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Interleukin-18 enhances IL-18R/Nox1 binding, and mediates TRAF3IP2-dependent smooth muscle cell migration. Inhibition by simvastatin

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Abstract

We investigated the role of TRAF3 interacting protein 2 (TRAF3IP2), a redox-sensitive adapter protein and an upstream regulator of IKK and JNK in interleukin (IL)-18 induced smooth muscle cell migration, and the mechanism of its inhibition by simvastatin. The pleiotropic cytokine IL-18 induced human coronary artery SMC migration through the induction of TRAF3IP2. IL-18 induced Nox1-dependent ROS generation, TRAF3IP2 expression, and IKK/NF- κ B and JNK/AP-1 activation. IL-18 induced its own expression and that of its receptor subunit IL-18R α . Using co-IP/IB and GST pull-down assays, we show for the first time that the subunits of the IL-18 heterodimer physically associate with Nox1 under basal conditions, and IL-18 appears to enhance their binding. Importantly, the HMG-coA reductase inhibitor simvastatin attenuated IL-18-induced TRAF3IP2 induction. These inhibitory effects were reversed by mevalonate and geranylgeranylpyrophosphate (GGPP), but not by farnesylpyrophosphate (FPP). Interestingly, simvastatin, GGPP, FPP, or Rac1 inhibition did not modulate ectopically expressed TRAF3IP2. The promigratory effects of IL-18 are mediated through TRAF3IP2 in a redox-sensitive manner, and this may involve IL-18R/Nox1 physical association. Further, Simvastatin inhibits inducible, but not ectopically-expressed TRAF3IP2. Targeting TRAF3IP2 may blunt progression of hyperplastic vascular diseases *in vivo*.

Keywords

Interleukin-18; TRAF3IP2; Nox1; oxidative stress; SMC migration; vascular diseases

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Introduction

Inflammation is a major component in the initiation and progression of vascular proliferative diseases. Migration and proliferation of smooth muscle cells are two key events in atherosclerosis and in restenosis following angioplasty.[1–3] The pleiotropic cytokine interleukin (IL)-18 plays a key role in atherosclerosis and injury-induced neointima formation, and exerts both mitogenic and migratory effects on vascular smooth muscle cells [4, 5]. IL-18 signals via a heterodimeric receptor comprising of ligand binding α subunit and signal transducing β subunit. Previously we reported that IL-18 is a potent inducer of SMC migration [6], and this response is dependent on the activation of nuclear factor-kappa B (NF- κ B) and activator protein (AP)-1, two redox-sensitive nuclear transcription factors known to positively regulate various pro-atherogenic cytokines and chemokines. NF- κ B and AP-1 also positively regulate adhesion molecule expression, and transcription of matrix-degrading metalloproteinases (MMPs).

Chronic oxidative stress also contributes causally to vascular diseases. In aortic smooth muscle cells (SMC), reactive oxygen species (ROS) are generated by various enzyme systems, including the NADPH oxidases (Nox)[7, 8]. Among the 5 known Nox isoforms, SMC express Nox1 and Nox4. Nox1-dependent superoxide has been shown to play a role in SMC hypertrophy and proliferation, and both Nox1 and Nox4 have been implicated in the induction of SMC migration. The small GTPase Rac1 has been shown to regulate the activation of the Nox1 enzyme complex[9]. We have also reported that IL-18 can regulate Nox1 transcription via AP-1, and NF- κ B has also been shown to regulate Nox1 expression[10]. This suggests that Nox1-induced superoxide generation that results in NF- κ B and AP-1 activation, may further promote Nox1 transcription, and positive amplification of Nox1 induction and persistent ROS generation may contribute causally to progression of vascular proliferative diseases.

TRAF3 interacting protein 2 (TRAF3IP2), also known as CIKS (Connection to IKK and SAPK/JNK) or Act1 (NF- κ B activator 1), is a cytoplasmic adapter protein and upstream regulator of IKK and JNK[11, 12]. Although its role in various inflammatory diseases has been well described, less is known about its expression, regulation and role in the cardiovascular diseases. We have recently reported a causal role for TRAF3IP2 in angiotensin (Ang)-II-induced cardiomyocyte hypertrophy [13], and its critical role in high glucose-induced endothelial dysfunction[14]. In those studies, we demonstrated that TRAF3IP2 is a potent inducer of NF- κ B and AP-1 activation, and its induced expression is Nox/ROS dependent.

Statins are widely used in the treatment of cardiovascular diseases mainly due to their cholesterol-lowering effects[15, 16]. They inhibit HMG-coA reductase, which catalyzes the conversion of HMG-coA to mevalonate, and the subsequent synthesis of cholesterol. However, statins also exert anti-inflammatory and antioxidant effects independently of cholesterol lowering[15, 16]. In SMC, we previously reported inhibition of NF- κ B and AP-1 by a statin [6]. Since TRAF3IP2 is redox-sensitive, regulates IKK and JNK activation, and plays a role in inflammation, we hypothesized that statins could inhibit its expression and downstream signaling. Here, we report that IL-18 induces Nox1/Rac1/ROS-mediated TRAF3IP2 induction, and TRAF3IP2-dependent IKK/NF- κ B and JNK/AP-1 activation, its own induction and SMC migration. For the first time, we also show that IL-18 receptor physically associates with Nox1, and IL-18 appears to enhance their binding. Further, simvastatin inhibits IL-18-induced TRAF3IP2 expression and SMC migration. However, simvastatin failed to inhibit ectopically expressed TRAF3IP2, suggesting that its inhibitory effects on IL-18-induced TRAF3IP2 expression may be indirect, and result from its

inhibition of Rac1 activation. Targeting IL-18/TRAF3IP2 signaling may attenuate progression of vascular proliferative diseases.

Materials and Methods

Simvastatin, sodium salt (#567021) and NSC23766 (#553502; 30 μ M in water for 3 h) were purchased from EMD4 Biosciences. Recombinant human IL-18 (#B001–5), recombinant mouse IL-18 (#B004–2), anti-IL-18 antibodies that detect mature IL-18 (#D043–3), neutralizing IL-18 (#D044–3), mouse IgG1 isotype control (#MAB002), IL-18R α (#AF840) antibodies, and normal goat IgG (#AB-108-C), and human IL-18 ELISA kit (#7620) were from R&D Systems (Minneapolis, MN). Anti-IL-18 antibodies that detect both pro and mature IL-18 (#sc-7954), JNK2 (p54; sc-827) and IL-18R α (#sc-50519) antibodies used in immunoprecipitation (IP) and immunoblotting (IB), Polybrene β (sc-134220; 5 μ g/ml in water), and lentiviral particles expressing shRNA that target human TRAF3IP2 (#sc-29634-V), IKK β (#sc-35644-V), p65 (#sc-29410-V), JNK2 (#sc-39101-V), and c-Jun (#sc-29223-V) were from Santa Cruz Biotechnology, Inc. The shRNA sequences for TRAF3IP2, IKK β , JNK2, p65 and c-Jun were previously described by us [14]. The polyclonal antibodies to IL-18R β used in both IP and IB (#bs-2616R) were purchased from Bioss (Woburn, MA). Anti-TRAF3IP2 (#IMG-563) and peroxidase-conjugated affinity purified goat anti-rabbit IgG (#111–035–046), and goat anti-mouse IgG were purchased from Imgenex (San Diego, CA) and Jackson Immuno Research (Baltimore, PA), respectively. SAPK/JNK Kinase Assay Kit (Nonradioactive; #8794) and antibodies against NF- κ B p65 (#3034), IKK β , phospho-JNK (#9251), JNK (#9252), c-Jun, Lamin A/C, GAPDH, and α -Tubulin were purchased from Cell Signaling Technology, Inc. (Beverly, MA). The nitric oxide donor S-Nitroso-N-acetyl-D,L-penicillamine (SNAP; 500 μ M in DMSO) was purchased from AXXORA (San Diego, CA). Affinity-purified polyclonal antibodies that detect both the latent (92 kDa) and active (88 kDa) forms of MMP9 (#RP1MMP9) were purchased from Triple Point Biologics, Inc. (Forest Grove, OR). Discovery Labware BD BioCoatTM MatrigelTM invasion chambers (#354481) were purchased from BD Biosciences (San Jose, CA). Anti-Myc, smooth muscle α -actin, and Nox1 antibodies used in IP and IB (#SAB4200097), actinomycin D (ActD), cycloheximide (CHX), gelatin (#G2500), and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Cell Culture-Normal human coronary artery SMC

Human coronary artery SMC were obtained from Clonetics Corp. (San Diego CA) and grown in SmGM-2 medium supplied by the manufacturer. At 70–80% confluency, the culture medium was replaced with basal medium containing 0.5% bovine serum albumin (conditioning medium). After 48 h incubation, recombinant human IL-18 was added and cultured for the indicated time periods. At the end of the experimental period, culture supernatants were collected into slick tubes, snap-frozen, and stored at –80 °C.

Isolation and culture of carotid artery SMC from wild type (WT) and TRAF3IP2-null mice

This investigation conforms to the *Guide for the Care and Use of Laboratory Animals*, published by the National Institutes of Health (NIH/National Institutes of Health, 1996), and all protocols were approved by the Institutional Animal Care and Use Committees of the University of Texas Health Science Center at San Antonio and Tulane University, New Orleans. The TRAF3IP2-null mice (C57B1/6 background) used in this study were previously described [17]. Wild type (WT) C57B1/6 mice were purchased from the Jackson Laboratory. All mice used in the study were male, ~ 3 months old, and weighed ~ 25 g. The TRAF3IP2-null mice exhibited no basal phenotypic abnormalities. Breeding, litter size, sex ratio, growth, and body weights were comparable between TRAF3IP2-null and WT control mice. SMC were isolated from the right and left carotid arteries from naïve mice by an

explant method described previously [18]. In brief, vessels were opened longitudinally, scraped free of endothelium, and cut into small pieces. The endothelium-denuded tissue was placed with luminal surface facing down on a 0.1% gelatin-coated culture flask containing DMEM/F12 + 20% FCS + antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL amphotericin B), and cultured at 37°C in a humidified atmosphere of 95% air/5% CO₂. After 12–14 days, the SMC outgrowths from the explants were trypsinized and replated in tissue culture dishes. Cells were pooled from both vessels from several animals, and used between passages 4–7. Around 96% of cells were positive for smooth muscle cell α -actin. At ~70% confluency, the cells were washed twice with serum-free medium and then maintained in DMEM/F12 with 0.1% BSA for 48 h before use.

Adenoviral vectors, lentiviral shRNA particles, and siRNA

Adenoviruses encoding green fluorescent protein (Ad.GFP), dnRac1 (Ad.N17rac1), and CuZnSOD (Ad.CuZnSOD) have all been described before [10, 13, 14, 18]. Adenovirus expressing shRNA directed to Nox1 (Ad.shNox1) was obtained from the Gene Transfer Vector Core (University of Iowa, Iowa City, IA) after permission from Dr. Robin L. Davisson [19]. Scrambled shRNA with GFP (#1642) was purchased from Vector Biolabs (Philadelphia, PA). SMC were infected with adenoviruses at indicated MOI at ambient temperature. After 1 h, the adenovirus-containing medium was replaced with medium containing 0.5% BSA. Lentiviral particles expressing shRNA that target human TRAF3IP2, IKK β , p65, JNK2, and c-Jun have been previously described [14]. SMC were infected with lentiviral particles for 48 h. To increase infection efficiency, cells were co-treated with the cationic polymer Polybrene β (5 µg/mL in water). Neither shRNA nor Polybrene β affected cell viability. Polybrene β also failed to modulate protein expression. At the indicated MOI and duration, adenoviral vectors and lentiviral shRNA particles failed to modulate SMC adherence to culture dishes, shape and viability (trypan blue-dye exclusion, data not shown). MMP9 expression was targeted by siRNA (MMP9, sense, 5'-CAUCACAUCUGGAUCCAAUU-3'), and has been previously described [6]. siRNA that does not target any genes in the human genome (5'-UUCUCCGAACGUGUCACGUA-3'; # 1022076, Qiagen, Inc.) served as a negative control. SMC were transfected with the indicated siRNA (20 nM) using the Neon transfection system (MPK-5000; Invitrogen) with the following parameters: pulse voltage 1,475 V, pulse width 20 ms, pulse number 2, and the tip type 10 µL. Cell viability was 96%. Seventy-two hours after transfection, knockdown of MMP9 was confirmed by RT-PCR. No off-target effects were observed. The shRNA and siRNA at the indicated concentrations and duration had no off-target effects.

Superoxide generation

Superoxide (O₂^{•-}) generation was quantified using the lucigenin-enhanced chemiluminescence assay as previously described [10]. In brief, SMC were rinsed twice in PBS and resuspended in Krebs/HEPES buffer (in mM: 115 NaCl, 20 HEPES, 1.17 K₂HPO₄, 1.17 MgSO₄, 4.3 KCl, 1.3 CaCl₂, 25 NaHCO₃, and 11.7 glucose pH 7.4) that contained no NADPH. After incubation at 37°C for 30 min, the tubes were placed in a Sirius luminometer (Berthold Detection Systems, Pforzheim, Germany). Dark-adapted lucigenin (5 µM) and either IL-18 alone or IL-18 combined with DPI or Tiron were added. Luminescence was measured for 10 s with a delay of 5 s. After background luminescence was subtracted, results were expressed as relative light units (RLU) per second per 5 × 10⁴ cells. Studies were also performed using SMC infected with Ad.CuZnSOD (MOI100 for 24 h) or Ad.shNox1 (MOI100 for 48 h) prior to the assay. Ad.GFP and Ad.shGFP served as controls.

RT-PCR and RT-qPCR

Total RNA was isolated using the TRIzol method. One microgram of RNA was used for the first strand cDNA synthesis using Quantitect cDNA Synthesis Kit. MMP9 mRNA expression was analyzed by RT-PCR [6]. IL-18 and IL-18R α mRNA expressions were analyzed by RT-qPCR using TaqMan β probes (IL-18, # 4331182; IL-18R α , # 4331182) and the Eppendorf Realplex⁴ system. Data are shown as fold change ($2^{-\Delta\Delta C_t}$).

Immunoblotting and ELISA

The methods of preparation of whole cell homogenates, cytoplasmic, and nuclear protein extraction, immunoblotting, detection of the immunoreactive bands by enhanced chemiluminescence (ECL Plus; GE Healthcare), and their quantification by densitometry were all described before [10, 13, 14]. α -Tubulin (whole cell homogenates), GAPDH (cytoplasmic), and lamin A/C (nuclear) served as loading and purity controls. IL-18 levels in culture supernatants were analyzed by an ELISA. The sensitivity of the assay is 12.5 pg/ml.

GST pull-down assay

The interactions between the C-terminal domain of human Nox1 and the cytoplasmic domains of the human IL-18 receptor subunits were investigated in a GST pull-down assay as described previously by us [10, 13, 14]. A DNA fragment encoding the C-terminal domain of human Nox1 was amplified from plasmid cDNA using the sense primer 5'-gagctcCCGCTCCCAGCAGAAG-3' and the antisense primer 5'-AACTCAAAAATTTCTTTGTTGAAGTA-3', cloned into pCR2.1-TOPO (Life Technologies, Grand Island, NY) and a SacI fragment excised and inserted into the SacI site of pET23bMBPH6 to encode MBP-Nox1₍₂₉₀₋₅₆₄₎. The cDNAs encoding the cytoplasmic domains of human IL-18R α and IL-18R β were amplified from the respective plasmids (Cat # SC117718, and SC117717, OriGene Technologies, Inc, Rockville MD), using the primer pair 5'-gaattcAGATGGAAAAACATATGATGCTTTTGTGTCTTACCT-3'/5'-CTGAAGATTAAGACTCGGAAAGAAGCAAG-3' for IL-18R α , and primer pair 5'-GAATTCAGGGGATAAAAAGGATTTTGATGCTTTCGTATCC-3'/5'-ATTTCACCATTCCTTAGGCTGGGAG-3' for IL-18R β . The products were cloned into pCR2.1-TOPO, and EcoRI fragments from these cloned into the EcoRI site of pGEX-3X (GE Healthcare Biosciences, Piscataway, NJ) to encode for GST-IL18R α ₍₃₇₁₋₅₄₁₎ and GST-IL18R β ₍₄₀₃₋₅₉₉₎. Binding assays between [³⁵S]-labeled MBP-Nox1₍₂₉₀₋₅₆₄₎ and the GST fusion proteins immobilized on GSH-Sepharose were carried out as we have detailed previously.

Binding interaction of IL-18R with Nox1 by reciprocal immunoprecipitation (IP)/immunoblotting (IB)

IP/IB experiments were carried out as previously described [10, 13, 14]. For IP, equal amounts of membrane extracts or whole cell lysates were incubated overnight at 4°C with specific antibodies attached to agarose beads under slow rotation. After washing three times in a buffer containing 50 mM Tris-HCl, 150 mM NaCl, and 0.1% Nonidet P-40, the bound proteins were eluted from the beads by boiling in SDS sample buffer for subsequent SDS-PAGE and IB. The IL-18R α , IL-18R β , and Nox1 antibodies used in IP and IB are detailed above.

IKK β , JNK, and Rac1 activity—IKK activity in SMC treated with IL-18 was determined by an *in vitro* kinase assay using glutathione *S*-transferase (GST)-I κ B fusion protein as the substrate [20]. JNK activity was measured using a commercially available kit (Stress-activated protein kinase/JNK assay kit) [21]. A c-Jun fusion protein (GST fused to the N terminus of c-Jun, amino acids 1–89) was used to pull-down JNK enzyme from cell extracts,

which in the presence of kinase buffer and ATP phosphorylates c-Jun (1–89). Phosphorylated c-Jun was detected by immunoblotting using anti-phospho-c-Jun (Ser63) antibody. Rac1 activity was analyzed according to the manufacturer's protocol using an EZ-detect Rac1 activation kit (Pierce, Rockford, Ill) using equal amounts of membrane extracts, and has been previously described [21].

Gelatin zymography

Protein levels in cell-free culture supernatants were quantified, and equal amounts were analyzed by SDS-PAGE essentially as described previously[6]. Gels were prepared with 1 mg/ml gelatin co-polymerized into the gel matrix. After electrophoresis, gels were agitated in 2.5% Triton X-100 to remove the SDS and to restore enzyme activity and then incubated for 24 h at 37 °C in 50 ml of 50 mM Tris-HCl (pH 7.6) containing 0.2 M NaCl, 5 mM CaCl₂, 0.02% Brij 35, and 0.02% sodium azide. Finally, gels were stained for 1 h in 50% methanol and 1% acetic acid with 0.125% Coomassie Blue G-250 dye and destained in 10% acetic acid. Zones of proteolytic activity were evident as clear bands against a dark blue background.

Cell migration

SMC migration was quantified using Discovery Labware BD BioCoat™ Matrigel™ invasion chambers (Cat. #354481, BD Biosciences) and 8.0-μm pore polyethylene terephthalate membranes with a thin layer of Matrigel™ basement membrane matrix [6, 10]. Cultured SMCs were trypsinized and suspended in SmGM-2 medium and 0.5% BSA, and 1 ml containing 2.0×10^5 cells/ml was layered on the coated insert filters. Cells were stimulated with IL-18 (1 ng/ml). The lower chamber contained similar levels of IL-18. After incubation at 37°C for 18 h, the membranes were removed and washed with PBS, and the noninvading cells on the upper surface were removed with a cotton swab. The cells migrating to the lower surface of the membrane were quantified using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described[10, 14]. In a subset of experiments, the layered cells were incubated with anti-IL-18 (αIL-18 Ab) or IL-18Ra (αIL-18Ra) neutralizing antibodies, or IL-18BP-Fc (10 μg/ml for 1 h) prior to IL-18 addition. Studies were also performed in a similar way with Tiron and simvastatin. To determine the role of TRAF3IP2, cells infected with lentiviral TRAF3IP2 shRNA (MOI0.5 for 48 h) were layered on the membrane, and then treated with IL-18. Studies were also performed by layering cells infected Ad.CuZnSOD, Ad.shNox1, or Ad.N17rac1, or transfected with MMP9 siRNA on membranes prior to IL-18 addition.

Cell viability and cell death analysis

Cell viability following various treatments and transfections was determined by the MTT assay. To determine whether transduction of viral vectors and exposure to pharmacological inhibitors negatively affected cell viability, cell death was analyzed using the Cell Death detection ELISA^{PLUS} [10, 14]. Cell viability was also tested by trypan blue dye exclusion and microscopic visualization of cell morphology and detachment.

Statistical Analysis

Comparisons between controls and various treatments were performed by ANOVA with post hoc Dunnett's *t*-tests. All assays were performed at least three times, and the error bars in the figures indicate the S.E. Though a representative immunoblot is shown in the main figures, changes in protein/phosphorylation levels from three independent experiments were semi-quantified by densitometry, and were shown as ratios and fold changes from untreated or respective controls at the bottom of the panels whenever the results are less clear.

Results

IL-18 induces primary aortic smooth muscle cell (SMC) migration via TRAF3IP2 and TRAF3IP2-dependent NF- κ B and AP-1 activation

Previously, we demonstrated that Ang-II mediated SMC migration is mediated in part by IL-18 [10]. Since TRAF3IP2 is a cytoplasmic adapter molecule and an upstream regulator of IKK and JNK [11, 12], and IL-18 activates both NF- κ B and AP-1, we hypothesized that IL-18 induces SMC migration in part via TRAF3IP2. Results show that IL-18 induced a significant increase in SMC migration (Fig. 1A), an effect markedly attenuated by IL-18 or IL-18R α neutralizing antibodies (Fig. 1A), and by IL-18 binding protein (data not shown). Further, TRAF3IP2 knockdown markedly attenuated IL-18-induced SMC migration (Fig. 1A). Confirmation of TRAF3IP2 knockdown is shown on the right. Expression levels of Akt, which served as an off-target gene, were not modulated by TRAF3IP2 shRNA. To confirm the role of TRAF3IP2, we investigated SMC isolated from the carotid arteries of wild type (WT) and TRAF3IP2-null mice. While IL-18 increased migration of WT-SMC, its effects were markedly attenuated in SMC isolated from the null mice (Fig. 1B).

We next investigated whether IL-18-induced NF- κ B and AP-1 activation in SMC is mediated through TRAF3IP2. In an *In vitro* kinase assay, IL-18 stimulated the phosphorylation of IKK β as well as its kinase activity, responses that were markedly attenuated by TRAF3IP2 knockdown (Fig. 1C). Similarly, IL-18 induced the JNK phosphorylation and kinase activity, and knockdown of TRAF3IP2 attenuated this activation response (Fig. 1D). As expected, IL-18 induced time-dependent p65 and c-Jun phosphorylation and nuclear translocation in these cells, and this too was inhibited by TRAF3IP2 knockdown (Fig. S1- E, F). Further, knockdown of IKK β , JNK2, p65 and c-Jun each inhibited IL-18-induced SMC migration (Fig. 1E). Thus IL-18 induces SMC migration at least in part via TRAF3IP2 and TRAF3IP2-dependent IKK/NF- κ B and JNK/AP-1 activation (Fig. 1).

IL-18 induces SMC migration and TRAF3IP2 expression via Nox1/Rac1/ROS

Previously we showed that Ang-II-induced SMC migration and proliferation are Nox1 dependent [10]. Therefore, we investigated whether Nox1 and ROS mediate IL-18-induced SMC migration. IL-18-induced SMC migration was markedly attenuated by Nox1 knockdown (Fig. 2A). Since the small GTPase Rac1 activates the Nox1 complex, we next examined its role in IL-18-induced SMC migration. Forced expression of a dominant negative mutant of Rac1 (N17rac1) markedly attenuated IL-18-induced SMC migration (Fig. 2A) as did the Rac1 inhibitor NSC23766 (data not shown). Further, Nox1 knockdown or N17rac1 overexpression each attenuated IL-18-induced superoxide generation (Fig. 2B), as did the flavoprotein inhibitor DPI, the superoxide scavenger Tiron or forced expression of the antioxidant enzyme CuZnSOD (Fig. 2C). We next investigated whether IL-18-induced TRAF3IP2 is Nox1-dependent. Results show that IL-18 induced TRAF3IP2 expression in SMC, with peak levels detected at 3 h (Fig. 2D). Knockdown of Nox1 or forced expression of dominant negative Rac1 (N17rac1) each significantly inhibited IL-18-induced TRAF3IP2 expression (Fig. 2E), as did DPI and Tiron (Fig. 2F). Further, co-treatment with actinomycin D (ActD), but not cycloheximide (CHX), inhibited IL-18-induced TRAF3IP2 expression (Fig. S2), indicating that IL-18-induced TRAF3IP2 is transcriptionally regulated. Thus IL-18 induces SMC migration in part via Nox1, Rac1 and ROS and enhanced TRAF3IP2 transcription (Fig. 2).

Nox1 physically associates with IL-18 receptor

Although IL-18 induces TRAF3IP2 expression through Nox1-dependent ROS generation (Fig. 2), it is not known whether IL-18 receptor physically associates with Nox1. We carried

out reciprocal IP/IB of solubilized membrane preparations from SMC treated or not with IL-18 for 15 min. With antibodies to IL-18R α , Nox1 was found to associate at low levels with IL-18R *in vivo* in SMC under basal conditions, but this association was increased following IL-18 treatment (Fig. 3A, left panel). These results were confirmed in the converse IP/IB with antibodies to Nox1 (Fig. 3A, right panel). Similar results were obtained when co-IP/IB experiments were performed using antibodies to IL-18R β and Nox1 (Fig. 3B). Previously we identified the C-terminal cytoplasmic region of AT1 as an interacting binding partner of the C-terminal region of Nox1 [10]. To determine whether the same region of Nox1 also interacts with the IL-18R heterodimer, we carried out GST pull-down assays using the C-terminal cytoplasmic domain of human Nox1 linked to maltose binding protein (MBP-Nox1_(290–564)) as the prey, and the C-terminal regions of the human IL-18 receptor subunits linked to GST (GST-IL18R α _(371–541) and GST-IL18R β _(403–599)) as the bait. Strong binding was observed between ³⁵S-labeled MBP-Nox1_(290–564) and both GST-IL18R α _(371–541) and GST-IL18R β _(403–599) (Fig. 3C, upper panel), whereas GST alone did not bind MBP-Nox1_(290–564). No binding was seen between MBP itself and either GST, GST-IL18R α _(371–541) or GST-IL18R β _(403–599) (Fig. 3C, middle panel), demonstrating the specificity of the interaction. Together, these results demonstrate for the first time that the IL-18 receptor heterodimer physically associates with Nox1 *in vivo* and this interaction is increased with IL-18 treatment (Fig. 3).

IL-18 induces its own expression and that of IL-18R α and that of MMP9 in part via TRAF3IP2

In addition to being a potent activator of NF- κ B and AP-1, IL-18 is also highly responsive to NF- κ B and AP-1, suggesting that once induced, IL-18 may perpetuate its own expression. In SMC, IL-18 treatment induced its own expression (Fig. 4A) and secretion (Fig. 4B, C), effects that were attenuated by TRAF3IP2 knockdown (Fig. 4D, E). Furthermore, treatment with IL-18 also induced IL-18R α mRNA (Fig. 4F) and protein (Fig. 4G) in a TRAF3IP2-dependent manner (Fig. 4H, I). These results indicate that IL-18 induces its own expression and that of IL-18R α in part via TRAF3IP2. Induction of both the ligand and the ligand-binding receptor subunit suggests a positive amplification in IL-18 inflammatory signaling (Fig. 4). Furthermore, treatment with IL-18 induced expression of MMP9, and TRAF3IP2 knockdown inhibited its expression (Fig. S3). Importantly, knockdown of MMP9 attenuated IL-18-induced SMC migration demonstrating that TRAF3IP2 mediates IL-18-induced MMP9 expression in SMC, which in turn modulates SMC migration (Fig. S3).

Simvastatin inhibits IL-18 autoregulation and SMC migration

Although statins are used in the treatment of cardiovascular diseases primarily for their cholesterol-lowering effects, they also exert anti-oxidative and anti-inflammatory effects [15, 16]. Therefore we investigated whether statins inhibit TRAF3IP2 expression. Simvastatin attenuated IL-18-induced TRAF3IP2 expression in SMC in a dose-dependent manner (Fig. 5A). This result was not due to an effect on the viability of the cells, since simvastatin did not appear to induce SMC death, as measured by the generation of oligonucleosomal fragmented DNA in cytoplasmic extracts (Fig. 5B). Simvastatin also inhibited IL-18-induced activation of Rac1 activation (Fig. 5C), superoxide generation (Fig. 5D), p65 and c-Jun nuclear translocation (Fig. 5E), IL-18 expression and secretion (Fig. 5F), MMP9 activation (Fig. 5G), and importantly, SMC migration (Fig. 5H). These results indicate that simvastatin inhibits IL-18-induced SMC migration by attenuating TRAF3IP2 expression and its down-stream signaling (Fig. 5).

Simvastatin inhibits inducible, but not forced expression of TRAF3IP2

Statins inhibit HMG-CoA reductase, which catalyzes the conversion of HMG-CoA to mevalonic acid (mevalonate), an initial rate-limiting step in cholesterol biosynthesis [15,

16]. Simvastatin inhibition of IL-18-induced TRAF3IP2 expression was reversed by co-treatment with L-mevalonate (Fig. 6A). Statins are known to inhibit the production of farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP), precursors of the post-translational modification known as prenylation. To determine which might have contributed to TRAF3IP2 suppression, SMC were treated with GGPP or FPP prior to incubation with simvastatin. GGPP, but not FPP, reversed the simvastatin inhibition of IL-18-induced TRAF3IP2 expression (Fig. 6B). However, neither the PSORT (www.psорт.hgc.jp/form2.html) nor the Prenylation Prediction Suite (<http://mendel.imp.ac.at/PrePS/index.html>) software identified potential prenylation sites on TRAF3IP2, suggesting an indirect effect for simvastatin. To confirm this possibility, SMC were transfected with a Myc-tagged human TRAF3IP2 expression vector, and the effect of simvastatin or simvastatin + mevalonate investigated. Neither treatment modulated the levels of the ectopically expressed TRAF3IP2 (Fig. 6C), and neither did GGPP and FPP (Fig. 6D). Further, while forced expression of TRAF3IP2 increased IL-18 secretion (Fig. 6E), treatment with simvastatin, or pharmacological inhibition of Rac1 by NSC23766 failed to affect its secreted levels (Fig. 6E). However, NSC23766 blunted IL-18-induced IL-18 secretion (Fig. 6F), suggesting that simvastatin attenuates TRAF3IP2 expression possibly by targeting Rac1 activation in IL-18-treated SMC and the generation of ROS (Fig. 6).

Discussion

Here we show that i) the proatherogenic cytokine IL-18 induces SMC migration via Nox1/ROS-dependent TRAF3IP2 induction, ii) the IL-18 receptor heterodimer binds Nox1 under basal conditions, and IL-18 enhances this association, iii) TRAF3IP2 gene deletion or knockdown inhibits IL-18-induced IKK/NF- κ B and JNK/AP-1 activation and SMC migration. Notably, (iv) simvastatin inhibits IL-18-induced TRAF3IP2 expression and SMC migration via Rac1 inhibition. Thus, TRAF3IP2 is a potentially important therapeutic target in hyperplastic vascular diseases.

The cytoplasmic adapter protein TRAF3IP2 is an upstream regulator of NF- κ B and AP-1 [11, 12]. IL-18-induced its own expression, as well as that of the ligand binding α subunit of the IL-18 receptor heterodimer, an AP-1 responsive gene [22]. These results imply a positive amplification in IL-18 inflammatory signaling. Consistent with this, we observed that IL-18-induced TRAF3IP2 expression persisted for prolonged periods of time in the SMC, with elevated levels still observed 12 h after stimulation. The effect after IL-18 stimulation may differ from that with IL-17A, which signals exclusively via TRAF3IP2 [23], and has been reported to result in the binding and phosphorylation of TRAF3IP2 by the IKK-related kinases IKK α and TBK1, and subsequent TRAF3IP2 degradation [24, 25]. Although IKK α , IKK β and IKK γ also bind TRAF3IP2, they do not mediate TRAF3IP2 phosphorylation [24], and therefore may not induce its degradation. Consistent with this, we did not observe a shift in the mobility of TRAF3IP2 following IL-18 treatment, even after prolonged exposure, suggesting minimal or no post-translational modification. Thus phosphorylation and degradation of TRAF3IP2 may be stimulus- and cell type-specific, and may not be a critical mechanism in IL-18-induced SMC migration.

We also show that IL-18-induced TRAF3IP2 expression is Nox1 dependent. Human vascular smooth muscle cells typically express Nox1 and Nox4 isoforms that show differential localization in the cellular compartments. However, Schröder et al. recently reported that Nox4 is expressed predominantly in endothelium rather than smooth muscle cells in normal arteries [26], and Clempus et al. have demonstrated that while Nox1 associates with, and promotes SMC proliferation, Nox4 plays a role in the maintenance of differentiated phenotype [7]. In the present study we focused our investigation on Nox1. Knockdown of Nox1 inhibited IL-18 induced TRAF3IP2 expression. Using GST pull-down

assays, we show for the first time that the TIR (Toll/Interleukin-1 receptor) domains of the subunits of the heterodimeric IL-18 receptor each can physically associate with the C-terminal cytoplasmic domain of Nox1. Reciprocal IP/IB confirmed these results, and further showed that IL-18 treatment enhanced their binding of Nox1 and IL-18R *in vivo* in SMC. Importantly, Nox1 knockdown blunted IL-18-induced superoxide generation and SMC migration. Studies are in progress to identify the amino acid sequence(s) that precisely bind Nox1, and determine whether mutation of these residues abrogates Nox1-dependent ROS generation and inhibits IL-18-induced expression of TRAF3IP2 and SMC migration. We will also investigate whether the IL-18R heterodimer binds other Nox homologues in a cell type-specific manner.

An important observation in this study was that simvastatin inhibits IL-18-induced TRAF3IP2 expression and SMC migration. Statins inhibit HMG-coA reductase, which catalyzes the conversion of HMG-coA to mevalonate, a rate-limiting step in cholesterol biosynthesis [15, 16]. The isoprenoids FPP and GGPP are downstream intermediates in the mevalonate pathway, and mediate protein prenylation, a post-translational modification of a target protein. Here we show that IL-18 induced TRAF3IP2 expression is inhibited by simvastatin in a dose-dependent manner, and that its inhibitory effects are reversed by mevalonate. Co-treatment with GGPP, but not FPP, reversed the inhibitory effect of simvastatin on TRAF3IP2 expression, implying a role for geranylgeranylation in TRAF3IP2 expression. However neither PSORT nor Prenylation Prediction Suite software identified prenylation sites on TRAF3IP2. Thus the inhibitory effects of simvastatin on TRAF3IP2 appear to be indirect. To test this hypothesis, we forced the expression of TRAF3IP2 in SMC by transfection of a plasmid containing TRAF3IP2 under control of the CMV promoter, and then treated with simvastatin. Under these conditions, simvastatin had no effect on ectopically expressed TRAF3IP2, and further, ectopically expressed TRAF3IP2 was not affected by co-treatment with mevalonate, GGPP, or FPP. These results indicate that simvastatin most likely targets one or more upstream intermediates in IL-18 signaling rather than TRAF3IP2 itself. Since statins have been shown to inhibit the prenylation of Rac1 [27], and prenylation of Rac1 results in its translocation from cytoplasm to the membrane and is an important step in Nox1 activation, our data suggest that activation of Nox1 and the generation of ROS is the target of simvastatin. In fact, our results show that forced expression of a dominant negative mutant of Rac1 (N17rac1) or knockdown of Nox1 markedly inhibited IL-18-induced superoxide generation and TRAF3IP2 expression. NSC23766, a pharmacological Rac1 inhibitor, similarly blunted IL-18-induced TRAF3IP2 expression as did the ROS scavenger Tiron. These results indicate that simvastatin attenuates inducible, but not forced expression of TRAF3IP2, possibly by targeting Rac1 prenylation and inhibiting ROS generation.

Our results demonstrate a number of possible positive feed forward mechanisms in IL-18-mediated TRAF3IP2 induction and SMC migration. For example, (i) IL-18 induced its own expression, (ii) Nox1/ROS-mediated NF- κ B and AP-1 activation and further induction of Nox1, and (iii) IL-18/Nox1/ROS-mediated TRAF3IP2 induction. These amplification mechanisms in IL-18, Nox1 and TRAF3IP2 induction might perpetuate oxidative stress and inflammation in a vessel wall, resulting in a persistent injurious stimulus and SMC migration. IL-18/TRAF3IP2 signaling represents a potential therapeutic target in vascular proliferative diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Ab	antibody
ActD	actinomycin D
Act1	activator of NF- κ B
AP-1	activator protein-1
C/EBP	CCAAT/enhancer-binding protein
CHX	cycloheximide
CIKS	Connection to IKK and SAPK/JNK
dn	dominant negative
DPI	diphenylene iodonium
FPP	farnesylpyrophosphate
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFP	green fluorescent protein
GGPP	geranylgeranylpyrophosphate
GST	glutathione-S-transferase
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
IκB	inhibitory κ B
IKK	I κ B kinase
IL	interleukin
IP/IB	immunoprecipitation/immunoblotting
IRF	IFN regulatory factor
JNK	c-Jun amino-terminal kinase
MOI	multiplicity of infection
MMP	matrix metalloproteinase
NF-κB	nuclear factor kappa B
Nox	NADPH oxidase
NADPH	nicotinamide adenine dinucleotide phosphate
N17rac1	a dominant negative form of rac1
Rac1	ras-related C3 botulinum toxin substrate 1
ROS	reactive oxygen species
SAPK	stress-activated protein kinase

siRNA	small interfering RNA
shRNA	small hairpin RNA
TIR	Toll-IL-1 receptor
TRAF	TNF Receptor Associated Factor
TRAF3IP2	TRAF3 interacting protein 2
TNF	tumor necrosis factor
WT	wild-type

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Highlights

- Interleukin-18 induces SMC migration via Nox1/ROS-dependent TRAF3IP2 induction
- TRAF3IP2 inhibition blocks IL-18-induced IKK/NF- κ B and JNK/AP-1 activation
- TRAF3IP2 inhibition blunts IL-18/TRAF3IP2-dependent SMC migration
- The IL-18 receptor heterodimer physically associates with Nox1
- TRAF3IP2 is a potential therapeutic target in hyperplastic vascular diseases

Fig. 1. IL-18 induces primary human coronary artery smooth muscle cell (SMC) migration via TRAF3IP2-dependent NF- κ B and AP-1 activation

A, TRAF3IP2 knockdown inhibits IL-18-induced SMC migration. SMC were trypsinized, suspended in SmGm-2+0.5% BSA, and layered on Matrigel™ basement membrane matrix-coated filters. Cells were incubated with neutralizing IL-18 or IL-18R α antibodies (10 mg/ml for 1 h) prior to the addition of recombinant human (rh) IL-18 (1 ng/ml). Normal mouse IgG1 or goat IgG served as respective controls. The lower chamber contained similar levels of IL-18. After 18 h at 37°C, cells migrating to the lower side of the membrane were quantified using MTT assay. In a subset of experiments, SMC infected with lentiviral TRAF3IP2 shRNA (MOI 0.5) for 48 h were assayed. Knockdown of TRAF3IP2 was confirmed by immunoblotting (panel on right). Akt served as an off-target control. Tubulin served as a loading control (n=3). * P < 0.001 vs. untreated, † P < 0.01 vs. IL-18 $\pm$ respective controls (n=12). **B**, TRAF3IP2 gene deletion blunts IL-18-induced mouse carotid artery SMC migration. SMC were isolated and cultured from carotid arteries from male TRAF3IP2-null and wild type (WT) C57B1/6 mice, and made quiescent in medium supplemented with 0.5% BSA for 48h. The quiescent SMC were then layered on Matrigel™ membrane, treated with recombinant mouse IL-18 (1 ng/ml), and migration quantified as in **A**. Absence of TRAF3IP2 expression in TRAF3IP2-null SMC was confirmed by immunoblotting (panel on the right). * P < at least 0.05 vs. respective untreated, † P < at least 0.01 vs. IL-18-treated WT-SMC (n=12). **C**, **D**, IL-18 induces IKK β and JNK phosphorylation and kinase activities in part via TRAF3IP2. SMC infected with lentiviral TRAF3IP2 shRNA (MOI 0.5 for 48 h) were treated with rhIL-18 (1 ng/ml for 1 h). Phospho-IKK (**C**) and Phospho-JNK (**D**) levels were analyzed by immunoblotting (upper panels). Their kinase activities were analyzed using *in vitro* kinase assays (lower panels) (n=3). **E**, IL-18-induced SMC migration is IKK β , JNK, NF- κ B and AP-1 dependent. SMC infected with lentiviral IKK β , JNK, p65 or c-Jun (MOI 0.5 for 48 h) were layered on Matrigel™ membrane, treated with rhIL-18 (1 ng/ml), and migration quantified as in Fig. 1A. Knockdown was confirmed by immunoblotting, and is shown on the right. * P < 0.001 vs. untreated, † P < 0.01 vs. IL-18 $\pm$ GFP (n=12).

Fig. 2. IL-18 induces human coronary artery SMC migration via Nox1 and Rac1

A, IL-18 stimulates SMC migration via Nox1 and Rac1. SMC infected with adenovirus expressing Nox1 shRNA (MOI 100 for 48 h) or Myc-tagged dominant negative Rac1 (N17rac1; MOI 100 for 24 h) were assayed for migration to rhIL-18 (1 ng/ml) as in Fig. 1A. Nox1 knockdown and Myc expression from N17rac1 were confirmed by immunoblotting (right panel). * $P < 0.001$ vs. untreated, † $P < 0.01$ vs. IL-18 ($n = 6$). **B**, **C**, IL-18 stimulates superoxide ($O_2^{\cdot -}$) generation via Nox1 and Rac1. SMC infected with Ad.shNox1 (MOI 100 for 48 h) or Ad.N17rac1 (MOI 100 for 24 h) were loaded with dark-adapted lucigenin (5 μ M), and incubated with rhIL-18 (1 ng/ml). ($O_2^{\cdot -}$) generation was quantified using the lucigenin-enhanced chemiluminescence assay. Luminescence was measured for 10 s with a delay of 5 s. After background luminescence was subtracted, results [relative light units (RLU)/second/ 5×10^4 cells] were expressed as fold increase from untreated controls. Studies (**C**) were also performed in SMC infected with Ad.CuZnSOD (MOI 100 for 24 h) or treated with the flavoprotein inhibitor DPI (10 μ M in DMSO for 30 min) or the superoxide scavenger Tiron (5 mM for 1 h) prior to rhIL-18 addition. **B**, **C**, * $P < 0.001$ vs. untreated, † $P < 0.01$ vs. IL-18 ($n = 12$). **D**, IL-18 induces TRAF3IP2 expression. Quiescent SMC were incubated with rhIL-18 (1 ng/ml). At the indicated time periods, TRAF3IP2 expression was analyzed by immunoblotting using cleared whole cell lysates. **E**, IL-18 induces TRAF3IP2 expression via Nox1 and Rac1. SMC infected with Ad.shNox1 (MOI 100 for 48 h) or Ad.N17rac1 (MOI 100 for 24 h) were incubated with rhIL-18 (1 ng/ml for 3 h). TRAF3IP2 expression was analyzed as in **D**. **F**, IL-18 induces TRAF3IP2 expression via Nox and superoxide. Quiescent SMC treated with DPI (10 μ M in DMSO for 30 min) or Tiron (5 mM for 1 h) were incubated with rhIL-18 (1 ng/ml for 3 h). TRAF3IP2 expression was analyzed as in **D**. **D**, **E**, **F**, * $P < 0.01$ vs. untreated, † $P < 0.05$ vs. IL-18 ($n = 3$).

Fig. 3. IL-18 enhances IL-18R/Nox1 physical association in human coronary artery SMC
A, IL-18 increases IL-18Rα/Nox1 physical association. Quiescent SMC were treated with rhIL-18 (1 ng/ml) for 10 min, and IL-18Rα and Nox1 binding was analyzed by reciprocal IP/IB (left and right panels) using solubilized membrane fraction (n=3). **B**, IL-18 increases IL-18Rβ/Nox1 physical association. Quiescent SMC were treated with rhIL-18 (1 ng/ml) for 10 min, and IL-18Rβ and Nox1 binding was analyzed by reciprocal IP/IB (left and right panels) using solubilized membrane fraction (n=3). **C**, The C-terminal cytoplasmic domain of Nox1 binds the cytoplasmic domains of IL-18Rα and IL-18Rβ. Equal volumes of GSH-sepharose were preloaded with GST, GST-IL-18Rα₍₃₇₁₋₅₄₁₎ or GST-IL-18Rβ₍₄₀₃₋₅₉₉₎ and binding assays carried out with equal amounts of [³⁵S]methionine-labeled fusion protein MBP-Nox1₍₂₉₀₋₅₆₄₎ or the fusion partner MBP. After washing three times in buffer (PBS + 0.05% TX-100), the gels were treated with 2X SDS-treatment buffer and 25% of the total

eluate analyzed by SDS-PAGE and fluorography. An aliquot of the labeled proteins (2% of the total reaction) was included as control ("Input"). Upper panel, [^{35}S]-MBP-Nox1_(290–564) bound, middle panel, [^{35}S]-MBP bound, lower panel, Coomassie Blue stained gel of the eluates to show equivalent loading of the GSH-sepharose.

Fig. 4. TRAF3IP2 mediates IL-18 autoregulation and IL-18R α upregulation in human coronary artery SMC

A–C, IL-18 induces its own expression. Quiescent SMC treated with rhIL-18 (1 ng/ml) were analyzed for IL-18 mRNA by RT-qPCR (A), pro and mature IL-18 by immunoblotting (B, n=3) and secreted IL-18 levels by ELISA (C, 24 h). A, C, * P < at least 0.05 vs. untreated (n=3–6). D, F, TRAF3IP2 knockdown inhibits IL-18 expression. SMC infected with lentiviral TRAF3IP2 shRNA (MOI 0.5 for 48 h) were treated with rhIL-18. Mature IL-18 levels at 2 h and secreted IL-18 levels at 24 h were analyzed by immunoblotting (n=3) and ELISA (n=6), respectively. E, * P < 0.001 vs. untreated, † P < 0.01 vs. IL-18+GFP (n=6). F, G, IL-18 induces IL-18R α expression. Quiescent SMC treated as in A for up to 3 h were analyzed for IL-18R α mRNA expression by RT-qPCR and protein levels by immunoblotting (n=3). F, * P < at least 0.05 vs. untreated (n=6). H, I, TRAF3IP2 knockdown inhibits IL-18 expression. SMC infected with lentiviral TRAF3IP2 shRNA (MOI 0.5 for 48 h) were treated with rhIL-18. IL-18R α mRNA expression at 1 h (H) and protein levels at 2 h were analyzed by immunoblotting (n=3), respectively. H, * P < 0.001 vs. untreated, † P < 0.01 vs. IL-18+GFP (n=6).

Fig. 5. Simvastatin inhibits IL-18-induced TRAF3IP2 expression and human coronary artery SMC migration

A, Simvastatin inhibits IL-18-induced TRAF3IP2 expression. Quiescent SMC were treated with the indicated concentrations of simvastatin for 3 h prior to rhIL-18 addition (1 ng/ml for 2 h). TRAF3IP2 expression was analyzed by immunoblotting (n=3). **B**, Simvastatin treatment does not affect cell viability. Quiescent SMC were treated with simvastatin (2.5 μ M for 3 h) prior to rhIL-18 addition (1 ng/ml for 24 h). Mono and oligonucleosomal fragmented DNA in cytoplasmic extracts was quantified by a cell death ELISA. The nitric oxide donor SNAP (500 μ M in DMSO) served as a positive control. * P < 0.001 vs. untreated or DMSO (n=12). **C**, Simvastatin inhibits IL-18-induced Rac1 activity. Quiescent SMC were treated with simvastatin (2.5 μ M for 1 h) prior to rhIL-18 addition (1 ng/ml). Activated (GTP-bound) Rac1 in membrane extracts was analyzed using a commercially available kit (n=3). GAPDH levels in cytoplasmic extracts served as an internal control (n=3). **D**, Simvastatin inhibits IL-18-induced superoxide ($O_2^{\cdot -}$) generation. SMC loaded with dark-adapted lucigenin (5 μ M) were treated with simvastatin (2.5 μ M for 1 h) prior to rhIL-18 addition (1 ng/ml). Superoxide ($O_2^{\cdot -}$) production was quantified using the lucigenin-enhanced chemiluminescence assay. * P < 0.001 vs. untreated, † P < 0.001 vs. IL-18 (n=12). **E**, Simvastatin inhibits p65 and c-Jun activation. Quiescent SMC were treated with simvastatin (2.5 μ M for 1 h) prior to rhIL-18 addition (1 ng/ml for 1 h). Phospho-p65 and phospho-c-Jun levels were analyzed by immunoblotting using nuclear protein extracts (n=3). **F**, Simvastatin inhibits IL-18 autoregulation. Quiescent SMC were treated with simvastatin (2.5 μ M for 1 h) prior to rhIL-18 addition (1 ng/ml for up to 24 h). Secreted IL-18 levels at 24 h were analyzed by ELISA. Mature IL-18 levels at 2 h were analyzed by immunoblotting (inset). * P < 0.05 vs. untreated, † P < 0.05 vs. IL-18 (n=3). **G**, Simvastatin inhibits IL-18-induced MMP9 expression. Quiescent SMC were treated as in **F**. After 24 h, culture supernatants were analyzed for active MMP9 by ELISA. MMP9 levels at 2 h were analyzed by immunoblotting (inset). * P < 0.05 vs. untreated, † P < 0.05 vs. IL-18 (n=3). **H**, Simvastatin inhibits IL-18-induced SMC migration. Quiescent SMC were layered on MatrigelTM membrane, incubated with simvastatin (2.5 μ M) for 3 h and then with rhIL-18 (1 ng/ml for 18 h). Cell migration was analyzed as in Fig. 1A. * P < 0.001 vs. untreated, † P < 0.01 vs. IL-18 (n=12).

Fig. 6. Simvastatin inhibits IL-18-induced, but not ectopically-expressed TRAF3IP2 in human coronary artery SMC

A, Mevalonate reverses the inhibitory effect of simvastatin on IL-18-induced TRAF3IP2 expression. Quiescent SMC were incubated with simvastatin (2.5 µM) and mevalonate (100 µM) for 3 h prior to rhIL-18 addition (1 ng/ml for 3 h). TRAF3IP2 expression was analyzed by immunoblotting (n=3). **B**, GGPP, but not FPP, reverses simvastatin inhibition of IL-18-induced TRAF3IP2. Quiescent SMC were incubated with simvastatin (2.5 µM) and GGPP (1 µM) or FPP (1 µM) for 3 h prior to rhIL-18 addition (1 ng/ml for 3 h). TRAF3IP2 expression was analyzed as in **A** (n=3). **C**, Simvastatin and mevalonate do not affect ectopically expressed TRAF3IP2. SMC transfected with pCMV6-TRAF3IP2 (1 µg) for 24 h were incubated with simvastatin (2.5 µM) and mevalonate (100 µM) for 3 h. TRAF3IP2 expression was analyzed as in **A** (n=3). **D**, Simvastatin, GGPP and FPP do not affect ectopically expressed TRAF3IP2. SMC transfected as in **C** were incubated with simvastatin (2.5 µM) and GGPP (1 µM) or FPP (1 µM) for 3 h. TRAF3IP2 expression was analyzed as in **A** (n=3). **E**, Simvastatin or Rac1 inhibition do not affect ectopically expressed TRAF3IP2-induced IL-18 secretion. SMC transfected with pCMV6-TRAF3IP2 (1 µg) for 24 h were incubated with simvastatin (2.5 µM) or the Rac1 inhibitor NSC23766 (30 µM in water). Twenty-four hours later, culture supernatants were analyzed for IL-18 by ELISA. * $P < 0.01$ vs. untreated (n=6). **F**, Simvastatin or Rac1 inhibition attenuates IL-18-induced IL-18 secretion. Quiescent SMC were treated with simvastatin (2.5 µM for 3 h) or the Rac1 inhibitor NSC23766 (30 mM in water for 3 h) prior to rhIL-18 addition (1 ng/ml for 24 h). Secreted IL-18 levels were quantified by ELISA. * $P < 0.01$ vs. untreated, † $P < 0.05$ vs. IL-18 (n=6).