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Statins upregulate CD36 expression in human monocytes, an effect strengthened when combined with PPAR-γ ligands Putative contribution of Rho GTPases in statin-induced CD36 expression

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Abstract

Scavenger receptor CD36 plays important roles in atherosclerosis, inflammation, thrombosis, and angiogenesis. Statins besides lowering serum cholesterol levels, exhibit a variety of effects on inflammation, coagulation and atherosclerosis lesion stability. PPAR-γ ligands influence macrophage responses to many inflammatory stimuli. Herein, we investigated in human monocytes the effect of statins alone, and in combination with PPAR-γ ligands on CD36 expression, as well as the molecular mechanisms underlying the regulatory action of statins. Our results demonstrate that statins upregulate both CD36 surface protein and mRNA by potentiating the transcription of the CD36 gene. Furthermore, the combination of statins and PPAR-γ ligands has an additive effect on CD36 expression. Effects of statins on CD36 expression were prevented by mevalonate and geranylgeraniol, indicating the requirement of geranylgeranylated proteins for CD36 regulation. Rho GTPases inhibitor C3 exoenzyme reproduced the effect of statins, while Rho activator lysophosphatidic acid downregulated CD36. Transient expression of dominant-negative mutants of RhoA and RhoB induced a significant increased in CD36 promoter activity. Finally, the actin cytoskeleton disrupter cytochalasin D upregulated CD36. These data indicate that Rho proteins are important modulators of CD36 expression, and strongly suggest that statins increased CD36 expression by disrupting cytoskeleton organization by inactivating Rho GTPases. These features prompt to investigate the roles of Rho GTPases and actin cytoskeleton modulators on monocytic functions affected by statins.

Keywords: Statins; Thiazolidinediones; Monocytes; Gene regulation; Rho; Inflammation

1. Introduction

CD36 is a scavenger receptor that, besides other cell types, is expressed on the cell surface of monocyte/macrophages, where it is thought to play important functions in inflammation and vascular biology [1]. Hence, CD36 participates in the recognition of apoptotic cells [2], helping to the resolution of inflammatory responses. In addition, monocytes recruited to the injured subendothelial space mediate, through their scavenger receptors, the uptake of oxidized low-density lipoprotein (OxLDL). A massive OxLDL uptake leads to the formation of foam cells, a critical step for the development of atherosclerosis lesions [3]. CD36 accounts for near a 50% of the total macrophage uptake of modified lipoproteins [4], and is abundantly expressed in macrophages located in the fatty streaks and in the core region of atherosclerotic lesions [5]. In this sense, the relevance of CD36 in atherosclerosis development is highlighted by the resistance to atherosclerosis found in CD36-null mice [6]. CD36 expression is highly regulated during monocyte/macrophage differentiation, and by

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Abbreviations: 15dPJ$_2$, 15-deoxy-$\Delta^{12,14}$-Prostaglandin J$_2$; AGE, advance glycation endproducts; eNOS, endothelial nitric oxide synthase; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; FCS, fetal calf serum; FOH, trans-trans farnesol; GGOH, all-trans geranyl geraniol; LDL, low-density lipoprotein; LPA, lysophosphatidic acid; OxLDL, oxidized low-density lipoprotein; PAI-1, plasminogen activator inhibitor-1; PPAR, peroxisome proliferator-activated receptor; SREBP, sterol regulatory element-binding protein; tPA, tissue plasminogen activator.

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cytokines, growth factors and modified lipoproteins present in atheroma and inflammation sites [7–9]. CD36 also binds native lipoproteins, although the physiological meaning of these interactions are so far unknown [10]. Furthermore, interaction of CD36 present in macrophages located within the intima with advanced glycation endproducts (AGEs) proteins [11] and thrombospondin-1 [12] might also contribute to the development/progression of vascular lesions. The multifunctional activities of CD36 present in monocyte/macrophages suggest that modulation of CD36 expression might lead to a series of potential beneficial/harmful effects in atherosclerosis, inflammation and angiogenesis.

High plasma cholesterol levels are a principal risk factor for atherosclerosis. The pathologic effects of an excess of circulating cholesterol are at least in part exerted through its uptake by macrophages present within atherosclerotic lesions. Clinical benefits on coronary heart disease of reduction of plasma cholesterol levels by pharmacological treatment using statins have been extensively established [13,14]. Statins reduce endogenous cholesterol synthesis by selectively inhibiting the HMG-CoA reductase, the rate limiting step in cholesterol synthesis. Lowering of intracellular cholesterol levels leads to the nuclear translocation of sterol regulatory element-binding protein (SREBP) transcription factors, where they activate a number of genes mostly involved in regulation of cholesterol homeostasis [15]. However, statins have been shown to increase the survival rates even in normocholesterolemic or hypocholesterolemic patients [16], observations that lead to the discovery that many of the pleitropic effects of statins are beyond the lowering of serum cholesterol levels [17]. In this respect, statins restore impaired endothelial-mediated vasodilatation by upregulating the expression of the endothelial nitric oxide synthase (eNOS) [18], and by inhibiting the synthesis of the vasoconstructive factor endothelin-1 [19]. In addition, statins activate the fibrinolytic potential of endothelial cells by increasing tissue plasminogen activator (tPA) expression and activity, and by decreasing plasminogen activator inhibitor-1 (PAI-1) expression [20]. Statins also inhibit migration of leukocytes to subendothelium by regulating the binding activity of leukocyte integrins [21], a feature that might result in a deceleration of atherosclerosis development and a reduction of inflammatory responses. Besides inducing the translocation to the nucleus of SREBP [15], statins are known to regulate gene expression through the action of proteins belonging to the Rho GTPase family [20], and via activation of protein kinase Akt [22].

The nuclear receptor peroxisome proliferator-activated receptor-γ (PPAR-γ) is highly expressed in monocytes where it is thought to modulate the expression of genes involved in monocyte activation and lipid metabolism [23]. Thiazolidinediones, a class of synthetic PPAR-γ ligands, besides improving insulin sensitivity, exert potent antiatherogenic effects on the arterial wall, a feature that have raised the possibility of extending their use for atherosclerosis treatment [24,25].

The broad functionality of CD36 in the context of the effective therapeutic use of statins, and the eventual future use of PPAR-γ ligands as anti-atherogenic/anti-inflammatory drugs, prompted us to investigate their effects, both alone and in combination, on the regulation of CD36 expression in human monocytes. In addition, we have also examined the nature of the molecular mechanisms underlying the regulation of CD36 expression by statins.

2. Materials and methods

2.1. Cell culture and reagents

THP-1 cells (ATCC TIB-202) were grown in RPMI 1640 containing 10% FCS. Human monocytes from volunteer donors were isolated from buffy coats by Ficoll density gradient separation and subsequent adherence to tissue-culture plates for 120 min at 37°. Fluoresceinated monoclonal antibody FA6-152 against human CD36 was from Immunotech. Lovastatin, fluvastatin, and atorvastatin were obtained from Merk Sharp & Dohme, Novartis, and Pfizer, respectively. 15-Deoxy-$\Delta^{12,14}$-Prostaglandin J_2 ($15dPJ_2$) and Wy-14643 were purchased to Cayman Chemical. BRL46490 (rosiglitazone) was from SmithKline & Beecham. Cytochalasin D was from Sigma-Aldrich. All the above compounds were dissolved in dimethyl sulfoxide (final concentration in culture, 0.044%, v/v). Trans-trans farnesol (FOH) and all-trans geranyl geraniol (GGOH) were purchased from Fluka and American Radiolabeled Chemicals Inc., respectively. C3 exoenzyme from *Clostridium botulinum* and lysophosphatidic acid (LPA) were purchased from BioMol. C3 exoenzyme was dissolved in PBS. LPA was prepared as a 5 mM stock solution in water containing 10 mg/mL of fatty acid-free bovine serum albumin. For the experiments requiring LPA, final concentration of bovin serum albumin was kept at 0.1 mg/mL for all the experimental conditions assayed. Vectors containing fragments of human CD36 gene promoter coupled to the luciferase reporter plasmid pGL-2 (Promega) were previously described [26]. pcDNA-3 expression vectors encoding wild-type RhoA and RhoB, and dominant-negative mutants RhoAN19 and RhoBN19 were used in transactivation experiments [27].

2.2. Flow cytometry

Cells were incubated with FITC conjugated antibodies for 60 min on ice. A FITC-isotypic antibody was used as control. In experiments with human monocytes, besides anti-CD36, a phycoerithrine labeled anti-CD14 antibody (Becton Dickinson) was simultaneously added to select a gate for monocytes. Cells were acquired on a FACScalibur (Becton Dickinson) cytofluorometer. Data were analyzed by using the Cell Quest (Becton Dickinson) and WinMDI (http://facs.scripps.edu) software programs.

2.3. Relative quantification of CD36 mRNA

Total RNA was isolated using TRIzol reagent (GIBCO-BRL). For relative quantification of CD36 mRNA levels, isolated total RNA was reversed transcribed using MMLV reverse transcriptase (Promega) primed with random hexamers. CD36 mRNA was amplified in a Perkin-Elmer thermocycler 2400 using 20 pmol of the specific primers: AACAGAGGCTGACAACTTCACA (sense, nucleotides 597-618), and GCAGTGACTTTCCCAATAGGAC (antisense, nucleotides 939-918), and Taq Gold polymerase (Perkin-Elmer) pre-activated during 10 min at 93°, and according to the following thermal cycle: 15 s at 94°, 30 s at 63°, and 30 s at 72°, for 22–24 cycles (previously determined to lie within the linear amplification phase). 18S RNA was used as internal standard, and was co-amplified using primers and competimers from Ambion Inc., using a 3:7 primer:competimer ratio. Amplified products (343 bp for CD36, and 488 bp for 18S RNA) were run on 2% agarose gels and visualized by staining with silver green (Molecular Probes). Band intensities were analyzed densitometrically using the NIH Image program. CD36 mRNA levels were normalized with respect to 18S RNA.

2.4. Transient expression and measurement of promoter activity

THP-1 cells were transfected by using the DEAE-dextran method. Briefly, each 4×10^6 cells were transfected with 0.75 μg of luciferase plasmid and 30 ng of renilla reporter vector pRL-CMV (Promega), using 100 μg/mL of DEAE-dextran. After transfection, cells were divided in aliquots of 10^6 cells, and incubated at 5×10^5 cells/mL in complete medium supplemented with the compounds indicated. Twenty-four hours later, cells were harvested, lysed, and assayed for luciferase and renilla activities using the Dual Luciferase Reporter Assay System (Promega). Renilla activities were used to normalize for transfection efficiencies.

2.5. Statistics

Data are shown as means ± standard errors (SE). Statistical evaluation was done using the paired *t* test. A value of $P < 0.05$ was considered significant.

3. Results

3.1. Statins upregulate CD36 cell surface protein expression in human monocytes through regulation of the transcriptional rate of the CD36 gene

The effects of statins on CD36 cell surface expression levels in the monocytic cell line THP-1 and in human peripheral blood monocytes were initially assessed by flow cytometry. All the statins assayed (lovastatin, atorvastatin, and fluvastatin) increased CD36 surface protein expression in THP-1 cells in a dose and time-dependent manner (Table 1). The increase was observed in the 2–10 μM range, and was noticeable 48 hr after the addition of the drug. Likewise, statins also upregulated CD36 surface protein expression in human monocytes, where maximal induction occurred after 24 hr of incubation (Table 1).

Table 1
Statins, PPAR-γ ligands, and their combination increase CD36 surface protein expression

	24 hr	48 hr	72 hr
THP-1			
Isotypic antibody	2.70 ± 0.17	2.86 ± 0.14	2.60 ± 0.09
Control	17.97 ± 2.95	20.46 ± 2.73	21.87 ± 1.19
Lov 2 μM	17.59 ± 2.00	33.43 ± 3.55	38.20 ± 2.05
Lov 10 μM	16.15 ± 1.41	36.60 ± 4.88	55.17 ± 4.97
Ator 4 μM	22.28 ± 0.46	40.60 ± 3.49	38.17 ± 6.33
Ator 10 μM	21.19 ± 0.03	45.73 ± 4.27	56.14 ± 5.96
Fluv 5 μM	20.07 ± 1.86	43.17 ± 3.86	61.39 ± 4.13
$15dPJ_2$ 4 μM	25.50 ± 4.72	36.21 ± 7.60	58.82 ± 5.66
BRL 30 μM	23.14 ± 2.02	33.18 ± 3.75	48.33 ± 5.35
WY-14643 50 μM	18.27 ± 1.98	17.10 ± 2.14	18.88 ± 1.72
Lov + $15dPJ_2$	19.09 ± 1.72	59.41 ± 14.54	127.49 ± 8.66
Monocytes			
Isotypic antibody	2.72 ± 0.27	3.47 ± 0.21	3.95 ± 0.16
Control	24.02 ± 2.37	15.28 ± 1.47	15.06 ± 1.44
Lov 10 μM	31.46 ± 2.82	19.82 ± 1.02	14.19 ± 2.16
Fluv 5 μM	31.58 ± 5.39	17.12 ± 4.34	16.42 ± 2.81
$15dPJ_2$ 4 μM	37.01 ± 1.93	23.19 ± 3.96	14.65 ± 2.09
BRL 30 μM	30.63 ± 4.69	21.29 ± 3.95	14.86 ± 4.04
Lov + $15dPJ_2$	48.28 ± 3.16	35.02 ± 5.17	22.09 ± 4.97
Fluv + $15dPJ_2$	46.56 ± 4.98	33.98 ± 3.20	19.17 ± 2.87
Lov + BRL	41.05 ± 5.10	24.16 ± 4.68	25.42 ± 4.94
Fluv + BRL	39.95 ± 3.55	22.07 ± 3.09	17.35 ± 2.73

FACS analysis by FA6.152 antibody showing the effects of statins, PPAR ligands and their combination on CD36 surface protein expression levels in THP-1 cells, and in isolated human monocytes. Control cells were treated with vehicle. Values indicate the median intensity of fluorescence ± SE from four independent experiments.

To investigate whether the increase in CD36 mRNA expression was due to an increase in the promoter activity of the CD36 gene, THP-1 cells were transiently transfected with different luciferase reporter plasmids under the transcriptional control of several CD36 gene promoter deletion constructs, and incubated for 24 hr in the presence or absence of lovastatin. As shown in Fig. 1A, while no increase in promoter activity was observed in the presence of lovastatin for the control empty vector pGL-2-Basic, the promoter activity for all the CD36 constructs assayed was between 3- and 4-fold higher in the presence of 10 μM lovastatin than in its absence. The increase in CD36 promoter activity by statins was associated with a concomitant 2-fold increase of CD36 mRNA, observable 24 hr after drug addition, and maintained after 48 hr (Fig. 1B). Similar findings were obtained with human monocytes (data not shown). These observations indicated

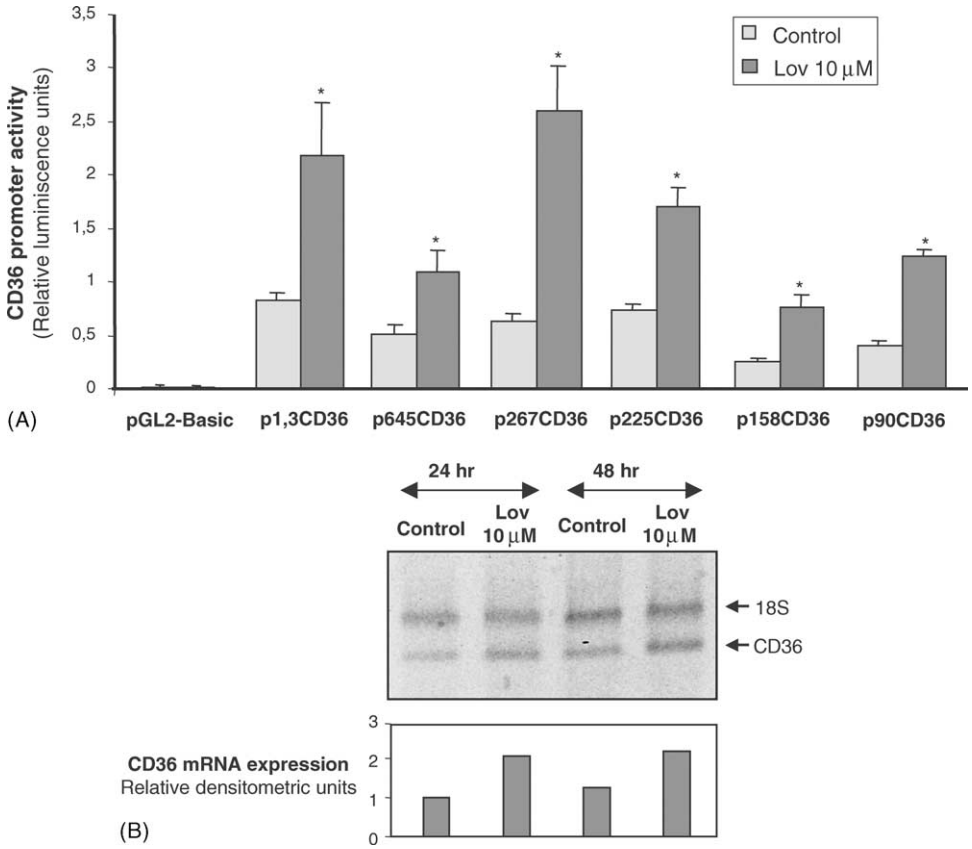

Fig. 1. (A) Lovastatin upregulates CD36 promoter activity. THP-1 cells were transfected with the empty reporter plasmid pGL-2-Basic or with the indicated reporter plasmids under the transcriptional control of the CD36 promoter [26] and with pRL-CMV, and treated with vehicle (control) or 10 μM lovastatin. After 24 hr of culture, cells were harvested and their firefly luciferase activity was normalized to Renilla luciferase activity. Results are means ± SE of at least four independent experiments. *Significantly different from their controls at $P < 0.05$. (B) Lovastatin increases CD36 mRNA levels. (Upper panel) Gel photograph of a representative experiment (out of three) of quantitative RT-PCR showing the time-dependent effects of lovastatin on the relative CD36 mRNA levels in THP-1 cells. Control cells were treated with vehicle. (Lower panel) Bar graph showing the relative band intensities of CD36 mRNA after densitometry and normalization to 18S ribosomal RNA of the gel shown in the upper panel.

that upregulation of CD36 by statins is due to an increase in the transcription rate of the CD36 gene. Furthermore, the above data point out the existence of at least one statin-response element located downstream from base pair 90 of the CD36 gene promoter.

3.2. Statins and PPAR-γ ligands have an additive effect on the expression of CD36

The upregulatory effect of several PPAR-γ ligands on CD36 expression has been previously addressed in some detail [28]. Here, we sought to investigate the combined effect of PPAR-γ ligands with statins. As shown in Table 1, upregulation of CD36 surface expression was observed after addition of $15dPJ_2$ to THP-1 cells. Upregulation was noticeable within the first 24 hr of treatment, and increased with time (Table 1), and was detectable at doses as low as 0.25 μM $15dPJ_2$ (data not shown). Thiazolidenidione BRL49653 at 30 μM also increased CD36 surface expression (Table 1). However, the fibrate and PPAR-α ligand Wy-14643, even at doses of 50 μM did not alter CD36 expression. Similarly to THP-1 cells, $15dPJ_2$ and BRL49653 upregulated CD36 surface protein expression in monocytes, where maximal expression was reached 24 hr after drug addition (Table 1). $15dPJ_2$ stimulated between 2- and 3-fold the promoter activity of all of the CD36 gene promoter deletion constructs assayed with the exception of construct p90CD36-luc (Fig. 2). These data corroborate that PPAR-γ ligands induce CD36 surface expression by potentiating the transcription of the CD36 gene [28], and indicate the presence of a $15dPJ_2$ responsive element between nucleotides −158 and −90 of the CD36 gene promoter.

Simultaneous addition of lovastatin and $15dPJ_2$ to THP-1 cells resulted in a higher increase in CD36 surface protein expression with respect to treatment with either one of the two drugs (Table 1). The increase was detectable after 48 hr and increased with time. Similar additive effects were also observed for monocytes treated with combinations of lovastatin/fluvastatin with $15dPJ_2$/BRL49643 (Table 1). However, for monocytes, the effects were more evident after 24 hr of treatment.

The additive effects of the combination of lovastatin and $15dPJ_2$ on CD36 expression were also revealed at the mRNA level and in the promoter activity (Fig. 3).

Taken together, the above results demonstrate that simultaneous addition of statins and PPAR-γ ligands have

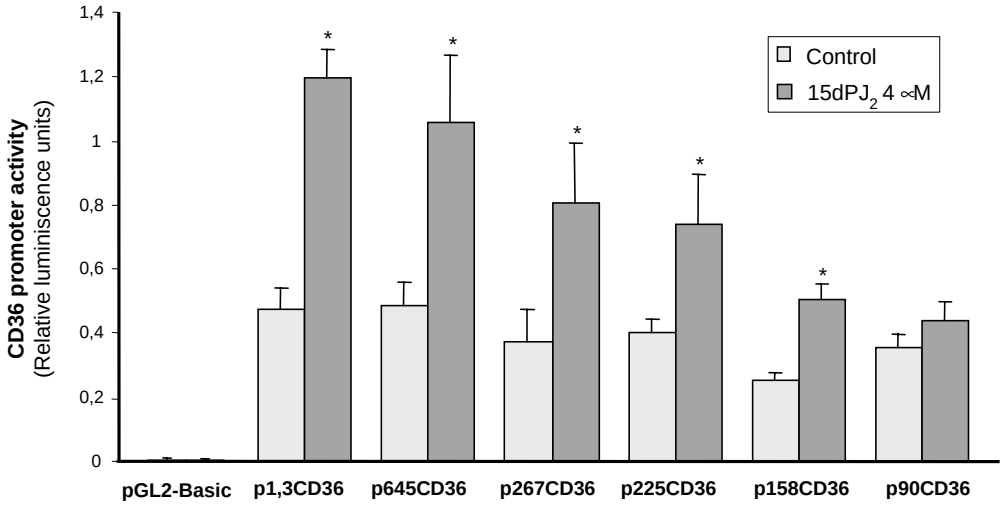

Fig. 2. 15dPJ$_2$ stimulates CD36 promoter activity. THP-1 cells were transfected with the empty reporter plasmid pGL-2-Basic or with the indicated reporter plasmids under the transcriptional control of the CD36 promoter [26] and with pRL-CMV, and treated with vehicle (control) or 4 μM 15dPJ$_2$. After 24 hr of culture, cells were harvested and their firefly luciferase activity was normalized to Renilla luciferase activity. Results are means ± SE of three independent experiments. *Significantly different from their controls at $P < 0.05$.

an additive effect on the expression of CD36 by stimulating the transcription of the CD36 gene, although through distinct promoter regulatory sites.

3.3. The upregulatory effect of lovastatin on CD36 expression is prevented by co-incubation with mevalonate or geranylgeraniol but neither with farnesol nor with LDL

Statins, through inhibition of the HMG-CoA reductase, deplete cellular pools of cholesterol and of its intermediate precursors. Therefore, we assayed whether the addition of exogenous cholesterol, as LDL particles, could revert the upregulatory effect on CD36 expression caused by statins. Addition of 50 μg/mL LDL to THP-1 cells did not prevent the inductive effect of lovastatin on both CD36 surface and mRNA expression levels, while the product of the HMG-CoA reductase, mevalonate, did (Table 2; Fig. 4). These observations suggested that statins regulate CD36 expression through inhibition of the HMG-CoA reductase and by a mechanism independent of their cholesterol-lowering action. Therefore, the intracellular levels of mevalonate or an intermediate/s compound/s within the cholesterol biosynthetic pathway might be directly or indirectly responsible for the upregulatory effects of statins on CD36 expression. Farnesyl and geranylgeranyl pyrophosphates are intermediate compounds downstream the cholesterol biosynthetic pathway. They deserve special interest because they constitute the substrates for protein prenylation, a post-translational feature which affects the functional activity of many proteins involved in cell metabolism and survival [29].

To evaluate the involvement of the isoprenoids farnesylpyrophosphate and geranylgeranyl pyrophosphate in the regulation of CD36 expression, cells were treated with lovastatin (which reduces/depletes the cellular pool of both prenylpyrophosphates) in the presence or absence of the hidroxylated forms of those isoprenoids compounds.

Table 2
Mevalonate but not LDL-cholesterol revert the inductive effect of statins on CD36 protein surface expression

	Vehicle	Lov 10 μM
Isotypic antibody	2.99 ± 0.12	3.14 ± 0.16
Control	20.25 ± 2.07	38.34 ± 2.98
Mev 0.2 mM	20.10 ± 1.62	20.44 ± 0.64
LDL 50 μg/mL	20.40 ± 1.97	31.47 ± 1.57

THP-1 cells were treated with vehicle or 10 μM lovastatin in the presence/absence of 0.2 mM mevalonate or 50 μg/mL LDL. After 48 hr, CD36 protein expression levels were evaluated by flow cytometry. Values indicate the median intensity of fluorescence ± SE from three independent experiments.

Table 3
GGOH but not FOH reverts the inductive effect of statins on CD36 protein expression

	Vehicle	Lov 10 μM
THP-1		
Isotypic antibody	2.99 ± 0.12	3.14 ± 0.16
Control	20.25 ± 2.07	38.34 ± 2.98
GGOH 20 μM	21.37 ± 3.99	24.97 ± 1.50
FOH 50 μM	20.83 ± 4.29	34.76 ± 2.25
Monocytes		
Isotypic antibody	4.21 ± 0.54	4.31 ± 0.62
Control	28.30 ± 2.21	42.90 ± 2.11
GGOH 20 μM	30.09 ± 0.37	34.60 ± 1.02
FOH 50 μM	28.66 ± 0.32	38.89 ± 1.82

THP-1 cells or monocytes were treated with vehicle or 10 μM lovastatin in the presence or absence of FOH 50 μM or GGOH 20 μM. After 48 hr for THP-1 cells or 24 hr for monocytes, CD36 protein expression levels were evaluated by flow cytometry. Values indicate the median intensity of fluorescence ± SE from three independent experiments.

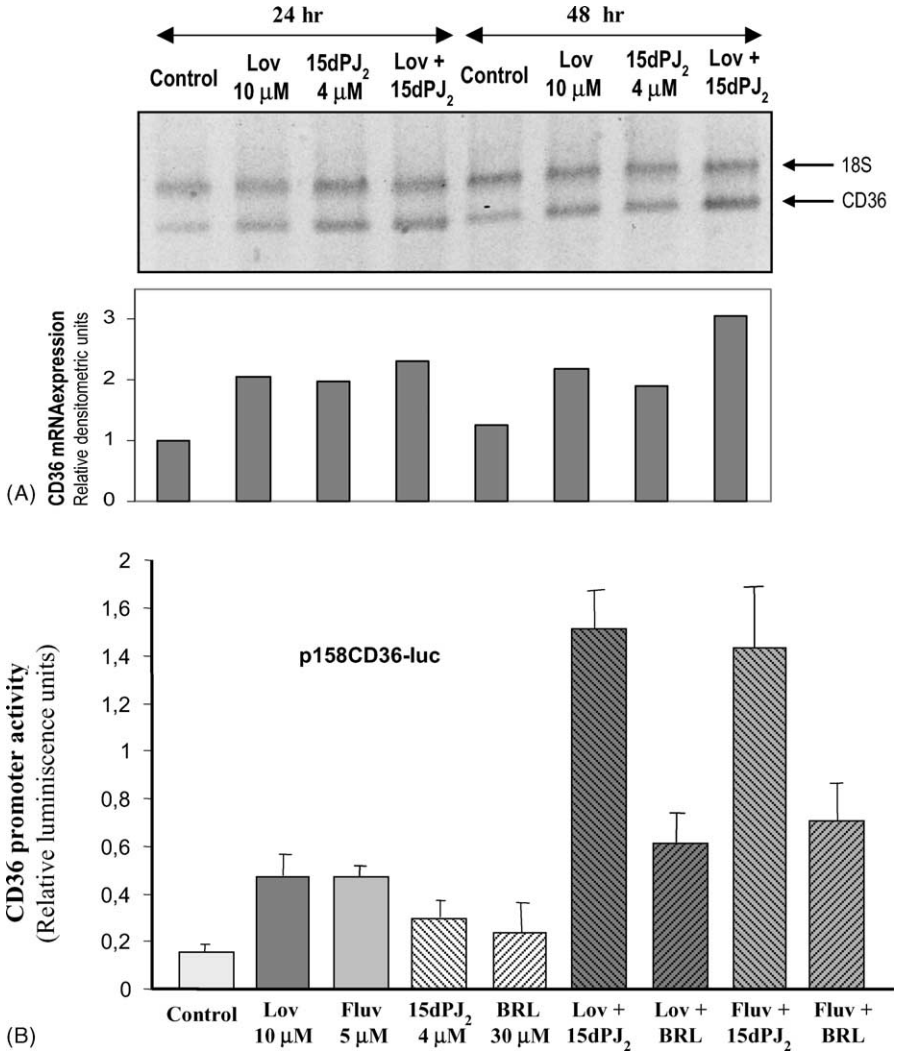

Fig. 3. Statins and PPAR ligands have an additive effect on upregulation of CD36 mRNA levels and CD36 promoter activity. (A) (Upper panel) Gel photograph of a representative experiment of quantitative RT-PCR showing the time-dependent effects of lovastatin, 15dPJ$_2$, and both together on the relative CD36 mRNA expression levels in THP-1 cells. Control cells were treated with vehicle. (Lower panel) Bar graph showing the relative band intensities of CD36 mRNA after densitometry and normalization to 18S ribosomal RNA of the gel shown in the upper panel. (B) THP-1 cells were transfected with p158CD36-luc (that responds to both statins and PPAR-γ ligands) plus pRL-CMV, and treated with vehicle (control) or with the drugs and doses indicated. After 24 hr of culture, cells were harvested and their firefly luciferase activity was normalized to Renilla luciferase activity. Results are means ± SE of three independent experiments. All drug-treated samples were significantly different from the control at $P < 0.05$.

As shown in Table 3, both isoprenoids alone had no effect on CD36 expression. However, GGOH prevented the inductive effect of lovastatin on CD36 surface protein expression at times when lovastatin achieved a maximal induction both in THP-1 cells and in monocytes. In contrast, co-treatment with FOH had essentially no effect on the upregulation of CD36 expression induced by lovastatin in THP-1 cells and monocytes. Altogether, these results indicate that a geranlylgeranylated protein/s could be important for expression of the CD36 gene.

3.4. Involvement of Rho proteins in the control of CD36 gene expression

The small GTPases Rho, Rac, and Ras are only active when prenylated. For endothelial cells, it has been demonstrated the involvement of Rho proteins in the regulation of expression by statins of tPA [20], eNOS [30], and pre-proendothelin-1 [27]. Rho GTPases also play key roles in controlling cytoskeleton organization [31]. In our experiments, we have consistently observed that both THP-1 cells and monocytes treated with statins acquired a round shape consistent with a disruption of their cytoskeletal structure (data not shown). The above reasonings prompted us to explore the contribution of Rho proteins in CD36 expression. For this purpose, we used C3 exoenzyme from *C. botulinum*, which when added to cells specifically ADP-ribosylates RhoA and RhoB proteins, rendering them biologically inactive [32]. Moreover, C3 exoenzyme from *C. botulinum* is known to enter monocytic cells and inactivate Rho proteins [33]. Incubation of THP-1 cells with C3 exoenzyme resulted in a strong induction of protein surface

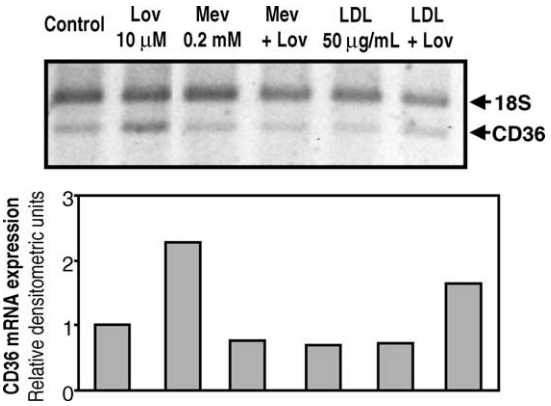

Fig. 4. Mevalonate but not LDL-cholesterol revert the inductive effect of statins on CD36 mRNA expression levels. THP-1 cells were treated with vehicle or 10 μM lovastatin in the presence/absence of 0.2 mM mevalonate or 50 μg/mL LDL. After 24 hr, relative CD36 mRNA levels were determined by quantitative RT-PCR. (Upper panel) Gel photograph of a quantitative RT-PCR representative experiment of three independent experiments. Control cells were treated with vehicle. (Lower panel) Bar graph showing the relative band intensities of CD36 mRNA after densitometry and normalization to 18S ribosomal RNA of the gel shown in the upper panel.

CD36 expression, specially noticeable 48 hr after the addition of the drug (Fig. 5A). This result indicates that selective inhibition of Rho activity by C3 exoenzyme leads to an increase in CD36 expression, a feature consistent with the CD36 induction expected to occur by inhibiting Rho activity by statin-mediated depletion of the intracellular pool of geranylgeranyl.

To further confirm the involvement of Rho proteins in CD36 expression we evaluated the effect of the Rho activator LPA. In the context of the previous results, it can be reasoned that activation of Rho proteins would result in a decrease of CD36 expression. As predicted, both in THP-1 cells and in monocytes, LPA at 5 and 50 μM downregulated CD36 expression in a dose-dependent manner (Fig. 5B). Although the biological effects of LPA are not only Rho-specific, its behavior on CD36 expression strengthens the involvement of Rho proteins in the regulation of CD36 expression. If as proposed on the basis of the results of this study, statins are "inactivating" Rho proteins, these should affect CD36 promoter activity. With this aim, THP-1 cells were co-transfected with a CD36 promoter construct together with expression vectors encoding for wild-type RhoA or RhoB, or for their corresponding dominant-negative mutants, N19RhoA or N19RhoB, respectively. As shown in Fig. 5C, dominant-negative RhoA and RhoB mutants were able to induce a significant increase in CD36 promoter activity with respect to the wild-type Rho forms. Thus, as predicted, inactivation of Rho proteins lead to an increase of the transcriptional activation of the CD36 gene.

3.5. Disruption of actin cytoskeleton triggers CD36 expression

Besides involvement of Rho proteins in regulation of gene transcription, they actively participate in cytoskeleton organization [31]. Thus, we wonder whether disruption of actin cytoskeletal would mimic the effect on CD36 expression observed when Rho activity was inhibited by statins or C3 exoenzyme. For this aim, we used cytochalasin D, a well-known disrupter of actin filaments. As shown in Table 4, cytochalasin D increased surface CD36 expression in THP-1 cells in a dose-dependent manner. In agreement with induction of CD36 increase by statins, maximal induction occurred 48 hr after the addition of the drug. In monocytes, the inductive effect was even more pronounced, and was maximal at 24 hr, dropping down progressively when time increased (Table 4). These results indicate that disruption of actin cytoskeleton induces an increase in CD36 expression.

Table 4
Cytochalasin D increases CD36 expression

	24 hr	48 hr	72 hr
THP-1			
Isotypic antibody	2.42 ± 0.41	3.24 ± 0.62	2.67 ± 0.91
Control	17.71 ± 2.20	18.37 ± 3.50	18.02 ± 2.68
Cyto D 0.5 μM	18.21 ± 1.36	27.39 ± 4.01	28.90 ± 3.65
Cyto D 2 μM	24.00 ± 1.40	35.51 ± 2.11	31.57 ± 3.03
Monocytes			
Isotypic antibody	2.85 ± 0.21	3.22 ± 0.25	3.12 ± 0.30
Control	15.76 ± 0.31	10.85 ± 0.23	15.54 ± 0.29
Cyto D 2 μM	43.95 ± 1.03	16.83 ± 0.70	17.00 ± 1.58

THP-1 cells or monocytes in culture medium were treated with vehicle or with cytochalasin D at the doses shown in the figure. At the times indicated, CD36 expression levels were evaluated by flow cytometry. Values shown indicate the median intensity of fluorescence ± SE from three independent experiments.

4. Discussion

In this study, we first demonstrate that statins increase surface protein expression of CD36 in human monocytes (Table 1) by inducing the transcription of the CD36 gene through a response element located downstream from base pair 90 of the promoter (Fig. 1). Our data showing the increased expression of monocytic CD36 after statin treatment are apparently in contrast to previous results by Pietsch *et al.*, which reported a downregulation of CD36 expression in the pro-monocytic cell line U937 [34] and in isolated monocytes after statin treatment [35]. However, like Pietsch *et al.*, we have found that lovastatin slightly downregulates CD36 expression in U937 cells (see below). Nevertheless, it should be noted that in this cell line, which is considered less differentiated than THP-1 [36], the expression levels of CD36 are extremely low (for a FACS experiment measured 48 hr after the addition of 10 μM lovastatin the following median values were obtained for expression of CD36: isotypic antibody, 2.62; CD36 antibody: control, 3.59; 10 μM lovastatin, 2.69; no changes were observed during the first 24 hr). With respect to the experiments performed with isolated monocytes, we have

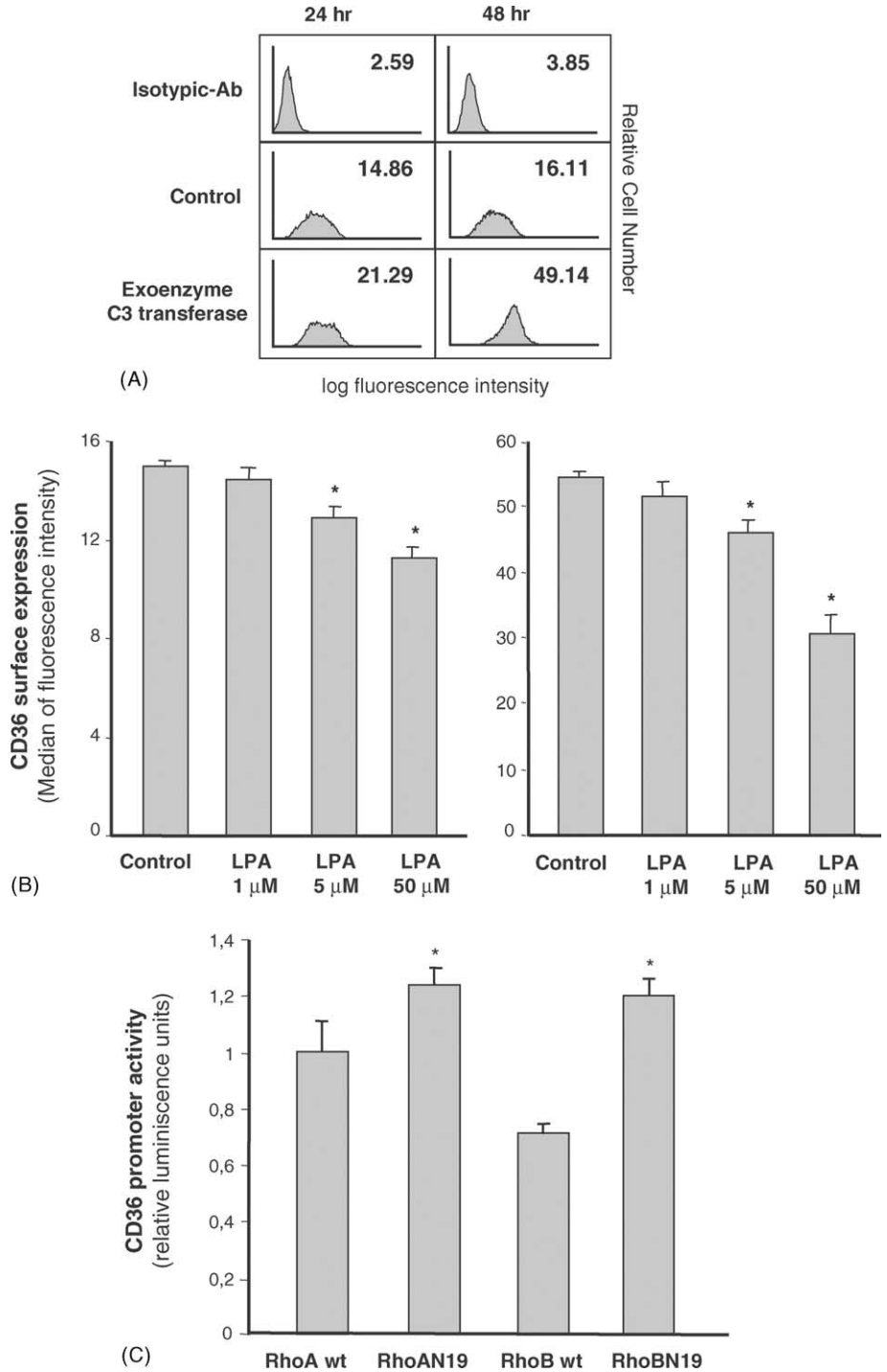

Fig. 5. Rho GTPases regulate CD36 expression. (A) Exoenzyme C3 transferase increases CD36 expression. THP-1 cells were treated with vehicle or 5 μg/mL of exoenzyme C3 transferase. Cells were collected after 24 and 48 hr and analyzed for CD36 expression levels by flow cytometry. Numeric values inside graphics indicate the median fluorescence intensity. The experiment shown is representative of two separate experiments. (B) LPA downregulates CD36 expression. THP-1 cells (left panel) or monocytes (right panel) in culture medium containing 1% FCS were treated with vehicle or with LPA at the doses shown in the figure. Promoter construct p158CD36-luc is known to control CD36 expression in monocytic cells [26], and to contain response element/s for statin induction (Figs. 1, 2, and 3B). At the times indicated, CD36 expression levels were evaluated by flow cytometry. Median intensity values of fluorescence of each graph expressed in channels are shown. Results are means ± SEM of three independent experiments. (C) Effect of Rho protein overexpression on CD36 promoter activity. THP-1 cells were co-transfected with p158CD36-luc plus pRL-CMV and pDNA3 (in one experiment to normalize data) and the vectors indicated in the figure. After 24 hr of culture, cells were harvested and their firefly luciferase activity was normalized to Renilla luciferase activity. Data obtained with the empty vector pcDNA-3 were used to normalized the data obtained using the other vectors. Results are means ± SE of three independent experiments. $^{*}P < 0.05$ vs. control.

systematically observed that statins induced an increase in CD36 expression. In contrast to control monocytes, statin-treated monocytes although do not detach they do lose some adherence to tissue-culture plates, what results in a reduction of their culture-induced differentiation with impaired upregulation of CD36 expression [7]. Thus, at times longer than 48 hr, untreated monocytes, due to their culture-induced differentiation to macrophages, may display higher expression levels of CD36 than monocytes treated with statins.

In agreement with previous reports, PPAR-γ ligands $15dPJ_2$ and BRL49653 increased the transcription rate of the CD36 gene (Table 1; Fig. 2), leading to an induction of CD36 surface expression in both THP-1 and human monocytes. Region encompassing nucleotides −273/−267 in the CD36 gene promoter have been suggested to contribute to the upregulation mediated by PPAR-γ ligands [28]. Our results, although do not contradict those data, suggest the presence of stronger response element/s for PPAR-γ ligands within the more proximal region −158/−90 of the CD36 promoter. The finding of a PPAR-γ response element lying within the above region in the mouse CD36 gene promoter [37], strength the above observation. Interestingly, the combination of statins and PPAR-γ ligands have an additive effect on CD36 expression (Table 1) by potentiating the transcription of the CD36 gene (Fig. 3), presumably through distinct promoter regulatory sites (Figs. 1A and 2).

Uptake of OxLDL by CD36 significant contributes to the transformation of macrophages towards foam cells [4]. During the earliest steps of atherosclerosis development, uptake of modified LDL within the atheroma plaque may have beneficial effects by precluding the adverse actions of OxLDL on surrounding cells [3]. However, a massive accumulation of lipids inside macrophages impairs their functions and lead to their dead, features that severely affect the composition and stability of the atheroma plaque. An increase expression of CD36 induced by statins is likely to result in an increase in OxLDL binding/uptake by blood monocytes, resulting in an increased susceptibility to form foam cells. However, here it should be noted that other scavenger receptors are also involved in OxLDL binding/uptake. In this respect it has been reported a downregulation of the expression of scavenger receptors SR-A and LOX-1 by statins [38,39]. This effect might compensate the OxLDL binding/uptake associated with the increase in CD36 expression, leading to a net unaltered OxLDL binding/uptake. In fact, some preliminary results from our laboratory suggest that this might be the case. In addition, a higher expression of CD36 in statin or PPAR-γ ligand-treated monocytes might favor the recognition of apoptotic cells, thereby facilitating their removal from the subendothelial space, a feature which might increase the stability of the plaque, as well as limit the tissue damage at the lesion site. In this sense, ingestion of apoptotic cells by monocytes inhibits monocyte production of proinflammatory cytokines [40]. Furthermore, the anti-angiogenic activity mediated through the interaction between CD36 and thrombospondin-1 present in microendothelium [12] might reduce subintimal neovascularization and plaque growth [41]. Like for microvascular endothelial cells, interaction of CD36 in monocytes with thrombospondin-1 might induce their apoptosis [12], a potential pro-atherogenic effect. CD36 acts also as a receptor for advanced glycation endproducts (AGEs) [11]. It is thought that interaction between AGEs and their receptors in vascular cells activates mechanisms which lead to the development of vascular lesions [42]. It is, therefore, tempting to speculate that overexpression of CD36 in monocyte/macrophages may help in the clearance of AGEs from blood and atherosclerosis lesions, and therefore display an anti-atherogenic role.

The multifunctional activity of monocytic CD36 makes difficult to predict the role of this scavenger receptor in atherogenesis. CD36 expressed in monocyte/macrophages could be contemplated as a component of the defense mechanism in the arterial wall that contributes to the elimination of noxious agents in the arterial intima. Only when the injuries are overwhelming, the macrophage will succumb. Overexpression of CD36 in macrophages using transgenic animals may provide definitive insights into the physiologic role in atherosclerosis of this scavenger receptor.

CD36 is likely to play, not only on monocytes but also in other cells types, important physiopathological roles on: (a) atherosclerosis and inflammation through its ability to mediate uptake of OxLDL [4], and by participating in the recognition of apoptotic cells [2], (b) angiogenesis via its interaction with thrombospondin-1 [12], (c) energy consumption through the uptake of free fatty acids [43], and (d) diabetes via its ability to interact with AGEs [11]. It is, therefore, promising that upregulation of CD36 expression by statin and PPAR-γ ligands might exert potent physiopathological effects.

In this study, we have also delved into the molecular mechanisms involved in the regulation of monocytic CD36 expression by statins. Our results indicate that regulation of CD36 expression by statins is independent of their lipid-lowering effects, and is caused by depletion of the cellular pool of geranylgeranyl, as evidenced by the preventive effect of GGOH, but neither by FOH nor by LDL, on the inductive action of lovastatin (Tables 2 and 3; Fig. 4). We have also found out that Rho GTPases are likely to be effectors of the action of statins on CD36 expression (Fig. 5). In fact, depletion of geranylgeranyl is known to impair the translocation of Rho GTPases from cytosolic compartments to membranes where they become activated through GDP/GTP exchanged [44]. Involvement of Rho GTPases on CD36 expression was supported by: (1) the upregulatory effect on CD36 expression by the specific Rho-inhibitor C3 exoenzyme, (2) the downregulation of CD36 expression by the Rho activator lysophospatidic acid, (3) the induction of CD36 promoter activity by dominant-negative mutant forms of both RhoA and RhoB. Although these data do prove the contribution of Rho

proteins on the regulation of CD36 expression, they do not rule out that other prenylated proteins also regulate CD36 expression. Rho proteins participate in various signaling pathways that lead to regulation of gene expression and alteration of cytoskeleton organization [33,44]. This later effect causes changes in cell adhesion, morphology and motility [45]. Induction of CD36 expression by cytochalasin D (Table 4), a disrupter of actin filaments, strongly suggest that Rho proteins affect CD36 expression via their action on the actin cytoskeleton. In this respect, the breakdown of actin cytoskeleton by statins has been demonstrated [46]. Taken together, the above data indicate that inactivation of Rho GTPases by statins causes an impairment in actin cytoskeleton organization, which by so far unknown mechanisms leads to upregulation of CD36 expression. In this regard, accumulation of nonisoprenylated Rho in the cytosol of aortic cells from mice treated with statins indicates that regulation of Rho GTPase activity by statins is likely to occur *in vivo* [47]. In monocyte/macrophages, aside from downregulation of CD36 expression, Rho, through its effector Rho-ROCK kinase, has been shown to be involved in transendothelial migration of monocytes and their accumulation in the adventitia, a feature that favors the formation of vascular lesions *in vivo* [48,49]. Inactivation of Rho GTPases by statins have been demonstrated to regulate the expression of several endothelial genes whose products play key roles in thrombosis and vascular biology: tPA [20], PAI-1 [30], eNOS [30], preproendothelin-1 [27], and apoprotein-1 [50]. However, like for CD36, the links connecting the regulation of their expression to Rho GTPases remains unknown. Clearly further studies are required to unravel the molecular mechanisms underlying the action of Rho on the expression of the above-mentioned genes.

In conclusion, our data strongly suggest a link between statins, Rho GTPases, actin cytoskeleton organization, and the regulation of the expression of the monocytic scavenger receptor CD36, and prompt to study the potential use of cell shape modulators to mimic some of the biological actions of statins, as well as to unravel the contributions of Rho GTPases and of alterations in actin cytoskeleton organization on monocytic functions affected by statins.

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