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The Nuclear Receptor Constitutive Androstane Receptor/NR1I3 Enhances the Profibrotic Effects of Transforming Growth Factor β and Contributes to the Development of Experimental Dermal Fibrosis

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Objective. **Nuclear receptors regulate cell growth, differentiation, and homeostasis. Selective nuclear receptors promote fibroblast activation, which leads to tissue fibrosis, the hallmark of systemic sclerosis (SSc). This study was undertaken to investigate the effects of constitutive androstane receptor (CAR)/NR1I3, an orphan nuclear receptor, on fibroblast activation and experimental dermal fibrosis.**

Methods. **CAR expression was quantified by quantitative polymerase chain reaction, Western blotting, immunohistochemistry, and immunofluorescence. CAR expression was modulated by small molecules, small interfering RNA, forced overexpression, and site-directed mutagenesis. The effects of CAR activation were analyzed in cultured fibroblasts, in bleomycin-induced dermal fibrosis, and in mice overexpressing a constitutively active transforming growth factor β (TGFβ) receptor type I (TβRI-CA).**

Results. **Up-regulation of CAR was detected in the skin and in dermal fibroblasts in SSc patients. Stimulation of healthy fibroblasts with TGFβ induced the expression of CAR messenger RNA and protein in a Smad-dependent manner. Pharmacologic activation or overexpression of CAR in healthy fibroblasts significantly increased the stimulatory effects of TGFβ on collagen synthesis and myofibroblast differentiation, and amplified the stimulatory effects of TGFβ on COL1A2 transcription activity. Treatment with CAR agonist increased the activation of canonical TGFβ signaling in murine models of SSc and exacerbated bleomycin-induced and TβRI-CA–induced fibrosis with increased dermal thickening, myofibroblast counts, and collagen accumulation.**

Conclusion. **Our findings indicate that CAR is up-regulated in SSc and regulates TGFβ signaling.**

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Activation of CAR increases the profibrotic effects of TGFβ in cultured fibroblasts and in different preclinical models of SSc. Thus, inactivation of CAR might be a novel approach to target aberrant TGFβ signaling in SSc and in other fibrotic diseases.

Fibrosis arises from excessive deposition of extracellular matrix components and results in scarring of various tissues. It is a hallmark of systemic sclerosis (SSc), which is a prototypical fibrotic disease affecting both the skin and many internal organs such as the lungs and gastrointestinal tract (1–3). Fibroblasts are the key players in the development of fibrosis. They show pathologic and persistent activation, with enhanced expression of contractile proteins and excessive release of extracellular matrix components. The profibrotic cytokine transforming growth factor β (TGFβ) has been identified as a central mediator of fibroblast activation in SSc (4). However, the intracellular signaling cascades by which this cytokine stimulates the production of extracellular matrix are incompletely understood.

Constitutive androstane receptor (CAR)/NR1I3 is an orphan nuclear receptor with no endogenous ligand identified so far (5). CAR is retained in the cytoplasm by forming a complex with phosphatase 2A, Hsp90, and cytosolic CAR retention protein (6). 1,4-Bis[2-(3,5-dichloro-pyridyloxy)]benzene (TCPOBOP) is a synthetic agonist for CAR (7), and 6-(4-chlorophenyl) imidazo[2,1-b][1,3]thiazole-5-carbaldehydeO-(3,4-dichlorobenzyl)oxime (CITCO) is an imidazothiazole derivative that functions as a selective agonist for human CAR (8). Upon activation, CAR translocates into the nucleus and binds to the response elements (5). The physiologic effects of CAR are pleiotropic. CAR has been shown to function as a xenobiotic receptor that regulates detoxification and clearance of toxic substances from the liver (9). In addition, recent studies suggest a role for CAR in inflammatory conditions, since deregulated CAR activity has been implicated in collagen-induced arthritis, liver cirrhosis, and cancer (10–12). However, its role in fibroblast activation and dermal fibrosis is not known. Thus, our aim was to investigate whether CAR might contribute to the pathologic activation of fibroblasts in SSc and to determine the role of CAR in the development of experimental skin fibrosis.

PATIENTS AND METHODS

Patients and fibroblast cultures. Fibroblast cultures were obtained from lesional skin biopsy specimens from 8 SSc patients and from 5 age- and sex-matched healthy volunteers. All SSc patients fulfilled the criteria for SSc as suggested by LeRoy et al (13). The median age of SSc patients was 48 years (range 22–65 years), and their median disease duration was 7 years (range 1–17 years). Four had limited cutaneous disease, and 4 had diffuse cutaneous SSc. None of the patients were treated with immunosuppressive or other potentially disease-modifying drugs. All patients and controls signed a consent form approved by the local institutional review boards.

Human fibroblasts were prepared by outgrowth cultures from skin biopsy specimens and cultured in Dulbecco's modified Eagle's medium (DMEM)–Ham's F-12 containing 10% heat-inactivated fetal calf serum (FCS), 25 m*M* HEPES, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 m*M* L-glutamine, and 2.5 μg/ml amphotericin B (all from Invitrogen) as previously described (14,15). In selected experiments, fibroblasts were stimulated with recombinant TGFβ (10 ng/ml; PeproTech). This latter concentration represents the standard concentration used for the stimulation of dermal fibroblasts and is based on the serum levels in SSc patients (16). Fibroblasts from passages 4–8 were used for the experiments.

Activation of human and murine CAR. We used the selective agonist CITCO (Sigma-Aldrich) to activate human CAR. CITCO is an imidazothiazole derivative with a 50% maximum response concentration of 49 nm and 450-fold higher selectivity for CAR than for pregnane X receptor (PXR), and no activity on other nuclear receptors (8). Human dermal fibroblasts were incubated with CITCO in concentrations ranging from 0.5 to 10 μM. CITCO was dissolved in DMSO. In our experiments, the final concentration of DMSO in the cell culture medium was 0.1%. Control fibroblasts were treated with the same DMSO concentration. In a subset of experiments, recombinant human TGFβ was added with CITCO. To activate murine CAR, we used TCPOBOP, which is a synthetic agonist for murine CAR (7). DMSO, the solvent of TCPOBOP, was used as a vehicle.

Cloning of the human CAR gene. Primer pairs used for the amplification of the human CAR gene were 3′-GATCGGATCCATGGCCAGTAGGGAAGATGAGCTGAGGAACTGTGTGGTATGTGG-5′ (forward) and 3′-GATCCTCGAGTCAGCTGCAGATCTCCTGGAGCAGCGGCATCA-TGGCAGAC-5′ (reverse). The mutation of human CAR with truncation of the DNA binding domain was created using site-directed mutagenesis (primer pair 3′-GATCGGATCCAGGAAAGACATGATACTGTCGGCAGAAGCCCTGGCATTG-5′ [forward] and 3′-GATCGGATCCCCACACAGTTCCTCAGCTCATCTTCCCTACTGGCCAT-5′ [reverse]) (17). The polymerase chain reaction (PCR) products were purified by electrophoresis, digested with *Bam* HI and *Xho* I (New England Biolabs), and ligated into a pcDNA3.1(+) expression vector (Life Technologies) that had previously been digested with the same restriction enzymes. The recombinant plasmids were introduced into *Escherichia coli* DH5α (Novagen) electrocompetent cells and selected on LB agar plates containing 50 μg/ml ampicillin. Single colonies were picked and tested for positive PCR products by colony PCR. LB medium containing ampicillin was inoculated with positive-tested colonies, which were grown overnight at 350 rpm and 37°C. Cells were harvested by centrifugation at 2,500*g* for 5 minutes, and plasmids were isolated using an isolation kit (Promega). For CAR overexpression experiments, 5 μg of the

plasmid constructs were transfected into dermal fibroblasts using a 4D Nucleofector (Lonza) (18,19).

Reporter assay. Dermal fibroblasts were transfected with the −353 COL1A2-Luc construct or a common pSv-β-galactosidase control vector as a transfection control using Lipofectamine 2000 (Life Technologies). The −353 COL1A2-Luc construct contains the fragment between −353 and +58 nucleotides of the COL1A2 gene and was kindly provided by M. Trojanowska (Boston University School of Medicine, Boston, MA) (20). Dermal fibroblasts were also transduced with 1,000 multiplicities of infection of Ad-CAGA-Luc construct or AdLacZ as a control for transduction efficiency (21). Transfected cells were then stimulated with TGFβ in the presence or absence of CITCO. Luciferase activity was determined after 24 hours using a microplate luminometer (Berthold Technologies).

Quantitative real-time PCR. Total RNA was isolated from SSc skin tissue and dermal fibroblasts using a NucleoSpin RNA II extraction system (Machery-Nagel) according to the instructions of the manufacturer. Reverse transcription (RT) into complementary DNA (cDNA) was performed as previously described using random hexamers (22,23). Gene expression was quantified by TaqMan or by SYBR Green real-time PCR using an ABI Prism 7300 sequence detection system (Applied Biosystems). Specific primer pairs for each gene were designed with Primer 3 software. The following primer pairs were used for the analyses: for human α1(I) procollagen (COL1A1), 5′-TCAAGAGAAGGCTCACGATGG-3′ (forward) and 5′-TCACGGTCACGAACCACATT-3′ (reverse); for human α2(I) procollagen (COL1A2), 5′-GGTCAGCAC-CACCGATGTC-3′ (forward) and 5′-CACGCCTGCCCTTC-CTT-3′ (reverse); for human fibronectin, 5′-TTCTAAGATT-TG-GTTTGGGATCAAT-3′ (forward) and 5′-TCTTGGT-TGGCTGCATATGC-3′ (reverse); and for human CAR, 5′-AGCAAACACCTGTGCAACTG-3′ (forward) and 5′-TGTCCGGATCAGCTCTTCTT-3′ (reverse).

Samples without enzymes in the RT reaction (non-RT controls) were used as negative controls. Nonspecific signals caused by primer dimers were excluded with the use of no-template controls and by dissociation curve analysis. β-actin (Applied Biosystems) was used as a housekeeping control to normalize for the amounts of cDNA within each sample. Differences were calculated using the threshold cycle (C_t) and the comparative C_t method for relative quantification (24).

Western blot analysis. Western blot analysis was performed via standard techniques, as previously described (25–27). Primary antibodies used were polyclonal rabbit anti-human antibodies against CAR (Abcam) and polyclonal goat anti-human antibodies against phospho-Smad2/3 (Santa Cruz Biotechnology). Secondary antibodies were horseradish peroxidase (HRP)–conjugated goat anti-rabbit or rabbit anti-goat antibodies (Dako).

Collagen measurements. The collagen content in cell culture supernatants and in lesional skin samples was analyzed with the hydroxyproline assay (28,29). Confluent fibroblasts were incubated for 24 hours with 1 ml DMEM/1% FCS containing ascorbic acid before collecting supernatants. Punch biopsy specimens (3 mm in diameter) were digested in 6*M* HCl for 3 hours at 120°C. The pH of supernatants and digested skin samples was adjusted to 7 with 6*M* HCl or NaOH. Afterward, samples were mixed with 0.06*M* chloramine T and incubated for 20 minutes at room temperature. Next, 3.15*M* perchloric acid and 20% *p*-dimethylaminobenzaldehyde were added, and samples were incubated for an additional 20 minutes at 60°C. Absorbance was determined at 557 nm with a SpectraMax 190 microplate spectrophotometer (Molecular Devices).

Immunofluorescence staining for phospho-Smad2/3, CAR, α-smooth muscle actin (α-SMA), and stress fibers. The expression of phospho-Smad2/3, CAR, and α-SMA was detected by staining with polyclonal goat anti-human antibodies against phospho-Smad2/3 (Santa Cruz Biotechnology), polyclonal rabbit anti-human antibodies against CAR (dilution 1:100; Abcam), and monoclonal mouse anti-human antibodies against anti–α-SMA antibody (clone 1A4; Sigma-Aldrich), respectively (27).

Donkey anti-goat Alexa Fluor 594–, goat anti-rabbit Alexa Fluor 488–, and donkey anti-mouse Alexa Fluor 594–conjugated antibodies were used as secondary antibodies. Counterstaining was performed with DAPI (Santa Cruz Biotechnology) for 10 minutes at room temperature. Slides were then viewed by microscopy using appropriate fluorescence filters. Actin stress fibers were visualized with rhodamine-conjugated phalloidin (Molecular Probes), as previously described (27).

Immunohistochemistry for α-SMA and CAR. For immunohistochemistry, skin sections were deparaffinized, followed by incubation with 5% bovine serum albumin in phosphate buffered saline for 1 hour to block nonspecific binding and incubation with 3% H_2O_2 for 10 minutes to block endogenous peroxidase activity. Staining was visualized with aminoethylcarbazole, using a peroxidase substrate kit (Vector).

Myofibroblasts were identified by staining for α-SMA as previously described (30). Cells positive for α-SMA in mouse skin sections were detected by incubation with monoclonal mouse anti-human antibodies against anti–α-SMA. Polyclonal rabbit anti-mouse antibodies labeled with HRP were used as secondary antibodies for 1 hour at room temperature. Irrelevant isotype-matched antibodies served as controls. The number of myofibroblasts was determined at 200-fold magnification using a Nikon Eclipse 80i microscope. Myofibroblasts were defined as single, α-SMA–positive spindle-shaped cells. Two experienced examiners (JA and JHWD) performed myofibroblast quantification in 6 randomly selected high-power fields for each section. Three sections were evaluated for each slide (31,32).

The expression of CAR was detected by staining with polyclonal rabbit anti-human antibodies against CAR (Abcam) at a respective dilution of 1:100 overnight at 4°C. Polyclonal goat anti-rabbit antibodies (Dako) labeled with HRP were used as secondary antibodies for 1 hour at room temperature. The intensity of CAR immunostaining was quantified with ImageJ software (National Institutes of Health), as described online at http://rsbweb.nih.gov/ij/docs/examples/stained-sections/index.html.

Bleomycin-induced experimental fibrosis. Skin fibrosis was induced in 6-week-old, pathogen-free, male C57BL/6 mice (Janvier) by injection of bleomycin as previously described (24,33). Subcutaneous injections of 100 μl 0.9% NaCl, the solvent for bleomycin, were used as controls. To investigate the effects of CAR activation on fibrosis, one group with 6 mice was treated for 3 weeks with TCPOBOP at a concentration of 5 μ*M* once a day by intraperitoneal injections. Two control

groups with 6 mice each were used. One group was treated intraperitoneally with DMSO (the solvent of TCPOBOP) and injected with bleomycin, and one group was treated intraperitoneally with DMSO and injected with NaCl. After 21 days, animals were killed by cervical dislocation.

Induction of dermal fibrosis in mice by local injections of attenuated adenoviruses overexpressing a constitutively active TGFβ receptor type I (TGFβRI). The effects of constitutive activation of TGFβ signaling using a constitutively active TGFβRI mutation in mice have been described previously (34,35). In the model used in this study, a mutation of Thr204 in the GS domain yields constitutively active receptors that can signal transcriptional responses in the absence of TGFβ (36,37). Dermal fibrosis was induced in pathogen-free, male C57BL/6 mice (Janvier) by intracutaneous injections of replication-deficient type V adenoviruses overexpressing a constitutively active TGFβRI (TβRI-CA) in defined areas of 1 cm^2 on the upper back.

Adenoviral vectors (6.7×10^7 infectious units) were injected at 4 weeks and 8 weeks of age. At 12 weeks of age, mice were killed by cervical dislocation. Six mice were infected with TβRI-CA and treated with TCPOBOP at a concentration of 5 μM once a day by subcutaneous injection. Six mice infected with TβRI-CA and treated with DMSO and another 6 mice infected with an adenovirus carrying a LacZ reporter gene (AdLacZ) and treated with DMSO were used as positive and negative controls, respectively. Constitutive activation of TGFβ signaling was confirmed in LacZ- and TβRI-CA–infected mice by immunofluorescence staining for phospho-Smad2/3 (data not shown).

Histologic analysis. Lesional skin was fixed in 4% formalin and embedded in paraffin. Histologic sections were stained with hematoxylin and eosin for the determination of dermal thickness. Dermal thickness at the injection sites was analyzed with a Nikon Eclipse 80i microscope as previously described (27,38). Mice were evaluated by 2 examiners (JA and JHWD) who were blinded with regard to treatment.

Statistical analysis. Data are expressed as the mean ± SEM. The Wilcoxon rank test for related samples and the Mann-Whitney U test were used for statistical analyses. *P* values less than 0.05 were considered significant.

RESULTS

Increased expression of CAR in the skin and dermal fibroblasts of SSc patients. First we analyzed the expression of CAR in 8 patients with SSc and in 5 controls. An overexpression of CAR protein was observed in SSc patients compared to controls (Figures 1A and B). Positive staining for CAR was detected in perivascular cells, endothelial cells, and fibroblasts (Figures 1C and D). In addition, staining for CAR was more intense in SSc patients than in controls ($P = 0.003$) (Figure 1E). We did not find any difference in CAR expression related to age, disease duration, or cutaneous subset of SSc. Next, we investigated CAR expression in explanted early-passage SSc and healthy control skin

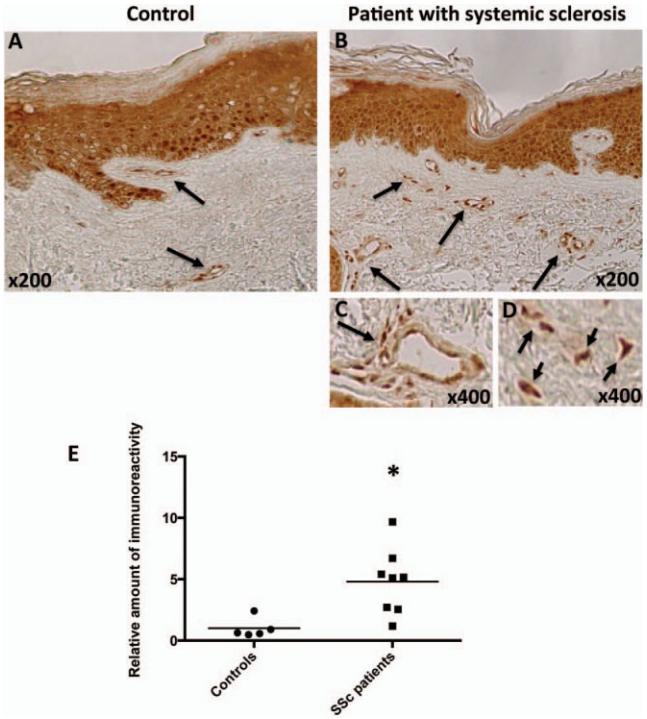

Figure 1. Overexpression of the nuclear receptor constitutive androstane receptor (CAR) in the skin of patients with systemic sclerosis (SSc). **A** and **B,** Overexpression of CAR protein in an ex vivo skin biopsy specimen from an SSc patient (**B**) compared to a control (**A**), detected by immunohistochemistry. **C** and **D,** Positive staining for CAR in perivascular cells (**C**) and fibroblasts (**D**) in skin biopsy specimens from SSc patients. In **A–D, arrows** indicate CAR-positive cells. **E,** Increased intensity of immunostaining for CAR in SSc patients compared to controls, as assessed with ImageJ software. Symbols represent individual patients; horizontal lines show the mean. * = $P < 0.05$ versus controls.

fibroblasts. Consistent with the up-regulation of CAR in the skin of SSc patients, the levels of messenger RNA (mRNA) for CAR were increased by a mean ± SEM of 189 ± 11% ($P = 0.02$) (Figure 2A). Similar results were observed at the protein level (Figure 2B) (additional results are available from the author upon request).

Induction of CAR expression by TGFβ. We next investigated whether the overexpression of CAR in SSc fibroblasts might be mediated by TGFβ. Stimulation of healthy fibroblasts with TGFβ increased CAR mRNA and protein levels. The maximal induction of CAR by TGFβ was observed after 24 hours (Figures 2C and D). To determine whether the induction of CAR by TGFβ depends on canonical Smad signaling, dermal fibroblasts were treated with SD208 (1 μM), a potent TGFβRI kinase inhibitor, or with SIS3 (3 μM), a novel Smad3-specific inhibitor, prior to stimulation with

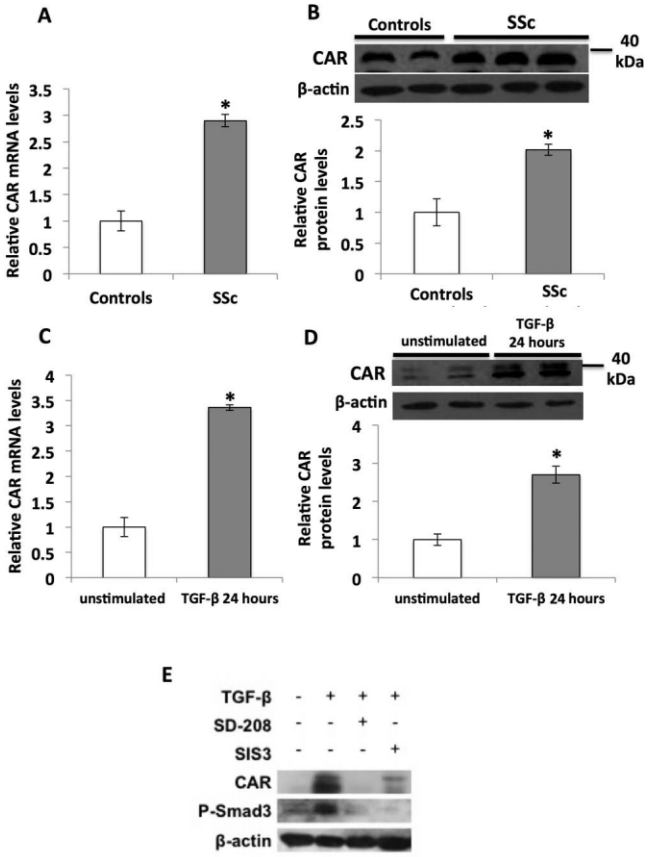

Figure 2. Induction of the expression of constitutive androstane receptor (CAR) by transforming growth factor β (TGFβ) in a Smad-dependent manner. **A,** Constitutive up-regulation of CAR mRNA in systemic sclerosis (SSc) fibroblasts compared to control fibroblasts. **B,** Western blot analysis showing overexpression of CAR protein in SSc fibroblasts. **C,** Up-regulation of CAR mRNA in healthy fibroblasts stimulated with TGFβ. **D,** Western blot analysis showing up-regulation of CAR protein levels in healthy fibroblasts stimulated with TGFβ. In **A–D,** bars show the mean ± SEM (n = 5 samples per group). $* = P < 0.05$ versus controls or unstimulated cells. **E,** Decreased TGFβ-induced phosphorylation of Smad3 and significant reduction in CAR protein expression in dermal fibroblasts treated with SD208, a potent TGFβ receptor type I kinase inhibitor, or with SIS3, a novel Smad3-specific inhibitor.

TGFβ. Treatment of fibroblasts with SD208 or SIS3 led to decreased TGFβ-induced phosphorylation of Smad3 and to a significant reduction in CAR protein expression (Figure 2E). These data suggest that TGFβ-induced CAR expression is mediated by canonical Smad signaling.

Sensitization of fibroblasts to the profibrotic effects of TGFβ by CAR activation. We next determined whether specific CAR activation by CITCO in healthy dermal fibroblasts could modulate collagen production. Treatment with 5 μM of CITCO resulted in a significant increase in the transcription of CAR in healthy and SSc dermal fibroblasts within 24 hours (results are available from the author upon request). Stimulation of healthy and SSc dermal fibroblasts with CITCO for 24 hours also led to an up-regulation of CAR protein, both in the cytoplasm and the nucleus (results are available from the author upon request). These data demonstrate the potent ability of CITCO to induce CAR mRNA and protein in healthy and SSc dermal fibroblasts.

Treatment of fibroblasts with 5 μM of CITCO led to slightly increased levels of mRNA for COL1A2 and increased release of collagen protein into cell culture supernatants (Figures 3A–C). However, although the profibrotic effects of CITCO alone were mild, CITCO sensitized fibroblasts to the profibrotic effects of TGFβ. The levels of mRNA for COL1A1 and COL1A2 were higher in fibroblasts costimulated with CITCO and TGFβ than in fibroblasts stimulated with TGFβ only (Figures 3A and B). This result was confirmed at the protein level, with a significantly increased release of collagen in culture supernatants of fibroblasts treated with CITCO and TGFβ compared to culture supernatants of fibroblasts stimulated with TGFβ only (Figure 3C). Similar results were observed in SSc fibroblasts (data not shown). To further demonstrate that CAR potentiates the stimulatory effects of TGFβ on the transcription of type I collagen, we performed transfection assays with a luciferase reporter construct under control of the −353-bp to +58-bp COL1A2 promoter and stimulated transfected fibroblasts with TGFβ in the presence or absence of CITCO. Consistent with previous results, coincubation with CITCO further increased the stimulatory effects of TGFβ on COL1A2 promoter activity (Figure 3D).

Although CITCO is considered to be specific for CAR, we aimed to exclude the possibility that the observed sensitization toward TGFβ is mediated by off-target effects. We therefore overexpressed CAR in dermal fibroblasts. Overexpression of CAR enhanced the susceptibility of fibroblasts to the profibrotic effects of TGFβ, with more pronounced increases in COL1A1 and COL1A2 mRNA, collagen protein, α-SMA expression, and stress fiber formation in fibroblasts overexpressing CAR that were stimulated with TGFβ than in fibroblasts transfected with control plasmids (Figures 4A–C) (additional results are available from the author upon request). In addition, we performed site-directed mutagenesis to delete the DNA binding domain of

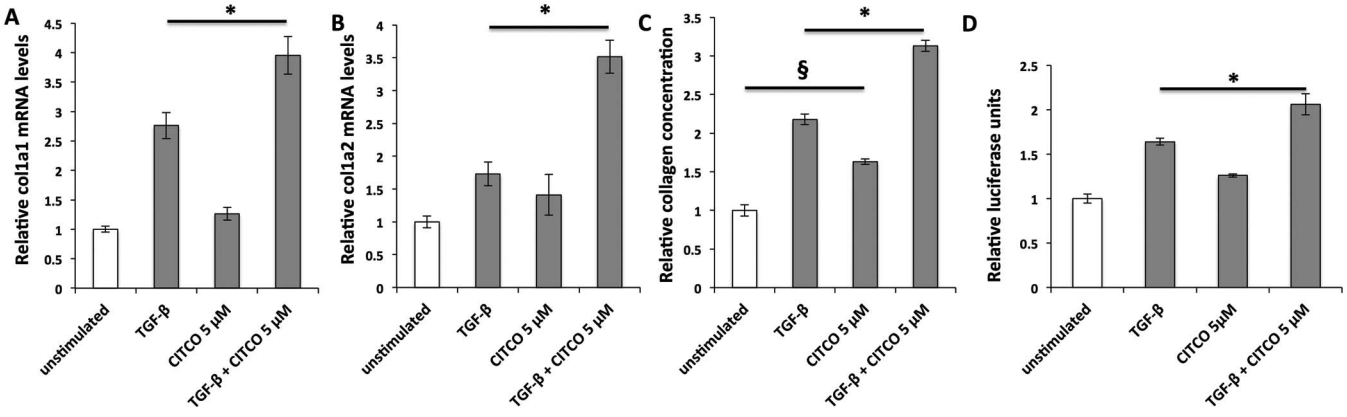

Figure 3. Sensitization of fibroblasts to the profibrotic effects of transforming growth factor β (TGFβ) upon activation of constitutive androstane receptor (CAR). **A–C,** Up-regulation of COL1A1 mRNA (**A**), COL1A2 mRNA (**B**), and collagen protein (**C**) in fibroblasts coincubated with CITCO and TGFβ compared to fibroblasts stimulated with TGFβ alone. **D,** Increase in the stimulatory effects of TGFβ on COL1A2 reporter assay transcriptional activity after activation of CAR by CITCO. Bars show the mean ± SEM (n = 5 samples per group). $* = P < 0.05$; § = $P < 0.05$.

CAR. We observed that fibroblasts overexpressing this mutated CAR had reduced collagen synthesis (Figures 4A–C) and myofibroblast differentiation (results available from the author upon request) upon TGFβ stimulation compared to fibroblasts overexpressing CAR and fibroblasts transfected with control vectors.

Enhanced Smad signaling upon stimulation with CITCO. Since we showed that CAR activation increased the susceptibility of dermal fibroblasts to TGFβ stimulation, we next focused on the effect of CAR activation on canonical Smad signaling. To evaluate the effects of CITCO on Smad signaling, we transduced fibroblasts with Ad-CAGA-Luc constructs, in which luciferase expression is controlled by Smad-binding elements (SBE). As expected, stimulation of fibroblasts with TGFβ markedly increased SBE reporter activity (Figure 4D). CITCO alone modestly increased SBE reporter activity. Stimulation with TGFβ in the presence of CITCO resulted in significantly enhanced activation of SBE reporter activity compared with TGFβ alone (Figure

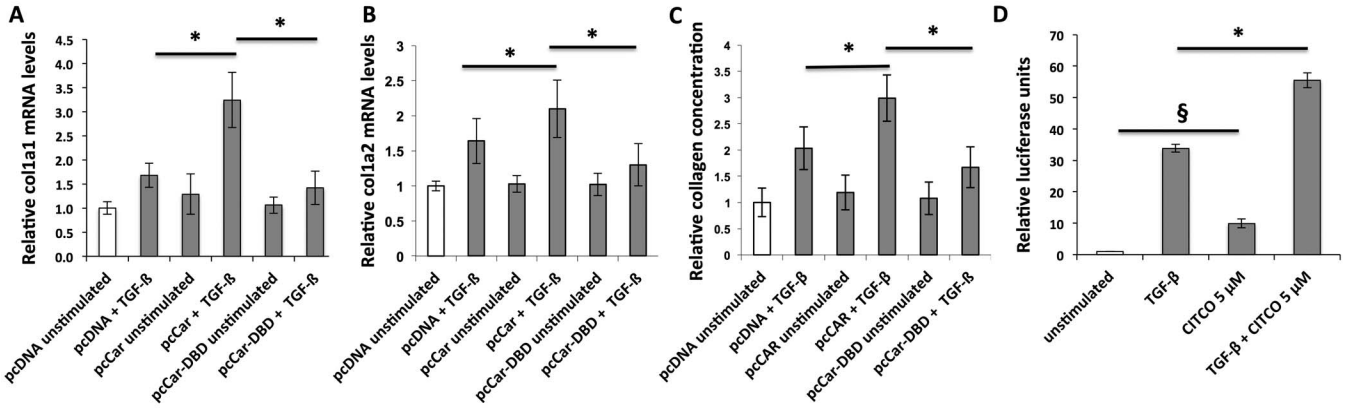

Figure 4. Enhanced transforming growth factor β (TGFβ) signaling in fibroblasts overexpressing constitutive androstane receptor (CAR). **A–C,** Enhanced susceptibility of fibroblasts to the profibrotic effects of TGFβ upon overexpression of CAR. Fibroblasts overexpressing CAR and stimulated with TGFβ had pronounced increases in COL1A1 mRNA (**A**), COL1A2 mRNA (**B**), and collagen protein (**C**) compared to fibroblasts transfected with control vectors and stimulated with TGFβ. Fibroblasts overexpressing mutated CAR (truncation of DNA binding domain [DBD]) displayed less susceptibility to TGFβ compared to fibroblasts overexpressing CAR or transfected with control vectors. **D,** Increase in the stimulatory effects of TGFβ in Smad reporter assays after treatment with CITCO. Bars show the mean ± SEM (n = 5 samples per group). $* = P < 0.05$; § = $P < 0.05$.

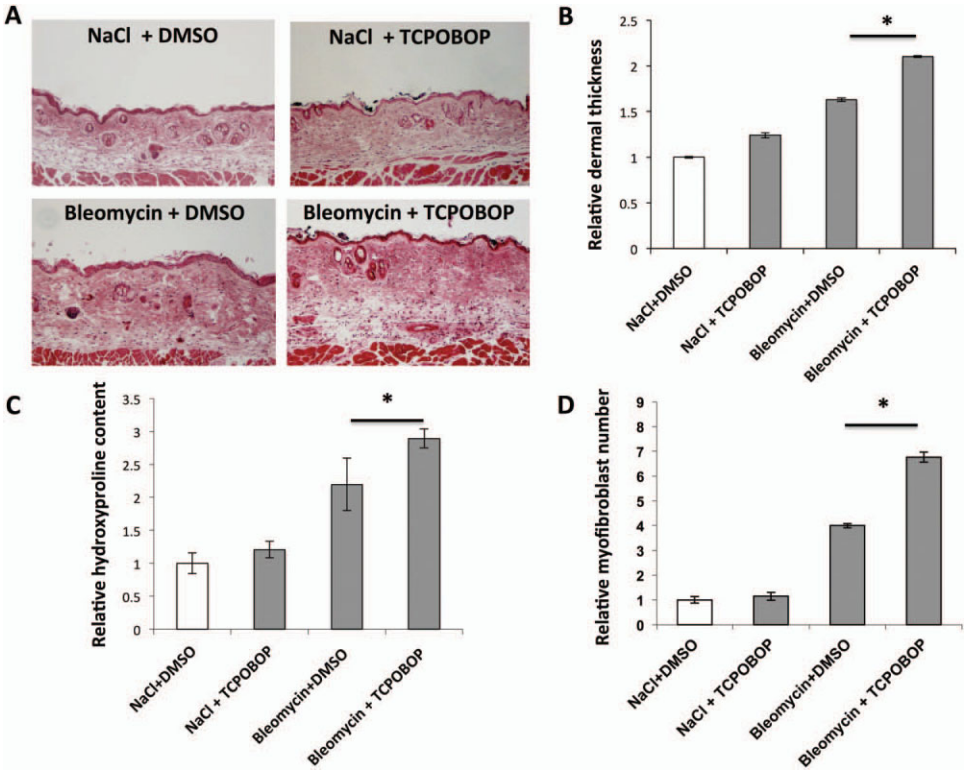

Figure 5. Exacerbation of bleomycin-induced skin fibrosis upon activation of constitutive androstane receptor (CAR) by TCPOBOP. **A,** Representative mouse skin sections stained with hematoxylin and eosin. Original magnification × 100. **B–D,** Increased dermal thickness (**B**), hydroxyproline content (**C**), and myofibroblast count (**D**) in mice treated with TCPOBOP and challenged with bleomycin compared to mice that received bleomycin and sham treatment. In mice challenged with NaCl, TCPOBOP treatment resulted in a modest increase in dermal thickness and collagen content compared to mice treated intraperitoneally with DMSO. Bars show the mean ± SEM (n = 6 mice per group). * = $P < 0.05$. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.38819/abstract.

4D). These results indicate that CAR activation enhances canonical Smad signaling in response to TGFβ in fibroblasts.

Enhanced bleomycin-induced dermal fibrosis in mice upon activation of CAR by TCPOBOP. To determine the profibrotic potential of CAR activation, we first evaluated the effects of the CAR agonist TCPOBOP in the bleomycin-induced dermal fibrosis model. In mice challenged with NaCl, TCPOBOP increased CAR protein expression (results are available from the author upon request) and resulted in a modest increase in dermal thickness and collagen content compared to mice treated intraperitoneally with DMSO (Figures 5A–C). In mice challenged with bleomycin, TCPOBOP markedly increased CAR protein expression (results are available from the author upon request), and this resulted in a significant increase in dermal thickening, by a mean ± SEM of 74 ± 6% ($P = 0.0003$) (Figures 5A and B). Analyses of the hydroxyproline content and of myofibroblast counts confirmed increased accumulation of collagen and increased activation of fibroblasts upon CAR activation (Figures 5C and D) (additional results are available from the author upon request).

Enhanced TβRI-CA–induced fibrosis in mice upon activation of CAR by TCPOBOP. Since we demonstrated that CAR activation sensitized the profibrotic effects of TGFβ in vitro, we next aimed to confirm the profibrotic effects of CAR activation by TCPOBOP in a murine model of TGFβ-driven fibrosis, which also serves as a model of later, less inflammatory stages of SSc with endogenous activation of fibroblasts (39). AdLacZ-infected mice that received TCPOBOP showed a modest increase in dermal thickness, collagen content, and myofibroblast count compared to AdLacZ-infected mice that received DMSO (Figures 6A–D) (additional results are available from the author upon request). Treatment with TCPOBOP significantly en-

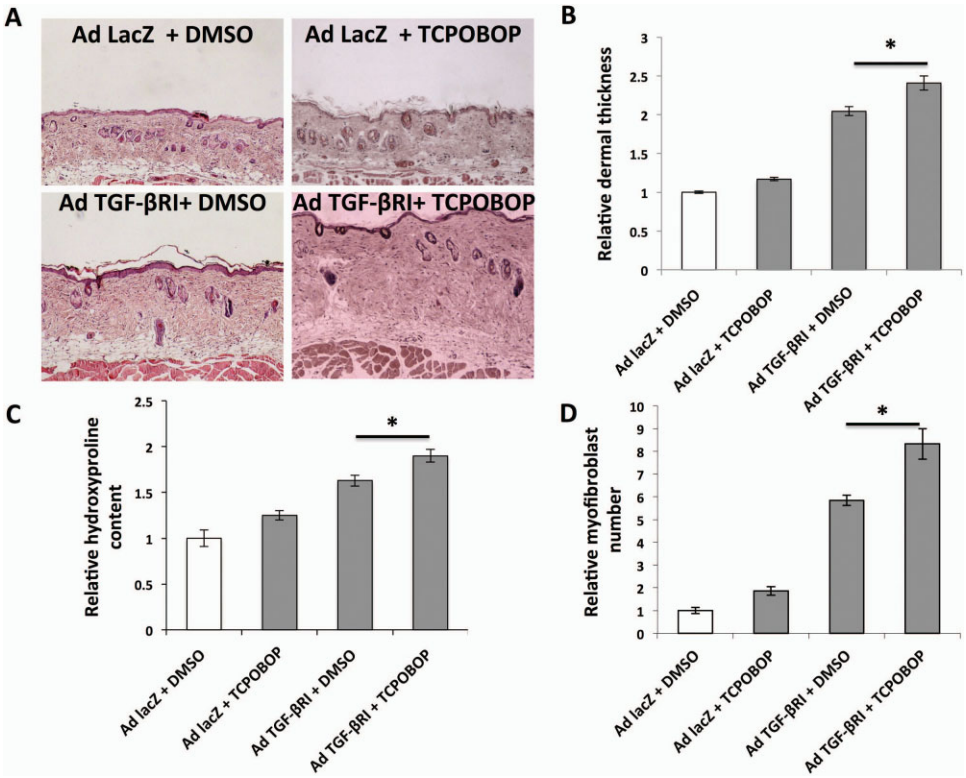

Figure 6. Enhanced constitutively active transforming growth factor β receptor type I (TβRI-CA)–induced fibrosis in mice with constitutive androstane receptor (CAR) activated by TCPOBOP. **A,** Representative images of mouse skin sections stained with hematoxylin and eosin. Original magnification $\times$ 100. **B–D,** Increased dermal thickness (**B**), hydroxyproline content (**C**), and myofibroblast count (**D**) in TCPOBOP-treated TβRI-CA–infected mice compared to sham-treated TβRI-CA–infected mice. In AdLacZ-infected mice, TCPOBOP treatment resulted in a modest increase in dermal thickness, collagen content, and myofibroblast count compared to mice treated with DMSO. Bars show the mean $\pm$ SEM (n = 6 mice per group). $*$ = $P < 0.05$. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.38819/abstract.

hanced TβRI-CA–driven fibrosis, with a 35 $\pm$ 5% increase in dermal thickening compared to sham-treated TβRI-CA–infected mice (P = 0.002) (Figures 6A and B). The hydroxyproline content and myofibroblast count were also higher in TβRI-CA–infected mice treated with TCPOBOP than in sham-treated TβRI-CA–infected mice (Figures 6C and D) (additional results are available from the author upon request).

Enhanced TGFβ signaling in experimental fibrosis upon activation of CAR signaling. We demonstrated that activation of CAR enhances TGFβ/Smad signaling in cultured fibroblasts to stimulate the release of collagen. To show that CAR signaling also up-regulates canonical Smad signaling in experimental fibrosis, we analyzed the nuclear levels of phospho-Smad2/3 signaling in our experimental mouse models. Levels of phospho-Smad2/3 were increased upon challenge with bleomycin or TβRI-CA. Treatment with the CAR agonist TCPOBOP further stimulated canonical Smad signaling, with increased nuclear levels of phospho-Smad2/3 compared to sham-treated controls (results are available from the author upon request).

DISCUSSION

We demonstrate for the first time that the nuclear receptor CAR/NR1I3 is overexpressed in fibrotic skin of patients with SSc. Our results also suggest a constitutive overexpression of CAR in SSc fibroblasts that might depend on stimulation by TGFβ, since the stimulation of healthy fibroblasts with TGFβ led to an increased expression of CAR at the mRNA and protein levels. These data suggest that TGFβ might be an important stimulus for the activation of CAR in SSc fibroblasts.

We assessed for the first time the effects of the selective CAR activator CITCO on fibroblasts. CAR activation displayed potent profibrotic effects in vitro. In particular, we demonstrated that CITCO sensitizes

fibroblasts to the profibrotic effect of TGFβ on collagen synthesis. CITCO markedly increased the induction of extracellular matrix proteins by TGFβ at the mRNA and protein levels and increased transcription activity. These findings were confirmed by increased collagen synthesis and myofibroblast differentiation after overexpression of CAR in healthy fibroblasts followed by stimulation with TGFβ, and decreased susceptibility to TGFβ after overexpression of mutated CAR (truncation of the DNA binding domain). Moreover, we demonstrated that CITCO-mediated CAR activation in fibroblasts directly affects the canonical Smad pathway of TGFβ signaling. Altogether, these results suggest that CAR is involved in a positive feedback loop which amplifies TGFβ signaling, since CAR is activated by TGFβ in SSc and further enhances the stimulatory effects of TGFβ in fibroblasts.

Nuclear receptors are progressively emerging as crucial players in fibrotic diseases. Our results now add CAR to the list of nuclear receptors with regulatory effects on fibroblast activation. Previously, studies have highlighted the regulatory effects of peroxisome proliferator–activated receptor γ (PPARγ) and PXR on fibroblasts. However, all of those nuclear receptors display different functions and effects. PXR and CAR are the most important members of the NR1I nuclear receptor family (40). They both act as a sensor of toxic byproducts of endogenous metabolism and of exogenous chemicals and enhance their elimination. Effects of PXR on fibrosis differ from those of CAR, since PXR interferes with interleukin-13 release from Th2 cells, which leads to decreased TGFβ signaling, fibroblast activation, and collagen release. PPARγ also regulates fibrotic responses by modulating TGFβ signaling in fibroblasts. The levels of PPARγ are markedly diminished in skin biopsy specimens from patients with SSc and in fibroblasts explanted from the lesional skin in a TGFβ-dependent manner. Activation of PPARγ by synthetic ligands reduces fibroblast activation and prevents bleomycin-induced skin fibrosis (41–43). Further studies are required to determine whether the different nuclear receptors modulate each other, e.g., by co-binding to the same promoters or by competition for cofactors.

In this study, we provide evidence of the profibrotic effects of CAR for the development of skin fibrosis in two mouse models of SSc. We first used the mouse model of bleomycin-induced dermal fibrosis. This model is characterized by dense inflammatory infiltrates in lesional skin. Inflammatory cells are thought to contribute to the initial activation of resident fibroblasts by the release of profibrotic mediators. Thus, the mouse model of bleomycin-induced dermal fibrosis mimics early stages of SSc, but is less representative of later stages of SSc, when inflammatory infiltrates are scarce and fibroblasts are endogenously activated (2). We next aimed to confirm our findings in another model in which fibrosis is induced by selective activation of TGFβ signaling. This mouse model was also of interest because persistent overproduction of extracellular matrix proteins occurs in the absence of inflammatory infiltrates, which better represents later stages of SSc (39). We also demonstrated potent profibrotic effects of CAR activation in this model. Since we showed that the exacerbation of bleomycin-induced fibrosis and AdTβRI-CA–induced fibrosis upon CAR activation are mediated by increased TGFβ signaling, our data indicate that CAR activation increases the profibrotic effects of TGFβ in vivo in both inflammation-dependent and -independent fibrosis.

Interestingly, CAR may modulate fibroblast activation not only in the skin, but also in other organs. CAR has been implicated in liver fibrosis, although its precise role is not completely elucidated. CAR has been found responsible for exacerbating hepatic injury and fibrosis in a dietary model of nonalcoholic steatohepatitis via up-regulation of lipid peroxidation (11). On the other hand, liver expression of CAR has been reported to be decreased in patients with stage 3 fibrosis related to chronic hepatitis C, and TCPOBOP has been shown to attenuate Fas-induced murine liver damage and fibrosis by altering Bcl-2 proteins (44,45).

In summary, we present the first evidence that CAR is activated in a TGFβ-dependent manner in SSc and further enhances the stimulatory effects of TGFβ on fibroblasts, thereby creating a positive feedback loop. Activation of CAR also enhances TGFβ signaling in vivo, and treatment with CAR agonists exacerbates fibrosis in mouse models of SSc. Thus, CAR might contribute to enhance TGFβ signaling and persistent activation of fibroblasts in SSc.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Avouac had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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