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Lipophilic statins inhibit YAP nuclear localization, co-activator activity and migration in response to ligation of HLA class I molecules in endothelial cells: role of YAP multi-site phosphorylation

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Abstract

Solid organ transplant recipients, exhibiting HLA donor specific antibodies (Abs) are at risk for graft loss due to chronic antibody-mediated rejection (cAMR). HLA Abs bind HLA molecules expressed on the surface of endothelial cells (ECs) and induce intracellular signaling pathways, including the activation of the transcriptional co-activator Yes-associated Protein (YAP). Here, we examined the impact of lipid-lowering drugs of the statin family on YAP localization, multi-site phosphorylation and transcriptional activity in human ECs. Exposure of sparse cultures of ECs to cerivastatin or simvastatin induced striking re-localization of YAP from the nucleus to the cytoplasm and inhibited the expression of the YAP/TEAD-regulated genes *C*onnective *T*issue *G*rowth *F*actor (*CTGF)* and Cysteine-rich angiogenic inducer 61 (*CYR61*). In dense cultures of ECs, statins prevented YAP nuclear import and expression of *CTGF* and *CYR61* stimulated by the mAb W6/32 that binds HLA I. Exposure of ECs to either cerivastatin or simvastatin completely blocked the migration of ECs stimulated by ligation of HLA I. Exogenously supplied mevalonic acid or geranylgeraniol (GGOH) reversed the inhibitory effects of statins on YAP localization either in low-density ECs or in high-density ECs challenged with W6/32. Mechanistically, cerivastatin increased the phosphorylation of YAP at Ser^{127}, blunted the assembly of actin stress fiber and inhibited YAP phosphorylation at Tyr^{357} in ECs. Using mutant YAP, we substantiated that YAP phosphorylation at Tyr^{357} is critical for YAP activation. Collectively, our results indicate that statins restrain YAP activity in EC models, thus providing a plausible mechanism underlying their beneficial effects in solid organ transplant recipients.

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Keywords

Human endothelial cells; anti-HLA I antibodies; transcription factors; YAP localization; statins; signal transduction; migration; YAP tyrosine phosphorylation

INTRODUTION

Solid organ transplant recipients, exhibiting HLA donor specific antibodies (DSA) [2] are at a higher risk for graft loss due to chronic antibody-mediated rejection (cAMR) and develop a progressive vascular disease known as transplant vasculopathy (TV). Is increasingly recognized that bivalent binding of Abs to HLA molecules expressed on the surface of endothelial cells (ECs) crosslink HLA and induce intracellular signaling pathways regulating cell survival, proliferation and migration, which contribute to TV. These cellular effects are independent of complement activation (1, 2). We advanced the notion that HLA molecules, which have a short intracellular domain without recognizable signaling motifs, associate with co-receptors to elicit endothelial cell activation (3, 4) and reported that integrin β4 and TLR4 form a molecular complexes with Class I that transduce signals leading to EC proliferation, migration and monocyte adhesion (3, 4). Accordingly, we showed that Ab-induced ligation of HLA Class I on the surface of ECs stimulate a set of signaling pathways, including activation of focal adhesion kinase (FAK), Src non-receptor tyrosine kinases (5), phosphatidylinositol 3-kinase (PI3K)/AKT, mechanistic target of rapamycin (mTOR) complex 1 (mTORC1) and mTORC2, p70 S6 Kinase (S6K) and extracellular-signal-regulated kinases (ERK1/2) (6-8). These HLA class I signaling cascades, mediated in part by HLA I complex formation with integrin β4, promote actin cytoskeleton reorganization, stress fiber formation, (7, 9) leading to migration and proliferation of ECs (6, 10-12). The gene-regulatory programs that operate downstream of these signaling nodes are of fundamental significance and translational importance but remain incompletely understood.

The transcriptional co-activators Yes-Associated Protein (YAP) and WW-domain-containing Transcriptional co-Activator with PDZ-binding motif (TAZ) are central effectors of the highly conserved Hippo pathway and emerged as novel sensors of the mevalonate pathway (13-15). Canonical Hippo signals are transduced through a serine/threonine kinase cascade where MST1/2 and MAP4K kinases phosphorylate and activate LATS1/2, which phosphorylate YAP at specific serine residues, including Ser^{127} and Ser^{397}, that regulate their localization and protein stability, respectively (16, 17). In the absence of inhibitory phosphorylation, YAP localizes to the nucleus where it binds and activates predominantly the TEA-domain DNA-binding transcription factors (TEAD 1-4) thereby stimulating the expression of multiple genes, including *C*onnective *T*issue *G*rowth *F*actor (*CTGF)* and Cysteine-rich angiogenic inducer 61 (*Cyr61*). In addition to regulation through the Hippo pathway, YAP/TAZ localization and activity is highly responsive to actin organization and thus, represents a point of convergence in the signaling by Rho, tyrosine kinase receptors, G protein-coupled receptors (GPCRs), integrins, mechanical cues and cell density (16-24). Several studies have demonstrated the importance of YAP/TAZ for angiogenesis and vascular homeostasis (25-27). Recently, we reported that Ab-induced ligation of HLA

I induces robust YAP nuclear localization and dephosphorylation at residues targeted by LATS1/2 in human aortic ECs (28). Mechanistically, we showed that Src family kinases (SFK) play a major role in mediating YAP nuclear localization and activation in response to Ab-induced HLA I signaling in ECs (28). These results identified YAP as a potential novel target in ECs for therapeutic interventions to prevent cAMR-associated TV.

Although inhibition of the activity of transcription factors or their co-activators has proven a difficult strategy, recent studies from several laboratories, including ours, led to the identification of the statins as YAP/TAZ inhibitors (13, 14, 21) but these inhibitory effects depend on cell type and the mechanism(s) involved remain incompletely understood. Statins are specific inhibitors of the 3-hydroxy-methylglutaryl (HMG) CoA reductase (29, 30), the rate-limiting enzyme in the generation of mevalonate, the first step in the biosynthesis of isoprenoids, leading to farnesyl pyrophosphate (FPP), geranylgeranyl pyrophosphate (GG-PP) and cholesterol (31). The transfer of the geranylgeranyl moiety to a COOH-terminal cysteine of Rho GTPases is critical for their function in signal transduction through actin remodeling (17). Accordingly, YAP and TAZ act as novel sensors of the mevalonate pathway and the statins inhibit their nuclear localization and transcriptional activity, at least in some cell types (13, 14, 32). Statins, which are usually well tolerated and generally safe, are widely used to treat hypercholesterolemia and prevent cardiovascular diseases. Importantly, epidemiological studies indicate a protective effect of statins in clinical transplant populations (33-35) but the mechanism(s) involved remain understudied.

In the present study, we determined the impact of different statins on YAP localization, multi-site phosphorylation and transcriptional activity in cultures of human aortic ECs. Exposure of low-density ECs to lipophilic statins, including cerivastatin, simvastatin or atorvastatin induced YAP redistribution from the nucleus to the cytoplasm and markedly reduced the mRNA levels of the YAP/TEAD-regulated gene *CTGF* and *Cyr61*. In confluent and post-confluent cultures of ECs, statins prevented YAP nuclear localization, increase in the expression of YAP/TEAD-regulated genes and EC migration stimulated by exposure to mAb W6/32 that binds HLA I. These inhibitory effects were reversed by exogenously supplied mevalonic acid or geranylgeraniol, a precursor of GGPP. Further mechanistic studies support the notion that statins prevent Src-mediated tyrosine phosphorylation of YAP at Tyr^{357} and indicate that this posttranscriptional modification plays a critical role in the regulation of YAP localization. Collectively, our results provide evidence that statins restrain YAP activity induced by engagement of HLA I in EC models by regulation of multi-site phosphorylation, thus providing a plausible molecular mechanism underlying their beneficial effects in solid organ transplant recipients.

Material and Methods

Materials

DMEM, FBS, goat anti-mouse IgG secondary antibody conjugated to Alexa Fluor 488 and all RT-qPCR reagents were obtained from Invitrogen (Carlsbad, CA). Phalloidin-TRITC were obtained from Sigma Chemical (St. Louis, MO). Dasatinib (S1021) was from Selleckchem. Rho Inhibitor I, a cell-permeable C3 transferase from *Clostridium botulinum,* was from Cytoskeleton. Primary antibodies used were as follows: YAP (H-9, sc-271134

and 63.1, sc-101199, final dilution 1:200); GAPDH (G-9, sc-365062) from Santa Cruz Biotechnology, Inc.; phospho-YAP Ser^{127} (D9W2I, 13008; final dilution 1:1000); phospho-YAP Ser^{397} (D1E7Y, 13619; final dilution 1:1000); Flag Antibody (DYKDDDDK Tag (D6W5B), 14793 final dilution 1:400) were all from Cell Signaling Technology (Danvers, MA); phospho-YAP Tyr^{357} (ab62751; final dilution 1:1000) from Abcam. Horseradish peroxidase–conjugated anti-rabbit IgG and anti-mouse IgG were from GE Healthcare Bio-Sciences Corp (Piscataway, NJ). pcDNA Flag Yap1 (Addgene plasmid # 18881) and pcDNA Flag Yap1 Y357F (Addgene plasmid # 18882) were gifts from Yosef Shaul; pCMV-flag YAP S127A was a gift from Kunliang Guan (Addgene plasmid # 27370). Anti-HLA I mAb W6/32 (mouse IgG2a), recognizing a conformational epitope on all HLA-A, B, and C heavy chains when are in association with β2-microglobulin was purified from cultured supernatants of the hybridoma HB-95 (ATCC, Manassas, VA). Purified allele-specific human mAbs HLA-A2/A28 (clone SN607D8, IgG1) was a gift from Dr. Sebastiaan Heidt. All other reagents were of the highest grade available.

Cell Culture

Primary human aortic endothelial cells were isolated from the aortic rings of explanted donor hearts as described previously (36) or commercial (lot no. EC5555) from Lonza/Clonetics (Walkersville, MD). Most experiments were performed using ECs from Lonza/Clonetics. Selected experiments were confirmed using primary human aortic endothelial cells from a different lot or isolated from the aortic rings. The cells were cultured in M199 medium (Mediatech, Manassas, VA) supplemented with 20% (v/v) FBS (HyClone), 90 mg/ml Heparin (Sigma-Aldrich), 20 mg/ml Endothelial Cell Growth Supplement (BD Biosciences), 100 U/mL penicillin, 100 μg/mL streptomycin, sodium pyruvate (1 mmol/l) at 37°C with 5% CO2 in a humidified incubator. Cells were cultured in flasks or dishes coated with 0.1% Gelatin (Sigma-Aldrich). For experimental purposes, cells from passage 6 to 8 were used at either low or high density (confluence) as indicated in the individual experiments and were transferred to medium M199 without serum for 4 h prior to use, unless otherwise indicated.

Western blot analysis

Cultures of ECs, grown on 35 mm tissue culture dishes, were washed twice with DMEM and incubated in serum-free medium for 4 h and then treated as described in individual experiments. The cultures were then directly lysed in 2 × SDS-PAGE sample buffer [200 mM Tris-HCl (pH 6.8), 2 mM EDTA, 0.1 M Na_3VO_4, 6% SDS, 10% glycerol, and 4% 2-mercaptoethanol], followed by SDS-PAGE on 4-15% gels and transfer to Immobilon-P membranes (Millipore, Billerica, MA). For detection of proteins, membranes were blocked using 5% nonfat dried milk in PBS, pH 7.2, and then incubated overnight with the desired antibodies diluted in PBS containing 0.1% Tween. Primary antibodies bound to immunoreactive bands were visualized by enhanced chemiluminescence (ECL) detection with horseradish peroxidase-conjugated anti-mouse, anti-rabbit antibody and a FUJI LAS-4000 mini luminescent image analyzer. Quantification of the bands was performed using the FUJI Multi Gauge V3.0 analysis program.

Immunofluorescence

Immunofluorescence of ECs was performed by fixing the cultures with 4% paraformaldehyde followed by permeabilization with 0.4% Triton X-100. After extensive PBS washing, fixed cells were incubated for 2 h at 25°C in blocking buffer (BB), consisting of PBS supplemented with 5% bovine serum albumin and then stained at 4°C overnight with a YAP mouse mAb (1:200) diluted in BB. Subsequently, the cells were washed with PBS at 25°C and stained at 25°C for 60 min with Alexafluor 488 - conjugated goat-anti mouse diluted in BB (1:100) and washed again with PBS. Nuclei were stained using a Hoechst 33342 stain (1:10,000). For staining of F-actin, fixed cells were blocked with 5% bovine serum albumin in PBS. The cells were then incubated with TRITC-conjugated phalloidin (0.25 μg/ml) in PBS for 30 min at room temperature and washed five times with PBS. Images were captured (X 40 magnification) as uncompressed 24-bit TIFF files captured with an epifluorescence Zeiss Axioskop and a Zeiss (Achroplan 40/.75W objective) and a cooled (−12 °C) single CCD color digital camera (Pursuit, Diagnostic Instruments) driven by SPOT version 4.7 software. AlexaFluor 488 signals were observed with a HI Q filter set 41001 and TRITC images with a HI Q filter set 41002c (Chroma Technology). In selected experiments, images were also captured using a Zeiss LSM 710 confocal microscope with a Plan-Apochromat 63/1.4 oil objective, 4 steps at 0.5 μm/z-step.

Image Analysis

For YAP localization the average fluorescence intensity in the nucleus and just outside the nucleus (cytoplasm) was measured using ImageJ software to determine the nuclear/cytoplasmic ratios. For analysis of stress fibre intensity, we utilized the ImageJ (NIH, Bethesda, MD) and analyzed an area that encapsulated most of the visible F-actin bundles and equal areas in cells with no visible F-actin bundles. The region of interest (ROI) manager was then used to analyze the stress fiber intensities. The selected cells displayed in the appropriate figures were representative of 80% of the population.

Plasmid Transfections

Bovine aortic endothelial cells (bovine ECs) were transfected with the plasmid containing a cDNA encoding FLAG- tagged YAP wild type and mutants from Addgene by using Lipofectamine 3000 (Invitrogen) as suggested by the manufacturer’s protocol. Analysis of the cells transiently transfected was performed 24 h after transfection.

Real time quantitative reverse transcription PCR (RT-qPCR).

Relative transcript expression levels of CTGF and CYR61 were determined by RT-qPCR using a TaqMan Gene Expression Assay. Briefly, total RNA was extracted from cells by using a PureLink RNA Mini Kit. Reverse transcription was performed with the High-Capacity cDNA Reverse Transcription Kit using 1μg of total input RNA. The synthesized cDNA samples were used as templates for the real-time (RT) PCR analysis. All reactions were performed using the Applied Biosystems StepOne system and the amplifications were done using the TaqMan Fast Advanced Master Mix. The following primers were used; gene-specific Homo sapiens oligonucleotides for *CTGF* (Assay ID: Hs01026927_g1), *CYR61*

(Assay ID: Hs99999901_s1) and the internal control was *18s* (Assay ID: Hs99999901_s1) all were from Life Technologies, Carlsbad, CA.

Cell migration assay by wound healing

Confluent ECs grown in 35-mm culture dishes were starved with 5% FBS for 4 h. Starved cells were then treated with 10 μg/ml mitomycin C for 2 h to inhibit cell proliferation. A scratch wound was created with a sterile 200-μl pipette tip and dishes were rinsed twice with M199 to remove detached cells. Cells were treated with or without statins and anti-HLA I mAb W6/32 for 16 h. The cells were then fixed with 4% paraformaldehyde, stained with Wright–Giemsa (Sigma-Aldrich) and wound closure was monitored by microscopy. The cell number between two initiated front edges was counted (10 fields). Migration rate was determined as relative fold of wound healing as compared to unstimulated ECs.

Statistical analysis

Each experiment was repeated three times independently. Unless otherwise noted, data are presented as mean ± SEM. Differences in protein phosphorylation, cell proliferation, or cell migration were determined using Student's t-test and were considered significant if $p < 0.05$.

RESULTS

Statins inhibit YAP nuclear localization and activity in low-density cultures of ECs.

Recently, we reported that EC density regulates YAP localization in the nucleus and cytoplasm, reflecting their active versus inactive states, respectively (28). Consequently, to examine the impact of statin on YAP localization in human aortic ECs, cultures of these cells were plated at either low or high densities, exposed to 0.3 μM cerivastatin and YAP localization was visualized by immunofluorescence staining. At low cell densities, YAP was localized prominently in the nucleus of ECs (Fig. 1A), in agreement with our previous results (28). Exposure to cerivastatin induced a striking re-localization of YAP from the nucleus to the cytoplasm (Fig. 1A), as verified by quantification of YAP nuclear/cytoplasmic ratio by image analysis (Fig. 1B). Similar results were reported in other cell types and verified by nuclear/cytoplasmic fractionation (13, 14). In contrast, ECs at higher densities display YAP primarily localized in the cytoplasm and exposure to cerivastatin produced a slight further increase in YAP cytoplasmic localization (Fig. 1A, B).

In other experiments, ECs were plated at a low density and grown to confluence over a period of 6 days. Exposure to cerivastatin for 18 h induced a marked redistribution of YAP from the nucleus to the cytoplasm in cells cultured for 1-4 days (low-density cultures). Subsequently (days 5 and 6), as cell density increased, YAP translocated from the nucleus to the cytoplasm and cerivastatin did not exert any further effect on YAP localization (Supplementary Fig. S1). Thus, our results demonstrate that cerivastatin induces robust YAP cytoplasmic localization in low density cultures, when YAP is primarily in the nucleus. In other experiments, we verified that the concentration of cerivastatin used in Fig. 1 and Supplementary Fig. 1 induced a maximal effect on YAP localization (data not shown).

Next we determined the effect of different FDA-approved statins on YAP localization in low-density cultures of ECs. Treatment of these cells with the lipophilic statins simvastatin or atorvastatin also induced robust YAP cytoplasmic localization whereas exposure to the hydrophilic statin pravastatin even at 3 μM, did not induce any detectable effect (Fig. 1C), as verified by quantification of YAP nuclear/cytoplasmic ratio by image analysis (Fig. 1D). Hydrophilic statins, such as pravastatin, require a specific transport system to enter the cells, which is expressed primarily in liver cells. Thus, lipophilic statins induce cytoplasmic localization of YAP in low-density cultures of ECs.

In line with the cytoplasmic localization induced by exposure to cerivastatin, simvastatin or atorvastatin in ECs, treatment of these cells with these statins markedly reduced the mRNA levels of the YAP/TEAD-regulated gene *C*onnective *T*issue *G*rowth *F*actor (*CTGF*), as shown in Fig. 1E. *CTGF* is one of the best-characterized direct target gene of YAP that contains three putative YAP-TEAD binding sites (GGAATG) in its promoter region. We also found that exposure to cerivastatin, simvastatin or atorvastatin inhibited the expression of Cysteine-rich angiogenic inducer 61 (*CYR61*), another YAP/TEAD-regulated gene (Fig. 1 E). These results indicated that statins regulate YAP localization and co-activator transcriptional activity in human ECs.

Statins inhibit the stimulation of YAP activity induced by anti-HLA class I monoclonal antibody W6/32 in dense cultures of ECs.

Recently, we reported that stimulation of dense cultures of ECs with the mAb W6/32 that binds HLA I, promoted YAP nuclear localization and stimulated the expression of YAP/TEAD-regulated genes (28). Here, we determined whether exposure to statins prevents YAP nuclear localization and transcriptional activity in confluent cultures of ECs in response to mAb W6/32. ECs were transferred to medium containing low serum, treated with or without cerivastatin at 0.3 μM and then stimulated with W6/32 at 0.1 μg/ml for 60 min. In line with our recent results (28), crosslinking of HLA I on the surface of ECs with W6/32 induced robust translocation of YAP to the nucleus. Prior exposure of these cells to 0.3 μM cerivastatin or 3 μM simvastatin prevented mAb W6/32-induced YAP translocation to the nucleus (Fig. 2A), as verified by quantification of YAP nuclear/cytoplasmic ratio by image analysis (Fig. 2B). We confirmed the increase in YAP nuclear translocation in response to W6/32 and the inhibitory effects of cerivastatin and simvastatin on YAP nuclear accumulation using either confocal microscopy (Fig. 2A; quantification Fig. 2 B) or fluorescence microscopy (quantification Fig. 2 C; images in Supplementary Fig. S2) and in post confluent cultures of ECs (Supplementary Fig. S3). In addition, we verified that stimulation with human mAbs HLA-A2/A28 (clone SN607D8) instead of W6/32 also induced YAP nuclear accumulation in ECs and that prior cell exposure to 0.3 μM cerivastatin or 3 μM simvastatin prevented YAP nuclear translocation in response to this allele-specific human mAb (Supplementary Fig. S4).

Nuclear extrusion of YAP induced by statin is expected to reduce the transcriptional activity of TEAD. Consequently, we determined the effect of statin on YAP/TEAD-regulated gene expression elicited by stimulation with W6/32. As shown in Fig. 2 D, engagement of HLA I in ECs with 0.1 μg/ml W6/32 increased the level of *CTGF* and *CYR61* transcripts, as

determined by RT-qPCR. Treatment with 0.3 μM cerivastatin or 3 μM simvastatin reduced the level of expression in unstimulated ECs and prevented the increase in the expression of these genes in response to ligation of HLA I. Thus, statins induced YAP cytoplasmic localization and inhibited YAP/TEAD-regulated gene expression either in low-density cultures of EC cells or in confluent cultures of these cells re-stimulated via engagement of HLA I with W6/32.

Statins inhibit HLA I-induced migration of ECs cells.

Previously, we demonstrated that HLA I signaling induces migration of ECs into a denuded area of the monolayer in a YAP-dependent manner (28). Having established here that statins promote cytoplasmic localization of YAP and inhibit YAP/TEAD-regulated gene expression in ECs, we next determined whether statins inhibit HLA I-stimulated migration in ECs. Because statin-mediated inhibition of YAP could reduce the number of cells in the denuded area of the wound by inhibiting cell proliferation rather than migration, we examined migration of ECs pretreated with mitomycin C, a DNA cross-linking agent, to prevent cell proliferation (12, 37). Exposure of ECs to either 0.3 μM cerivastatin or 3 μM simvastatin completely blocked the migration of ECs into the denuded area of the monolayer stimulated by ligation of HLA I, as shown using a scratch wound assay (Fig. 3A; quantification in Fig. 3B). These results indicate that statins inhibit the increase in cell migration induced by crosslinking of HLA I in ECs.

Exogenously supplied mevalonic acid or geranylgeraniol prevents statin inhibition of HLA I-mediated signaling in ECs

As an initial step to elucidate the mechanism(s) by which statins inhibit YAP nuclear localization and coactivator transcriptional activity, we determined whether treatment with cerivastatin interferes with the binding of W6/32 mAb to ECs. Exposure of ECs to cerivastatin did not reduce the binding of W6/32 mAb to ECs, implying that the inhibitory effects of statins on HLA I-induced YAP signaling are not due to a decrease in HLA I expression but occur at a post-receptor locus (Fig. 3 C).

As mentioned above, statins inhibit the 3-hydroxy-methylglutaryl (HMG) CoA reductase, the rate-limiting enzyme in the generation of mevalonic acid, the first step in the biosynthesis of isoprenoids, leading to FPP and GG-PP. If statins induce YAP nuclear localization via suppression of HMG-CoA reductase and depletion GG-PP in ECs, exogenously supplied mevalonic acid or geranylgeraniol (GGOH), which is converted into GGPP within cells via a salvage pathway (38, 39) should reverse the inhibitory effects of statins on YAP localization. In line with results shown above, treatment with cerivastatin induced cytoplasmic localization of YAP in low-density cultures of ECs and prevented YAP nuclear import promoted by engagement of HLA I with W6/32 in high-density cultures of ECs. Exogenously added mevalonic acid (100 μM) or GGOH (10 μM) largely reversed the inhibitory effect of cerivastatin on YAP localization either in low-density ECs (Fig. 4 A; quantification in Fig. 4 B) or in high-density ECs challenged with W6/32 (Fig. 4 C; quantification in Fig. 4 D). These results suggest that statins inhibit YAP nuclear localization via inhibition of HMG CoA reductase and consequent depletion of GGPP.

Mechanism by which statins induce cytoplasmic localization and inhibit transcriptional activity of YAP: role of actin organization and YAP multi-site phosphorylation.

GGPP is a critical isoprenoid in Rho prenylation via geranylgeranyl transferases (GGTase) that catalyzes the transfer of the GG moiety of GGPP to Rho, a key modification for Rho in cell signaling. Accordingly, Rho is as a major target of statins in a variety of cell types (13, 14, 40) and is implicated in YAP activation but the precise mechanism linking Rho to YAP activity in ECs remains incompletely understood. We verified that suppression of Rho activity by exposure to a cell-permeable C3 transferase from *Clostridium botulinum* prevented the nuclear import of YAP in response to HLA I ligation in ECs (Supplementary Fig. S5). Rho is known to promote the organization of the actin cytoskeleton and the formation of actin stress fibers, which are one of the major upstream players in YAP regulation. Previously, we showed that HLA class I ligation, mediated in part by HLA I complex formation with integrin β4, promotes Rho activation and actin stress fiber formation, (7, 9). Consequently, we examined whether statin exposure prevents actin organization in ECs in response to W6/32, as visualized by phalloidin staining. In agreement with our previous results (3, 7), stimulation of EC with W6/32 induced a marked increase in the assembly of actin stress fibers. Exposure to cerivastatin blunted stress fiber formation in ECs (Fig. 5A; quantification in 5B). Similar results were obtained in post confluent cultures of ECs (Supplementary Fig. S2). These results raise the possibility that, under our experimental conditions, statin-mediated disorganization of the actin cytoskeleton contributes to hinder YAP function in ECs.

In other cell types, Rho appears to downregulate YAP phosphorylation at Ser^{127} through a Hippo-independent pathway (13, 14). More recently, Rho-mediated inhibition of YAP phosphorylation has been attributed to inhibition of MST1/2 and MAP4K, the upstream kinases in the Hippo pathway, mediated through the protein phosphatase of the multiprotein complex STRIPAK (41). Consequently, we examined the impact of statins on YAP phosphorylation at Ser^{127} or Ser^{397} in ECs. Treatment of low-density ECs with cerivastatin, simvastatin or atorvastatin increased YAP phosphorylation at Ser^{127} (Fig. 5 C; quantification in 5D). In confluent ECs, exposure to cerivastatin or simvastatin increased the level of YAP phosphorylation at Ser^{127} and Ser^{397} in unstimulated cells and attenuated the decline in YAP phosphorylation on these residues in response to HLA I crosslinking with W6/32 (Fig. 5 E, F). Collectively, these results imply that statins induce YAP redistribution from the nucleus to the cytoplasm in low-density EC and in high-density ECs stimulated with W6/32, at least in part, by promoting YAP phosphorylation at Ser^{127} and Ser^{397}.

It is increasingly recognized that YAP localization and co-activator activity is also regulated via phosphorylation on tyrosine residues, including Tyr^{357} (42-44), mediated by Src family tyrosine kinases (SFK). Therefore, we determined whether crosslinking of HLA class I regulates YAP phosphorylation at Tyr^{357}. Stimulation of ECs with W6/32 induced a marked increase in YAP phosphorylation on Tyr^{357} (Fig. 6 A; quantification in Fig.6 B) in a time-dependent manner (Fig. 6 C). Exposure to cerivastatin or simvastatin prevented the increase in YAP Tyr^{357} phosphorylation induced in response to W6/32 in ECs (Fig. 6 A; quantification in Fig.6B).

In order to determine the contribution of serine and tyrosine phosphorylations to the regulation of YAP localization in ECs, we used wild type and mutant FLAG-tagged YAP expressed in cultures of bovine ECs, which are easier to transfect than the human counterparts. Initially, we verified that endogenous YAP was restricted to the nucleus of sub-confluent cultures of bovine ECs and that exposure to either cerivastatin or the SFK inhibitor dasatinib induced cytoplasmic relocalization of endogenous YAP in these cells, similar to results obtained with human ECs (Fig. 6 D).

Next, we transiently transfected wild type FLAG-YAP, FLAG-YAP with Ser^{127} mutated to Ala (FLAG-S127A-YAP) and FLAG-YAP with Tyr^{357} mutated to Phe (FLAG-Y357F-YAP) into bovine ECs and determined their nuclear/cytoplasmic distribution in the absence or presence of either cerivastatin or dasatinib. As shown in Fig. 6E, wild type FLAG-YAP localized predominantly in the nucleus of low-density ECs and redistributed to the cytoplasm in response to cerivastatin treatment. As expected, FLAG-YAP with Ser^{127} mutated to Ala also localized in the nucleus of ECs. Treatment with cerivastatin induced cytoplasmic localization of FLAG-S127A-YAP, implying that statins control YAP localization via a mechanism(s) that circumvents the phosphorylation of YAP on Ser^{127}. Crucially, FLAG-YAP with Tyr^{357} mutated to Phe (FLAG-Y357F-YAP) was excluded from the nucleus of most ECs (Fig. 6E) and treatment with the SFK inhibitor dasatinib induced cytoplasmic localization of either wild type FLAG-YAP or FLAG-S127A-YAP. These results imply that YAP phosphorylation on Tyr^{357} plays a major role in controlling YAP localization in ECs and suggest that statins promote cytoplasmic YAP via inhibition of its phosphorylation on Tyr^{357}.

DISCUSSION

Statins are among the most widely prescribed medications in the world. Although most research on the pharmacological effects of statins has been in the context of cardiovascular or metabolic diseases, recent epidemiological studies indicate a protective effect of statins in clinical transplant populations (33-35). An early study concluded that simvastatin therapy initiated early after heart transplantation leads to significantly better 8-year survival rates and a significantly lower incidence of TV without impairment of organ function or severe adverse effects (33). Statins also attenuated rejection of other organs, including lung and kidney (45). Furthermore, statin use has been associated with improved cancer-free and overall survival after cardiac transplantation (46). Thus, statins administration not only leads to significantly lower incidence of TV without impairment of organ function but also appears to be associated with improved cancer-free and overall survival after transplantation. A recent meta-analysis including early small randomized controlled trials and retrospective nonrandomized studies concluded that statins prevent fatal rejection episodes and reduce the incidence of coronary TV (34). Consequently, mechanistic studies on the effects of statins on Ab-induced signaling in ECs are of significance as a step leading to the identification of new targets and pharmacological approaches to prevent DSA-induced cAMR.

The highly conserved transcriptional co-activators YAP and TAZ, originally identified in *Drosophila*, are attracting intense interest as key regulators of organ-size, tissue regeneration, inflammation and tumorigenesis (16, 17). Canonical Hippo signals in vertebrate cells

proceed through a serine/threonine kinase cascade wherein MST1/2 kinases phosphorylate and activate LATS1/2. In turn, LATS1/2 phosphorylate YAP and TAZ at multiple serine residues. The phosphorylation of YAP by LATS1/2 at Ser^{127} creates binding sites for 14–3-3 proteins, which localize and anchor YAP in the cytoplasm. In turn, phosphorylation of YAP by LATS1/2 at Ser^{397} promotes proteolytic degradation (16, 17, 19, 22, 24).

Our recent results showed that growing cultures of ECs display nuclear localization of YAP and that knock down of YAP/TAZ strikingly impairs the migration into a denuded area of the monolayer and entry into the S phase of the cell cycle of ECs (28). Furthermore, we demonstrated that stimulation of confluent cultures of ECs with the mAb W6/32 directed against HLA-I induces rapid YAP translocation from the cytoplasm to the nucleus and concomitantly decreases YAP phosphorylation at Ser^{127} and Ser^{397}, residues phosphorylated primarily by LATS1/2 (28). In line with the stimulation of YAP nuclear import, Ab-induced HLA I activation promotes expression of YAP/TEAD-regulated genes, including *CTGF* and *CYR61*. The products of these genes (i.e. CTGF and CYR61) are matricellular proteins that are involved in cell adhesion and migration (47). These results indicated that activation of the YAP/TEAD axis is an early point of transcriptional convergence in HLA I signaling in human ECs leading to migration.

In the present study, we examined the impact of statins on YAP localization, phosphorylation and transcriptional activity in ECs. Exposure of growing cultures of ECs to cerivastatin or simvastatin induced a striking re-localization of YAP from the nucleus to the cytoplasm and inhibited the expression of the YAP/TEAD-regulated genes *CTGF* and *CYR61*. In confluent and post-confluent cultures of ECs, statins prevented YAP nuclear import and expression of *CTGF* and *CYR61* stimulated by mAb W6/32-induced HLA I activation. Exposure of ECs to either cerivastatin or simvastatin blocked the migration of ECs into a denuded area of the monolayer stimulated by ligation of HLA I.

As discussed above, statins inhibit the 3-hydroxy-methylglutaryl (HMG) CoA reductase (29, 30), the rate-limiting enzyme in the generation of mevalonate, the first step in the biosynthesis of isoprenoids, leading to FPP, GG-PP and cholesterol (31). The transfer of the geranylgeranyl moiety to a COOH-terminal cysteine of Rho GTPases is critical for their function in signal transduction (17). In line with this notion, we demonstrate that exogenously added mevalonic acid or GGOH, a precursor of GG-PP, largely reversed the inhibitory effect of statins on YAP localization either in growing ECs or confluent ECs challenged with W6/32. These results support the notion that statins inhabit YAP nuclear localization via inhibition of HMG CoA reductase and consequent depletion of GGPP.

GGPP is a critical isoprenoid in Rho prenylation and function. Our previous results demonstrated that engagement of HLA I induces Rho activation and assembly of stress fibers in ECs (9). In turn, actin organization regulates YAP localization, through Hippo-dependent and Hippo independent pathways (15, 48). Here, we show that exposure to statins disrupted HLA I-mediated stress fiber formation in ECs. These results imply that one of the mechanisms by which statins inhibit YAP nuclear import and co-activator activity is via disorganization of the actin cytoskeleton, a major cellular response induced by Rho in

response to engagement of HLA I in ECs. Accordingly, we verified that suppression of Rho function in ECs prevented YAP nuclear accumulation induced by ligation of HLA I.

In previous studies, we demonstrated that antibody-meditated crosslinking of HLA I induces rapid SFK activation in ECs (5). More recently we demonstrated that isoforms of the SFK play a critical role in YAP activation and phosphorylation at Ser^{127} and Ser^{397} in ECs (28). In the present study, we show that HLA I crosslinking induces phosphorylation of YAP on Tyr^{357} in ECs. Importantly, statins prevented YAP tyrosine phosphorylation suggesting a novel mechanism by which these drugs regulate YAP activity in ECs.

To elucidate the contribution of YAP phosphorylation on Ser^{127} and Tyr^{357} on the regulation of its localization, we expressed wild type and mutant FLAG-tagged YAP expressed in growing cultures of bovine ECs. As expected, wild type FLAG-YAP and FLAG-S127A-YAP localized predominantly in the nucleus. Interestingly, cerivastatin treatment induced cytoplasmic localization of both wild type and mutant YAP, implying that statins regulate YAP nuclear-cytoplasmic shuttling via a mechanism that appears to bypass Ser^{127} phosphorylation. It is also noteworthy that the SFK inhibitor dasatinib also induced cytoplasmic localization of wild type FLAG-YAP and FLAG-S127A-YAP, suggesting that SFKs play a critical role in the control of YAP localization independently of Ser^{127} phosphorylation. Crucially, FLAG-YAP with Tyr^{357} mutated to Phe was excluded from the nucleus of most ECs and were not responsive to either statins or dasatinib. These results indicate that YAP phosphorylation on Tyr^{357} plays a major role in controlling YAP localization in ECs and imply that statins promote cytoplasmic YAP via inhibition of SFK-mediated phosphorylation on Tyr^{357}. Given that phosphorylation of Ser^{127} and Tyr^{357} regulate YAP localization in ECs in an opposite manner, it is conceivable that statins antagonize this reciprocal regulation leading to YAP inactivation (i.e., cytoplasmic localization) in these cells, primarily through inhibition of SFK-mediated phosphorylation of YAP on Tyr^{357}.

It is increasing recognized that donor specific HLA antibodies, either existing prior to transplantation or develop *de novo* after transplantation are an important problem in poorer graft outcomes (49). Mounting evidence indicates that Ab-mediated crosslinking of HLA class I and II molecules expressed on the surface of ECs induce intracellular signaling pathways regulating cell survival, proliferation and migration (1, 3, 4, 10, 37, 50), which contribute to TV. In a previous study, we identified the transcriptional co-activator YAP as a central mediator of HLA I-induced EC migration and proliferation, processes that are critical in the pathogenesis of TV (28). Furthermore, CTGF, which has been implicated in the development of TV (51) and transplant-related fibrosis (52), is a well-known target of YAP/TEAD induced by HLA I ligation in ECs (28). The results presented here demonstrate, for the first time, that statins promote YAP cytoplasmic localization and inhibit YAP activity induced by ligation of HLA I in EC models, including the increase in *CTGF* expression in human ECs. Mechanistically, we identified YAP phosphorylation on Tyr^{357} as a critical posttranscriptional modification in the control of YAP localization in ECs and put forward the hypothesis that statins promote cytoplasmic YAP via SFK-mediated phosphorylation on Tyr^{357}. We propose that the inhibitory effect of statins on YAP function is a major mechanisms mediating the beneficial effects of these drugs in solid organ transplant

recipients, especially in cAMR promoted by donor specific HLA antibodies. Since DSA-induced AMR and TV occurs across multiple transplanted organs (49), our findings raise the possibility of expanding the use of lipophilic statins for attenuating rejection in transplant medicine, a proposition that warrants further mechanistic and clinical work. In this context, it will be important to determine whether statins also inhibit YAP transcriptional function in ECs stimulated by HLA II antibodies, that have been associated with severity of TV (53).

There is increasing recognition that physical forces produced by blood flow deliver important environmental cues that control EC function, including actin cytoskeleton (54). Given that the Hippo/YAP pathway has been implicated in mechanosensing (48, 55) at least in part via the actin cytoskeleton, it will be important to examine, in future studies, the inhibitory effect of statins on YAP localization and function in response to HLA I crosslinking under conditions that replicate physical forces created by blood flow.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used in this paper

Ab	Antibody
AMR	antibody-mediated rejection
ANOVA	analysis of variance
CTGF	Connective Tissue Growth Factor
CYR61	Cysteine-rich angiogenic inducer 61
DSA	donor specific antibodies
EC	endothelial cells
HLA I	HLA class I
RT-qPCR	Real time quantitative reverse transcription PCR
SFK	Src family of tyrosine kinases
siRNA	small interfering RNA
TAZ	WW-domain–containing transcriptional coactivator with PDZ-binding motif

TEAD	TEA domain DNA-binding transcription factor
TV	transplant vasculopathy
YAP	Yes-Associated Protein

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Key Points:

1. Statins induced cytoplasmic YAP localization and inhibit its activity in human ECs
2. Mevalonic acid or geranylgeraniol reversed the inhibitory effect of statins on YAP
3. Statins inhibit YAP Tyr^{357} phosphorylation, which is critical for YAP activation

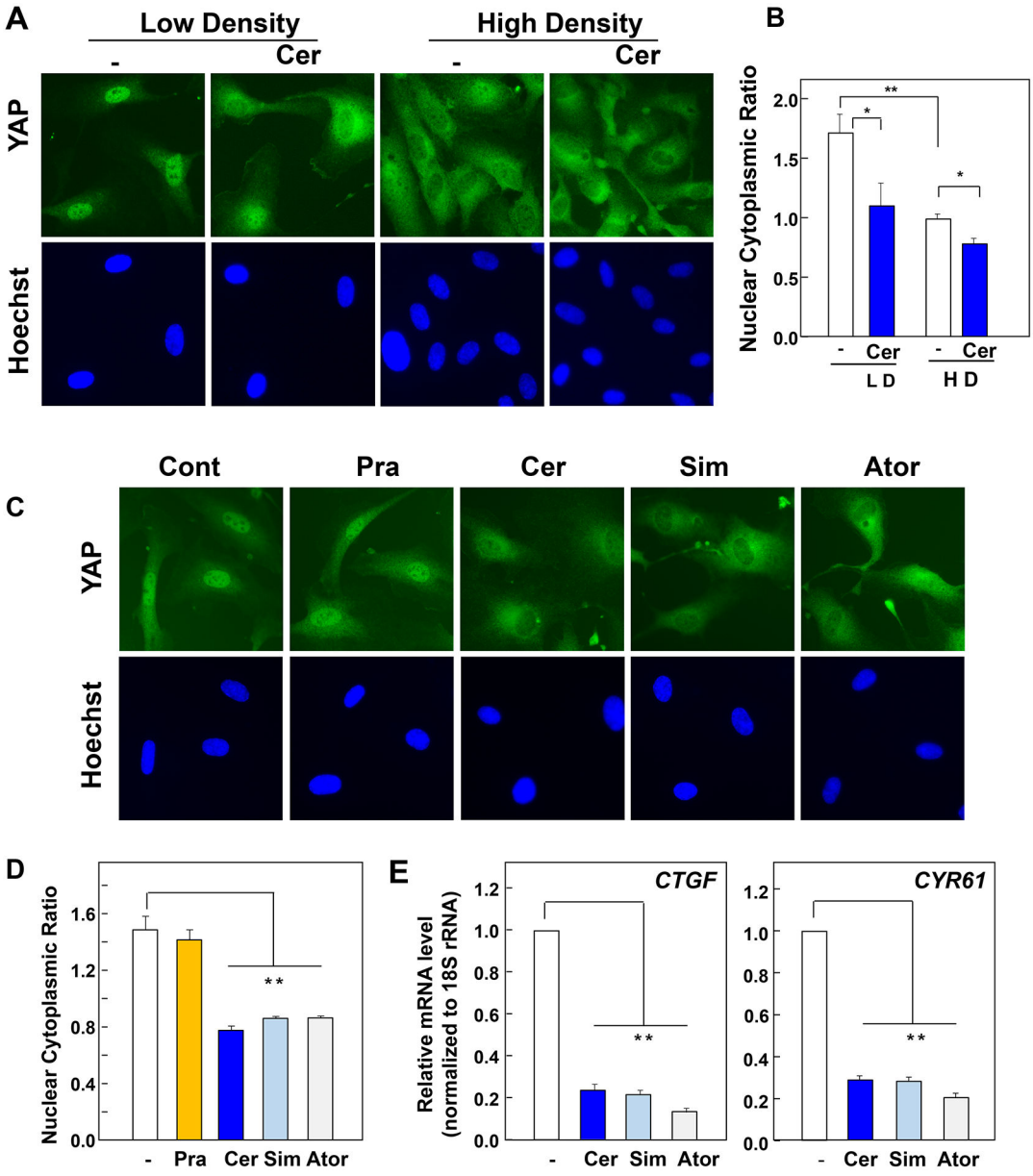

Figure 1. Statins induce YAP translocation to the cytoplasm and inhibit YAP/TEAD-regulated genes in human ECs.

(**A)** Human aortic ECs were plated at low density (10^5 cells), or at higher density ($2.5x10^5$ cells) in 35 mm cell culture dishes. Cells were incubated in the absence or presence of 0.3 μM Cerivastatin (Cer) 1 day after plating for 18 h, and were fixed with 4% paraformaldehyde at day 2 after plating. The cultures were then stained with an Ab that detects total YAP and with Hoechst 33342 to visualize the cell nuclei. Images were captured at original magnification X 40. **(B)** Bars represent the nuclear/cytoplasm ratio of fluorescence (200–300 cells) mean ± SE with similar results obtained in 4 independent experiments (*p< 0.05; **p< 0.01). **(C)** Human aortic ECs were plated at low density (10^5 cells) in 35 mm cell culture dish. Cells were incubated in either without or with the lipophilic statins 0.3 μM cerivastatin (Cer); 3 μM simvastatin (Sim); 3 μM atorvastatin

(Ator) or the hydrophilic statin 3 μM pravastatin 1 day after plating for 18 h, and were fixed with 4% paraformaldehyde at day 2 after plating. The cultures were then stained with an Ab that detects total YAP and with Hoechst 33342 to visualize the cell nuclei. **(D)** Bars represent the nuclear/cytoplasm ratio of YAP fluorescence (200–300 cells) mean ± SE with similar results obtained in three independent experiments (**p< 0.01). **(E)** Low density cultures of human aortic ECs were incubated in either absence or presence of 0.3 μM cerivastatin (Cer); 3 μM simvastatin (Sim) or 3 μM atorvastatin (Ator) as indicated. Statins were added 1 day after plating and the incubation continued for 18 h. RNA was then isolated and the relative levels of CTGF or CYR61 mRNA using 18S mRNA as internal control were measured by RT-qPCR. Data are presented as mean ± SEM of 3 independent experiments and compared with untreated controls (**p< 0.01).

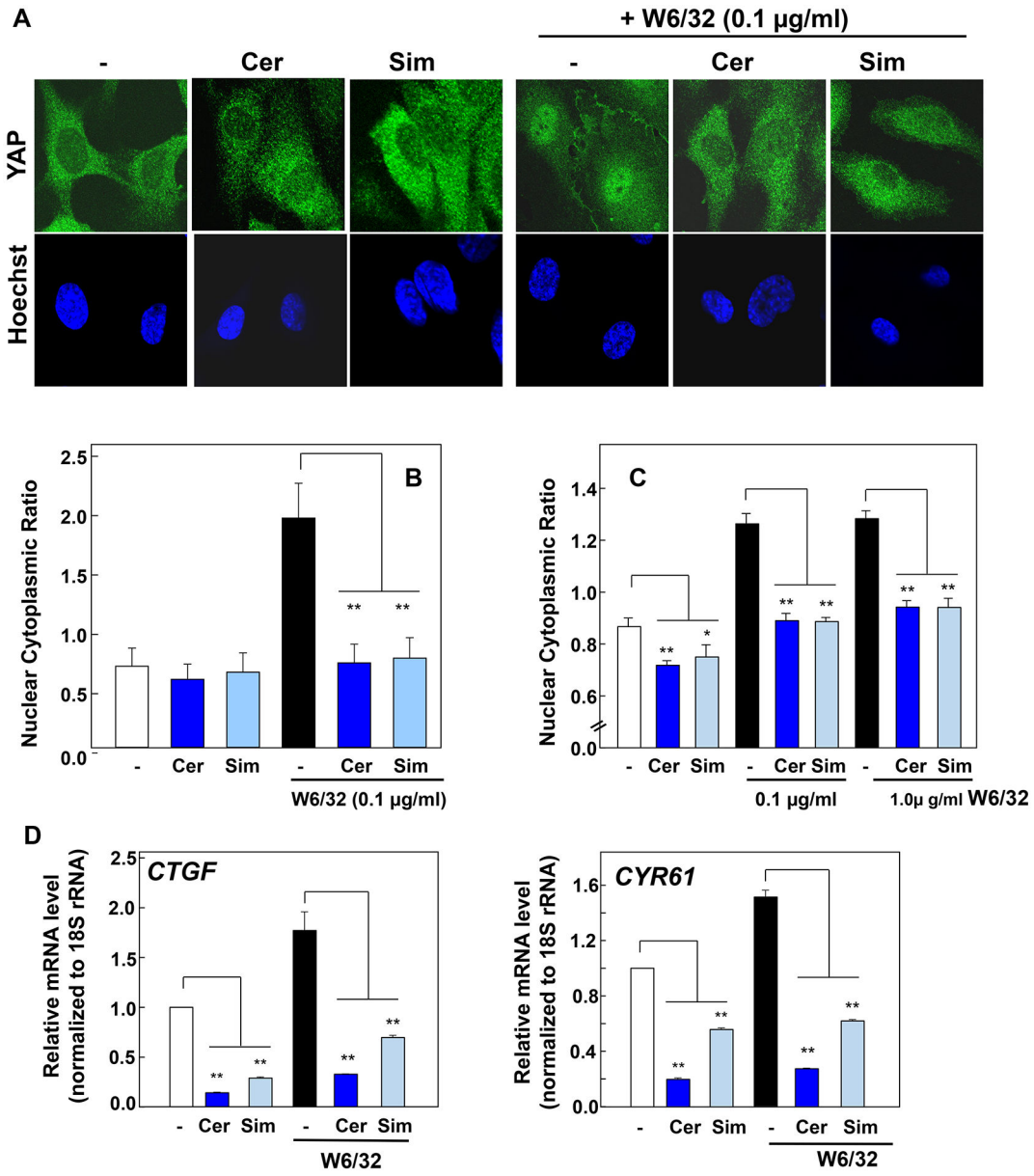

Figure 2. Statins inhibit YAP nuclear localization and YAP/TEAD-regulated genes induced by Ab-mediated crosslinking of HLA I in human ECs.

(A) Confluent cultures of human aortic ECs were incubated either in the absence or presence of 0.3 μM cerivastatin (Cer) or 3 μM simvastatin (Sim) for 18 h. The cultures were then stimulated without (−) or with anti- HLA I mAb W6/32 (0.1 μg/ml) for 60 min. The cultures were then washed, fixed with 4% paraformaldehyde, and stained with an antibody that detects total YAP and with Hoechst 33342 to visualize the cell nuclei. Confocal microscopy was performed using a Zeiss LSM 710 confocal microscope with a Plan-Apochromat 63/1.4 oil objective (Zeiss). **(B)** Bars represent the nuclear/cytoplasm ratio of fluorescence (50-75 cells) mean + SE with similar results obtained in 3 independent experiments **p< 0.01). **(C)** Bars represent the ratio of nuclear/cytoplasm (200 to 300 cells) of ECs imaged by fluorescence microscopy. Cerivastatin (Cer) and simvastatin (Sim) significantly decreased

nuclear localization of YAP as compared with untreated controls (*p< 0.05, **p<0.001) and significantly decreased HLA-I induced nuclear localization of YAP (**p< 0.001) in 5 independent experiments. (**D)** Confluent ECs were treated without (−) or with 0.3 μM cerivastatin (Cer) or 3 μM simvastatin (Sim) for 18 h. ECs were then stimulated with W6/32 (0.1 μg/ml) for 60 min. RNA was isolated, and relative levels (n = 3) of CTGF and CYR61 mRNAs (compared with 18S mRNA) were measured by quantitative RT-qPCR. Data are presented as mean ± SEM (** p< 0.01).

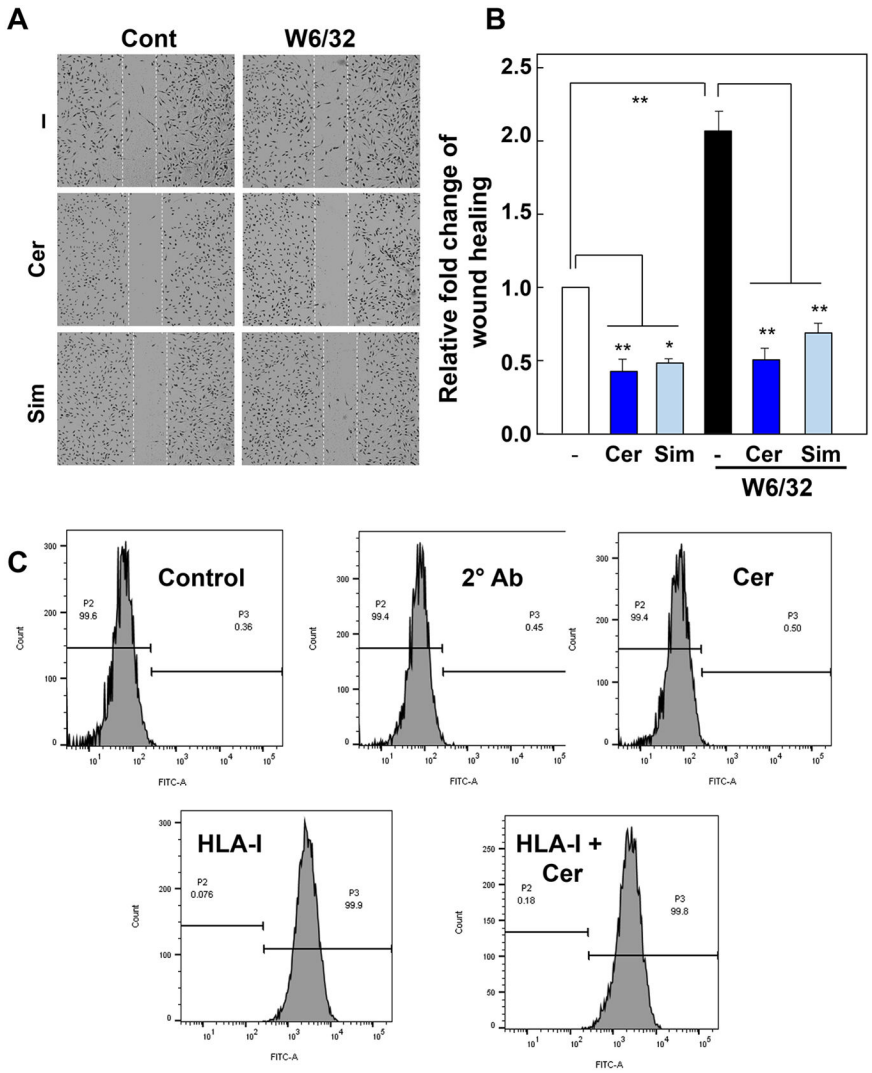

Figure 3. Statins inhibit HLA I induced migration of human ECs.

(A) Confluent ECs were incubated either in the absence or presence of cerivastatin (0.3 μM Cer) or simvastatin (3 μM Sim) for 18 h. Cells were pretreated with 10 mg/ml mitomycin C for 2 h to inhibit cell proliferation. A scratch wound was then created with a sterile 200-ml pipette tip. After washing, wounded cells were stimulated with or without 1 μg/ml HLA I mAb W6/32 for 16 h. The cultures were then fixed with 4% paraformaldehyde and stained with Giemsa stain. Representative microscopy fields are shown. Images were captured at original magnification X10. **(B)** Bars represent relative migration (average of 10 fields/experiment) of ECs unstimulated or stimulated with HLA I mAb W6/32 incubated in the absence or presence of Cer or Sim. Data are presented as mean ± SE of three independent experiments. HLA I mAb W6/32 increase relative migration in ECs as compared with untreated controls (**$p < 0.01$). Cer and Sim significantly decreased relative migration as compared with untreated controls (*$P<0.05$, **$p < 0.01$) and abrogated the increase in EC migration induced by HLA I cross-linking (**$p < 0.01$). **(C)** Statins do not decrease the binding of HLA-I to human ECs. Confluent cultures of ECs were incubated either in the absence or presence of cerivastatin (0.3 μM) for 18 h. Cells were then trypsinized

and incubated with 1 mg/ml HLA I mAb W6/32 for 30 min on ice. After washing, cells were incubated with 1:100 diluted FITC conjugated anti-mouse IgG, $F(ab')_2$ fragment and incubated on ice for 30 min. HLA I binding capacity on Human aortic ECs was measured by flow cytometry.

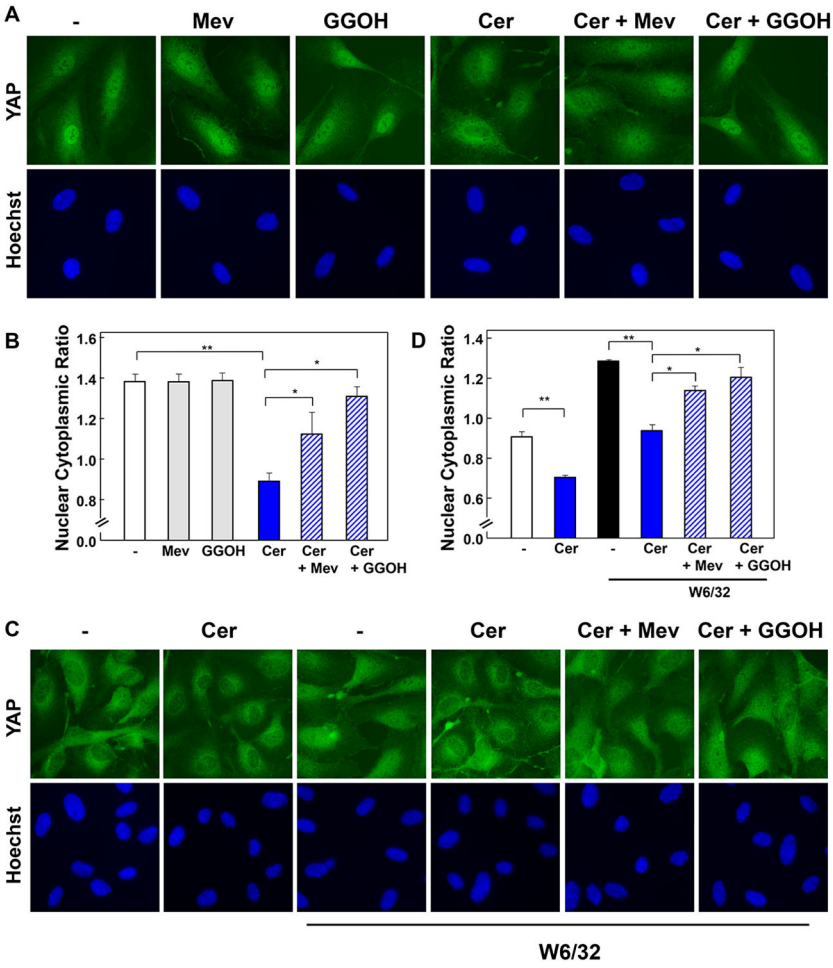

Figure 4. The inhibitory effect of statins on YAP nuclear localization is reversed by exogenously added mevalonic acid or geranylgeraniol (GGOH) in human ECs.

(A) Low density culture of human aortic ECs was incubated in either absence or presence of 0.1 μM cerivastatin (Cer) added 1 day after plating and for 18 h either with or without 100 μM mevalonic acid (Mev) or 10 μM GGOH. The cells were fixed with 4% paraformaldehyde at day 2 after plating. The cultures were then stained with an Ab that detects total YAP and with Hoechst 33342 to visualize the cell nuclei. Images were captured at original magnification x40. **(B)** Bars represent the nuclear/cytoplasm ratio (100–200 cells) mean ± SE with similar results obtained in three independent experiments. (*$p < 0.05$; **$p < 0.01$). **(C)** Confluent culture of human aortic ECs was incubated in either absence or presence of 0.1 μM cerivastatin (Cer) for 18 h either with or without 100 μM mevalonic acid (Mev) or 10 μM GGOH. The cells were then stimulated with or without 0.1 μg/ml HLA I mAb W6/32 for 1 h and fixed with 4% paraformaldehyde. Fixed cells were then stained with an Ab that detects total YAP and with Hoechst 33342 to visualize the cell nuclei. Images were captured at original magnification X40. **(D)** Bars represent the nuclear/cytoplasm ratio (200–300 cells) mean ± SE with similar results obtained in three independent experiments (*$p < 0.05$).

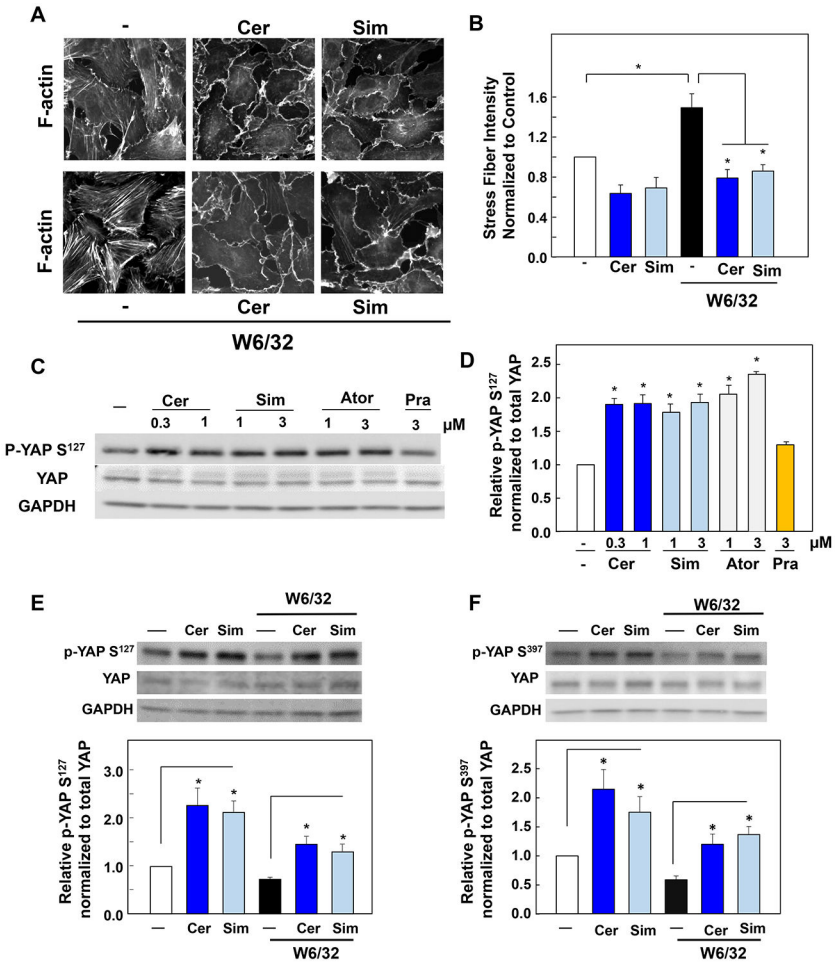

Figure 5. Statins inhibit the assembly of actin stress fibers induced by stimulation with HLA I and increase YAP phosphorylation at Ser^{127} and Ser^{397} in human ECs

(A) Confluent cultures of human aortic ECs were incubated either in the absence or presence of 0.3 μM cerivastatin (Cer) or 3 μM simvastatin (Sim) for 18 h. The cultures were then stimulated without (−) or with W6/32 HLA I mAb (1 μg/ml) for 60 min. The cultures were then washed, fixed with 4% paraformaldehyde, and stained with TRITC-conjugated phalloidin to visualize F-actin. **(B)** Quantification of stress fibers was performed as describes in Methods and normalized to the control values. HLA I mAb W6/32 increase stress fibers in ECs as compared with untreated controls (N=5; *p< 0.05). Cerivastatin (Cer) and simvastatin (Sim) significantly decreased HLA-I induced stress fibers as compared with untreated controls. (**C),** Low density cultures of human aortic ECs were incubated in either absence or presence of cerivastatin (Cer), simvastatin (Sim), atorvastatin (Ator) or pravastatin (Pra), at the indicated concentrations. The statins were added to the medium 1 day after plating for 18 h. The cultures were then lysed with 2X SDS–PAGE sample buffer and analyzed by immunoblotting with Abs that detect YAP phosphorylated at Ser^{127}; total YAP and GADPH (used as loading control). (**D**) Quantification of phosphorylated YAP Ser^{127} was performed using Multi Gauge V3.0. Lipophilic statins increased YAP phosphorylation as compared with untreated controls (*p< 0.05) **(E, F)** Confluent ECs were treated without (−) or with 0.3 μM cerivastatin (Cer) or 3 μM simvastatin (Sim) for

18 h. The cultures were then stimulated with W6/32 (0.1 mg/ml) for 60 min, lysed with 2X SDS–PAGE sample buffer and analyzed by immunoblotting with Abs that detect YAP phosphorylated at Ser^{127} **(E)** and Ser^{397} **(F)**; total YAP and GADPH (used as loading control). Quantification of phosphorylation at Ser^{127} and Ser^{397}, normalized to total YAP. The results represent the mean ± SE for 4 independent experiments and are expressed as ratio of YAP phosphorylated at Ser^{127} or Ser^{397} and total YAP. Lipophilic statins increased YAP phosphorylation in untreated cultures or in cultures stimulated with HLA I mAb W6/32 (*$p < 0.05$).

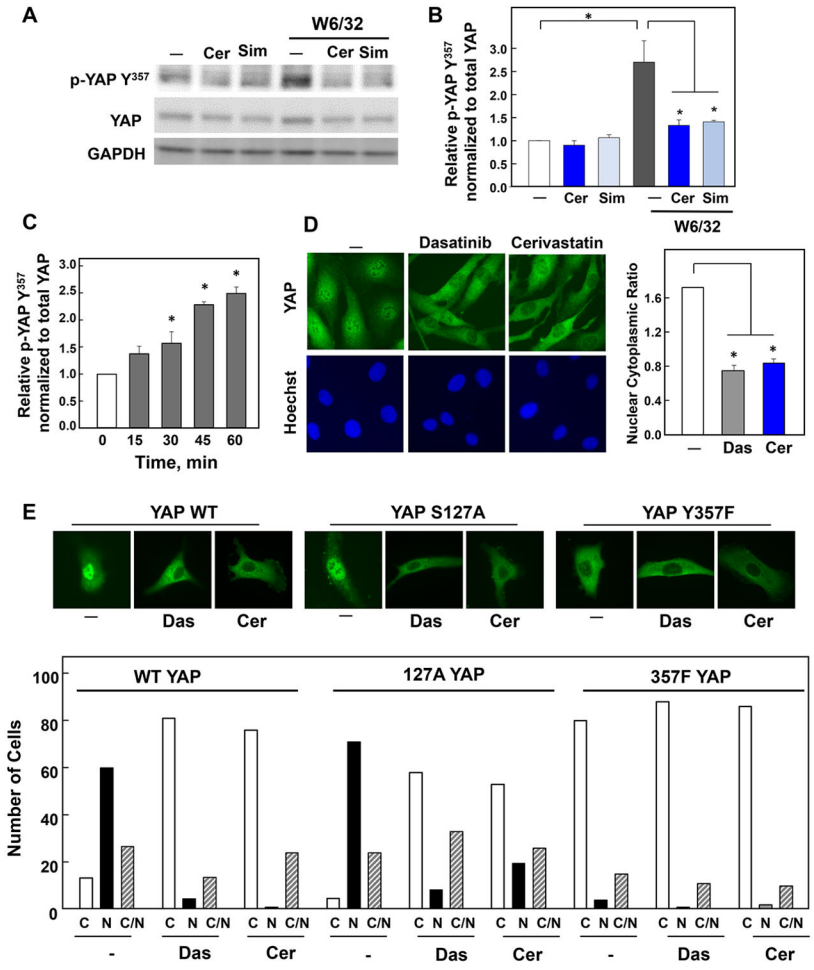

Figure 6. Statins inhibit YAP tyrosine phosphorylation in aortic ECs.

(A) Confluent ECs were treated without (−) or with cerivastatin (0.3 μM) or simvastatin (3 μM) for 18 h. ECs were then stimulated with W6/32 (0.1 μg/ml) for 60 min. The cultures were then lysed with 2X SDS–PAGE sample buffer and analyzed by immunoblotting with Abs that detect YAP phosphorylated at Tyr^{357}; total YAP and GADPH (used as loading control)**. (B)** Quantification of phosphorylation at Tyr^{357} using total YAP to normalize. The results represent the mean ± SE OF 5 independent experiments and are expressed as ratio of YAP phosphorylated at Tyr^{357} to total YAP. HLA I mAb W6/32 increase YAP phosphorylation at Tyr^{357} in ECs as compared with untreated controls (*p< 0.05). Cerivastatin (Cer) and simvastatin (Sim) significantly decreased phosphorylation at Tyr^{357} as compared with cultures stimulated with HLA I mAb W6/32 (*p< 0.05). **(C)** Stimulation of confluent ECs with W6/32 (0.1 μg/ml) induces time-dependent increase in YAP phosphorylated at Tyr^{357}. The results represent the mean ± SE OF 5 independent experiments and are expressed as ratio of YAP phosphorylated at Tyr^{357} to total YAP (*p< 0.05). **(D)** Statins prevent nuclear YAP localization in growing bovine aortic endothelial cells. Low density cultures of bovine aortic endothelial cells were treated with dasatinib (1 μM, 2 h) or cerivastatin (0.3 μM, 18 h) and then then fixed and stained for YAP. Bars represent the ratio of nuclear/cytoplasm immunofluorescence (100 to 200 cells) of

bovine aortic endothelial cells after dasatinib (Das) or cerivastatin (Cer) treatment. Statins significantly decreased nuclear localization of YAP in bovine ECs as compared with untreated controls (*$p < 0.05$). **(E)** YAP Y357F mutation prevents YAP nuclear localization. Bovine aortic endothelial cells were transiently transfected with FLAG epitope-tagged WT or phospho-deficient mutants of YAP (S127A or Y357F). The growing cultures (1 day after transfection) were treated with dasatinib (1 μM, 2 h) or cerivastatin (0.3 μM, 18 h) and then fixed with 4% paraformaldehyde and stained for the FLAG epitope-tag. The bars represent the proportion of cells displaying wild type or mutant YAP in the cytoplasm (C), nucleus (N) or in both compartments (CN).