FACULDADE DE MEDICINA DA UNIVERSIDADE DE COIMBRA

MESTRADO INTEGRADO EM MEDICINA – TRABALHO FINAL

BRIGITE TEIXEIRA RIBEIRO VAN DEN WILDENBERG GOOSKENS

Stents, statins and sirolimus:

a new approach to prevent restenosis

ARTIGO CIENTÍFICO ORIGINAL

ÁREA CIENTÍFICA DE BIOLOGIA CELULAR

Trabalho realizado sob a orientação de:

PROFESSOR DOUTOR HENRIQUE MANUEL PAIXÃO DOS SANTOS

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TABLE OF CONTENTS

ABSTRACT

Introduction: Cardiovascular diseases remain the leading cause of death worldwide, being coronary artery disease accountable for up to 20% annually in Europe. The treatment encompasses conservative management, coronary artery bypass graft and the much less invasive percutaneous coronary intervention. The drug-eluting stents deployed by percutaneous coronary intervention were a milestone that significantly reduced the stent restenosis rate and stent thrombosis associated to the first-ever used bare-metal stents; however, drug-eluting stents still present some drawbacks. Besides smooth-muscle cells proliferation inhibition, achieving complete reendothelialization of injured vessel after stenting, with a regenerated endothelium showing normal morphologic characteristics and functions, is a strategic objective for a stent. One major disadvantage of the use of drug-eluting stents with compounds such as rapamycin, also known as sirolimus, a mTOR inhibitor, is that not only acts on the vascular smooth muscle cells, but also on endothelial cells, which might exacerbate the endothelial function impairment when the stent is deployed. Although autophagy can be directly activated by mTOR inhibition, it can be activated indirectly, namely through 5' adenosine monophosphate-activated protein kinase (AMPK). Since statins can activate AMPK and improve endothelial function, reducing inflammation, we hypothesize that statins can reduce the detrimental effects of sirolimus-eluting-stents on endothelial cells promoting a beneficial increase in endothelial function and consequentially a better arterial healing response, and reduced stent restenosis.

Methods: To address this question, we used a cell-based approach, with an immortalized mouse cardiac endothelial cell line and an endothelial cell primary culture. These cells were treated with statins (simvastatin) or rapamycin separately or in combination. Afterwards we evaluated endothelial activity by matrigel tubulation assay, migration assays and autophagy flux/activity by western blot.

Results: Our results demonstrated that when in combination with rapamycin (or sirolimus), simvastatin partially reverts the negative effects of rapamycin in the endothelial cells angiogenic potential, observed by an increased tubulation and migration capacity. Moreover, we observed that simvastatin potentiates autophagy regulation.

Discussion: The increase on angiogenesis might be attributable to an increase of thin-walled and fragile neovessels that may serve as a pathway for recruitment of leukocytes to high-risk areas of the plaque. On the other hand, the capacity of endothelial cells for angiogenesis correlates with a proper arterial healing response and functional endothelium. There was an increase of the ability of endothelial cells to migrate and subsequently close a wound, what means that they were able to follow chemo-attractant and migrate through a physical barrier toward it, which is a desirable quality when there is endothelial damage, such as when a stent is deployed.

Comparing to rapamycin alone, the addition of simvastatin seems to further increase autophagy, that has been associated with angiogenesis in endothelial cells, and with a better arterial healing response.

Conclusion: These results will pave the way for new understandings on the effects of statins on the vascular cells, and potentially a statin-eluting stent the default one on clinical practice, for a lower risk of restenosis and endothelial dysfunction.

KEYWORDS

Hydroxymethylglutaryl-CoA Reductase Inhibitors; Endothelial Cells; Sirolimus; Drug-Eluting Stents; Autophagy; Endothelium, Vascular

RESUMO

Introdução: As doenças cardiovasculares continuam a ser a principal causa de morte no mundo, sendo a doença arterial coronária, responsável por 20%, anualmente, na Europa. O tratamento abrange abordagem conservadora, revascularização miocárdica cirúrgica e intervenção coronária percutânea, muito menos invasiva. Os *stents* farmacológicos implantados pela intervenção coronária percutânea foram um marco que reduziu significativamente a taxa de reestenose do *stent* e a trombose do stent associada aos primeiros *stents* metálicos usados; no entanto, os *stents* farmacológicos ainda apresentam algumas desvantagens. Além da inibição da proliferação das células musculares lisas, a completa reendotelização do vaso lesado após o implante do *stent*, com endotélio regenerado, mostrando características e funções morfológicas normais, é um objetivo estratégico para o *stent*. Uma grande desvantagem do uso de *stents* farmacológicos com compostos como a rapamicina (sirolimus), um inibidor do mTOR, é que não apenas atua nas células musculares lisas vasculares, mas também nas células endoteliais, o que poderá exacerbar a disfunção endotelial provocada pelo *stent*. Embora a autofagia possa ser ativada diretamente pela inibição da mTOR, ela pode ser ativada indiretamente, através da proteína quinase ativada por 5 ' monofosfato de adenosina (AMPK). Como as estatinas podem ativar a AMPK e melhorar a função endotelial, reduzindo a inflamação, nós colocamos a hipótese que as estatinas possam reduzir os efeitos deletérios dos *stents* farmacológicos de sirolimus, nas células endoteliais, melhorando a resposta arterial e reduzindo a taxa de reestenose.

Métodos: Para abordar esta questão, utilizámos uma abordagem celular, com uma linhagem celular endotelial cardíaca imortalizada de ratos e uma cultura primária de células endoteliais. Estas células foram tratadas com estatinas (sinvastatina) ou rapamicina separadamente ou em combinação. Em seguida, avaliou-se a atividade endotelial por ensaio de tubulação de matrigel, ensaios de migração e fluxo de autofagia/atividade por western blot.

Resultados: Os nossos resultados demonstraram que, quando em combinação com a rapamicina (ou sirolimus), a sinvastatina reverte parcialmente os efeitos negativos da rapamicina no potencial angiogénico das células endoteliais, observado por um aumento na capacidade de migração e tubulação. Além disso, observámos que a sinvastatina promoveu a autofagia.

Discussão: O aumento da angiogénese pode ser atribuído a um aumento de neovasos de paredes finas e frágeis, que podem servir para o recrutamento de leucócitos para áreas de alto risco da placa. Por outro lado, a capacidade das células endoteliais para a angiogénese correlaciona-se com uma resposta arterial adequada e um endotélio funcional. Observou-se ainda um aumento da capacidade de migração das células endoteliais, o que significa que elas responderam a um estímulo quimiotácito e migraram através de uma barreira física em direção a ele, o que é desejável quando há lesão endotelial, nomeadamente aquela induzida pela impantação de um *stent*.

Os resultados sugerem que comparando com a rapamicina, a adição de sinvastatina promove a autofagia, que tem sido associada à angiogénese nas células endoteliais, com melhor resposta arterial.

Conclusão: Estes resultados abrirão caminho para novos entendimentos sobre os efeitos das estatinas nas células vasculares, e potencialmente um *stent* com eluição de estatina, o padrão na prática clínica, para um menor risco de reestenose e disfunção endotelial.

PALAVRAS-CHAVE

Inibidores de Hidroximetilglutaril-CoA Redutases; Células Endoteliais; Sirolimus; Stents Farmacológicos; Autofagia; Endotélio vascular

INTRODUCTION

Cardiovascular diseases remain the leading cause of death summing up to one third of deaths worldwide, being coronary artery disease (CAD) accountable for up to 20% of all deaths annually in Europe.[1]

The CAD treatment encompasses the conservative management, coronary artery bypass graft and the revolutionary less invasive percutaneous coronary intervention (PCI).[2] At first, the hallmark percutaneous treatment was the balloon angioplasty; however, it had a 6-month restenosis rate of 30-40%.[3]

To counteract the vessel recoil and seal dissections after this technique,[4] permanent bare-metal stents (BMS) were implanted in the coronary arteries, shortening the stent restenosis (SR) to 20-30%, which still remains a major drawback.[3]

SR has been associated with vascular smooth muscle cell (VSMC) proliferation into the media, migration to the intima, and subsequently formation of the neointima,[5] which is exacerbated by the stent application with a high-pressure technique.[6] Importantly, given the anti-proliferative role of endothelial cells (EC) on VSMC, the endothelial denudation caused by the injury performed during balloon inflation in PCI impacts negatively this intervention.[7]

In order to prevent neointima hyperplasia, drug-eluting stents (DES) were created, coated with polymers, which allow a slow release of immunomodulatory and antiproliferative drugs, such as rapamycin (also known as sirolimus) that targets the mammalian target of rapamycin (mTOR).[8]

Despite the efficacy of second generation DES, in reducing the incidence of SR, the high frequency and complexity of PCI performed worldwide may explain the unremitting prevalence of SR. Nevertheless, the effects of DES readily translated into a significant decrease in the clinical need for subsequent repeat revascularization.[5]

The first generation DES were associated with stent thrombosis and late restenosis. This has led to a prolonged requirement for dual anti-platelet therapy when using DES.[9] Third generation, with biodegradable polymers and fourth generation, bioresorbable scaffolds, both "limus"- family eluting stents have not been proven superior.[10]

Besides the inhibition of VSMC proliferation, mTOR inhibitors also induce self-digesting autophagy in vascular EC, which might be responsible for the endothelial impairment, and consequently for the late stent thrombosis, myocardial infarction, and mortality in patients with DES.[11]

Achieving complete reendothelialization of injured vessel after stenting, with a regenerated endothelium showing normal morphologic characteristics and functions, is a strategic objective for a stent, as well as obtaining complete apposition, reduction or elimination of late and very late stent thrombosis and minimizing the need for long-term dual antiplatelet therapy.[10]

Two distinct multiprotein complexes, with different cellular functions, constitute the serine/threonine kinase mTOR: mTORC1 and mTORC2. Under normal conditions, mTORC1 promotes protein synthesis, proliferation, lipogenesis, growth and energy metabolism.[12]

Loss of mTORC1 signalling results in inhibition of cellular growth, arresting SMC proliferation in G1-phase; inhibition of protein synthesis and metabolism as well as induction of autophagy, a process for the destruction of proteins and organelles in lysosomes aimed at restoring or maintaining energy and nutrient levels in the cell.[13]

Sirolimus is a potent inhibitor of mTORC1, however its effects on mTORC2 are rather controversial and will only appear following chronic administration. The mechanisms involved in sirolimus-induced autophagy remain elusive.

Inhibition of mTORC1 can also be achieved via indirect mechanisms such as activation of the 5' adenosine monophosphate-activated protein kinase (AMPK).[12] AMPK is a potent stimulator

of autophagy that responds to declining ATP levels by inhibiting mTORC1 and directly activating several proteins involved in autophagy initiation.

It has been shown that statins exhibit non-cholesterol-dependent pleiotropic effects, including the induction of autophagy via activation of AMPK, inhibition of VSMCs proliferation and platelet activation, improving endothelial function, and reducing inflammation.[14] Therefore, it is conceivable that statins reinforce the autophagic effects of mTOR inhibitors.[13]

Moreover, there is also emerging evidence suggesting that chronic inflammation and impaired endothelial function could promote late neoatherosclerosis inside the neointimal hyperplasia in BMS and DES. Neoatherosclerosis seems to be a significant mechanism for very late SR and thrombosis, which occurs more frequently and earlier after DES than after BMS implantation.[5] Given the pivotal role of statins as vulnerable plaque stabilizers, they could also have an influence in very late SR and thrombosis.

Although it has been reported that autophagy activation has a number of important effects upon endothelial cell function, with implications on angiogenesis, particularly within ischaemic microenvironments, the exact mechanism whereby autophagy regulates the angiogenic process remains elusive.[15]

It is plausible that the combined effect of statins and sirolimus modulate autophagy in EC. It is still elusive if statins can influence the autophagy by affecting mTOR activity.

The purpose of this study is to determine the cooperative effect of statins and sirolimus on EC in what regards angiogenic activity, migration, and autophagy activity.

METHODS AND MATERIALS

Cell Culture and treatments

The immortalized mouse cardiac endothelial cell (MCEC-1) line was developed by Faculty of Medicine, National Heart & Lung Institute in London, United Kingdom and nobly provided by Professor Justin Mason. MCEC-1 was maintained in Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, Carlsbad, CA, USA), supplemented with 10% (volume/volume) Fetal Bovine Serum (FBS) (Life Technologies, Carlsbad, CA, USA), 10 U/ml heparin (Sigma-Aldrich, St. Louis, MO, USA) and 75 µg/ml endothelial cell growth factor (Sigma-Aldrich, St. Louis, MO, USA).

Human pulmonary arterial endothelial cells (PAECs) were obtained from GIBCO (Life Technologies, Carlsbad, CA, USA) and cultured with Medium 200 supplemented with Low Serum Growth Supplement (LSGS) (both from Life Technologies, Carlsbad, CA, USA).

All cell medium (MCEC-1 and PAECs) were supplemented with 1% Penicillin/Streptomycin (100 U/mL:100 µg/mL) and 1% GlutaMAX (both from Life Technologies, Carlsbad, CA, USA).

ECs (MCEC-1 and PAECs) were cultured in 1% gelatin (Sigma-Aldrich, St. Louis, MO) coated plates, expanded and plated at 37ºC with 5% CO_2, 24 to 48 hours prior to experimentation.

At the time of the experiments, MCECs and PAECs were between passage 10-25 and 6-10, respectively.

When appropriate, cells were treated with the following agents: 50 nM of rapamycin (Tocris Bioscience, Bristol, UK), 1 µM of activated simvastatin (Sigma Aldrich, St. Louis, MO, USA) and 50 nM Bafilomycin A1 (Millipore, Bedford, MA, USA).

Simvastatin activation

To cell culture use, simvastatin needs to be activated by opening of the lactone ring. Briefly, simvastatin (0.019 mM) was dissolved in 100% ethanol, with subsequent addition of 0.1 N NaOH until a final volume of 0.5 mL.

The solution was heated at 50°C for 2 hours in a sand bath and then neutralized with HCl (pH 7.2) before being brought to a final volume of 1 ml with distilled water. Activated simvastatin was stored at −80°C until use.

Matrigel angiogenesis assay

The angiogenic potential of ECs was tested by Matrigel angiogenesis assay. For that, MCEC-1 or PAECs (15000 cells per well) were seeded onto 10 µL solidified Matrigel (Corning® Matrigel® Matrix Growth Factor Reduced, BD Biosciences, Fairleigh, NJ, USA) containing or not a piece of bare-metal (Tsunami® Gold, TERUMO, Tokyo, Japan) or sirolimus-eluting stent (Ultimaster™, TERUMO, Tokyo, Japan) and treated as indicated in Table 1 in a µ-Slide Angiogenesis (IBIDI, Martinsried, Germany).

Phase-contrast images were collected 6 hours after seeding, by using Zeiss Axio HXP IRE 2 microscope (Carl Zeiss AG, Jena, Germany) and were processed with Angiogenesis Analyzer (Gilles Carpentier) in ImageJ (National Institute of Health, Bethesda, MD, USA).

Table 1: Experimental conditions used in the matrigel angiogenesis assay. 0: no additional treatment; BMS: bare-metal stent; DES: drug-eluting stent; Rap: rapamycin; Simv: simvastatin.

Matrigel	0	0	0	0	BMS	BMS	DES	DES
Treatment	0	Simv	Rap	Simv + Rap	0	Simv	0	Simv

Migration assay

ECs migration was assessed by a scratch assay.

Briefly, pretreated confluent cell monolayers (for 4 hours before the beginning of the assay with rapamycin (50 nM), simvastatin (1µM), or rapamycin (50 nM) plus simvastatin (1µM)) were scratched using the extremity of a 20 to 200 µL sterile pipette tip. Detached cells were removed by a PBS (Phosphate Buffer Saline) wash, and 1mL test medium was added, followed by incubation at 37ºC in 5% CO_2.

Phase contrast images were taken immediately after the scratch, 2 and 4 hours later using a Zeiss Axio HXP IRE 2 microscope (Carl Zeiss AG, Jena, Germany). The area of wound reduction was quantified using ImageJ (National Institute of Health, Bethesda, MD, USA).

Western blot analysis

After appropriate treatments, confluent cell monolayers (12-well plate) cells extracts were washed twice in ice-cold PBS, and directly lysed on the culture plate in 2x Laemmli Sample buffer - 80 µL per well of 12-well plate - (125 mM Tris, pH 6.8, 4% [weight/volume] SDS, 20% [volume/volume] glycerol, 10% [volume/volume] ß-mercaptoethanol, 0.006% bromophenol blue), scraped off the dishes and heated at 95oC for 5 minutes before loading.

Cell lysates were separated by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis), and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked with 5% non-fat milk in Tris-buffered saline-Tween 20 (TBS-T) (20 mM Tris, 150 mM NaCl, 0.2% Tween 20, pH 7.6) and probed with primary antibodies against the following proteins: rabbit anti-LC3 (PA1-16931, Thermo Fisher Scientific, Carlsbad, CA, USA), rabbit anti-p62/SQSTM1 (#5114s, Cell Signaling Technology,

Beverly, MA, USA), goat anti-GAPDH (AB0049, SICGEN, Cantanhede, Portugal), goat anti-Calnexin (AB0041, SICGEN, Cantanhede, Portugal).

After the reaction with the secondary antibody (horseradish peroxidase-conjugated secondary goat anti-rabbit (170-6515) or rabbit anti-goat (172-1034), both from Bio-Rad Laboratories, Hercules, CA, USA), the protein of interest was detected using Clarity™ western ECL substrate (Bio-Rad Laboratories, CA, USA) according to the manufacturer's instructions. The chemiluminescent blots were imaged ImageQuant™ LAS 500 (GE Healthcare Life Sciences, Piscataway, NJ, USA). Densitometric analysis was carried out using ImageJ software (National Institute of Health, Bethesda, MD, USA) and calnexin or GAPDH (glyceraldehyde 3-phosphate dehydrogenase) were used as a protein loading control.

Statistical Analysis

Tests and parameters used are indicated in the figures. All statistical analyses were performed using GraphPad Prism 6 for Windows, version 6.01 (GraphPad Software, Inc.).

In all cases, $p < 0.05$ was considered statistically significant.

RESULTS

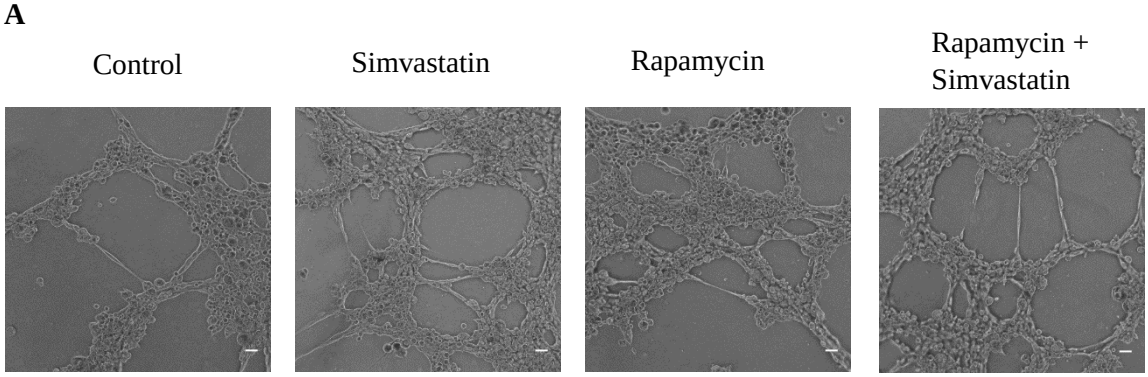
Although the use of drug-eluting stents (DES) has been an important contribution to prevent restenosis due to neointima hyperplasia, the drugs used, namely sirolimus, can also affect endothelial cells (EC), compromising reendothelialization following angioplasty-induced denudation of endothelium. One of the main objectives of this study was to identify strategies that mitigate the effect of sirolimus on EC in order to permit reendothelialization. To address this question we evaluated the impact of statins, incubated either alone or in combination with sirolimus, hereby mentioned as rapamycin, on key aspects of the angiogenic process.

The addition of simvastatin to rapamycin promoted angiogenesis

First, we performed a tubulation assay, which assesses the capacity of MCEC in culture to undergo structural changes that resemble the formation of capillary-like structures, incubated in the presence of rapamycin and/or simvastatin. After 6 hours of incubation, we evaluated different parameters implicated in the formation of tube-like structures *in vitro*, such as the number of meshes, nodes and master segments length formed by MCEC.

The results obtained and plotted in a graph (Figure 1 A and B) show that cells treated with rapamycin, presented modest differences in comparison with control cells incubated in the absence of any drug, whereas simvastatin resulted in the formation of higher number of meshes, nodes and longer master segments. Importantly, when the cells were treated simultaneously with the two drugs, we observed a tendency for a better response, with the formation of more tube-like structures, when comparing with rapamycin alone.

A



B

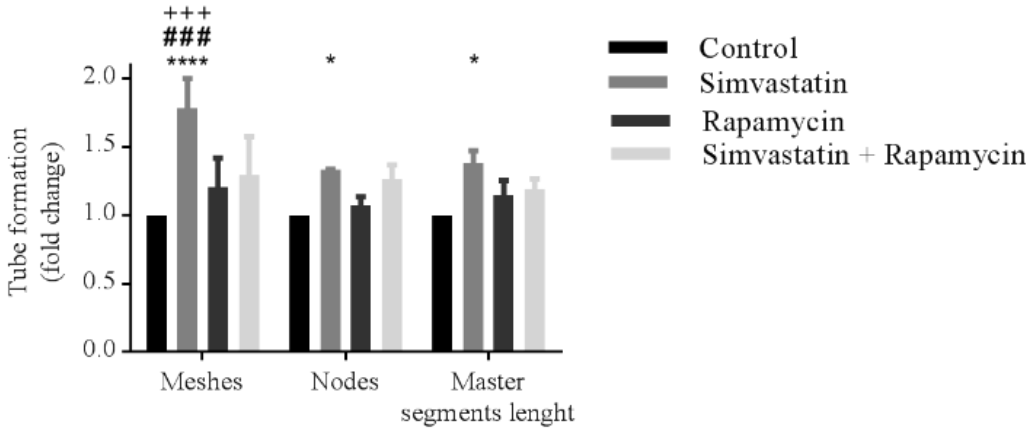


Figure 1: The addition of simvastatin to rapamycin on MCEC promotes tubulation. (**A)** Representative phase-contrast images of capillary tube formation of MCECs upon treatment with simvastatin, rapamycin or rapamycin plus simvastatin. Scale bar: 50 µm. **(B)** Quantitative assessment of capillary tube formation (total number of meshes, nodes and master segments (length-sum of the length of the detected master segments in the analysed area)). Results represent mean ± SD, normalized to control (n=3). t-test (Holm-Sidak's). *p<0.05, ***P< 0.001, and ****P<0.0001. Comparison statistically significant in relation with: control (*), rapamycin (#) and simvastatin+rapamycin (+).

After, we measured the same parameters in MCEC cultured in matrigel containing either a bare-metal stent or a sirolimus-eluting stent.

The results presented in Figure 2 show that after 6 hours in culture, cells incubated with sirolimus-eluting stent (DES) performed worst compared to cells incubated in matrigel containing a bare-metal stent (BMS), in terms of tubulation assay. However, when statin is

added to the medium, cells incubated in matrigel containing sirolimus-eluting stent, form more meshes, nodes and longer master segments, in comparison with cells maintained in the absence of statin (data plotted in a graph, Figure 2 B).

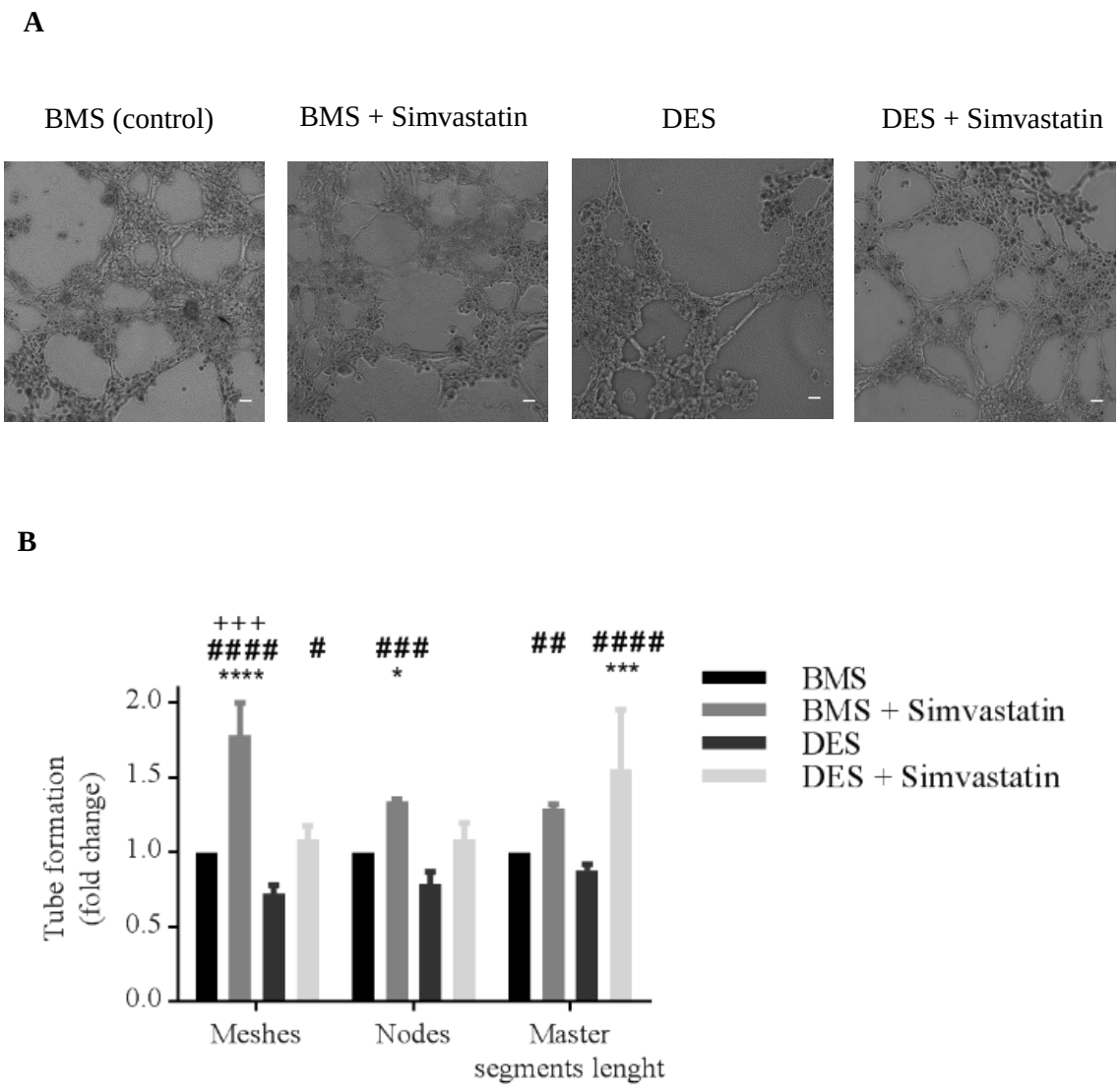


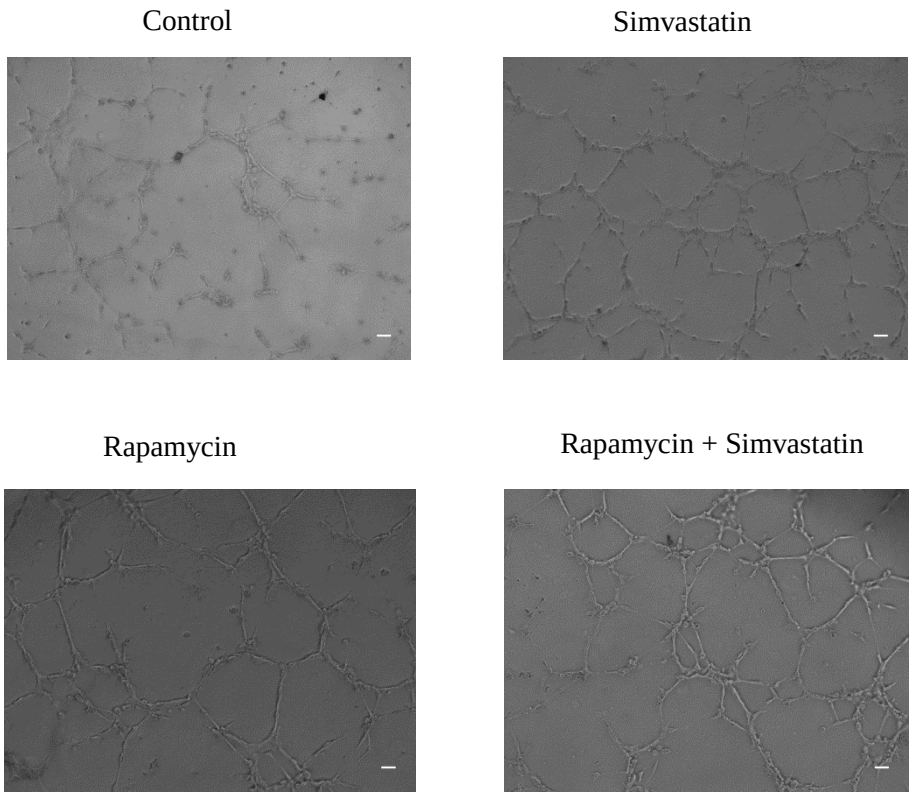
Figure 2: Simvastatin promoted tubulation on MCEC with a sirolimus-eluting-stent. (**A)** Representative phase-contrast images of capillary tube formation of MCECs, treated with simvastatin in the presence of a stent (BMS: bare-metal stent or DES: drug-eluting stent). Scale bar: 50 µm. **(B)** Quantitative assessment of capillary tube formation (total number of meshes, nodes and master segments (length-sum of the length of the detected master segments in the analysed area)). Results represent mean ± SD, normalized to control (n=3). t-test (Holm-Sidak's)*p<0.05, **P<0.01, ***P< 0.001, and ****P<0.0001. Comparison statistically significant in relation with: control (*), DES (#) and DES+simvastatin (+).

Once we have investigated the impact of statin in sirolimus-induced impairment of tubule formation, we proceeded the study using primary culture of pulmonary arterial endothelial cells (PAEC), which constitute a closer model to human cells.

As with MCEC, the matrigel tubulation assay on PAEC demonstrated that simvastatin promoted the formation of more capillary-like tubes (meshes, nodes and master segments length), when compared with control cells, incubated in the absence of any drug.

However, when simvastatin is incubated in the presence of rapamycin, no significant differences were detected, in comparison with cells treated with either simvastatin or rapamycin alone.

A



B

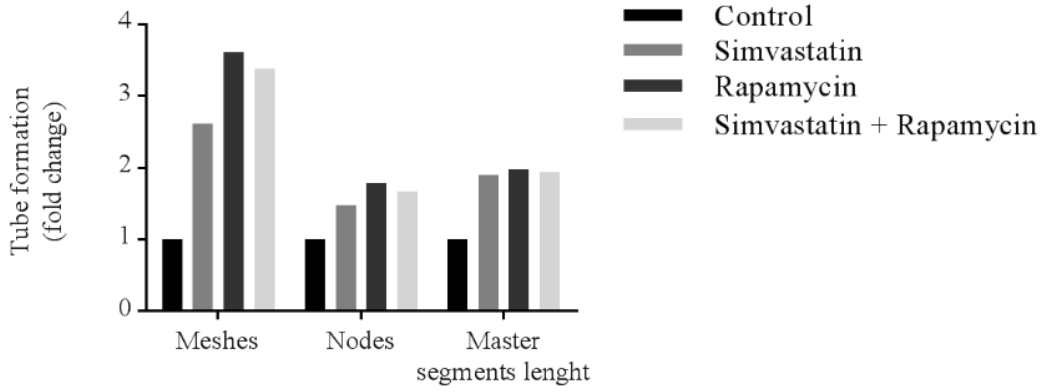


Figure 3: Simvastatin improved angiogenesis on PAECs. (**A)** Representative phase-contrast images of capillary tube formation of PAECs, without stent, treated with simvastatin, rapamycin or rapamycin plus simvastatin. Scale bar: 50 µm. **(B)** Quantitative assessment of capillary tube formation (total number of meshes, nodes and master segments (length-sum of the length of the detected master segments in the analysed area). Results normalized to control. n=1.

However, when PAECs were incubated in the presence of sirolimus-eluting stent, as a source of rapamycin, the addition of simvastatin to the medium resulted in an improved capacity of cells to form capillary-like structures, suggesting that statin is enhancing the angiogenic potential.

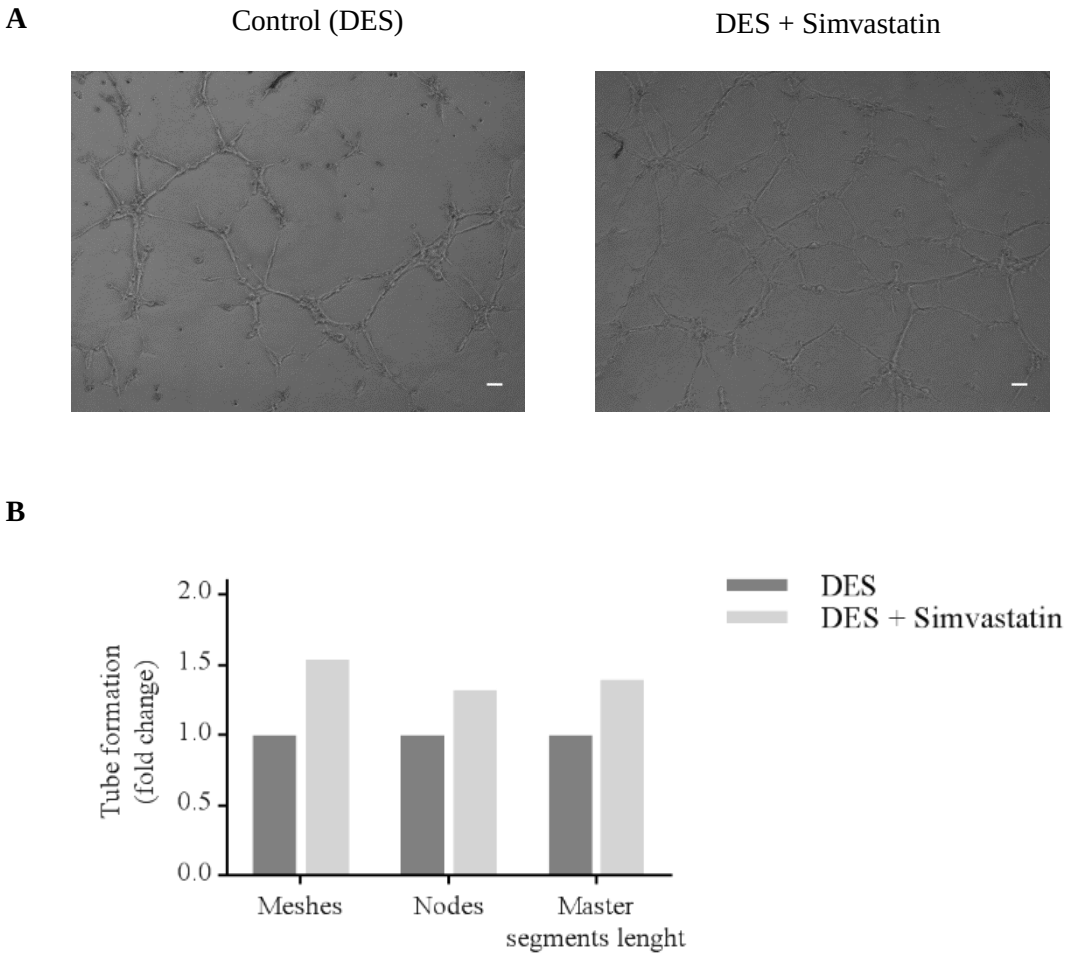


Figure 4: Simvastatin promoted tubulation on PAEC with a sirolimus drug-eluting-stent (DES). **(A)** Representative phase-contrast images of capillary tube formation of PAECs, with 15000 cells per well, after 12 hours of treatment with simvastatin 1 µM on a drug-eluting stent (DES). Scale bar: 50 µm. **(B)** Quantitative assessment of capillary tube formation (meshes, nodes and master segments length). Results normalized to control, DES. n=1. t-test (Holm-Sidak's)*p<0.05, **P<0.01, ***P< 0.001, and ****P<0.0001

Altogether, the data obtained up to this stage, with MCEC and PAECs in this matrigel assay, with stent, indicates that simvastatin treatment in the presence of BMS promoted more capillary tube formation. Moreover, the presence of the DES seems to decrease the angiogenic potential of EC, being this effect partially reverted upon treatment with simvastatin, attested by the higher number of capillary-like tubes formed.

Simvastatin promotes ECs migration

After, we evaluated the impact of simvastatin and rapamycin on EC migration, a key aspect of the reendothelialization process. The migration is assessed by measuring the area of wound reduction after a scratch performed in the cell monolayer.

The results presented in Figure 5 show that the incubation with rapamycin resulted in an inhibition of cell migration, and that the co-presence of simvastatin reverted this effect, with cells migrating faster than in controls or cells incubated with rapamycin alone.

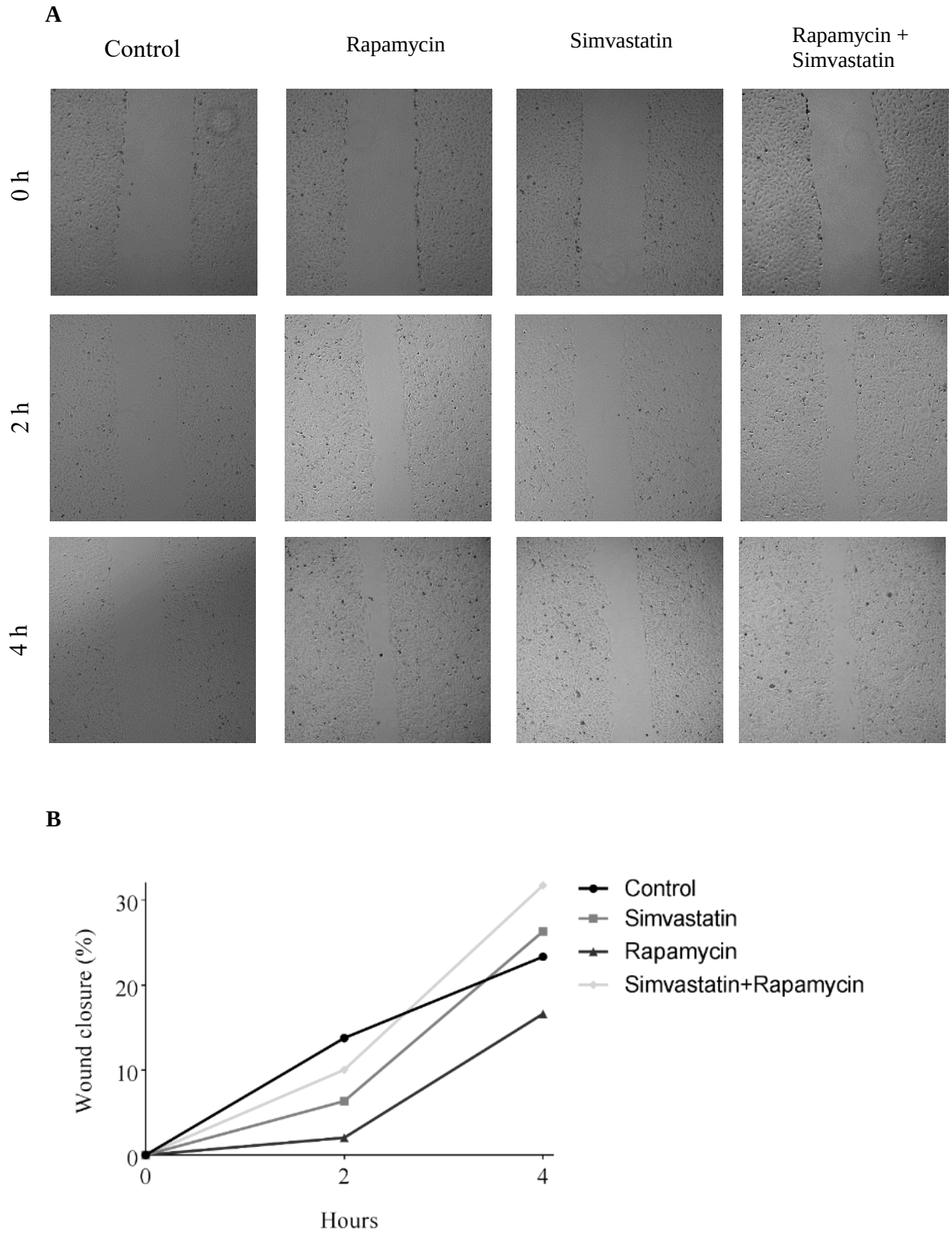


Figure 5: Simvastatin seems to potentiate migration of MCEC. **(A)** Representative images of MCEC migration taken at 0, 2 and 4 hours after scratch in the presence of rapamycin, simvastatin or rapamycin plus simvastatin, after pre-treatment for 6 hours. **(B)** Distance measurements were performed at the indicated times after the scratch, percentage of closure was calculated and plotted in a graph (n=3).

Simvastatin modulates autophagy on endothelial cells

It has been established that sirolimus, an analogous of rapamycin, besides preventing SMC proliferation and neointima formation, can also impact on autophagy in EC, which can underlie the undesired effect of sirolimus in hampering reendothelialization .

The effect on autophagy activity was assessed by the conversion of LC3-I (the cytosolic unlipidated form of LC3) to LC3-II (the phagophore-attached lipidated form LC3), as a mean to measure the formation of new autophagic vesicles. In addition to LC3, we also evaluated the levels of p62, an autophagy substrate, as an indicator of autophagy activity.

Based on the results presented above, we hypothesized that statins could attenuate the effect of sirolimus on autophagy of ECs. For that, we evaluated the autophagy activity in MCEC incubated either in the presence or absence of rapamycin and/or simvastatin, by measuring the levels of LC3-I and LC3-II, by western-blot, since these two forms of LC3 have different migrating patterns giving rise to two distinct bands.

As expected, the incubation with rapamycin resulted in an initial increase of LC3-II, (Figure 6 C), up to 12h, likely corresponding to autophagy induction and the formation of autophagy vesicles, after which these levels started to decrease, as the vesicles fuse with the lysosome.

When cells were incubated with simvastatin 1 µM no significant changes on LC3 levels were observed in any of the time points tested (12, 24 and 48 h). However, the presence of simvastatin 5 µM, for 48 hours, lead to an accumulation of LC3-II (2.91-fold increase) and p62 (Figure 6 C and B), which might be indicative of induction of autophagy or autophagosome-lysosome fusion impairment. However, since p62 (a downstream step on the autophagy cascade) is also increased, it seems more likely that there is an inhibition on the fusion of autophagosomes, which suggests that simvastatin on a higher concentration is inhibiting the last steps of autophagy.

Importantly, in the presence of rapamycin, simvastatin 1 µM affects autophagy (Figure 6 B and D), although to a lesser extent when compared with simvastatin 5 µM, with the accumulation of p62 and LC3-II, compared to LC3-I.

In addition to LC3-II levels, the autophagy activity was assessed by the LC3-II/ LC3-I ratio, that reflects the conversion of LC3-I into the lipidated LC3-II, that corresponds to the LC3 incorporated into the newly formed autophagy vesicles.

The results depicted in Figure 6 D confirmed that cells treated with simvastatin 1µM, compared to rapamycin, presented a decrease of the LC3-II/I ratio, which might be ascribed to an increase of autophagy, with more degradation of LC3-II after fusion with lysosomes, or an inhibition of the catabolic pathway, due an impairment of lipidation process that converts LC3 I in II. However, since p62, under the same conditions, (except for the 48 hours simvastatin condition) is decreased (Figure 6 B), then it is more likely that simvastatin on lower concentration is promoting autophagy.

Moreover, LC3-II itself is decreased with simvastatin 1µM, when compared to rapamycin, (Figure 6 C), which is another indicator that the autophagy is indeed being activated with simvastatin.

When cells were incubated with rapamycin and simvastatin, there was a time dependent increase of LC3-II/I ratio, which might be ascribed to an induction of autophagy flux.

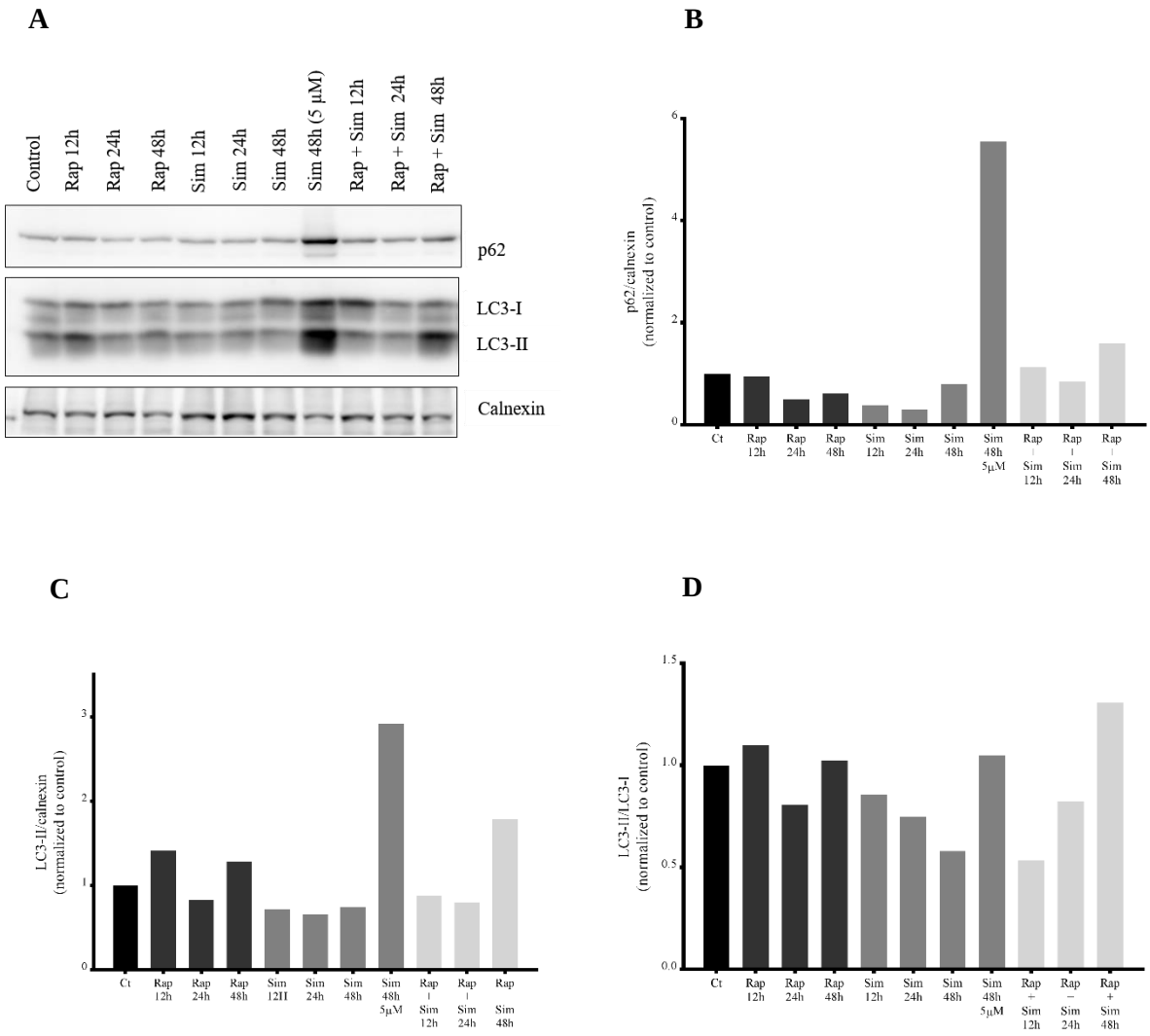


Figure 6: Simvastatin modulates autophagy. **(A)** Representative immunoblot images of MCEC treated for 12/24/48 hours with rapamycin, simvastatin 1 and 5 µM; and rapamycin plus simvastatin. **(B)** Changes in the levels of p62 calculated by immunoblot densitometry, calnexin was used as loading control. **(C)** Changes in the levels of LC3-II calculated by immunoblot densitometry, calnexin was used as loading control. **(D)** LC3-II/I ratio, calculated by immunoblot densitometry. **(A-D)** Nontreated controls are set as 1. n=1.

To evaluate the effect of a certain molecule on autophagy flux, the levels of LC3-II were determined in cells incubated either in the presence or in absence of an autophagy inhibitor, such as bafilomycin A1 (BafA1), which prevents the fusion between autophagosomes and lysosomes.

As expected, BafA1 lead to a high accumulation of p62 and LC3-II, whereas incubation with simvastatin resulted in a more modest increase of both autophagy proteins. Importantly, when the two compounds are added simultaneously, we observed a cumulative effect, as the ratio LC3-II/I is higher than individually BafA1 and simvastatin, suggesting that simvastatin can act as an activator of autophagy on ECs.

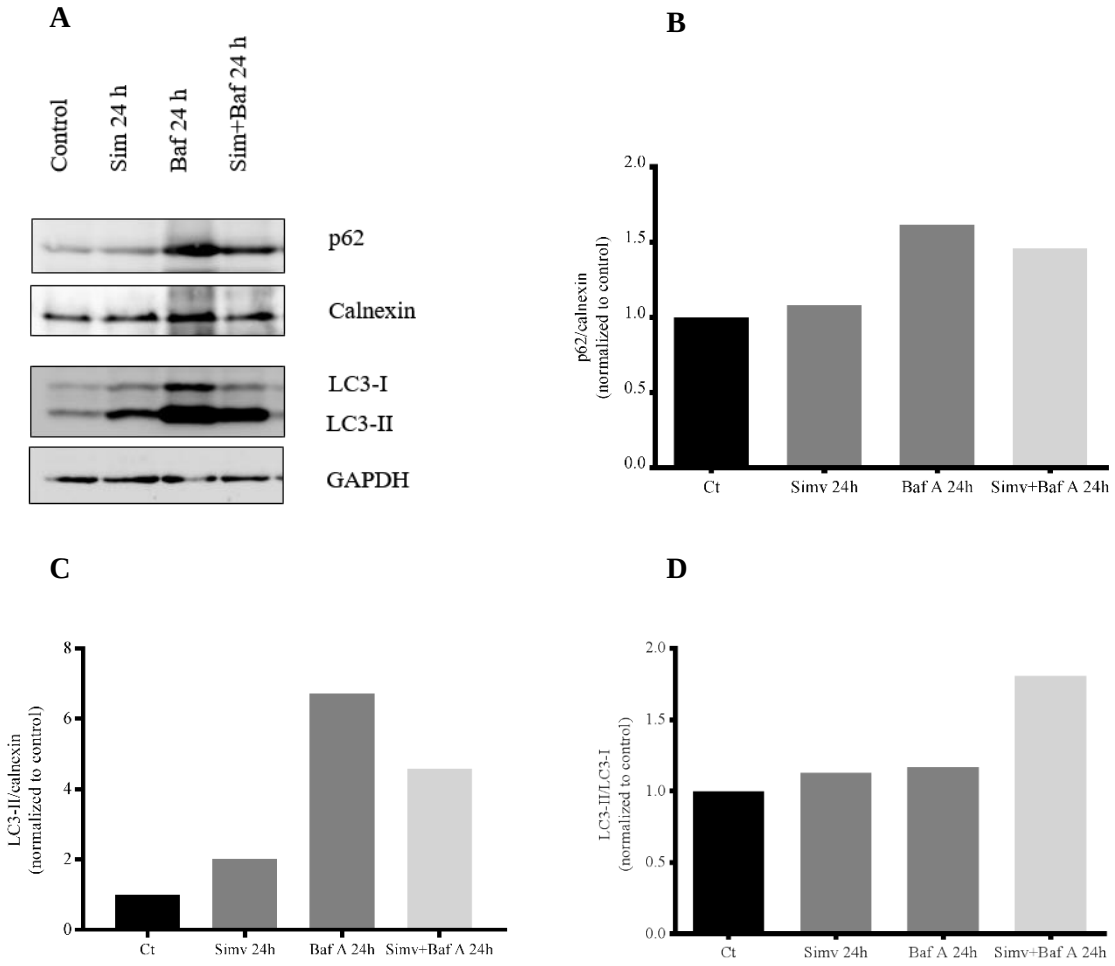


Figure 7: The addition of simvastatin partially reverts the inhibition of autophagy by bafilomycin A1. **(A)** Representative immunoblot images of MCEC treated 24 hours with simvastatin, bafilomycin A1, or both. **(B)** Changes in the levels of p62 calculated by immunoblot densitometry, calnexin was used as loading control. **(C)** Changes in the levels of LC3-II calculated by immunoblot densitometry, GAPDH was used as loading control. **(D)** LC3-II/I ratio, calculated by immunoblot densitometry. **(A-D)** Nontreated controls are set as 1. n=1.

DISCUSSION AND CONCLUSION

This set of results indicate that indeed simvastatin, when added to rapamycin promotes angiogenesis. This might be subject of interpretation. Neovessels may serve as a pathway for recruitment of leukocytes to high-risk areas of the plaque. Furthermore, thin-walled neovessels are often leaky and fragile, and disruption of microvessels might result in intraplaque hemorrhage.[13] On the other hand, the capacity of EC for angiogenesis correlates with a proper arterial healing response that promotes vascular repair and regeneration, after stent deployment.[7]

Simvastatin also reverted the decrease of migration observed with rapamycin, which suggests that rapamycin plus simvastatin might increase the reendothelialization rate *in vivo*.

This suggests that simvastatin may have a beneficial effect when endothelial damage occurs, such as when a stent is deployed. EC dysfunction also seems to determine neoatherosclerosis, more associated to late stent failure, and which pathogenesis is being speculated that incomplete or immature endothelialization permits a larger amount of lipoproteins to enter the neointima. Neoatherosclerosis generally increases the neointimal burden and contributes to development of stent restenosis. Therefore, statins might also impact neoatherosclerosis and decrease very late stent thrombosis.[16]

Rapamycin and simvastatin seem to differentially induce autophagy over time. Comparing to rapamycin alone, the addition of simvastatin seems to further stimulate autophagy, which could impact in ECs survival upon stress and angiogenesis.[15]

However, additional experiments and experimental approaches are necessary to confirm these results, like immunofluorescence or an autophagy detection kit that measures autophagic vacuoles and monitors autophagic flux.

Moreover, to further proceed with this work, cell viability assays, such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT assay) could be performed to assess endothelial cell metabolic activity in the presence of rapamycin and/or simvastatin, despite during all the experiments performed, no signs of cell viability loss or stress were observed.

ECs proliferation is a crucial feature of angiogenesis, and, taking that into account, further experiments assessing the modulation of this process by rapamycin and/or simvastatin can help to clarify the role of these compounds in the modulation of the angiogenic process and/or reendothelialization after stent arterial injury.

Another important issue to be addressed is the investigation of the effect of a lower dose of rapamycin with higher doses of simvastatin. The ultimate goal is to investigate a lower dose of rapamycin that, nonetheless, still inhibits the proliferation of vascular smooth muscle cells, further inhibiting mTOR, but without the collateral side effect of endothelial dysfunction.

Moreover, on the matrigel assay, the condition DES (stent with sirolimus), the concentration of this compound is unknown, as it is a fragment of DES. In part of this assay, when assessing the condition drug-eluting stent plus simvastatin, it was normalized to drug-eluting stent (sirolimus), due to material shortage. However, it should have been compared to BMS and BMS plus simvastatin.

Overall, intact autophagic responses are key to preserving cardiovascular homeostasis in physiological conditions and hence preventing premature degenerative changes in cardiovascular tissues.[17] Notably, autophagy activation is also associated with angiogenesis in EC particularly within ischaemic microenvironments.[15]

Previous studies have shown that VSMCs, macrophages, and EC display characteristics of autophagy activation when exposed to proatherogenic factors. Moreover, increase in autophagy

levels of vascular cells was found in atherosclerotic lesions, indicating that autophagy is involved in regulating the process of atherosclerosis, seemingly delaying its progression.[17]

Autophagy plays a vital role in the maintenance of ECs populations in response to a series of extracellular insults.[18] The inhibition of autophagy impairs the secretion of von Willebrand Factor from EC, suggesting that EC autophagy may also have an anti-thrombotic function.[15]

These sets of results were taken with several independent experiences (n=3), except for PAECs (n=1), however with technical triplicates showing consistent results. Nonetheless those experiments should be repeated, and complemented with different experimental approaches, eventually with higher concentration of statins.

Moreover, to clarify the potential beneficial synergy of rapamycin and statins on vascular recovery upon stent placement, further studies with vascular smooth muscle cells, endothelial cells and vascular smooth muscle cells co-cultures and *in vivo* animal studies are required.

However, to the best of our knowledge, this is a pilot study showing that statins, when added to sirolimus, promotes angiogenesis, and a higher migration rate, as well as the potentiation of the autophagy regulation. A functional endothelium will have the potential to reduce the requirement for long-term dual antiplatelet therapy and the bleeding risk that accompanies it, as well as the proliferation of vascular smooth muscle cells that sets restenosis, as growing evidence suggests that DES-associated endothelial dysfunction is likely to be essential for early/late stent thrombosis, in-stent restenosis, and myocardial infarction.[16]

These results will pave the way for new understandings on the effects of statins on the vascular cells, and potentially a statin-eluting stent the default one on clinical practice, for a lower risk of restenosis and endothelial dysfunction.

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