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Acetylsalicylic Acid Inhibits IL-18-Induced Cardiac Fibroblast Migration Through the Induction of RECK

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Abstract

The pathogenesis of cardiac fibrosis and adverse remodeling is thought to involve the ROS-dependent induction of inflammatory cytokines and matrix metalloproteinases (MMPs), and the activation and migration of cardiac fibroblasts (CF). Here we investigated the role of RECK (reversion-inducing-cysteine-rich protein with Kazal motifs), a unique membrane-anchored MMP regulator, on IL-18 induced CF migration, and the effect of acetylsalicylic acid (ASA) on this response. In a Matrigel invasion assay, IL-18 induced migration of primary mouse CF was dependent on both IKK/NF-κB- and JNK/AP-1-mediated MMP9 induction and Spl-mediated RECK suppression, mechanisms that required Nox4-dependent H_2O_2 generation. Notably, forced expression of RECK attenuated IL-18 induced MMP9 activation and CF migration. Further, therapeutic concentrations of ASA inhibited IL-18 induced H_2O_2 generation, MMP9 activation, RECK suppression, and CF migration. The salicylic acid moiety of ASA similarly attenuated IL-18 induced CF migration. Thus, ASA may exert potential beneficial effect in cardiac fibrosis through multiple protective mechanisms.

Extracellular matrix (ECM) in heart is unique, and is highly regulated. In addition to providing a scaffold for normal cardiac structure and function, the ECM serves as a reservoir of various growth factors and cytokines that influence the function of cardiac fibroblasts (CF) and cardiomyocytes. CF are the principal cells responsible for ECM deposition in the

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heart. Under physiological conditions, CF express various matrixins (matrix-degrading metalloproteinases or MMPs) and their tissue inhibitors (TIMP metallopeptidase inhibitors or TIMPs) that regulate ECM deposition, degradation and turnover, and thus cardiovascular homeostasis (MacKenna et al., 2000; Iyer et al., 2012; Chen and Frangogiannis, 2013; van Nieuwenhoven and Turner, 2013). Under pathological conditions however, CF secrete increased amounts of MMPs, leading to a disruption in the delicate balance between MMPs and their endogenous inhibitors, that ultimately leads to increased ECM degradation, adverse remodeling of cardiac interstitium, myocardial dysfunction, fibrosis, and increased risk of heart failure (Villarreal et al., 2003; Iyer et al., 2012; Chen and Frangogiannis, 2013; Spinale et al., 2013; van Nieuwenhoven and Turner, 2013). Therefore, targeting MMP expression and/ or activation may attenuate cardiac fibrosis and adverse remodeling.

Reversion-inducing-cysteine-rich protein with Kazal motifs (RECK) is a unique membrane-bound glycoprotein and a MMP regulator (Takahashi et al., 1998; Siddesha et al., 2013). It is anchored to the plasma membrane via a COOH-terminal hydrophobic region and a GPI (glycophosphatidylinositol) interaction (Takahashi et al., 1998). RECK inhibits various MMPs, including MMPs 2, 7, 9, and 14 (MT1-MMP) (Takahashi et al., 1998; Noda et al., 2003; Omura et al., 2009; Siddesha et al., 2013), that are known to play a role in cardiac fibrosis and adverse remodeling. RECK was originally identified as a transformation suppressor of v-Ki-ras-transformed NIH 3T3 mouse embryo fibroblasts (Takahashi et al., 1998). While normal cells express RECK under basal conditions, many tumors and tumor-derived cells express either low or undetectable levels of RECK, probably contributing to enhanced MMP expression/activation, ECM destruction, angiogenesis, and malignant transformation. RECK is expressed in various organs, including the heart (Takahashi et al., 1998; Siddesha et al., 2013), however, its role and regulation in cardiovascular diseases has not been fully investigated.

Recently, we reported that angiotensin II (Ang II)-induced myocardial hypertrophy and fibrosis in a mouse model are characterized by sustained MMP induction and a marked reduction in RECK expression. Further, Ang II induced CF migration in vitro, and RECK and MMPs differentially regulated its promigratory effects. Of note, Ang II exerts its biological effects in part via proinflammatory cytokine induction. We previously demonstrated that Ang II induces the expression of IL-18, a proinflammatory cytokine, in cardiomyocytes (Valente et al., 2012a). Further, IL-18 stimulates cardiac fibroblast migration in part via MMP9 induction and activation (Fix et al., 2011; Valente et al., 2013). Aspirin or acetylsalicylic acid (ASA) is a widely used analgesic and antipyretic. Because of its inhibitory effects on cyclooxygenase (COX) and on platelet aggregation, it is also used in the treatment of cardiovascular diseases (Hennekens et al., 1997). In addition to their anti-inflammatory effects, ASA and its salicylic acid moiety also exert antioxidant effects (Muller et al., 2001; Mehta et al., 2004). It inhibits various redox-sensitive transcription factors involved in MMP induction, specifically NF-κB and AP-1 (Mehta et al., 2004). The RECK gene is responsive to the redox-sensitive transcriptional regulator Sp1 (Sasahara et al., 1999), and ASA has been shown to suppress Sp1 DNA-binding activity or degradation (Abdelrahim and Safe, 2005; Fiorucci et al., 2005). Since RECK is a negative regulator of MMP9 (Takahashi et al., 1998), we hypothesized that IL-18 stimulates CF migration by

suppressing RECK, but by inducing MMP9, and ASA will reverse IL-18-induced CF migration by inhibiting these responses.

Materials and Methods

Materials

Acetylsalicylic acid (aspirin; A5376), salicylic acid (SA; 247588), anti-MMP9 antibodies that detect both pro and active forms of MMP9 (M9570), lentiviral shRNA targeting p65 (RELA; #TRCN0000055346), c-Jun (JUN; #TRCN0000229528), Sp1 (#TRCN0000071603) and GFP (#SHC005V), β-actin and smooth muscle actin (SMA) antibodies, and all other chemicals were purchased from Sigma–Aldrich (St. Louis, MO). Recombinant mouse (rm) IL-18 (#B002-5), anti mouse IL-18 antibodies (#D048-3; used in neutralization and detection of mature IL-18 by immunoblotting), rat IgG1 isotype control (Clone 43414; #MAB005), recombinant human IL-18BPα-Fc chimera (#119 BP-100; 10 μg/ml for 1 h), and Fc were purchased from R & D Systems (Minneapolis, MN). The Stress-activated protein kinase/JNK assay kit (#9810) and antibodies against RECK (#3433), IKKβ (#8943), p65 (#4764), p-p65 (#3033), c-Jun (#9165), p-c-Jun (#9164), Akt (#9272), α-tubulin (# 2144), and Lamin A/C (# 2032) were purchased from Cell Signaling Technology, Inc. (Beverly, MA) Anti-RECK antibodies used in immunofluorescence studies were from Bioss (#bs-2901R; Woburn, MA). Anti-MMP9 antibodies that detect total MMP9 (#AB19016) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; #MAB374) were purchased from Millipore (Billerica, MA). Enhanced chemiluminescence detection kit was from Amersham Pharmacia Biotech. Anti-Sp1 (p-Thr453) antibody was from Novus Biologicals (Littleton, CO). Anti-hemagglutinin antibodies were from Covance. Antibodies against JNK1 (#sc-1648), CD-31 (platelet-endothelial cell adhesion molecule-1 or PECAM-1; #sc-28188) and vimentin (#sc-5565) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mithramycin A (#1489) was purchased from Tocris Bioscience (Minneapolis, MN). DPI (#300260), SP600125 (#420119), and DMSO were purchased from EMD Biosciences (Gibbstown, NJ). EUK-134 (#10006329) is a synthetic manganese-porphyrin complex that scavenges reactive oxidative species such as H_2O_2, superoxide and peroxynitrite, and was purchased from Cayman Chemical Company (Ann Arbor, MI). Sodium pyruvate is a H_2O_2 scavenger, and was obtained from Invitrogen/Life Technologies (Grand Island, NY).

Animals and isolation of adult mouse cardiac fibroblasts

All animal studies were approved by the Institutional Care and Use Committees of Tulane University in New Orleans, and the University of Texas Health Science Center in San Antonio, and conformed to the Guide for the Care and Use of Laboratory Animals published by the NIH. Fibroblasts were isolated from the hearts of 8–10-wk-old wild type male C57B1/6 as previously described (Venkatachalam et al., 2008; Valente et al., 2013). Cell phenotype was confirmed by immunofluorescence using β-actin, CD-31, vimentin, and α-smooth muscle actin antibodies. CF are positive for vimentin and β-actin immunoreactivity, but are negative for CD-31 and αSMA, and display typical fibroblast-like morphology (flat, spindle-shaped; Fig. 1A, ×40; actin immunofluorescence is shown in the inset, ×400). Freshly isolated and up to second passage CF were used in all experiments. At 50–70%

confluency, the cells were made quiescent by incubating in medium containing 0.5% BSA (serum-free) for 48 h.

Adeno- and lentiviral transductions

Adenovirus that expresses full-length mouse RECK ORF (GenBank accession No. NM_016678.2) under control of the CMV promoter (Ad.RECK) has been previously described (Siddesha et al., 2013). Ad.siNox4 and Ad.siGFP were also previously described (Valente et al., 2013). Adenoviral vector expressing siRNA against MMP9 (Ad.siMMP9) was provided by Jasti S. Rao (University of Illinois College of Medicine, Peoria, IL). CF were infected at ambient temperature with the adenovirus in PBS at the indicated multiplicity of infection (moi). After 1 h, the medium containing adenovirus was replaced with culture media supplemented with 0.5% BSA. Assays were carried out after 48 h. The transfection efficiency with adenoviral vectors was near 100% as evidenced by the expression of GFP in CF infected with Ad.GFP (data not shown). For lentiviral infection, CF at 50–60% confluency were infected with lentiviral particles at moi 0.5 in complete media for 48 h. At the indicated moi, adeno, or lentiviral infection displayed no off-target effects and did not affect cell viability and adherence (data not shown).

Detection of hydrogen peroxide by Amplex® Red assay

Quiescent CF were treated with IL-18 (10 ng/ml for 30 min). H_2O_2 production was measured according to the manufacturer's instructions using a commercially available kit (Amplex[1] Red hydrogen peroxide/peroxidase assay kit, Molecular Probes, Inc./Life Technologies). Fluorescence was recorded at 530 nm excitation and 590 nm emission wavelengths (CytoFluor II; Applied Biosystems, Foster City, CA). Standard curves were generated using known concentrations of H_2O_2. Studies were also performed after DPI pretreatment or Ad.siNox4 transduction.

Transcription factor activation

Nuclear extracts were prepared using the Panomics Nuclear Extraction kit (Cat. no. AY2002, Panomics/Affymetrix, Freemont, CA) according to the manufacturer's instructions. Nuclear p p65 (Ser536), p-c-Jun (Ser73), and Sp1 levels were analyzed by immunoblotting (Chandrasekar et al., 2006). Lamin A/C (nuclear) and GAPDH (cytoplasmic) served as loading and purity controls.

mRNA expression

Total RNA was isolated using the TRIzol method and treated with DNase I. One microgram of DNA free total RNA was reverse transcribed using Quantitect cDNA Synthesis Kit. MMP9 and RECK mRNA expressions were analyzed by RT-qPCR using TaqMan[1] probes (Applied Biosystems) and Eppendorf Realplex[4] system. 18S served as an invariant control. Data are shown as fold change ($2^{-\Delta\Delta Ct}$).

Protein expression

Preparation of whole cell homogenates, immunoblotting, detection of the immunoreactive bands by enhanced chemiluminescence (ECL Plus; GE Healthcare), and their quantification

by densitometry were as described previously (Venkatesan et al., 2013). MMP9 activation was analyzed by gelatin zymography using similar quantities of culture supernatants (Chandrasekar et al., 2006). Tubulin in whole cell homogenates served as a loading control.

IKK and JNK activity assays

IKK activity in CF-treated with IL-18 (10 ng/ml for 30 min) was determined by an in vitro kinase assay using glutathione S-transferase (GST)-IκB fusion protein as the substrate. JNK activity was measured using a commercially available kit. 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) antibodies.

Immunofluorescence

Intracellular actin and RECK surface expression was analyzed by immunofluorescence. In brief, cells were plated on chamber slides, treated or not with ASA (0.5 mM for 30 min) followed by IL-18 (10 ng/ml for 12 h), fixed in 3.7% paraformaldehyde, blocked with 1% BSA, incubated with antibody (1 h at 22°C, 16 h at 4°C), washed in TBST (Tris-buffered saline containing 0.05% Tween 20; ×3), incubated with Alexa Fluor 488 conjugated secondary antibody (green fluorescence), washed (×3) and then mounted in VECTASHIELD HardSet Mounting Medium. Cells were analyzed by fluorescent microscopy (Zeiss Axio Imager M1). Images were acquired at the same magnification (100×) and exposure time for comparison.

Cell Migration

CF migration was quantified as described previously (Chandrasekar et al., 2006) using BioCoat^{TM} Matrigel^{TM} invasion chambers and 8.0-μm pore polyethylene terephthalate mem branes with a thin layer of Matrigel^{TM} basement membrane matrix. Cultured CF were trypsinized and suspended in RPMI 1640 + 0.5% bovine serum albumin, and 1 ml containing 2.0×10^5 cells/ml was layered on the coated insert filters. Cells were stimulated with IL-18 (10 ng/ml). The lower chamber contained similar levels of IL-18. Plates were incubated at 37°C for 12 h. Membranes were washed with PBS, and non-invading cells on the upper surface were removed using cotton swabs. CF invading into and through the Matrigel^{TM} matrix were quantified by MTT assay. Results of IL-18 effects on CF migration were normalized with the migration of untreated cells and expressed as fold change from untreated controls.

Cell death Analysis

To determine whether IL-18, ASA, SA, transduction of viral vectors, and pharmacological inhibitors affected cell viability, cell death was analyzed using the Cell Death Detection ELISA^{PLUS}, trypan blue dye exclusion, microscopic visualization of cell shape and for cells floating in the media.

Statistical Analysis

Results are expressed as mean ± S.E. For statistical analysis we used analysis of variance followed by an appropriate post hoc multiple comparison test (Tukey method). Data were considered statistically significant at $P < 0.05$.

Results

Acetylsalicylic acid (ASA) inhibits interleukin-18-induced cardiac fibroblast (CF) migration

The cardiac cells used in this study were characterized as fibroblasts by their distinctive spindle-shaped morphology (Fig. 1A) and reactivity to anti-beta actin antibodies (Fig. 1A, inset). These cells were negative for aSMA and for vimentin (data not shown), which is also characteristic of CF.

Since ASA is known to inhibit CF proliferation (Wang et al., 2012), we hypothesized that ASA would also block IL-18-induced CF migration. Confirming our earlier results (Chandrasekar et al., 2006), IL-18 markedly increased CF migration (~2.52-fold), an effect attenuated by IL-18 neutralizing anti-bodies or its endogenous inhibitor IL-18 binding protein (IL-18BP; Fig. 1B). Notably, while therapeutic concentrations of ASA and SA had no significant inhibitory effects on basal CF migration, they both markedly attenuated IL-18-induced CF migration (~67% reduction; Fig. 1C), confirming that ASA attenuates the promigratory effects of IL-18 (Fig. 1).

ASA reverses IL-18-induced MMP9 expression and RECK suppression

CF migration follows ECM degradation, and enhanced MMP expression or activation contributes to ECM destruction (Iyer et al., 2012; Chen and Frangogiannis, 2013; Spinale et al., 2013; van Nieuwenhoven and Turner, 2013). RECK is a MMP regulator, and has been shown to inhibit various MMPs, including MMP9 (Takahashi et al., 1998). Therefore, we investigated whether ASA will block IL-18-induced MMP9 expression, but reverse RECK suppression. IL-18 induced MMP9 mRNA expression and activation, effects that were markedly attenuated by ASA (Fig. 2A). ASA, however, had no significant effect on either basal MMP9 mRNA expression or activity (Fig. 2A). Notably, MMP9 knockdown attenuated IL-18-induced CF migration (Fig. 2B). Further, IL-18 suppressed RECK expression in a time-dependent manner (Fig. 2C), and ASA partially restored its expression (Fig. 2C, left lower) and surface expression (Fig. 2C, right). In addition, adenoviral transduction with wild type RECK (Ad.RECK), which forced RECK expression in a dose-dependent manner (Fig. 2D), markedly attenuated IL-18-induced CF migration (Fig. 2E). Ad. RECK was used at moi 40 for 24 h in this experiment, but even at moi 80, RECK overexpression did not affect cell viability or adherence to culture dishes (data not shown). Importantly, forced expression of RECK attenuated IL-18-induced MMP9 activation (Fig. 2F). These results indicate that IL-18 differentially regulates MMP9 and its inhibitor RECK in CF, and RECK overexpression blunts IL-18-induced CF migration in part via suppression of MMP9 expression and activation (Fig. 2).

ASA inhibits IL-18-induced ROS generation

Signal transduction pathways induced by IL-18 are known to involve the generation of reactive oxygen species (ROS) by enzymes such as the Nox family of NADPH oxidases (Valente et al., 2013). We previously demonstrated that the induction of MMP9 by IL-18 in vascular smooth muscle cells is dependent on Nox1 (Valente et al., 2012b). We therefore investigated whether the suppression of RECK is also dependent on Nox NADPH oxidase and ROS, and the effect of ASA on this response. Nox4 is the dominant NADPH oxidase isotype in CF (Colston et al., 2005), and the usual measurable product of Nox4 is H_2O_2 (Takac et al., 2011). Addition of IL-18 induced a marked increase in H_2O_2 production by CF (Fig. 3A), and pretreatment with the flavoprotein inhibitor DPI and Nox4 knockdown each inhibited this response, as did ASA (Fig. 3A). Moreover, ASA inhibited IL-18-induced Nox4 mRNA expression (Fig. 3B). Further, DPI pretreatment and Nox4 knockdown each attenuated IL-18-induced MMP9 mRNA expression (Fig. 3C), but reversed RECK suppression (Fig. 3D) and CF migration (Fig. 3E). Since the Nox inhibitor DPI attenuated IL-18-induced MMP9 expression (Fig. 3C), implying a role for oxidative stress in IL-18 signaling, we next investigated whether other free radical scavengers exert similar inhibitory effects. Pretreatment with EUK-134, a synthetic manganese-porphyrin complex that scavenges ROS such as H_2O_2, superoxide and peroxynitrite (Rong et al., 1999), and sodium pyruvate, a H_2O_2 scavenger (Salahudeen et al., 1991), each attenuated IL-18-induced H_2O_2 production (Fig. 3F), MMP9 induction (Fig. 3G) and RECK suppression (Fig. 3H). Together, these results demonstrate that IL-18-induced MMP9 expression and RECK suppression are oxidative stress-responsive, and ASA inhibits these responses by blocking the generation of H_2O_2 (Fig. 3).

ASA inhibits IL-18-induced MMP9 expression through inhibition of NF-κB and AP-1 activation, and suppresses RECK via Sp1

Since IL-18 differentially regulated MMP9 and RECK, we investigated the mechanisms involved and the effect of ASA. MMP9 is a NF-κB and AP-1 responsive gene, and IL-18 is a potent inducer of both transcription factors. Therefore, we investigated whether IL-18-induced MMP9 expression is NF-κB- and AP-1-dependent, and whether ASA inhibits this response. We examined the phosphorylation status of p65 and c-Jun in IL-18-treated CF as markers of NF-κB and AP-1 activation. Our results indicate that IL-18 induced p65 (Fig. 4A) and c-Jun phosphorylation (Fig. 4B). Further, knockdown of p65 or c-Jun attenuated IL-18-induced MMP9 mRNA expression (Fig. 4C). Notably, ASA attenuated nuclear translocation of both p65 and c-Jun (Fig. 4D, upper and lower parts).

Since MMP9 mRNA expression was attenuated partially and to a similar extent following p65 or c-Jun knockdown (Fig. 4C), and by DPI treatment (Fig. 3C), we next investigated whether combining p65 or c-Jun knockdown with DPI would reduce IL-18-induced MMP9 mRNA expression further. CF transduced with lentiviral p65 (Fig. 4E) or c-Jun (Fig. 4F) for 48 h were incubated with DPI (10 μM) for 30 min, and then treated with IL-18 (10 ng/ml) for two additional hours. MMP9 mRNA expression was analyzed by RT-qPCR. Confirming our earlier results (Fig. 4C, 3C), while p65 knockdown or DPI pretreatment similarly attenuated IL-18-induced MMP9 mRNA expression (Fig. 4E), their combination (p65 knockdown + DPI) was more effective and further reduced MMP9 mRNA expression (Fig.

4E). Similarly, c-Jun knockdown coupled with DPI (Fig. 4F) markedly attenuated IL-18-induced MMP9 mRNA expression. However, the levels did not reach those seen at basal conditions in each of the six experiments performed independently. These results suggest contribution of other regulatory mechanisms, such as Nox-independent ROS generating systems, and activation of other cis-regulatory elements that could affect MMP9 mRNA expression in response to IL-18 (Fig. 4).

ASA inhibits IL-18-induced IKK and JNK activation

We have demonstrated that ASA inhibits IL-18-induced p65 or c-jun activation in CF. In the canonical signal transduction pathway, phosphorylation of p65 at Ser536 by the IκB kinase (IKKβ) results in its nuclear translocation (Sakurai et al., 1999) and transcriptional regulation of κB-responsive genes. Therefore, we next investigated whether ASA inhibits IL-18-induced IKKb activity. Since JNK mediates c-Jun phosphorylation/activation, we also investigated whether ASA inhibits c-Jun activation by targeting JNK. IL-18 induced a time-dependent increase in IKKb (Fig. 5A) and JNK (Fig. 5B) kinase activity, with increased levels detected as early as 15 min following IL-18 addition. Further, while IKKb knockdown attenuated IL-18-induced p65 phosphorylation (Fig. 5C), JNK knockdown attenuated c-Jun phosphorylation (Fig. 5D), and knockdown of IKKb (Fig. 5E) or JNK (Fig. 5F) attenuated IL-18-induced CF migration. Further, combining IKKb knockdown with JNK inhibition markedly reduced IL-18-induced MMP9 mRNA expression (Fig. 5G) and CF migration (Fig. 5H). The cell viability however, was not affected by their combined inhibition (data not shown). Notably, ASA inhibited IL-18-induced IKKb (Fig. 5I) and JNK (Fig. 5J) activity. These results indicate that ASA inhibits p65 and c-Jun activation by targeting their upstream regulators IKKb and JNK in CF (Fig. 5).

ASA reverses IL-18-induced Sp1 activation and RECK suppression

Since Sp1 activation has been shown to suppress RECK (Sasahara et al., 1999), we investigated whether IL-18 suppresses RECK via Sp1 activation. Of note, Sp1 is also an oxidative stress-responsive transcription factor. Treatment with IL-18 induced Sp1 phosphorylation (Fig. 6A, upper part) and nuclear translocation (Fig. 6A, lower part), and Nox4 knockdown attenuated these effects. Further, IL-18 suppressed RECK mRNA expression, an effect reversed by Sp1 knockdown or pretreatment with the Sp1 inhibitor mithramycin (Fig. 6B), demonstrating a negative role for Sp1 activation in RECK regulation.

We next investigated whether ASA inhibits IL-18-induced Sp1 activation. Pretreatment with ASA attenuated IL-18-induced Sp1 activation (Fig. 6C). Notably, ASA and SA alone both induced RECK expression (Fig. 6D), demonstrating that ASA and SA not only reverse IL-18-mediated RECK suppression, but also induce RECK expression. Of note, neither ASA nor SA modulated CF viability (data not shown). Together, these results indicate that IL-18 differentially regulates MMP9 and RECK; while inducing MMP9 via NF-κB and AP-1 activation, it suppresses RECK via Sp1, and ASA and SA reverse these responses (Figs. 5 and 6).

Disussion

RECK is a unique membrane-anchored MMP regulator (Takahashi et al., 1998). Here we show for the first time that IL-18 stimulates CF migration via RECK suppression and MMP9 induction. While IL-18 suppresses RECK through Nox4/ROS-mediated Sp1 activation, it also induces MMP9 via Nox4/ ROS-mediated IKK/NF-κB and JNK/AP-1 activation. Further, ASA reversed RECK suppression by inhibiting Sp1 activation, inhibited MMP9 expression by attenuating IKK/NF-κB and JNK/AP-1 activation, and CF migration. The salicylic acid (SA) moiety of ASA similarly reversed IL-18-mediated RECK suppression and CF migration. In addition, ASA and SA alone could induce RECK expression. Notably, forced expression of RECK inhibited IL-18-induced MMP9 expression and activity, and CF migration (Fig. 7). Together, these results suggest that strategies that upregulate RECK expression may attenuate the progression of cardiac fibrosis and adverse remodeling in vivo.

In this study, we used ASA at a concentration of 0.5 mM. At this therapeutic dose, ASA reversed IL-18-induced MMP9 expression, RECK suppression, and CF migration. The systemic levels of ASA have been shown to range between 0.38 and 10.26 mM in subjects receiving 25 mg/kg aspirin (Juarez Olguin et al., 2004). In vitro, ASA was used at various concentrations, ranging from 0.5 to 20 mM. In one study, ASA at a dose of 4 mM almost completely inhibited IFN + TNF-α-induced nitrite production in primary cardiac fibroblasts (Farivar et al., 1996). In another study, ASA at 10 mM or higher (20 mM) was shown to completely inhibit IL-1b or TNF-α-induced IL-8 secretion in lung epithelial cells (Yoo et al., 2001). In that study, IL-8 levels were almost undetectable at higher concentrations of ASA. This did not appear to be a result of cell death since even at these higher levels ASA had no effect on cell viability (Yoo et al., 2001). It is thus plausible that at higher concentrations, ASA might exert more pronounced effects, and might restore RECK to near basal levels and completely inhibit IL-18-induced CF migration. Here we demonstrate a marked decrease in MMP9 induction and CF migration at a therapeutic dose of 0.5 mM.

We also show for the first time that CF express RECK at basal conditions and IL-18 suppresses this expression. RECK is a 110 kDa glycoprotein with several serine protease inhibitor-like domains, and is bound to the plasma membrane surface via a glycophosphatidylinositol (GPI) anchor (Takahashi et al., 1998). RECK negatively regulates expression and activity of various MMPs, including MMP9 (Gelatinase B) (Takahashi et al., 1998; Noda et al., 2003; Omura et al., 2009). Here we show that IL-18 suppresses RECK, but induces MMP9, and forced expression of RECK inhibits MMP9 activity. In fact, RECK has been shown previously to inhibit MMP9 via inhibition of pro-MMP9 secretion and enzyme activity (Takahashi et al., 1998), indicating that RECK inhibits post-transcriptional regulation of MMPs. However, RECK has also been shown to block MMP9 transcription (Takagi et al., 2009) via mechanisms not fully understood.

In addition to MMP9, RECK also inhibits MMP2 (Gelatinase A) (Takahashi et al., 1998), another soluble MMP known to be involved in CF migration. MMP2 is synthesized as a latent or inactive proform, and is then processed to intermediate and active forms via proteolytic cleavage at the plasma membrane (proform→intermediate form→active form)

by a pro-MMP2/ MT1-MMP/TIMP2 ternary complex (Turck et al., 1996). RECK inhibits secretion of both the intermediate and active forms of MMP2 by negatively regulating the membrane-anchored MT1-MMP (MMP14) and the active form of MMP2 itself (Takahashi et al., 1998). These reports, along with the current data, indicate that RECK inhibits both the soluble type (MMP2 and 9) and the membrane-anchored (MT1-MMP) MMPs. In fact, we recently reported that RECK overexpression inhibits MMP2 activity (Siddesha et al., 2013). In addition, RECK has also been shown to inhibit ADAM10 (a disintegrin and metalloproteinase domain 10; Muraguchi et al., 2007) and ADAM17 (Hong et al., 2013), members of the metzincin subgroup of zinc proteases, that play a role in cytokine release, growth factor activation, and cell-cell, and cell-matrix interactions. ADAM17, also known as TNF-α-converting enzyme (TACE), contributes causally to myocardial hypertrophy and fibrosis in hypertensive heart disease (Wang et al., 2009). Expression of ADAM10 is found to be increased in dilated cardiomyopathy, and shows a positive correlation with LV dilatation and indices of systolic contractile dysfunction (Fedak et al., 2006). While TIMP3 inhibits ADAM17 (Lee et al., 2002), both TIMPs 1 and 3 inhibit ADAM10 (Amour et al., 2000), suggesting that RECK shares some functional similarities to TIMPs 1 and 3.

Interestingly, TIMP2, while playing a role in MMP2 maturation, also acts as a MMP2 inhibitor. Recently, TIMP2 has been shown to suppress endothelial cell migration via RECK upregulation, independent of its inhibitory effects on MMPs (Oh et al., 2004). In those studies TIMP1 failed to modulate RECK expression (Oh et al., 2004). Whether TIMPs 3 and 4 regulate RECK expression is not known. Of note, low doses of ASA inhibit MMPs 2 and 9 in macrophages via TIMP 1 and TIMP2 induction (Hua et al., 2009). Although we did not examine the expression levels of TIMPs in the present study, it is plausible that ASA might have inhibited IL-18-induced MMP expression via TIMP induction and TIMP-dependent RECK upregulation. In addition to MMPs 2, 9, and 14, RECK competitively inhibits MMP7 expression (Omura et al., 2009). However, MMP7 expression is undetectable in cardiac fibroblasts. Interestingly, RECK is also a target of MMP proteolytic activity. Using purified proteins, both MMPs 2 and 7 have been shown to cleave RECK in vitro (Omura et al., 2009), suggesting that increased MMPs may downregulate their endogenous inhibitor RECK in vivo, resulting in unopposed MMP activation and adverse remodeling.

The proximal promoter region of RECK gene contains two Sp1 binding sites, and while mutation of each site reduces RECK promoter-dependent reporter gene activation, mutations in both sites abrogated its activation (Sasahara et al., 1999), indicating a critical role for Sp1 in RECK induction. Here, we show that IL-18 suppresses RECK expression via Sp1 activation. IL-18 induced Sp1 activation, as evidenced by its phosphorylation at Thr453, and knockdown of Sp1 by lentiviral shRNA and treatment with the Sp1 inhibitor mithramycin each reversed IL-18-mediated RECK suppression. Interestingly, while Sp1 activation suppresses RECK, its activation induces MMP expression. The three known MMPs that are targeted by RECK, that is, *MMP2, MMP9*, and *MMP14 (MT1-MMP)*, are all Sp1-responsive genes, suggesting that IL-18-mediated Sp1 activation differentially regulates MMPs and their endogenous inhibitor RECK, that is, while inducing MMPs, it suppresses RECK. The mechanism whereby Sp1 negatively regulates RECK in CF is unclear. It has been previously reported that phosphorylation of Sp1 at Thr453 changes Sp1 from an inducer to a

transcriptional repressor (Bonello and Khachigian, 2004). In another study, the HER-2/neu oncogene has been shown to repress RECK transcription and tumor cell invasion via ERK-dependent Sp1 phosphorylation/activation, and trichostatin A, a histone deacetylase inhibitor reverses these effects (Hsu et al., 2006). Those authors suggest that the HER-2/neu induced RECK suppression may be mediated by binding of HDAC1 to the Sp1 site in RECK promoter, thus inhibiting its induction (Hsu et al., 2006). Whether IL-18-induced RECK suppression involves a similar a mechanism is currently under investigation.

We demonstrated that IL-18 suppresses RECK, but induces MMP9, via Nox4-dependent H_2O_2 production. Although the primary product of Nox4 activity is most likely superoxide, the usual measurable product is H_2O_2, and not superoxide (Takac et al., 2011). These authors identified the 28 amino acid third extracytosolic loop (E-loop) of Nox4 to be the critical component in Nox4-mediated H_2O_2 production. The mutant Nox4 without the E-loop generated superoxide and not H_2O_2, and had no effect on its expression or sub-cellular localization. They also demonstrated that the E-loop in Nox4, but not Nox1 and Nox2, contains a highly conserved histidine that could serve as a source for protons to accelerate spontaneous dismutation of superoxide to form H_2O_2. Mutation of this, but not of four other conserved histidines, also switched Nox4 from H_2O_2 to superoxide formation. They hypothesized that the structure of the E-loop may hinder superoxide exit and/or provide a source for protons, allowing dismutation to form H_2O_2. The authors concluded that H_2O_2 formation is an intrinsic property of Nox4 that involves its E-loop. Importantly, using cardiac-specific Nox4 gene deletion or overexpression, Kuroda et al. have recently demonstrated a critical role for Nox4 in pressure overload-induced myocardial hypertrophy and fibrosis (Kuroda et al., 2010). Here we demonstrate that Nox4 knockdown reverses IL-18-induced H_2O_2 production, MMP9 induction and RECK suppression. Similar results were obtained with the H_2O_2 scavengers sodium pyruvate and EUK-134, suggesting that IL-18-mediated MMP9 induction and RECK suppression are redox-sensitive. Of note, the IL-18R heterodimer binds Nox4 both in vitro and in vivo, and IL-18 enhances their physical association in vivo (Valente et al., 2013).

We also show that ASA, the active ingredient in aspirin, reverses IL-18-induced MMP9 induction and RECK suppression. In fact, ASA, a COX inhibitor, has been shown to induce RECK expression in lung cancer cells (Liu et al., 2002). However, COX-2 overexpression or prostaglandin E(2) treatment failed to affect RECK expression (Liu et al., 2002), suggesting a COX-independent mechanism. Interestingly, ASA has been shown to inhibit Sp1 activation (Fiorucci et al., 2005). Here we show that both ASA and its salicylic acid moiety induce RECK expression in CF. Further, ASA inhibited p65 and c-Jun activation by targeting IKKβ and JNK activities. Of note, ASA was shown previously to specifically inhibit IKKβ activity both in vitro and in vivo by inhibiting ATP binding to IKKβ (Yin et al., 1998). Here, we also show that ASA inhibits JNK activation, c-Jun phosphorylation, and CF migration, demonstrating that ASA inhibits two major signal transduction pathways in CF that are oxidative stress-responsive. Of note, ASA exerts potent antioxidant effects. Here we report that ASA inhibits IL-18-mediated Nox4 expression and Nox4-dependent H_2O_2 production in CF.

Taken together, our results demonstrate that IL-18 promotes fibroblast migration via MMP9 induction and RECK suppression, and ASA reverses these effects. These results suggest that ASA may exert dual protective effects: while inducing the MMP inhibitor RECK, it also suppresses MMPs, and thus may exert potential beneficial effects in cardiac fibrosis.

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Abbreviations

ADAM	a disintegrin and metalloproteinase
AP-1	activator protein 1
ASA	acetylsalicylic acid
AP-1	activator protein-1
CF	cardiac fibroblasts
CMV	cytomegalovirus
COX	cyclooxygenase
DPI	diphenyleneiodonium chloride
ECM	extracellular matrix
GFP	green fluorescent protein
GPI	glycophosphotidylinositol
HA	hemagglutinin
HER-2	human epidermal growth factor receptor 2
IKK	IκB kinase
IL	interleukin
IL-18BP	IL-18 binding protein
JNK	c-Jun amino terminal kinase
MAPK	mitogen activated protein kinase
MMP	matrix metalloproteinase

moi	multiplicity of infection
MT1-MMP	Membrane type 1 metalloprotease
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NF-κB	nuclear factor κB
NADPH	nicotinamide adenine dinucleotide phosphate
Nox	NADPH oxidase
ORF	open reading frame
PET	polyethylene terephthalate
RECK	reversion-inducing-cysteine-rich protein with Kazal motifs
ROS	reactive oxygen species
SA	salicylic acid
shRNA	short hairpin RNA
siRNA	small interfering RNA
Sp1	Specificity protein 1
TIMP	tissue inhibitor of metalloproteinase

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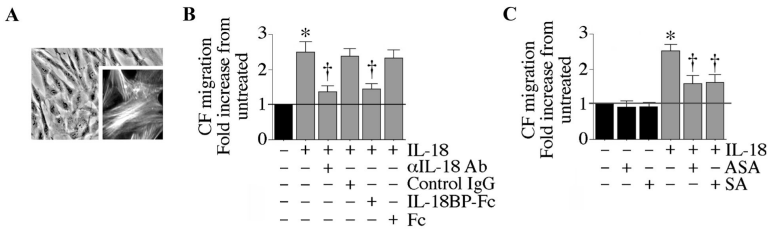

Fig. 1.

Acetylsalicylic acid (ASA) inhibits IL-18-induced cardiac fibroblast (CF) migration. A: Characterization of cardiac fibroblasts used in this study. Fibroblasts were isolated from the hearts of 8–10-wk-old wild type male C57Bl/6 mice. The cells were characterized as fibroblasts by their distinctive spindle-shaped morphology (×40) and expression of β-actin (inset; ×400). Freshly isolated and up to second passage CF were used in all experiments. B: IL-18 induces CF migration. At 50–70% confluency, the cells were made quiescent by incubating in medium containing 0.5% BSA (serum-free) for 48 h. IL-18 stimulates CF migration. At 70–80% confluence, CF were made quiescent by incubating in medium supplemented with 0.5% BSA for 48 h. The quiescent CF were trypsinized, re-suspended in medium containing 0.5% BSA, layered on Matrigel^{TM} basement membrane matrix-coated filters, and then treated with recombinant mouse IL-18 (10 ng/ml for 12 h). The lower chamber contained similar levels of IL-18. Specificity for IL-18 was verified by incubating the cells with IL-18-neutralizing antibodies or IL-18BP-Fc (10 μg/ml) for 1 h prior to IL-18 addition. Cells migrating to the other side of the membrane were quantified using MTT assay. C: ASA and its salicylic acid (SA) moiety inhibit IL-18-induced CF migration. The quiescent CF layered on matrix-coated filters were incubated with ASA (0.5 mM) or SA (1 mM) for 30 min prior to IL-18 addition (10 ng/ml for 12 h). Cell migration was quantified as described in B. B, C, * $P < 0.001$ versus untreated; †$P < 0.01$ versus IL-18 ± respective controls (n = 6).

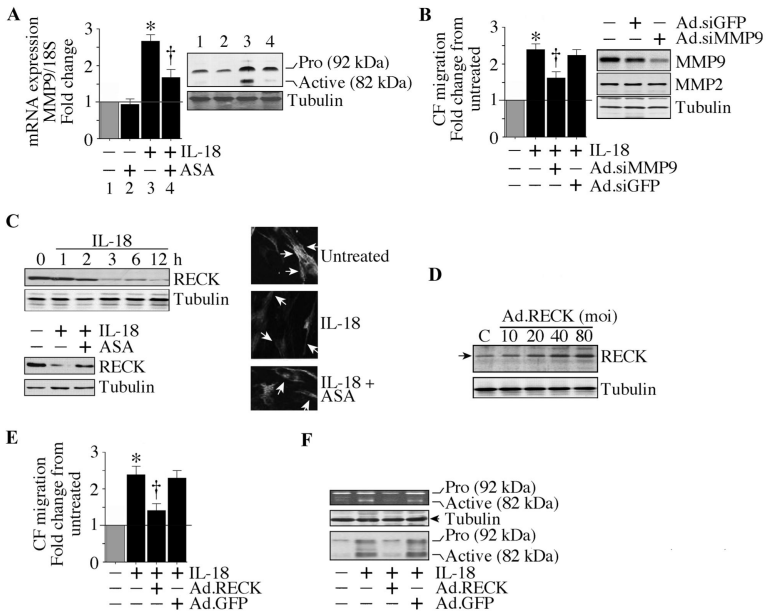

Fig. 2.

ASA reverses IL-18-induced MMP9 induction and RECK suppression. A: ASA inhibits MMP9 induction and activation. The quiescent CF were treated with ASA (0.5 mM for 30 min) prior to IL-18 addition (10 ng/ml for 2 h). MMP9 mRNA expression was analyzed by RT-qPCR (left part). Activation of MMP was analyzed by immunoblotting using antibodies that detect both pro and mature forms (inset; n = 3). B: IL-18 induces CF migration in part via MMP9. CF infected with Ad.siMMP9 (moi 100 for 24 h) were layered on matrix-coated filters, incubated with IL-18, and then analyzed for migration as in Figure 1B. Knockdown of MMP9 was confirmed by immunoblotting using cleared whole cell homogenates and is shown on the right. MMP2 served as an off-target (n = 3). C: IL-18 suppresses RECK in a time-dependent manner, and is reversed by ASA. The quiescent CF were treated with IL-18 (10 ng/ml) for up to 12 h. Experiments were also performed after ASA pre-treatment (0.5 mM for 30 min) followed by IL-18 addition for 6 h. RECK expression in whole cell homogenates was analyzed by immunoblotting, and its surface expression by immunofluorescence (right hand parts; n = 3). D: Adenoviral transduction of wild type RECK (Ad.RECK) induces RECK expression in a dose-dependent manner. CF infected with Ad.RECK at various moi for 24 h were analyzed for RECK expression by immunoblotting (n = 3). E: Forced expression of RECK attenuates IL-18-induced CF migration. CF infected with Ad.RECK (moi 40 for 24 h) were incubated with IL-18. CF migration was analyzed as in Figure 1B. F: Forced expression of RECK inhibits IL-18-induced MMP9 activity. CF transduced with Ad.RECK as in E, but for 2 h (immunoblotting) and 24 h (zymography) were analyzed for MMP9 activation by immunoblotting using whole cell homogenates and antibodies that detect both pro and mature forms, and by gelatin zymography using equal amounts of culture supernatants. Tubulin in cell homogenates served as a loading control. A, B, E: * $P <$ at least 0.01 versus untreated; †$P < 0.05$ versus IL-18 (n = 6).

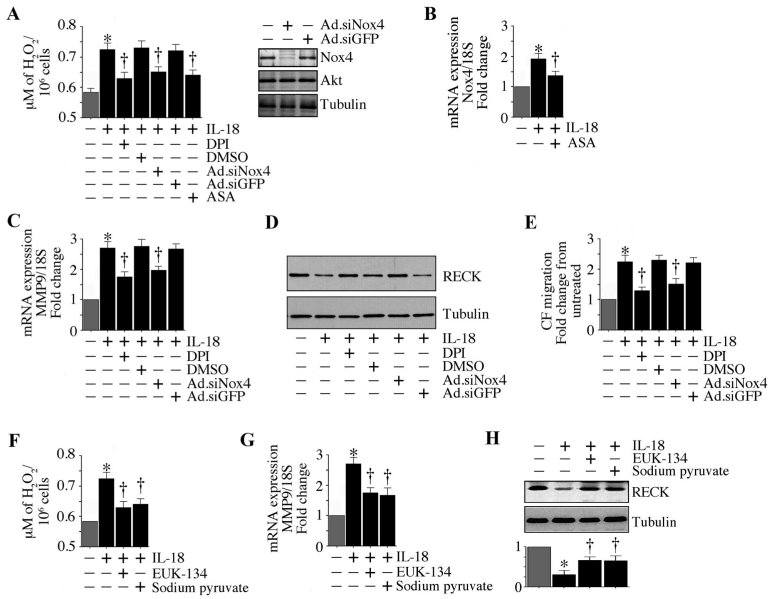

Fig. 3.

IL-18 induces CF migration via Nox4-dependent ROS production. A: IL-18 stimulates H_2O_2 generation via Nox4. CF infected with Ad. siNox4 (moi 100 for 48 h) or treated with DPI (10 μg/ml for 30 min) or ASA (0.5 mM for 30 min) prior to IL-18 addition (10 ng/ml) were analyzed for H_2O_2 production using the Amplex^W Red assay. Knockdown of Nox4 was confirmed by immunoblotting, and is shown on the right (n = 3). Akt served as an off-target. B: ASA inhibits IL-18-induced Nox4 mRNA expression. Quiescent CF were incubated with or without ASA as in A and then with IL-18 (10 ng/ml). Nox4 mRNA expression was analyzed after 6 h by RT-qPCR. C, D: IL-18-induced MMP9 mRNA expression (C) and RECK suppression (D) are mediated by Nox4. CF treated as in (A), but for 2 (C) or 6 h (D) with IL-18 were analyzed by RT-qPCR (MMP9) and immunoblotting (RECK; n = 3). E: IL-18 induced CF migration is Nox4 sensitive. CF treated as in A, but for 12 h with IL-18 were analyzed for migration. F–H: Free radical scavengers attenuate IL-18 induced H2O2 production (F) and MMP9 mRNA expression (G), and reverse RECK suppression (H). Quiescent CF were treated with EUK-134 (1 mM) or sodium pyruvate (10 mM) for 1 h prior to IL-18 addition (10 ng/ml) for 30 min (F), 2 h (G) or 6 h (H). H_2O_2 production was analyzed using the Amplex^W Red assay, MMP9 mRNA by RT-PCR, and RECK expression by immunoblotting (n = 3). A, B, C, E, F, G: * $P < 0.01$ versus untreated; †$P <$ at least 0.05 versus IL-18 ± respective controls (n = 6).

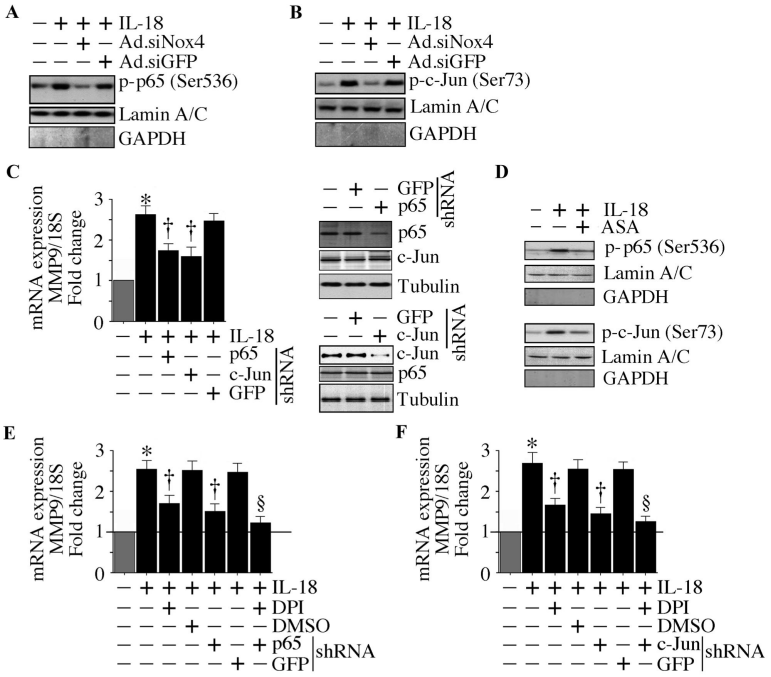

Fig. 4.

IL-18-induced NF-κB- and AP-1-mediated MMP9 expression is inhibited by ASA. A, B: IL-18 activates NF-κB and AP-1 via Nox4. CF transduced with Nox4 siRNA by adenoviral transduction (moi 100 for 48 h) were incubated with IL-18 (10 ng/ml) for 1 h, and then analyzed for phospho-p65 (A) or phospho-c-Jun (B) by immunoblotting using nuclear protein extracts (n = 3). Lamin A/C and GAPDH served as loading and purity controls (n = 3). C, IL-18 induces MMP9 expression via NF-κB and AP-1. CF transduced with lentiviral p65 or c-Jun shRNA (moi 0.5 for 48 h) were incubated with IL-18 (10 ng/ml) for 2 h, and then analyzed for MMP9 mRNA expression by RT-qPCR. Knockdown of p65 and c-Jun was confirmed by immunoblotting and is shown on the right. c-Jun (upper part) and p65 (lower part) served as off-targets (n = 3). * $P < 0.001$ versus untreated; †$P <$ at least 0.05 versus IL-18 ± GFP control (n = 6). D: ASA inhibits IL-18-induced p65 and c-Jun phophorylation. Quiescent CF were incubated with ASA (0.5 mM for 30 min) prior to IL-18 addition (10 ng/ml for 1h). Phospho-p65 and phospho-c-Jun levels in nuclear protein extracts were analyzed by immunoblotting (n = 3). E, F: Combining p65 or c-Jun knockdown with DPI treatment markedly reduces IL-18-induced MMP9 expression. CF transduced with lentiviral p65 (E) or c-Jun (F) for 48 h were treated with DPI (10 mM) for 30 min, and then incubated with IL-18 (10 ng/ml) for two additional hours. MMP9 mRNA expression was analyzed by RT-qPCR. E, F: * $P < 0.001$ versus untreated; y$P < 0.05$ versus IL-18 ± GFP control, xy$P < 0.01$ versus IL-18 (n = 6).

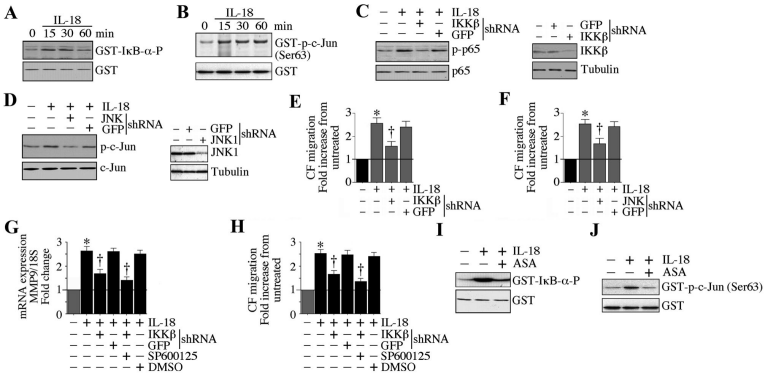

Fig. 5.

ASA inhibits IL-18-induced IKKβ and JNK activation. A, B: IL-18 stimulates time-dependent IKK and JNK activities. Quiescent CF treated with IL-18 for the indicated time periods were analyzed for IKK (A) or JNK (B) activity. IKKβ activity was analyzed by an in vitro kinase assay using glutathione S-transferase (GST)-IκB as the substrate (A; n = 3). JNK activity was analyzed by an in vitro kinase assay using GST-c-Jun as the substrate (B; n = 3). C, D: IL-18 induces p65 and c-Jun activation via IKKβ and JNK respectively. CF transduced with lentiviral IKKβ (C) or JNK1 (D) shRNA (moi 0.5 for 48 h) were treated with IL-18 (10 ng/ml) for 1 h, and then analyzed for phospho-p65 (C) or phospho-c-Jun (D) levels in cleared whole cell homogenates by immunoblotting using activation-specific antibodies (n = 3). Knockdown of IKKβ (C) and JNK1 (D) was confirmed by immunoblotting (respective right hand parts). E, F: IL-18 induces CF migration via IKKβ or JNK. CF transduced with IKKβ (E) or JNK (F) shRNA were layered on Matrigel™ basement membrane matrix-coated filters, treated with IL-18 (10 ng/ml for 12 h), and analyzed for migration as described in Figure 1B. E, F: * $P < 0.001$ versus untreated; †$P <$ at least 0.05 versus IL-18 ± GFP (n = 6). G, H: Combining IKK knockdown with JNK inhibition abrogates IL-18-induced MMP9 expression (G) and CF migration (H). CF were transduced with lentiviral IKKβ shRNA (moi 0.5) for 48 h, treated with the JNK inhibitor SP600125 (20 mM in DMSO) for 30 min, and then incubated with IL-18 for an additional 2 h. MMP9 mRNA expression was analyzed by RT-qPCR. In migration assays, CF transduced with lentiviral IKKβ shRNA were layered on Matrigel™ basement membrane matrix-coated filters, incubated with SP600125, and then treated with IL-18 for 12 h. Cell migration was analyzed as in Figure 1B. G, H: * $P < 0.001$ versus untreated; †$P < 0.05$, ‡ $P < 0.01$ versus IL-18 ± GFP or DMSO control (n = 6). I, J: ASA inhibits IL-18-induced IKKβ or JNK activity. Quiescent CF were incubated with ASA (0.5 mM for 30 min) prior to IL-18 addition (10 ng/ml for 15 min). IKKβ (I) and JNK (J) activities were analyzed as in (A) and (B), respectively (n = 3).

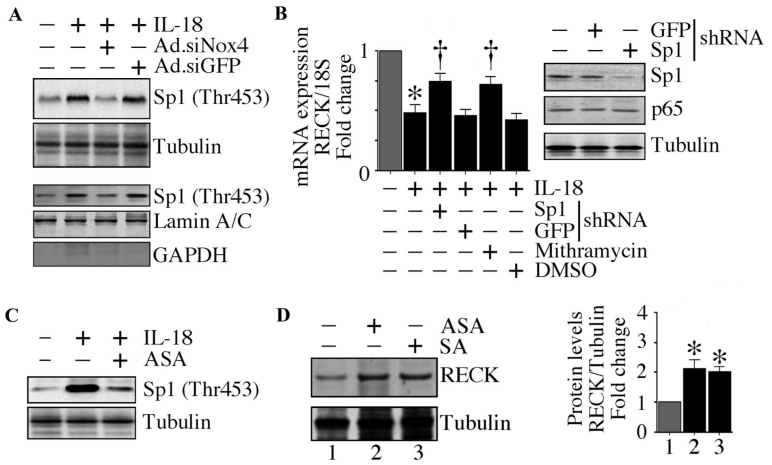

Fig. 6.

IL-18 suppresses RECK expression via Sp1 activation, and is reversed by ASA. A: IL-18 activates Sp1 via Nox4. CF treated as in Figure 4A were analyzed for phospho-Sp1 levels in whole cell homogenates (upper part) and nuclear extracts (lower part) by immunoblotting (n = 3). Tubulin (whole cell homogenates), Lamin A/C (nuclear) and GAPDH (cytoplasmic) served as loading and purity controls. B: IL-18 suppresses RECK via Sp1. CF transduced with lentiviral Sp1 shRNA (moi 0.5 for 48 h) or treated with mithramycin (100 nM in DMSO for 45 min) prior to IL-18 addition (10 ng/ml for 3 h) were analyzed for RECK mRNA expression by RT-qPCR. Knockdown of Sp1 was confirmed by immunoblotting and is shown on the right. p65 served as an off-target (n = 3). * $P < 0.001$ versus untreated; †$P <$ at least 0.05 versus IL-18 ± GFP control (n = 6). C, ASA inhibits IL-18-induced Sp1 activation. The quiescent CF were treated with ASA (0.5 mM for 30 min) prior to IL-18 addition (10 ng/ml for 1 h), and then analyzed for phospho-Sp1 levels in whole cell homogenates by immunoblotting (n = 3). D: ASA and SA induce RECK expression in CF. Quiescent CF treated with ASA (0.5 mM; D) or SA (1 mM; E) for 4 h were analyzed for RECK expression by immunoblotting. Densitometric analysis of immunoreactive bands from three independent experiments is summarized on the right. * $P < 0.05$ versus untreated (n = 3).

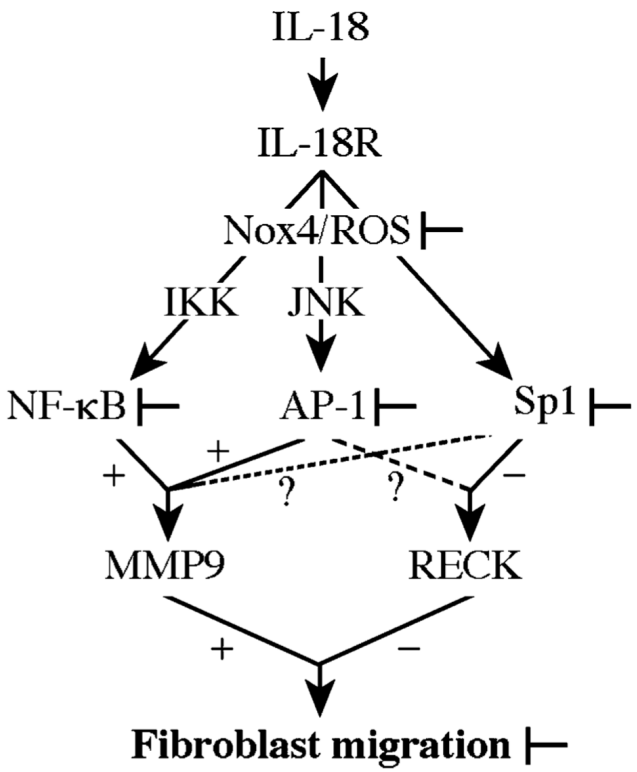

Fig. 7.

Schema showing multiple sites of action by acetylsalicylic acid (ASA) on IL-18 induced CF migration. +: Positive regulation, : negative regulation. Broken lines: roles for Sp1 in MMP9 induction and AP-1 in RECK suppression have been reported. Inhibition by acetylsalicylic acid is also indicated.