased the expression of mRNA specific for Mcl-1 whereas mRNA specific for the pro-apoptotic protein Bax was not affected resulting in a decreased Mcl-1/Bax ratio shifting the cells towards a pro-apoptotic state [40]. It should be emphasized that Mcl-1 is expressed at relatively high levels in human heart in comparison to other Bcl-2-related proteins [41]. It should also be noted that the Mcl-1 homologue Bcl-2 protected cardiac myocytes from a wide variety of death-promoting signals [42]. Our findings on the regulation of Mcl-1 and Bax by statins are in agreement with other studies showing that statins reduced Bcl-2 and Bcl-xL expression without affecting Bax expression in vascular smooth muscle cells, murine tubular cells, and human hepatocytes and decreased Mcl-1 expression and increased Bax in myeloma cell lines [36,43–45].

The pro-apoptotic effect of lipophilic statins in human cardiac myocytes was further confirmed by the qualitative and quantitative in vitro determination of cytoplasmic histone-associated-DNA-fragments. An increase in these fragments is a feature of cells undergoing apoptosis [46]. Atorvastatin, fluvastatin and simvastatin increased histone-associated-DNA-fragments release up to seven-fold whereas the hydrophilic statin pravastatin had no effect. The effect of simvastatin on histone-associated-DNA-fragments release was dose-dependent and was already seen with 0.5 μM and reached maximum between 2.5 and 5 μM of statin. It was shown earlier that cerivastatin and lovastatin dose-dependently elevated the level of cytoplasmic histone-associated-DNA-fragments in rat vascular smooth muscle cells and human endothelial cells, respectively [29,47]. In our study the effect of statins on histone-associated-DNA-fragments release was completely reversed by mevalonate and geranylgeranyl pyrophosphate, suggesting the involvement of protein prenylation. This is in agreement with previous in vitro studies [24,36,43].

Since the concentrations of statins used in this in vitro study are higher than observed plasma concentrations in patients treated with these HMG CoA reductase inhibitors caution should be used in extrapolating results of our experimental study to the clinical setting [48]. The concentrations used here are, however, in the same range as concentrations of statins used in numerous other tissue culture studies [24,25,29–31,33,36,37]. Furthermore, one could speculate that the local concentration in tissue in the in vivo situation might depend on the exposure time of the tissue to the HMG CoA reductase inhibitors. Considering the fact that in most clinical studies patients are treated with statins over a period of days or even weeks, these exposure times are much longer as compared to the in vitro setting where cells are treated with these drugs only for hours. In that respect it should be emphasized that when cardiac myocytes in our study were exposed to simvastatin at 100 nM for a prolonged exposure time of 96 h the reduction of Mcl-1 protein was also observed as well as with 1.25 μM of the statin after an exposure time of 48 h.

In conclusion we could show here for the first time that statins can induce a pro-apoptotic state in primary human cardiac myocytes in vitro. If such an effect is also operative in vivo one could speculate that, similar to findings in animal models, statins might be involved in the attenuation of cardiac hypertrophy and remodeling in humans by modulating the balance between cell survival and apoptosis [9–13,23].

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