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Simvastatin disrupts cytoskeleton and decreases cardiac fibroblast adhesion, migration and viability

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ABSTRACT

Statins reduce the isoprenoids farnesyl and geranylgeranyl pyrophosphate, essential intermediates, which control a diversity of cellular events such as cytoskeleton integrity, adhesion, migration and viability. Cardiac fibroblasts are the major non-myocyte cell constituent in the normal heart, and play a key role in the maintenance of extracellular matrix. The effects of simvastatin on cardiac fibroblast processes previously mentioned remain unknown. Our aims were to investigate the effects of simvastatin on cytoskeleton structure and focal adhesion complex assembly and their relationships with cell adhesion, migration and viability in cultured cardiac fibroblasts. To this end, cells were treated with simvastatin for 24 h and changes in actin cytoskeleton, levels of vimentin and paxillin as well as their subcellular localization were analyzed by Western blot and immunocytochemistry, respectively. Cell adhesion to plastic or collagen coated dishes, migration in Transwell chambers, and cell viability were analyzed after simvastatin treatment. Our results show that simvastatin disrupts actin cytoskeleton and focal adhesion complex evaluated by phalloidin stain and immunocytochemistry for paxillin and vinculin. All these effects occurred by a cholesterol synthesis-independent mechanism. Simvastatin decreased cell adhesion, migration and viability in a concentration-dependent manner. Finally, simvastatin decreased angiotensin II-induced phospho-paxillin levels and cell adhesion. We concluded that simvastatin disrupts cytoskeleton integrity and focal adhesion complex assembly in cultured cardiac fibroblasts by a cholesterol-independent mechanism and consequently decreases cell migration, adhesion and viability.

1. Introduction

Simvastatin inhibits 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and suppresses cholesterol biosynthesis. Statins exert beneficial pleiotropic effects on myocardial remodeling independently of their lipid-lowering properties (van der Harst et al., 2006). However, detrimental effects of statin have been observed in cardiac muscle cells, and in skeletal muscle (Rabkin and Kong, 2003; Sirvent et al., 2005; Rabkin et al., 2007).

Cellular effects of statins arise from the inhibition of HMG-CoA reductase, resulting in deprivation of intracellular mevalonate, and also isoprenoid intermediaries, including farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP). These isoprenoids provide lipophilic anchors which are essential for membrane attachment and biological activity of small GTP binding proteins (Elson et al., 1999). FPP and GGPP are required for Ras farnesylation and Rho geranylgeranylation, respectively. Both Ras and Rho are necessary for a diversity of cellular events including actin cytoskeleton organization, cell adhesion and migration (Etienne-Manneville and Hall, 2002). In addition, Rho effectors are implicated in the formation of stress fibers and focal adhesions in nonmuscle cells and contractility of smooth muscle cells (Riento and Ridley, 2003). Stress fibers are composed of actin and actin-associated proteins including myosin II which plays a critical role in controlling actomyosin contractility (Pellegrin and Mellor, 2007). Focal

Abbreviations: Ang II, angiotensin II; BSA, bovine serum albumin; CF, cardiac fibroblasts; ECM, extracellular matrix; FAK, focal adhesion kinase; FBS, fetal bovine serum; FPP, farnesylpyrophosphate; GGPP, geranylgeranyl pyrophosphate; HMG-CoA, 3-hydroxy 3-methylglutaryl coenzyme A; MVA, mevalonate; PBS, phosphate-buffered saline; PI, propidium iodide; RhoA, Ras homologue gene family member A; SQ, squalene; α-SMA, alpha-smooth muscle actin; TRITC, tetrarhodamine isothiocyanate.

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adhesion serves as organizing centers for regulatory and structural proteins so as to facilitate rapid, precise control of cell proliferation, differentiation and function (Sieg et al., 2000; Wozniak et al., 2004). Among these effectors are anchoring proteins such as vinculin, paxillin, talin and α-actinin, which link the actin cytoskeleton to transmembrane integrin receptors at focal adhesion (Dugina et al., 2001).

Cell adhesion is critical for cell migration to occur, and this process is mediated by the continuous formation of cytoplasmic protrusions at the leading edge of the cell and controlled retraction of adhesive contacts at its rear (Werner and Grose, 2003). Focal adhesions provide dynamic links between the actin cytoskeleton and the extracellular matrix (ECM), and β1 integrins and vinculin are considered key proteins that mediate this functional connection during migration and adhesion (Friedl and Wolf, 2009). Therefore, alterations in cardiac fibroblasts (CF) number, location, adhesion and migration could lead to changes in the amount and composition of the cardiac ECM conducting to pathological cardiac remodeling or can prevent proper healing after tissue damage.

In experimental studies, the effects of statins have largely focused on global myocardial remodeling *in vivo* or *in vitro* (Dechend et al., 2001; Patel et al., 2001; Ogata et al., 2002). There are considerably less data related to statin and CF. These cells are the major non-myocyte cell constituent in the normal heart, and they are responsible for maintaining its structural integrity. In human cardiac myofibroblast, statins inhibited proliferation and metalloproteases (MMPs) activity (Porter et al., 2004); disrupt cytoskeleton integrity and decrease cytokine expression (Turner et al., 2007a,b). We found that simvastatin alters cytoskeleton structure in human gingival fibroblasts (Cáceres et al., 2011); and statins inhibited Ang II-induced collagen synthesis in human and mouse CF (Martin et al., 2005; Chen and Mehta, 2006, respectively). Our recent findings showed that simvastatin triggers CF and myofibroblast apoptosis (Copaja et al., 2011). All these evidences suggest that statins could modulate negatively cell responses involved in the maintenance of tissue integrity, ECM turnover and tissue repair.

Finally, growth factors, cytokines and mechanical stress have been identified as regulators process associated to tissue repair such as CF adhesion, migration and growth (Eghbali, 1992; Camelliti et al., 2005). However the influence of statins on these processes in CF remained unexplored. Thus, our aims were to study the effects of simvastatin on CF cytoskeleton, the intracellular mechanisms underlying these effects, and whether these effects are linked to cell adhesion, migration and viability.

2. Materials and methods

2.1. Reagents

The following reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA): trypan blue, simvastatin, farnesyl pyrophosphate (FPP), geranylgeranyl pyrophosphate (GGPP), mevalonate (MVA), squalene (SQ). Trypsin/EDTA, prestained molecular weight standard and fetal bovine serum (FBS) were from Gibco BRL (Carlsbad, CA, USA). All organic and inorganic compounds were from Merck (Darmstadt, Germany). The enhanced chemo-luminescence (ECL) reagent was from PerKin Elmer Life Sciences, Inc. (Boston, MA, USA). Sterile plastic materials were purchased to Falcon® (NJ, USA). The primary antibodies for paxillin, vinculin, and phospho-paxillin were purchased from Cell Signaling Technology (Boston, MA, USA). Vimentin was from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Isolation and culture of cardiac fibroblasts

Rats were obtained from the Animal Breeding Facility of the Faculty of Chemical and Pharmaceutical Sciences, University of Chile. All studies were conformed the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and experimental protocols were approved by our Institutional Ethics Review Committee. CF were prepared from hearts of 1–3 day-old Sprague-Dawley rats as described previously, and the purity of our cultured CF (more than 95%) was analyzed by immunocytochemistry using antibodies against vimentin or alpha-smooth muscle actin (α-SMA) as previously described (Vivar et al., 2008).

Cells were treated without or with simvastatin (0.1–10 μM) for different times, dependent of each experiment. The intermediaries of cholesterol synthesis (MVA, 100 μM; SQ, 100 μM; GGPP, 10 μM; FPP, 10 μM) were added to cultures one day before treatment with simvastatin. Angiotensin II (Ang II, 100 nM) was added to cultures the last 5 min of treatment with simvastatin.

2.3. Staining for cytoskeletal proteins

Cell cultures were grown close to 70% of confluence on glass coverslips in complete medium containing 10% fetal bovine serum (FBS). Then cells were serum-deprived for 24 h, and treated with 0.1–10 μM simvastatin for 0–24 h. Simvastatin-induced morphologic changes were monitored by phase-contrast microscopy. After drug treatment, cells were fixed at room temperature for 10 min in 3.7% formaldehyde (v/v) in phosphate-buffered saline (PBS). They were then washed with cytoskeletal buffer with the following composition (in mM): 2-[N-morpholino] ethane sulfonic acid (MES) 10 containing NaCl 150, EGTA 5, $MgCl_2$ 5, and glucose 5 [pH 6.1]), followed by permeabilization for 10 min with 0.1% Triton X-100 in PBS and blocking with serum-containing buffer (10% FBS in PBS with 0.02% sodium azide). To stain cytoskeletal F-actin, the cells were incubated with tetrarhodamine isothiocyanate (TRITC)-phalloidin in buffer with 0.2% saponin for 45 min. For detection of focal adhesions, cells were double immunostained, with monoclonal antibodies anti-paxillin and anti-vinculin; and anti-phosphopaxillin for 2 h at room temperature. The secondary antibodies used were conjugated with TRITC (paxillin and phospho-paxillin) and FITC (vinculin). Coverslips were mounted on glass slides with Dako, and micrographs were obtained with a fluorescence microscope (Axioplan-2; Zeiss, Germany).

2.4. Western blot analysis

Cell proteins were extracted with a protease inhibitor cocktail-containing lysis buffer. Aliquots were resolved on 12% SDS-PAGE, transferred to a nitrocellulose membrane and incubated with primary antibodies against paxillin (1:1000), phospho-paxillin (1:1000), vimentin (1:500). All of them incubated at 4 °C overnight. Bound antibodies were detected by horseradish peroxidase secondary antibody and visualized by ECL method.

2.5. Migration assays

Cell migration was performed with the Costar Transwell system (Corning Life Sciences, Acton, MA, USA) which allows cells to migrate through 8 mm pore size polycarbonate membrane. Briefly, simvastatin-pretreated cells were trypsinized, washed, and resuspended in serum-free DMEM-F12 (300,000 cells/mL). This suspension (100 μL) was added into the Transwell upper chamber. The lower chamber was filled with 500 μL DMEM-12 containing 10% FBS as chemoattractant. After a 2-h stimulation serum, upper chambers were removed, and cells remaining on the upper surface of the membrane were removed with a cotton swab. Later, membranes were washed with PBS, and cells present beneath the membrane were fixed with cold methanol for 15 min and stained with crystal violet. Cells were counted in 10 high-power microscope fields. Analysis was performed on 3 wells for each condition, and each experiment was repeated 3 times. For experiments using MVA and SQ, cells were pretreated with this chemical 1 day before the simvastatin treatment.

2.6. Cell adhesion assays

Assays were performed to determine the effects of simvastatin treatment on the adhesiveness of CF. Attachment assays were performed in 24-well plates that had been coated with 5% (w/v) of bovine serum albumin (BSA: as control), collagen type I (100 μg/mL) overnight at 4 °C in PBS. Simvastatin-pretreated cells were detached using trypsin EDTA (0.5%), which was inactivated with media containing serum. Cells were resuspended in serum-free media at a concentration of 20×10^4 cells/mL and 10×10^3 cells were seeded per well. Unless stated cells were left to attach and spread for 2 h after which media were aspirated off and the cells were washed twice with PBS. Cells were stained for 20 min with crystal violet (0.3%, in ethanol 10% v/v), before washing twice with PBS. Lastly, the attached and stained cells were dissolved with SDS 1% and the absorbance was measured at 595 nm.

2.7. Cell viability

CF were seeded in 60 mm plates at a rate of 2×10^4 cell/cm^2, cultured in DMEM-F12 containing 10% FBS, which was subsequently replaced by DMEM-F12. After 24 h of starvation, simvastatin was applied to the times and concentrations indicated for each experiment. Then cells were released from the plates using 1× trypsin-EDTA which was inhibited using DMEM-F12 with 10% serum. Subsequently 20 μL cell suspension was mixed with 20 μL of trypan blue solution and the living cells were counted placed in a Neubauer chamber.

2.8. Statistical analysis

Data are mean ± SD of, at least, 3 independent experiments. Student's *t*-test for comparison between 2 groups or one-way ANOVA followed by a Tukey's post hoc test for multigroup comparisons was used. Significance was set at $p < 0.05$.

3. Results

3.1. Simvastatin alters morphology and cytoskeleton in cultured cardiac fibroblasts

Exposure to simvastatin induced morphological alterations in cultured CF, characterized by decreased cell size, cell rounding and stimulation of filamentous extension and process formation. Phalloidin staining for actin stress fibers in cultures of CF revealed the presence of regular actin stress fibers in control cells (Fig. 1A). The highly organized dense meshwork of actin stress filaments of CF stained very intensely and appeared to be organized in a transverse plane throughout the cell body. In contrast, simvastatin treatment (10 μM) from 16 h resulted in nearly complete disrupts and loss of actin stress fibers and instead less dense filaments were evident. Fig. 1B shows that more than 40% of cells were with disrupted cytoskeleton at 16 h with statin. After 24 h simvastatin treatment, an increase in damaged cytoskeleton cells (60%) and a reduction in cell number were observed.

3.2. Simvastatin-induced changes on cytoskeleton are prevented by isoprenoid supplementation

To determine whether the effects of simvastatin on CF are dependent of cholesterol synthesis, we examined if the isoprenoids (FFP, GGPP), MVA and SQ may prevent simvastatin effects. The addition of GGPP or FPP (10 μM), MVA (100 μM) and SQ (100 μM) to the medium of simvastatin-treated cells (10 μM for 24 h), revealed that SQ does not prevent the recovery of cell morphology to the normal state (Fig. 2A). Fig. 2B shows that 60% of simvastatin-treated cells had disrupted cytoskeleton; however, this effect was reduced by GGPP, FPP and MVA supplementation, but not with SQ.

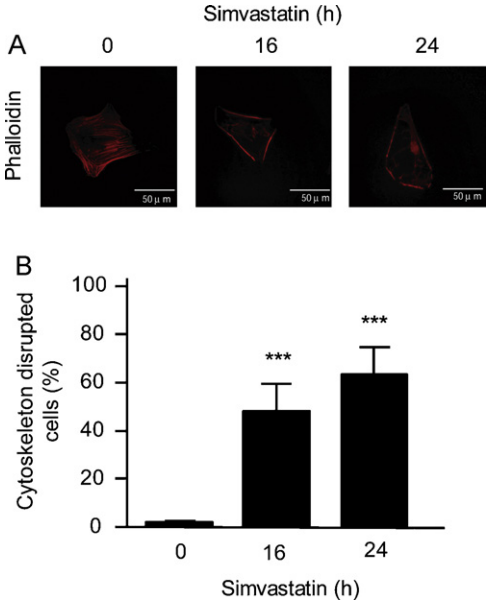

Fig. 1. Effect of simvastatin on actin cytoskeleton structure in cultured cardiac fibroblasts. Cells were pre-treated with simvastatin (10 μM) for 0–24 h. (A) Cells were fixed, permeabilized, stained with rhodamine–phalloidin and visualized by fluorescence microscopy; simvastatin disrupts actin cytoskeleton in a time dependent manner (scale bar = 50 μm). (B) Graphics analysis of disrupted cytoskeleton cell number. Data are shown as mean ± SD ($n = 3$ independent experiments), $^{***}p < 0.001$ vs. control.

3.3. Simvastatin modifies the distribution of vinculin and paxillin in cultured cardiac fibroblasts

To explore whether cytoskeleton changes induced by simvastatin are associated with redistribution or reduction in expression levels of focal adhesions proteins (detected by inmmunostaining for vinculin or paxillin). Fig. 3A shows a double labeling of vinculin and paxillin in cultured CF. The distribution profiles of vinculin and paxillin displayed a clear pattern mainly localized on cell membrane as punctuate pattern typical for focal adhesions complex. Simvastatin (10 μM for 24 h) caused a marked redistribution in staining for both vinculin and paxillin, indicating a loss from focal adhesions complex (Fig. 3A). This effect was significant from 16 h (Fig. 3B). Also, vinculin and paxillin protein levels were analyzed by Western blot. Fig. 3C depicts that vinculin and paxillin expression levels in simvastatin-treated CF did not change respect to the untreated cells (control). This result suggests that simvastatin disrupts focal adhesion complex but it also leads to vinculin and paxillin accumulation in the cytosol of CF without changes in their levels.

3.4. Simvastatin alters phospho-paxillin levels on cultured cardiac fibroblasts

To study if simvastatin-induced changes in paxillin localization on cell membrane are associated to changes in its assembly, phospho-paxillin localization on cell membrane was investigated. Ang II is a well known stimulus to induce paxillin phosphorylation, a key step for its assembly and localization on focal adhesion complex. As shown in Fig. 4A, a typical localization and low phospho-paxillin levels on cell membrane were detected under basal condition. Ang II (100 nM during the last 5 min) induced a significant increase in phospho-paxillin levels and their localization on cell membrane. In contrast, in CF incubated for 24 h with simvastatin (10 μM) and the last 5 min stimulated with Ang II (100 nM), this peptide was unable to induce paxillin phosphorylation and their localization on cell membrane. Also, phospho-paxillin levels were assessed to identify whether the changes associated to distribution profiles on focal adhesion are also linked to activation levels changes. Fig. 4B shows that phospho-paxillin levels did not change in untreated (control) and simvastatin-treated CF. However, the addition of Ang II (100 nM for the last 5 min) stimulated a significant increase in phospho-paxillin levels, but in CF pretreated with simvastatin (10 mM for 24 h) this peptide was unable to induce paxillin phophorylation. Finally, Fig. 4C shows that the pretreatment with MVA but not with SQ prevent the decrease in phospho-paxillin levels triggers by simvastatin, and also those induced by Ang II. These results suggest that simvastatin modify focal adhesion assembly and its localization on cell membrane by a mechanism involving protein prenylation.

3.5. Simvastatin reduces adhesion, migration, and cell viability in cultured cardiac fibroblasts

We study whether simvastatin effects on cytoskeleton and focal adhesion complex are linked to changes in cell adhesion and migration. Fig. 5A depicts that simvastatin-pretreatment (1 and 10 μM for 24 h) reduces cell adhesion to collagen or plastic matrix, although higher adhesion was observed on collagen. Simvastatin decreased cell migration (Fig. 5C). To study whether the mechanisms involved on these effects are dependent of cholesterol synthesis, MVA (100 μM) or SQ (100 μM) were added to the medium of simvastatin-treated cells (10 μM for 24 h). The results

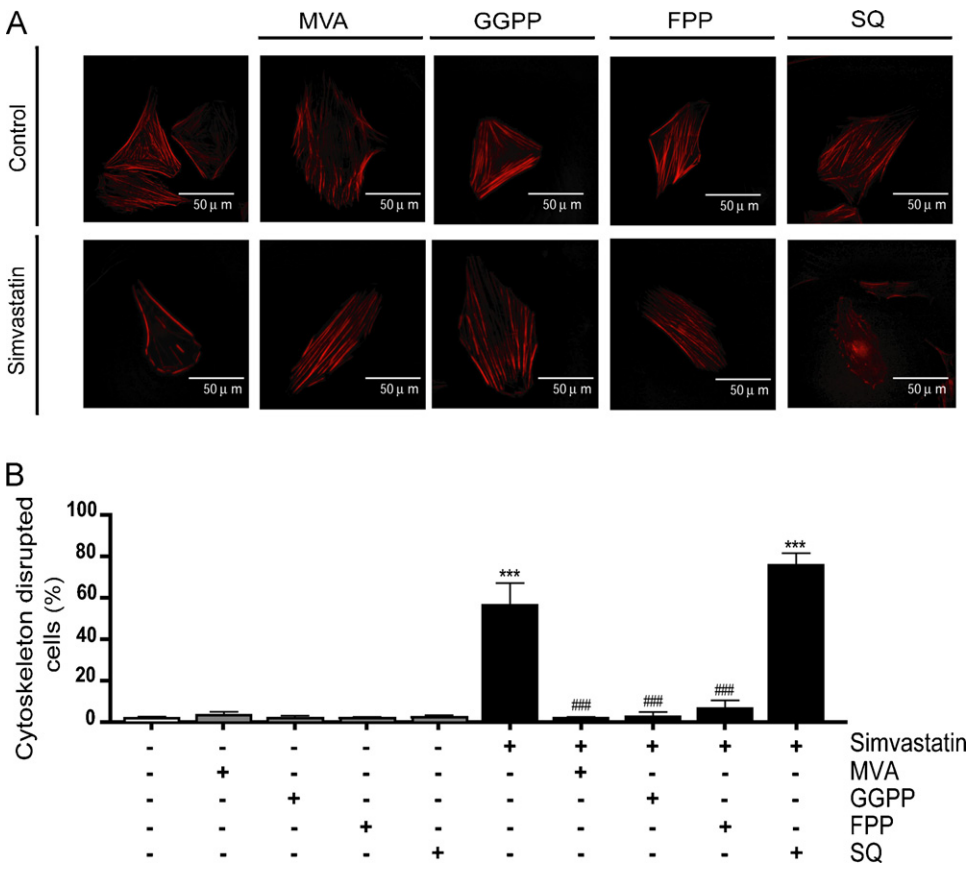

Fig. 2. Isoprenoid intermediaries prevent disruption of actin cytoskeletal structure induced by simvastatin in cultured cardiac fibroblasts. Cells were pretreated with farnesyl pyrophosphate (FPP) or geranylgeranyl pyrophosphate (GGPP) (10 μM), mevalonate (MVA) or squalene (SQ), and then treated with simvastatin (10 μM 24 h). (A) Cells were fixed, permeabilized, stained with rhodamine–phalloidin and visualized by fluorescence microscopy. SQ did not prevent actin cytoskeleton changes induced by simvastatin (scale bar, 50 μm). (B) Graphics analysis disrupted cytoskeleton cell number. Data are shown as mean ± SD ($n = 3$ independent experiments), $^{***}p < 0.001$ vs. control; $^{###}p < 0.001$ vs. simvastatin.

revealed that SQ does not prevent the decrease in cell adhesion to collagen matrix (Fig. 5B) and cell migration (Fig. 5D). These data show that both effects of simvastatin are independent cholesterol synthesis.

3.6. Simvastatin pretreatment modifies Ang II-induced cardiac fibroblast adhesion

After the morphological changes induced by simvastatin on cultured CF (Fig. 1A), we evaluate the minimum time where these effects of simvastatin become irreversible. For this aim, CF were treated with simvastatin (10 μM) for different times (4, 8, 16 and 24 h), after which the culture medium was replaced by fresh medium without simvastatin, and the cells were incubated up to 24 h, at which we measure the loss of cell viability. The results show that 16 h simvastatin treatment is the minimum time required to observe irreversible effects on cell viability (Fig. 6A). As shown in Fig. 5, simvastatin also inhibits paxillin phosphorylation induced by Ang II, which promotes focal adhesion complex assembly and increasing cell attachment. Therefore, our next aim was to study whether simvastatin modifies Ang II-induced CF adhesion. To this end, CF were treated with simvastatin for 8 and 24 h, time in which the effects of simvastatin on cell viability are non lethal and lethal respectively. Then the medium was replaced with fresh medium without simvastatin, but containing Ang II 100 nM, and CF were cultured up to 24 h, at which time cell adhesion assays were performed. Our results show that in CF pre-treated for 8 h with simvastatin, this drug did not reduce cell adhesion in a significant manner; in addition, in CF pretreated with simvastatin (8 h), Ang II increased cell adhesion, similar to those treated only with Ang II (Fig. 6B). As control, in CF treated with simvastatin for 24 h a significant decrease in cell adhesion was observed.

4. Discussion

Statins have shown to induce toxicity stimulating cardiomyocyte apoptosis (Demyanets et al., 2006). Also, statin produces a variety of myopathy ranging from muscle pain to rhabdomyolysis (Thompson et al., 2003), and *in vitro* studies depicts that simvastatin interferes with mitochondrial permeability transition (Velho et al., 2006). We have recently showed that simvastatin trigger CF apoptosis by a caspase-dependent mechanism, but independent of mitochondrial injury (Copaja et al., 2011), and also that affect actin cytoskeleton in gingival fibroblast (Cáceres et al., 2011).

Our results showed that simvastatin disrupts the actin cytoskeleton, and this effect was prevented by MVA, GGPP and FPP, but not by SQ. This result agrees with Turner et al. (2007a), who described that simvastatin disrupts actin fibers in human cardiac myofibroblasts. However our data are partially consistent with those works describing that simvastatin disrupts the actin fibers in endothelial cells and this disruption is reversed by MVA and GGPP but not by FPP (Pozo et al., 2006), suggesting that

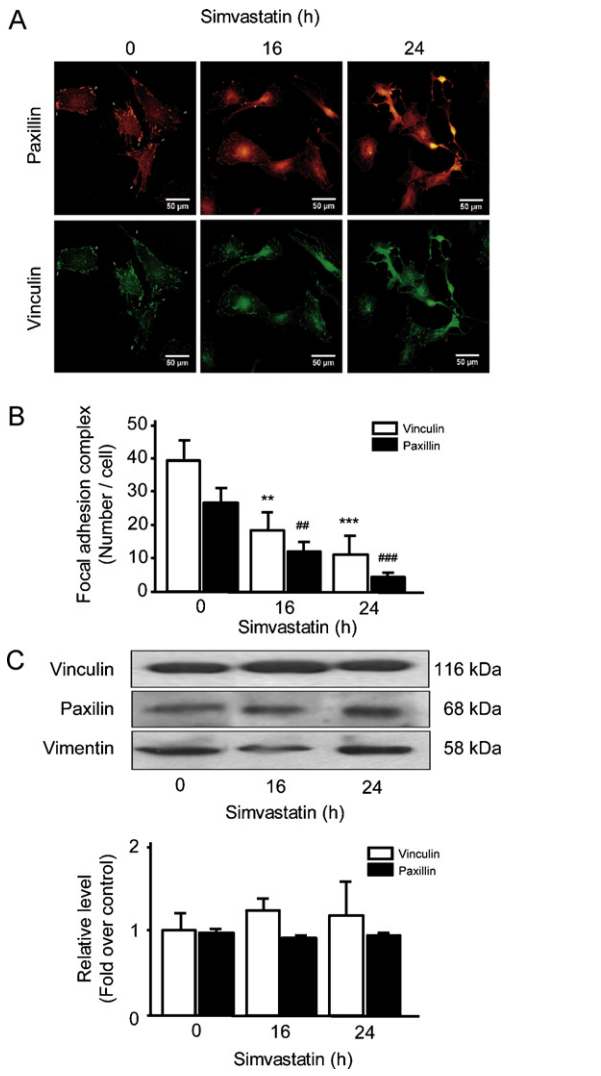

Fig. 3. Simvastatin changes levels and localization of paxillin and vinculin in cultured cardiac fibroblasts. (A) Cells treated with simvastatin (0.1–10 μM) for 24 h and then were fixed, permeabilized, and double immunostained with antibodies to paxillin (red) or vinculin (green), and visualized by fluorescence microscopy (scale bar, 50 μm). In control cells, vinculin and paxillin were localized in focal adhesion complex on cell membrane (arrow), while they were in the cytosol at 16 and 24 h. (B) Graphic analysis of focal adhesion complex number by cells, evaluated as number of positive immunostained dots for vinculin and paxillin respectively. (C) Cells were treated with simvastatin (0.1–10 μM) for 24 h. Then cells were lysed and paxillin and vinculin levels were analyzed by Western blot, vimentin was used as load control. The results are shown as mean ± SD ($n = 3$ independent experiments). $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. control to vinculin; $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ vs. control to paxillin. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

isoprenylated rather than farnesylated proteins are necessary. Rho and/or Ras proteins are required for maintenance of actin filaments and cytoskeletal structure (Fenton et al., 1992; Bifulco et al., 1993; Koch et al., 1997; Evers et al., 2000), and, Rho activity is required to maintain focal adhesions in cells attached to a matrix (Hall, 1998). Our data did not show changes in the levels of vinculin and paxillin; however, simvastatin stimulates the subcellular redistribution of vinculin and paxillin, and a decrease in focal adhesion complex number was observed. These effects were prevented by the addition of MVA, GGPP and FPP, whereas no such effect was observed with SQ. In this sense, our previous findings show that simvastatin did not modify RhoA levels in CF and myofibroblast, but reduced RhoA-GTP levels (Copaja et al., 2011). Collectively, these results suggest that isoprenylated proteins maintain focal adhesions and cytoskeleton integrity.

To understand the effects of simvastatin on focal adhesions assembly and to evaluate the role of the Rho family in this process, CF were exposed to Ang II and phospho-paxillin levels were assessed. Our results showed that simvastatin reduced the phospho-paxillin levels induced by Ang II. This effect was prevented by MVA but not by SQ. Thus, this effect was regulated by isoprenylated proteins. On the other hand, it has also shown that phosphorylation of paxillin is important for the assembly of focal adhesions, specifically triggered by focal adhesion kinase (FAK), and it has been showed that Ang II induce paxillin phosphorylation through FAK activation (Zouq et al., 2009). Thus, by inhibiting the synthesis of GGPP and FPP, simvastatin prevents the activation of these small GTPases, and it has been reported that the small GTPase Rho regulates FAK functions (Ren et al., 2000). Our results, demonstrate that simvastatin prevents paxillin phosphorylation Ang II-induced. In this way, it is possible that simvastatin affects multiple cellular functions by interfering with basic cell signaling pathways that lead to cytoskeletal organization, localization of paxillin and vinculin, cell adhesion, migration, and lastly survival. Our results show that simvastatin disrupt cytoskeleton and disassembly focal adhesions, conducting to loss in cell attachment, which could stimulate CF death, probably by anoikis. We find that pretreatment for 8 h with simvastatin is not enough to deplete of farnesyl and geranylgeranyl intermediates in CF; however, simvastatin effects on cell viability are irreversible from 16 h of treatment; thus mean that previous to cell death by anoikis a disassembly of focal adhesion complex conducting to loss of cell attachment take place. Thus, it is possible that after 16 h of treatment with simvastatin the depletion of farnesyl and geranylgeranyl intermediates triggers the loss of cell adhesion and viability. In this sense, has been showed that statin induces anoikis (Valentijn et al., 2004; Li et al., 2006), specifically, phospho-paxillin and the FAK play an important role in the suppression of anoikis (Zouq et al., 2009). Together these results suggest that simvastatin effects on CF viability could be associated with loss of cell attachment by decreasing the Rho-FAK signaling and to disassembly of focal adhesion complex conducting to anoikis.

Simvastatin decreased cell adhesion which could be explained by decreases in cell adhesion proteins levels like integrins, intracellular adhesion molecule-1, E-selectin and CD40 (Mulhaupt et al., 2003; Cernuda-Morollon and Ridley, 2006; Takeda et al., 2007; Eccles et al., 2008). This result is coincident with our data observed in human gingival fibroblast were after 16 h of exposition to statin a decrease in β1 integrin was observed (Cáceres et al., 2011). Other studies have shown that the isoprenylated Rho GTPase protein family, and specifically Rac regulates cell adhesion because of its ability to stimulate lamellipodium extension (Riento and Ridley, 2003). To this end we sought to prevent the effect of simvastatin on isoprenylated protein, pretreating CF with MVA and SQ. Supplementation with SQ did not overcome the inhibitory effect of simvastatin on cell adhesion, however, these effects were prevented by MVA, showing that isoprenylated intermediates are necessary and probably isoprenylated proteins could be involved. In this sense, we find that Ang II can to trigger cell adhesion, in cell where the intermediate levels are enough to ensure the assembly of focal adhesion complex. However, in CF where farnesyl or geranylgeranyl intermediates depletion was strongly induced by simvastatin, Ang II was unable to prevent the loss of cell adhesion simvastatin-induced. On the other hand, our results show that simvastatin reduces CF migration. In this regard, Porter et al. (2004), demonstrated that simvastatin inhibits geranylgeranylation of RhoA, preventing its translocation to the plasma membrane and subsequent activation of downstream activators, including ROCK, and through of this mechanism disrupts the cytoskeleton and inhibits human cardiac

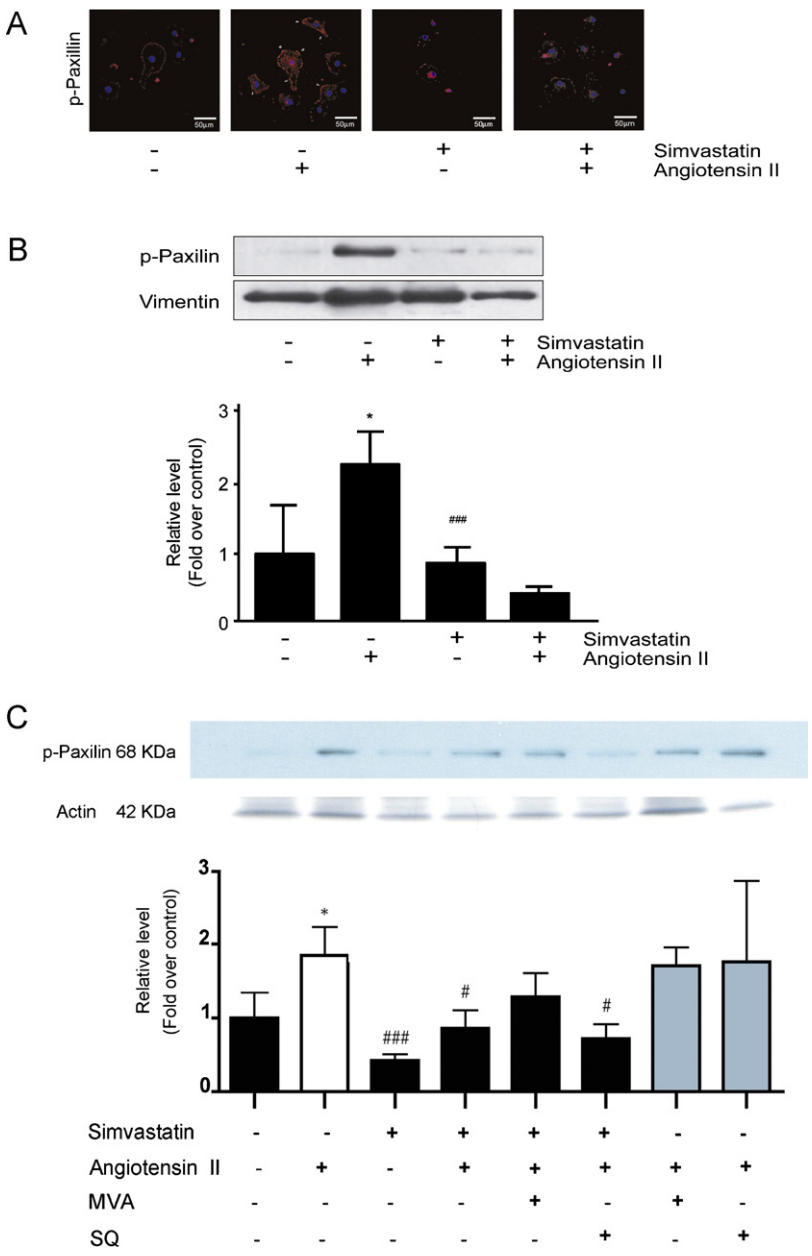

Fig. 4. Simvastatin reduces paxillin phosphorylation stimulated by angiotensin II in cultured cardiac fibroblasts. Cells were treated with simvastatin (10 μM for 24 h) and during the last 5 min were stimulated with Ang II 100 nM. (A) Cells were fixed, permeabilized and immunostained with antibody to phospho-paxillin, and visualized by fluorescence microscopy. (B) Cells were lysed and phospho-paxillin protein levels were analyzed by Western blot. The results of western blot analysis are shown as mean ± SD ($n = 3$ independent experiments). (C) Cells were pretreated with mevalonate (MVA) or squalene (SQ), and then treated with simvastatin (10 μM 24 h), and during the last 5 min were stimulated with Ang II 100 nM. Cells were lysed and phospho-paxillin protein levels were analyzed by Western blot, and the results of western blot analysis are shown as mean ± SD ($n = 3$ independent experiments). $^{*}p < 0.05$ Ang II vs. control; $^{\#}p < 0.05$ simvastatin plus SQ vs. Ang II; $^{\#\#\#}p < 0.001$ simvastatin vs. Ang II.

myofibroblast migration. In addition, they showed that the ROCK inhibitor Y27632 mimics the effect of simvastatin suggests that the anti-migratory effects of simvastatin are mediated via inhibition of the RhoA/ROCK pathway. Collectively, these results suggest that following the depletion of farnesyl and geranylgeranyl intermediates by statins, a wide range of cellular processes, including those triggered by Ang II, a major modulator of cardiac function and remodeling could be affected.

Finally, we do not believe that the effects of simvastatin on cardiac fibroblasts observed in the present study are reflective of all statin drugs, because other statins do not show toxicity to similar degree in CF (data not shown). The difference toxicity could be explained maybe for its different chemical structure, which is associated to different profile in potency and toxic effects; similar results had been observed in other cells (Skottheim et al., 2008).

One of our study limitations was that the results described here on the effects of simvastatin on cell adhesion, migration and viability were performed in cultured CF. The relevance of this finding reported in this paper for CF in intact hearts is unclear and this point should be addressed in further studies.

In conclusion, simvastatin disrupts cytoskeleton and disassembly focal adhesion complex in cultured CF. Based on these evidences, simvastatin shows toxic effects on CF decreasing cell adhesion, migration and viability. These biological processes are key components of a physiological response that must be preserved to allow cardiac tissue function under physiological and pathological states including those regulated by Ang II.

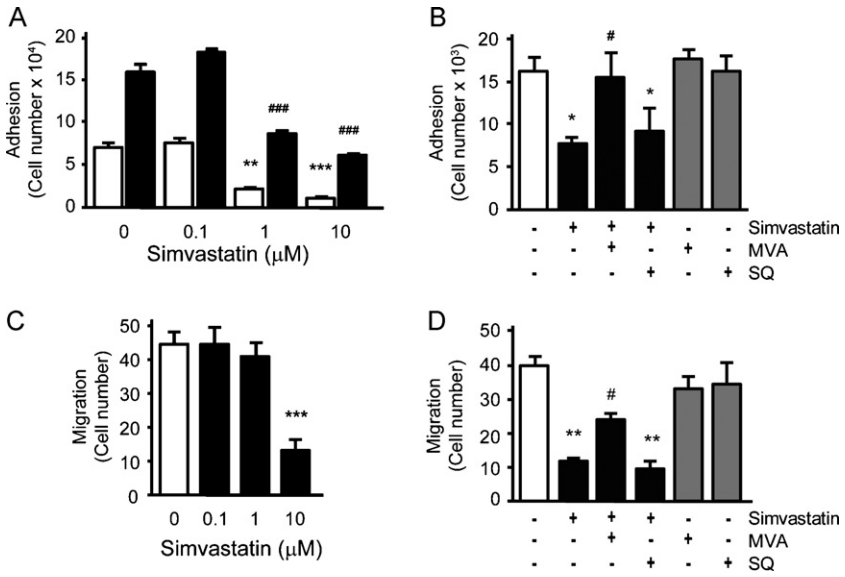

Fig. 5. Effects of simvastatin on cardiac fibroblasts adhesion, migration and viability. Cells were treated for 24 h with simvastatin. Then cells were detached and counted for cell viability assays. Viable cells were seeded on culture dishes or collagen coated dishes, or on Transwell chambers for attachment or migration assays, respectively, as indicated in Section 2. Simvastatin reduces cell attachment to plastic (white bar) or collagen (black bar) matrix (A), and cell migration (C) in a concentration dependent manner. Pretreatment with mevalonate (MVA) but not squalene (SQ) prevents decrease induced by simvastatin in cell attachment to collagen matrix (B) and migration (D). Data are shown as mean ± SD. Data are representative of three independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. control; $^{\#\#\#}p < 0.001$ vs. control collagen; $^{\#}p < 0.05$ vs. simvastatin.

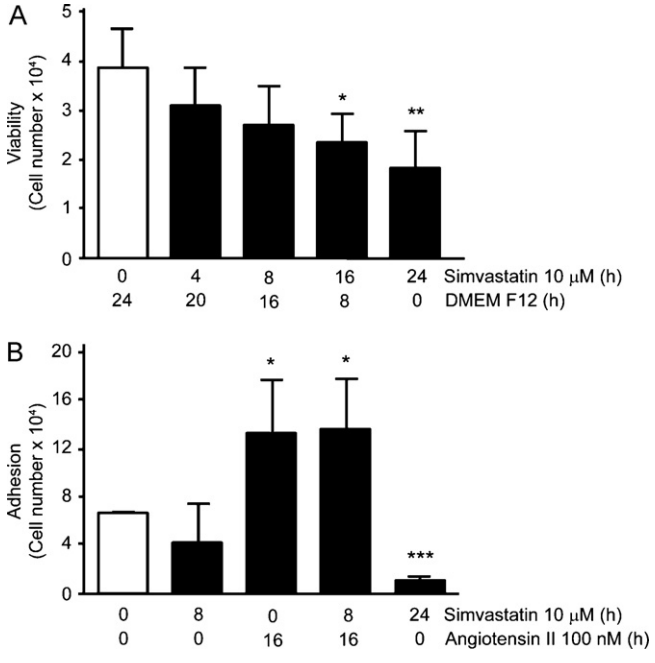

Fig. 6. Simvastatin modifies Ang II-induced cardiac fibroblast adhesion. (A) Cardiac fibroblasts were treated with simvastatin (10 μM) for 0, 4, 8, 16 and 24 h, after each time culture medium was replaced by a fresh medium without simvastatin and cells were incubated up to 24 h, and cell viability was determined by trypan blue method. The results are expressed as ±SD ($n = 3$), $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control. (B) Cells were treated with simvastatin (10 μM) for different times, and after the culture medium were replaced by fresh medium without simvastatin and cells were incubated up to 24 h with/without Ang II 100 nM. Then cells were detached and counted for cell adhesion assays. Viable cells were seeded on culture dishes for attachment as indicated in Section 2. The results are shown as mean ± SD ($n = 3$ independent experiments). $^{*}p < 0.05$, and $^{***}p < 0.001$ vs. control.

Conflict of interest

The authors have no conflicts of interest to disclose.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tox.2012.01.011.

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