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Perspective

Geranylgeraniol prevents the simvastatin-induced PCSK9 expression: Role of the small G protein Rac1

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ABSTRACT

Statins are known to increase the plasma levels of proprotein convertase subtilisin kexin type 9 (PCSK9) through the activation of the sterol responsive element binding protein (SREBP) pathway due to the inhibition of cholesterol biosynthesis. In the present study, we explore a possible role of the prenylated proteins on the statin-mediated PCSK9 induction in Caco-2 cells. Simvastatin (40 μM) induced both PCSK9 mRNA (10.7 ± 3.2 fold) and protein (2.2 ± 0.3 fold), after 24 h incubation. The induction of PCSK9 mRNA was partially, but significantly, prevented by the co-incubation with mevalonate (MVA), farnesol (FOH) and geranylgeraniol (GGOH), while a complete prevention was observed on secreted PCSK9, evaluated by ELISA assay. Under the same experimental conditions, MVA, GGOH, but not FOH, prevented the activation of the PCSK9 promoter by simvastatin in a SRE-dependent manner. Simvastatin reduced by −35.7 ± 15.2% the Rac1-GTP levels, while no changes were observed on RhoA- and Cdc42-GTP. This effect was prevented by MVA and GGOH. A Rac inhibitor, and N17Rac1 dominant negative mutant, significantly induced PCSK9 levels, and a suppression of Rac1 expression by siRNA, counteract the effect of simvastatin on the induction of PCSK9 mRNA. Finally, simvastatin, and Rac inhibitor inhibited the nuclear translocation of STAT3 and its knock-down by siRNA increased significantly the susceptibility of Caco-2 to simvastatin on PCSK9 expression. Taken together, the present study reveal a direct role of Rac1 on simvastatin-mediated PCSK9 expression via the reduction of STAT3 nuclear translocation.

1. Introduction

The clinical benefits of statins are strongly related to their low density lipoprotein cholesterol (LDL-C) lowering properties [1]. Their efficacy in reducing LDL-C is due to their inhibition of the 3-hydroxy-3-methyl-3-glutaryl coenzyme A (HMG-CoA) reductase and thus, hepatic cholesterol biosynthesis [2]. In response to their action, the intracellular pool of free sterols decrease and the transcription factors sterol responsive element binding proteins (SREBPs) undergo proteolytic activation resulting in the induction of the LDL receptor (LDLR) and HMG-CoA reductase, in order to restore the intracellular cholesterol levels [3].

More recently, it was discovered that statins significantly induce the proprotein convertase subtilisin/kexin type 9 (PCSK9), at both mRNA and protein levels [4,5]. PCSK9 is a circulating inactive protease capable of interacting with LDLR and leading to the intracellular degradation of the LDLR [5–8]. PCSK9 is mainly synthesized by the liver, but significant levels have also been observed in the intestine, kidney [9], and in the atherosclerotic plaque [10]. While the hepatic effect of PCSK9 is mainly involved in the regulation of the LDL-C levels, at the intestinal level, PCSK9 may mediate the synthesis of triglyceride-rich apoB containing lipoproteins [11], the *trans*-intestinal cholesterol excretion (TICE) process [12], and postprandial triglyceridemia [13].

The effect of statins on mRNA PCSK9 expression is mediated by the activation of SREBP transcription factors and is completely prevented by mevalonate (MVA) [5], the enzymatic product of the HMG-CoA reductase [2]. Indeed, sterol responsive elements (SRE)

Abbreviations: FOH, farnesol; GGOH, geranylgeraniol; HMG-CoA, 3-hydroxy-3-methyl-3-glutaryl coenzyme A; MVA, mevalonate; LDL-C, low-density lipoprotein cholesterol; LPDS, lipoprotein plasma-deprived serum; PCSK9, proprotein convertase subtilisin/kexin type 9; SOCS3, suppressor of cytokine signaling; SRE, sterol responsive elements; SREBPs, sterol responsive element binding proteins; TICE, trans-intestinal cholesterol excretion.

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have been identified in the human PCSK9 promoter [14], and the intracellular sterol concentrations have shown to regulate PCSK9 expression both *in vitro* and *in vivo* [14]. The statin-induced increase in PCSK9 plasma levels have also been observed in clinical trials conducted with atorvastatin [15,16], simvastatin [17], and rosuvastatin [18]. More interestingly, it has been observed that the induction of PCSK9 occurs within 24 h after a single administration of atorvastatin [19]

From a mechanistic point of view, the induction of PCSK9 could counteract the effect of statins on the upregulation of the LDLR, thus supporting the rationale for a combination of statins with PCSK9 inhibitors. Indeed, the monoclonal antibodies direct against PCSK9 were shown to have an additive effect with statins [20].

Since statins, by inhibiting the HMG-CoA reductase, reduce the intracellular synthesis of nonsteroidal isoprenoid compounds, their administration can result in pleiotropic effects beyond their hypocholesterolemic properties [21]. In particular, two isoprenoids farnesyl-pyrophosphate and geranylgeranyl-pyrophosphate are substrates of prenyltransferase enzymes involved in the post-translational modification of intracellular proteins, the so called prenylation process [22,23]. Among the several prenylated proteins, the Rho GTPase family is the best characterized and its role on atherosclerosis and cardiovascular diseases has been hypothesized [24,25].

The discrimination among the pleiotropic and LDL-C lowering effects may be more evident during the early phase of treatment since plasma MVA levels drop up to 70% within 1–2 h while a reduction of LDL-C, detectable after 24 h, became significant after 6–7 days [26–28]. Based on these premises, in the present study we investigated the role of isoprenoids and isoprenylated proteins on simvastatin-mediated PCSK9 expression in Caco-2 cell line.

2. Methods

2.1. Reagents

Eagle's minimum essential medium (MEM), trypsin EDTA, penicillin, streptomycin, nonessential amino acid solution, Fetal Calf Serum (FCS), plates and petri dishes were purchased from EuroClone. Farnesol (FOH) and geranylgeraniol (GGOH) were from Sigma and were dissolved in ethanol 70%. Mevalonate (MVA) was purchased from Sigma-Aldrich and dissolved in H_2O. Simvastatin (Merck, Sharp, & Dohme Research Laboratories) was dissolved in 0.1 M NaOH and the pH was adjusted to 7.4, this solution was then sterilized by filtration [29]; The Rac inhibitor was the compound **6** from Ferri et al. [30] and was dissolved in DMSO.

2.2. Cell culture

The colon adenocarcinoma Caco-2 cell line (ATCC® HTB-37) was cultured in MEM containing 10%FCS, non-essential aminoacids, penicillin/streptomycin. For the experiments, cells were incubated with MEM containing either 10% of lipoprotein plasma-deprived serum (LPDS).

2.3. Quantitative real time PCR analysis

Total mRNA was extracted using iScript reagent (BIO-RAD), according to manufacturer's instructions; the cDNA was obtained with reverse-transcription utilizing Maxima First Strand cDNA synthesis kit (Carlo Erba Reagents). The quantitative real-time PCR on human PCSK9 and human Rac-1 was performed with Thermo SYBR Green/ROX qPCR Master Mix kit (Carlo Erba Reagents). The sequences of the primers are: hPCSK9-FWD, 5′-CCTGCGCGTGCTCAACT-3′ and hPCSK9-REV 5′-GCTGGCTTTTCCGAATAAACTC-3′; for hRAC-1-FWD, 5′-TGCAGACACTTGCTCTCCTATGTAGT-3′ and hRAC-1-*R*EV 5′-TTCAATGGCAACGCTTCATTC-3′. The analysis was executed with the ABI Prism® 7000 Sequence Detection System (Applied Biosystems; Life Technologies Europe BV) with the following cycling conditions: 95 °C, 10 min; 95 °C, 30 s and 60 °C, 1 min for 40 cycles. 18S mRNA levels were determined in each samples as a normalizing gene. The mRNA levels of the genes were expressed with the relative quantity method as $\Delta\Delta$Ct.

2.4. ELISA assay

ELISA assay was performed from conditioned media of the cultured Caco-2 cells. The amount of PCSK9 was quantified with DuoSet® ELISA kit (R&D System) as previously described [31].

2.5. G-LISA assay

The activation of small G-proteins Rac1-GTP, RhoA-GTP and Cdc42-GTP was measured with G-proteins Linked ImmunoSorbet Assay form Cytoskeleton, Inc. as previously described [32].

2.6. Western blot analysis

Total protein extracts were prepared by washing the cell monolayers twice with PBS and lysed by incubation with a solution of 50 mM Tris, pH 7.5, 150 mM NaCl, and 0.5% Nonidet P-40, containing proteases inhibitor cocktails (Sigma-Aldrich), for 30 min on ice. Cell lysates were cleared by centrifugation at 14,000*g* for 10 min, and protein concentrations were determined using the bicinchoninic acid (BCA) protein assay (EuroClone).

Nuclear and cytosolic fractions were prepared with the Nuclear Extraction Kit (Cayman Chemical) according to manufacturer's instructions. Protein concentrations were determined using the BCA protein assay.

Equal amounts of total proteins per sample were separated by SDS–PAGE under reducing conditions and transferred to nitrocellulose membrane using the *trans*-Blot® Turbo™ Transfer System (BIO-RAD). Immunoblotting was then performed with primary antibodies following appropriate secondary fluorescent labeled antibodies and acquired with the Odissey FC system (LI-COR). Quantitative densitometric analysis was performed with Image Studio software (LI-COR). For the analysis, the following antibodies were used: anti-STAT3 (Abcam), anti-Rac1 (Millipore-Merck), anti-actin and anti α-tubulin (Sigma-Aldrich), anti-laminA (Cell signaling), anti-mouse IRDye® 800CW and anti-rabbit IRDye® 800CW (LI-COR) [33].

2.7. Transfection of small interfering RNA

ON-TARGET plus SMART pool siRNA directed to Rac1, STAT3 or scramble control were purchased from Dharmacon™ (Carlo Erba Reagents). Transfections were performed as previously described using SilentFectTM Lipid Reagent (BIO-RAD) according to the manufacturer's protocol [34].

2.8. Transfection of Rac1N17 construct

The plasmid pBM-IRES-Rac1N17 were previously synthesized according standard molecular biology techniques [29]. Caco-2 cells were transfected with plasmid by using turbofect reagent (Thermo Fisher) and Opti-MEM/2.5% FCS. 24 h post transfection, the cells were seeded in a 48-well tray (8×10^4 cells per well) in medium containing 10%LPDS and total RNA extracted using iScript reagent after an additional 24 h.

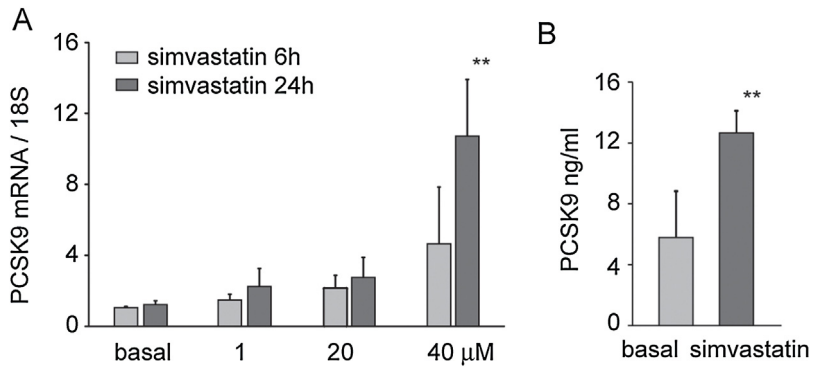

Fig. 1. Simvastatin induces PCSK9 expression in Caco-2 cells.
A and B) Caco-2 cells were seeded at density of 80.000 cells in a 48 well tray in MEM containing 10%FCS. The day after the medium was changed with MEM/10%LPDS in the presence or absence of indicated concentrations of simvastatin. A) At the indicated time points, total RNA was extracted and RT-PCR performed with specific primers for PCSK9 and 18S. B) Under the same experimental conditions, conditioned media was collected and PCSK9 levels determined by ELISA assay. **$p < 0.01$ Student's *t*-test.

2.9. Luciferase reported promoter activities assay

Caco-2 cells were transfected with pGL3-PCSK9-D4, pGL3-PCSK9-SREmut, and pGL3-PCSK9-HNFmut constructs, by using TurboFect Reagent (Thermo Fisher). The day after, the cells were seeded in a 48-well plate (8×10^4 cells per well) and after 24 h, the medium was changed with MEM/10%LPDS containing simvastatin, GGOH, FOH and MVA. Luciferase activity was measured after an additional 24 h using NeoLite reagent, according to manufacturer's instructions.

2.10. Analysis of the data

Statistical analysis was performed using the Prism statistical analysis package version 6.0 (GraphPad Software, San Diego, CA). Data are given as mean ± SD of at least 3 independent experiments. *p*-values were determined by Student's *t*-test. A probability value of $p < 0.05$ was considered statistically significant.

3. Results

To investigate the molecular mechanism underlying the induction of PCSK9 by statins, Caco-2 cells were incubated with increasing concentrations of simvastatin for 6 and 24 h. A significant induction of mRNA PCSK9 was observed at both 20 μM and 40 μM, with a maximal effect after the highest concentration at 24 h (10.7 ± 3.2 fold) (Fig. 1A). The ELISA analysis of the conditioned media also revealed a significant increase of secreted PCSK9 after 24 h incubation with 40 μM simvastatin (2.2 ± 0.3 fold; Fig. 1B).

To study the effect of isoprenoids on PCSK9, we performed the same analysis in Caco-2 cells incubated with simvastatin at 40 μM in the presence or absence of MVA, the enzymatic product of HMG-CoA reductase, or FOH and GGOH, the isoprenoids substrates of prenyltransferases. As documented by a previous study conducted with HepG2 cells [5], the co-incubation with MVA prevented the induction of both mRNA and secreted PCSK9 in Caco-2 cell line (Fig. 2A and B). More interestingly, the co-incubation of FOH and GGOH showed similar effect of MVA, with a full prevention on the induction of secreted PCSK9 by simvastatin (Fig. 2A) and significant effect on the mRNA levels (Fig. 2B).

To determine whether the effect of FOH and GGOH was due to an inhibitory effect at the transcriptional level of PCSK9, we performed a luciferase assay in Caco-2 cells transfected with the reporter construct pGL3-PCSK9-D4 [35]. This plasmid contains the 5′-flanking region of the PCSK9 gene, from nucleotide −440 to −94 (relative to the ATG start codon), in front of the luciferase coding sequence. The luciferase activity of the PCSK9 promoter was significantly induced by simvastatin (2.21 ± 0.28 fold, Fig. 3A). This induction was fully prevented by the co-incubation with MVA and GGOH, while FOH did not affect the PCSK9 promoter activity (Fig. 3A).

The role of SREBP and HNF-1α on the effect of isoprenoids on PCSK9 promoter activity was investigated in Caco-2 cells transfected with D4 construct containing the mutation of SRE or HNF-1 sites, thus mutually excluding the effect of SREBP or HNF-1α

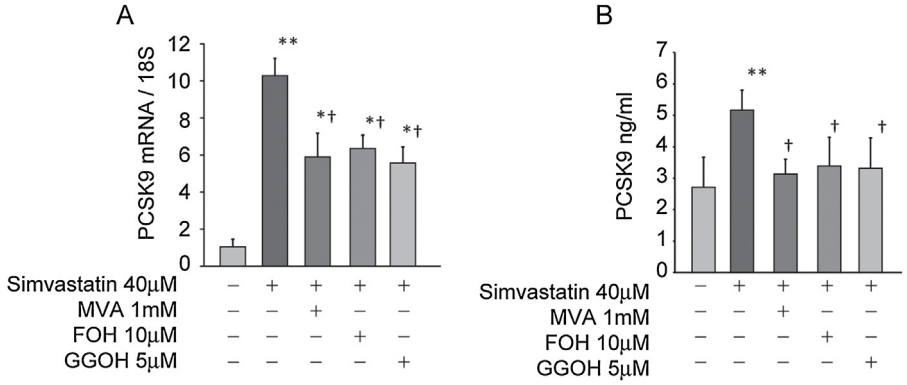

Fig. 2. MVA and its isoprenoid derivatives prevented the induction of PCSK9 by simvastatin in Caco-2 cells.
A and B) Caco-2 cells were seeded at density of 80.000 cells in a 48 well tray in MEM containing 10%FCS. The day after the medium was changed with MEM/10%LPDS in the presence or absence of simvastatin and the mevalonate derivatives. A) After 24 h, total RNA was extracted and RT-PCR performed with specific primers for PCSK9 and 18S. B) Under the same experimental conditions, conditioned media was collected and PCSK9 levels determined by ELISA assay. Differences between treatments were assessed by Student's *t*-test and one-way ANOVA (when necessary). **$p < 0.01$ and *$p < 0.05$ treated vs untreated; ‡$p < 0.05$ treated vs simvastatin alone.

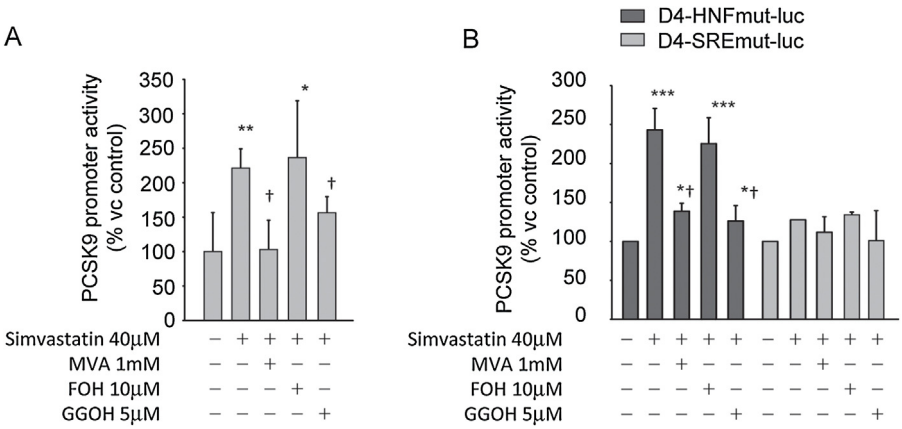

Fig. 3. Simvastatin induces PCSK9 promoter activity in Caco-2 cells.
Caco-2 cells were transiently transfected with the pGL3-PCSK9-D4 plasmid (A) or pGL3-PCSK9-SREmut (B) or pGL3-PCSK9-HNFmut (B). The day after the transfection, cells were harvested and seeded in 48 well tray plate. After 24 h, cells were incubated with MEM/10%LPDS in the presence or absence of simvastatin, MVA, FOH, and GGOH. After an additional 24 h, luciferase activities were determined by Neolite reagent (PerkinElmer). Differences between treatments were assessed by Student's *t*-test and one-way ANOVA (when necessary). *p < 0.05; ***p < 0.001 treated vs untreated. ‡p<0.05 treated vs simvastatin alone.

transcription factors (Fig. 3B). Importantly, the SRE mutation completely abrogated the induction of PCSK9 promoter activity by simvastatin (Fig. 3B), showing the absolute requirement of the SREBP transcription factor. In contrast, in the presence of the HNF-1 site mutation, the effect of simvastatin was preserved together with full prevention by the co-incubation with MVA and GGOH, while FOH did not affect the PCSK9 promoter activity induced by simvastatin (Fig. 3B).

Taken together, these data indicate the potential involvement of geranylgeranylated protein in preventing the effect of GGOH on PCSK9 expression induced by simvastatin. The effect of simvastatin on the intracellular levels of GTP-bound active form of geranylgeranylated proteins RhoA, Cdc42 and Rac1, was then determined by G-LISA assay. The incubation of simvastatin 40 μM for 24 h determined a significant reduction (-35.7 ± 15.2%) of Rac1-GTP while the levels of RhoA-GTP and Cdc42-GTP were not affected or even slightly increased (Fig. 4). As expected, the co-incubation with MVA or GGOH totally prevented the effect of simvastatin on Rac1-GTP, while FOH had no effect (Fig. 4C). The expression levels of Rac1, measured by western blot analysis, significantly increased in response to simvastatin (4.3 ± 0.4 fold); MVA and GGOH partially prevented this induction, while FOH showed not significant effect on Rac1 (Fig. 4D).

To further evaluate the role of Rac1 on PCSK9 expression, Caco-2 cells were incubated with increasing concentrations of the Rac1 inhibitor previously identified in our laboratory [30]. This inhibitor was shown to interfere with Rac1 activation by preventing the interaction with its guanine nucleotide exchange factor (GEF) and thus affecting the formation of Rac1-GTP with an IC_{50} value of 4.4 μM [30]. The incubation with 2.5 μM and 5 μM for 24 h determined a significant induction of PCSK9 mRNA levels by 2.25 ± 0.9 fold and 2.11 ± 0.6 fold, respectively (Fig. 5A). Similar results were obtained after transfection with a plasmid encoding for a dominant negative mutant of Rac1 (N17Rac1) (Fig. 5B), which cannot bind GTP and it is expressed as either a nucleotide free state or in its inactive, GDP-bound, state [36]. The combination of Rac inhibitor with simvastatin, did not significantly modify the induction of PCSK9 (Fig. 5C). Taken together, these results demonstrated a positive link between the intracellular levels of Rac-GDP and PCSK9 expression.

The involvement of Rac1 on simvastatin-mediated PCSK9 induction was then explored by using the siRNA against Rac1. As shown in Fig. 5D, siRNA anti Rac1 determined an almost complete suppression of Rac1 mRNA levels as compared to Caco-2 cells transfected with scramble siRNA. In line with western blot analysis (Fig. 4D), simvastatin produced a significant induction of Rac1 mRNA (2.09 ± 0.12 fold; Fig. 5D). The knock-down expression of Rac1 by siRNA did not affect the basal levels of PCSK9 mRNA, while there was a significant interference with the effect of simvastatin (Fig. 5E). The induction of PCSK9 mRNA in Caco-2 cells transfected with siRNA anti Rac1 was reduced by 54 ± 14% compared to cells transfected with scramble siRNA (Fig. 5E). Taken together, these results support the direct relationship between the induction of PCSK9 by simvastatin and the activity of the geranylgeranylated Rac1.

Since Rac1 and a GTPase-activating protein, MgcRacGAP, are required for nuclear translocation of STAT transcription factors [37], which is involved in the mRNA expression of PCSK9 [38], we investigated the effect of simvastatin and Rac inhibitor, on nuclear translocation, as well as the effect of siRNA anti STAT3 on PCSK9 expression. Both simvastatin and Rac inhibitor, significantly reduced the nuclear levels of STAT3, retaining it in the cytosol fraction (Fig. 6A and B). More interestingly, the induction of PCSK9 by simvastatin was dramatically enhanced in Caco-2 cells transfected with siRNA anti STAT3 compared to siRNA scramble control (Fig. 6C). These data indicate that STAT3 act as suppressor of PCSK9 transcription and that simvastatin, by inhibiting its nuclear translocation, induces the PCSK9 levels.

4. Discussion

In the present study, we provided evidences of the pivotal role of GGOH and Rac1 on simvastatin-mediated PCSK9 induction. The presence of the SRE in the human PCSK9 promoter clearly predisposes the regulation of PCSK9 levels at intracellular sterol levels. Indeed, both *in vitro* and *in vivo* evidences demonstrated that statins, by reducing the intracellular sterols levels, induced PCSK9 transcription as well as its circulating levels [5,14]. Nevertheless, the possible involvement of an additional mechanism, independent by the lipid lowering effect of statins, was hypothesized after the clinical observation that a single administration of atorvastatin increased the plasma levels of PCSK9 within 24 h, a time-frame non-sufficient to observe an LDL-C reduction after statin treatment [27]. Within this period, statin administration determines a very rapid reduction of urine and plasma levels of MVA and potentially its isoprenoids derivatives, such as farnesyl-PP and geranylgeranyl-PP [26–28]. Thus, in the present study we investigated the possible involvement of MVA-derived isoprenoids and prenylated proteins on PCSK9 expression.

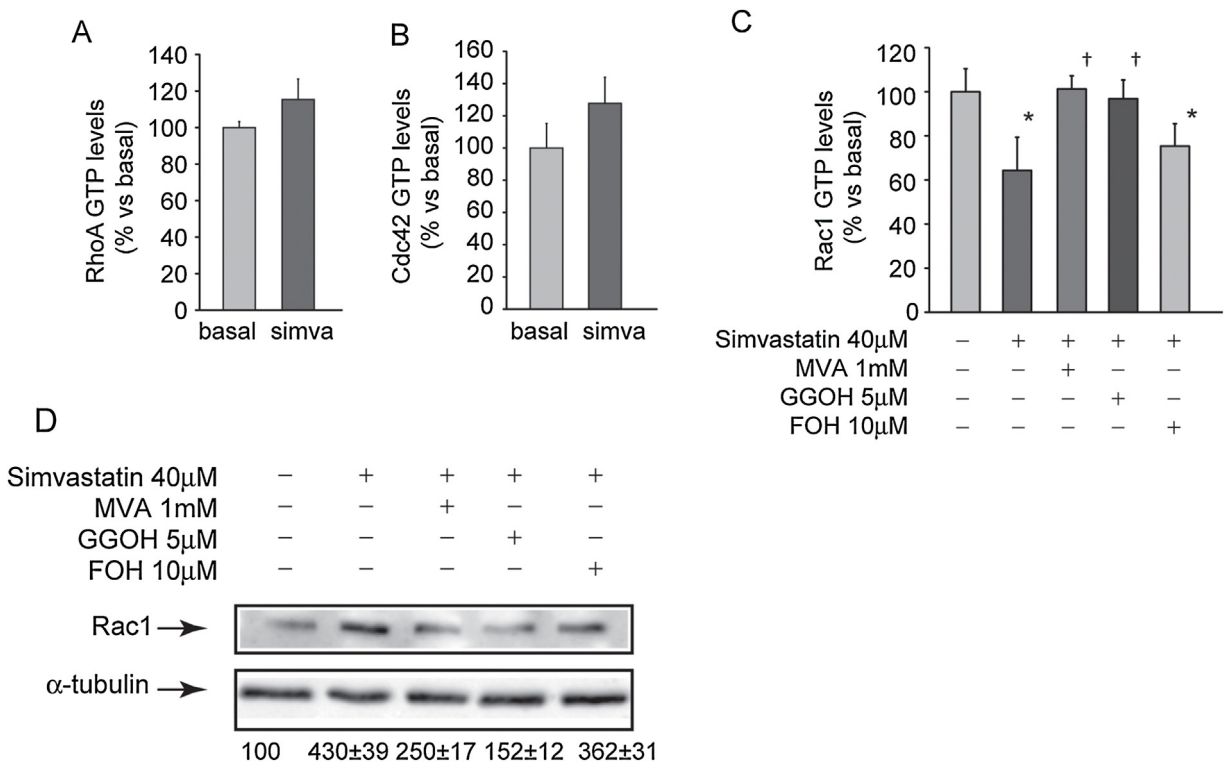

Fig. 4. Effect of simvastatin on RhoA-, Cdc42- and Rac1-GTP levels in Caco-2 cells.
A–C) Caco-2 cells were seeded at density of 300.000 cells in a 6-well tray in MEM containing 10%FCS. The day after the medium was changed with MEM/10%LPDS in the presence or absence of simvastatin (A–C) and the MVA derivatives (C). After 24 h, specific G-LISA assays were performed from total cell lysates. D) Under the same experimental conditions described for panel C, the total Rac1 expression levels were determined by western blot analysis. α-tubulin was used as loading control. Differences between treatments were assessed by Student's *t*-test and one-way ANOVA (when necessary). *$p < 0.05$ treated vs untreated. ‡$p < 0.05$ treated vs simvastatin alone.

Our data clearly demonstrated that simvastatin induces PCSK9 in Caco-2 cells at concentration levels significantly higher than those required to fully inhibit the cholesterol biosynthesis [21,29]. A strong and significant induction of PCSK9 was, indeed, observed at 40 μM after 24 h, while only minor changes where seen at 1 μM, a concentration sufficient to reduce by more than 50% the cholesterol biosynthesis [29]. Thus, the rapid induction of PCSK9 by simvastatin seemed to be unrelated to its effect on cholesterol biosynthesis and more likely linked to the inhibition of the isoprenoids synthesis that elicits the so-called pleiotropic effects of statins [23]. Indeed, the *in vitro* evidence of pleiotropic effects of statins has been always documented at concentrations approximately ten times higher than those required to inhibit the cholesterol biosynthesis [21,39,40].

Both FOH and GGOH were shown to prevent the induction of PCSK9 by simvastatin suggesting the involvement of prenylation process. However, significant differences between the two isoprenoids were observed. GGOH completely prevented the effect of simvastatin on mRNA, secreted protein, and promoter activity of PCSK9, while FOH was not able to prevent the transcriptional induction of PCSK9. On this regard, it is intriguing to note that oncostatin M [41] and suppressor of cytokine signaling 3 (SOCS3) [38] significantly regulate the mRNA levels of PCSK9 without altering its transcriptional regulation. Thus, it is conceivable to hypothesized a different role of the two isoprenoids, potentially related to the fact that FOH, and not GGOH, is a precursor of cholesterol biosynthesis [42].

The prevention of GGOH on the PCSK9 promoter activity induced by simvastatin was observed also by using the reported plasmid containing a mutation in the HNF-1 site, excluding an involvement of HNF-1α transcription factor. On the contrary, the mutation of the SRE on the promoter completely abrogated the induction by simvastatin and the effect of GGOH. Thus, GGOH seems to activate a PCSK9 promoter suppressor factor that antagonized the SREBP-dependent activation by simvastatin.

A possible negative regulatory mechanism could involve an intracellular geranylgeranylated protein. In a first attempt to identify a protein involved in a negative regulation of PCSK9 transcriptional regulation, we measured the effect of simvastatin on the intracellular levels of the small G proteins, RhoA, Cdc42 and Rac1. Interestingly, 24 h incubation of Caco-2 cells with simvastatin significantly affected the intracellular levels of the GTP-Rac1 with no effect on RhoA and Cdc42. The differential effect could be related to the half-life time of these proteins, with Rac1 having the faster turnover, and thus potentially affected by the short incubation time with simvastatin. The inhibition of simvastatin was associated with an induction of Rac1 expression. MVA and GGOH, but not FOH, prevented the reduction of Rac1-GTP levels in response to simvastatin, as well as the induction of total Rac1.

We then took advantage of a selective Rac inhibitor previously identified in our laboratory that selectively interferes with the interaction of Rac1-Tiam1, and thus with the exchange between GDP to GTP [30]. The inhibition of Rac1 by this chemical compound mimicked the effect of simvastatin by increasing the PCSK9 mRNA levels. These data suggest that the reduction of the intracellular levels of Rac1-GTP, either by simvastatin or Rac inhibitor, induces PCSK9. Consistently, the transfection of Caco-2 cells with a plasmid encoding for the dominant negative mutant N17Rac1, also induced PCSK9 mRNA levels. This point mutation determines the expression of Rac1 in either a nucleotide free state or in its inactive, GDP-bound, state [36]. The use of siRNA anti Rac1 that almost completely knock-down the expression of Rac1, did not induce PCSK9 mRNA, but rather inhibited the effect of simvastatin. These data suggest that Rac1 is a requested downstream protein for the induction of PCSK9 by simvastatin.

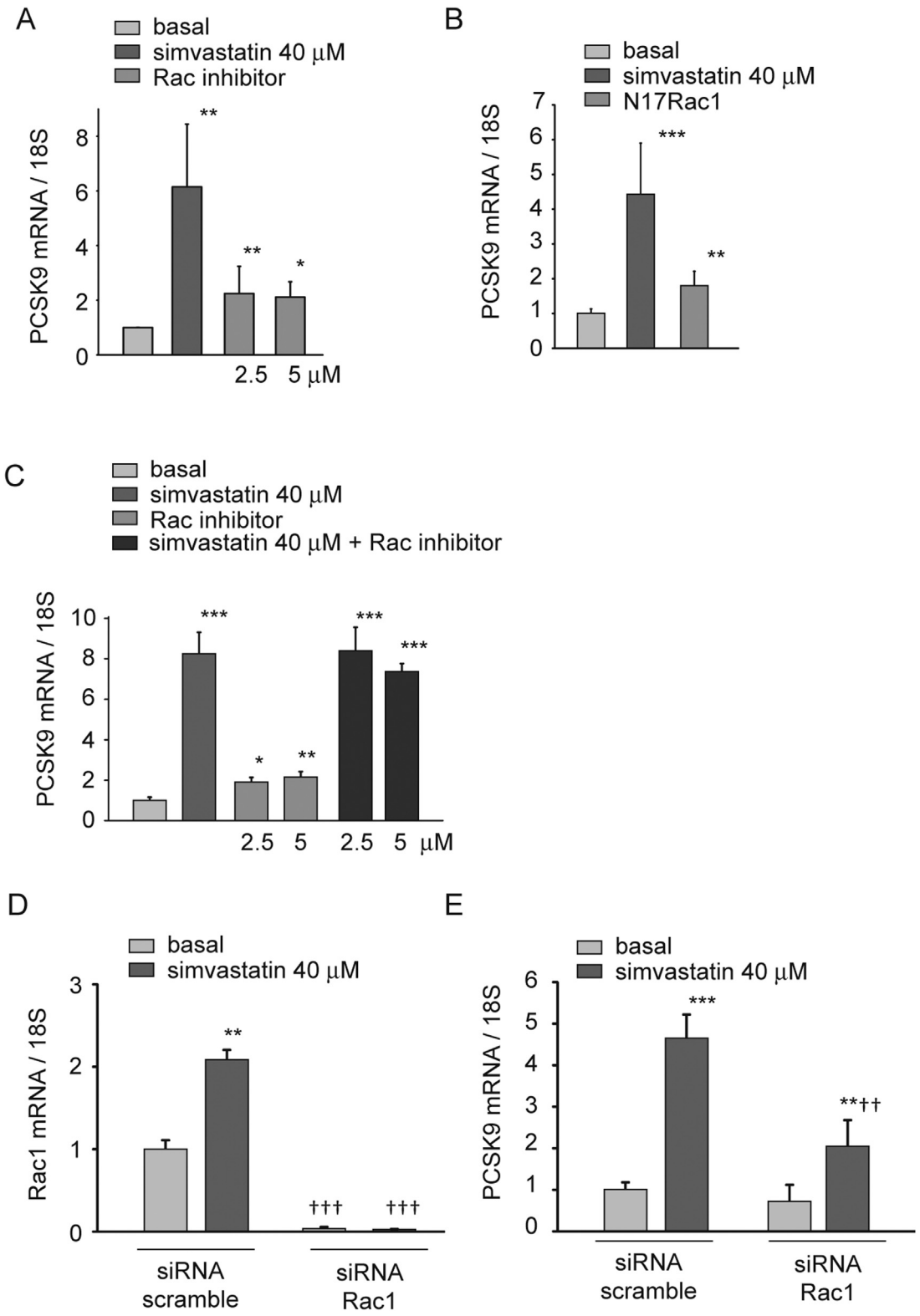

Fig. 5. Effect of Rac1 inhibition on PCSK9 mRNA levels in Caco-2 cells.
A) Caco-2 cells were seeded at density of 80.000 cells in a 48 well tray in MEM containing 10%FCS. The day after the medium was changed with MEM/10%LPDS in the presence or absence of simvastatin or indicated concentrations of Rac inhibitor. After 24 h, total RNA was extracted and RT-PCR performed with specific primers for PCSK9 and 18S. B) Caco-2 cells were transfected with plasmid encoding N17Rac1, and the day after seeded in 48 well tray plate in MEM/10%LPDS. After an additional 48 h RT-PCR was performed as indicated in panel A. C) Caco-2 cells were treated with simvastatin, Rac inhibitor and their combination, under the same experimental conditions described for panel A. RT-PCR analysis was then performed. D and E) Caco-2 cells were transiently transfected with siRNA anti Rac1 or siRNA scramble. The day after the transfection, cells were harvested and seeded in 48 well tray plate. After 24 h, cells were incubated with MEM/10%LPDS in the presence or absence of simvastatin. After an additional 24 h, total RNA was extracted and RT-PCR performed with specific primers for Rac1, PCSK9 and 18S. Differences between treatments were assessed by Student's *t*-test and one-way ANOVA (when necessary) $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ treated vs untreated; ‡‡$p < 0.01$; ‡‡‡$p < 0.001$ siRNA Rac1 vs siRNA scramble.

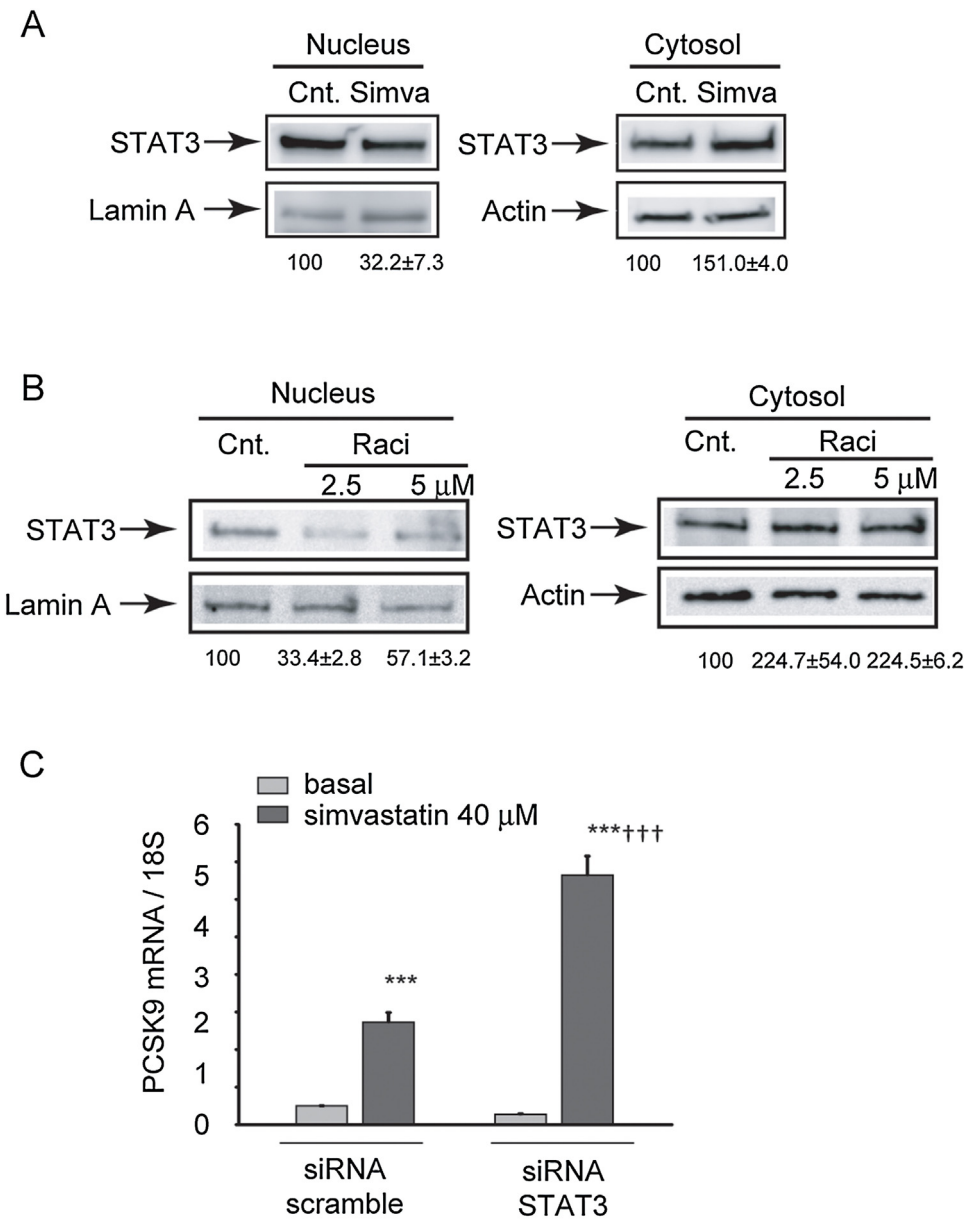

Fig. 6. STAT3 counteracts the induction of PCSK9 mRNA by simvastatin in Caco-2 cells.
A and B) Caco-2 cells were seeded at density of 1×10^6 cells in a 60 mm petri dish in MEM containing 10%FCS. The day after the medium was changed with MEM/10%LPDS in the presence or absence of 40 μM simvastatin and indicated concentrations of Rac inhibitor (Raci). After 24 h, nuclear and cytosol protein extracts were prepared and western blot analysis for STAT3 was performed. Actin and LaminA were used as loading control. C) Caco-2 cells were transiently transfected with siRNA anti STAT3 or siRNA scramble [38]. The day after the transfection, cells were harvested and seeded in 48 well tray plate. After 24 h, cells were incubated with MEM/10% LPDS in the presence or absence of 40 μM simvastatin. After an additional 24 h, total RNA was extracted and RT-PCR performed with specific primers for PCSK9 and 18S. Differences between treatments were assessed by Student's *t*-test and one-way ANOVA. ***$p < 0.001$ simvastatin vs basal; ‡‡‡$p < 0.001$ siRNA STAT3 vs siRNA scramble.

Interestingly, it is important to notice that prenylated Rac1 accumulates in the nucleus during the G2 phase of the cell cycle [43] and that only Rac1-GTP is found at a nuclear level [44]. Rac1-GTP nuclear accumulation becomes more evident in the presence of the proteasome inhibitor MG132 [44], a chemical compound that has been shown to suppressed PCSK9 expression in the HepG2 cells through a SREBP-1c related pathway [45]. From these observations, it is intriguing to speculate that simvastatin, by reducing the Rac1-GTP levels, could inhibit Rac1 nuclear localization allowing the transcription of PCSK9.

How nuclear Rac1 interferes with the PCSK9 transcription still need be fully determined, although it is important to notice that Rac1 is implicated in the nuclear import of STAT proteins [37], and that SOCS3, the endogenous inhibitor of the JAK/STAT pathway, strongly upregulates PCSK9 [38]. STAT3 has also shown to suppress the transcription of SREBP-1 and genes involved in the *de novo* lipogenesis [46,47], including PCSK9 [38]. Thus, we hypothesize that STAT3 was the final regulator of PCSK9 transcription by simvastatin. By analyzing the nuclear and cytosolic protein extracts, we demonstrated that both simvastatin and the Rac inhibitor, significantly inhibited the nuclear translocation of STAT3, an effect that could release the PCSK9 transcription by SREBP1 [47]. This hypothesis is supported by the fact that the effect of simvastatin on PCSK9 was strongly enhanced in Caco-2 cells transfected with siRNA anti STAT3.

5. Conclusions

In conclusion, in the present study we unrevealed a new mechanism of statin-mediated PCSK9 induction that involves the inhibition of the geranylgeranylated protein Rac1 and the nuclear translocation of STAT3, a negative regulator of SREBP-1c promoter activity and thus PCSK9 transcription [47]. This *in vitro* data provide a possible mechanism underlying the rapid increase of plasma PCSK9 observed after treatment with atorvastatin [19].

Author contributions

Nicola Ferri design the work, analyze and interpreted the data of the work and write the manuscript; Silvia Marchianò performed the real time PCR and the western blot analysis; Annalisa Trenti helped to perform the western blot analysis of nuclear and cytosol extracts; Giuseppe Biondo performed the real time PCR analysis and the G-LISA assays; Paola Castaldello performed the experiments with Rac1 inhibitors and siRNA; Alberto Corsini contributes to the design of the work and interpretation of the data.

Conflict of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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