Carbon Monoxide Modulates α -Smooth Muscle Actin and Small Proline Rich-1a Expression in Fibrosis

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Carbon monoxide (CO) is a biologically active molecule produced in the body by the stress-inducible enzyme, heme oxygenase. We have previously shown that CO suppresses fibrosis in a murine bleomycin model. To investigate the mechanisms by which CO opposes fibrogenesis, we performed gene expression profiling of fibroblasts treated with transforming growth factor- β_1 and CO. The most highly differentially expressed categories of genes included those related to muscular system development and the small proline-rich family of proteins. We confirmed *in vitro*, and in an *in vivo* bleomycin model of lung fibrosis, that CO suppresses α -smooth muscle actin expression and enhances small proline-rich protein-1a expression. We further show that these effects of CO depend upon signaling via the extracellular signal-regulated kinase pathway. Our results demonstrate novel transcriptional targets for CO and further elucidate the mechanism by which CO suppresses fibrosis.

Keywords: carbon monoxide; heme oxygenase-1; lung fibrosis; small proline-rich protein; α -smooth muscle actin

Previous studies from our laboratory indicate that matrix production by fibroblasts *in vitro* and *in vivo* is inhibited by exposure to a low concentration of carbon monoxide (CO) (1). CO is a biologically active molecule produced in the body via catabolism of heme by the stress-inducible enzyme, heme oxygenase. It has protective effects in the setting of lung injury from a number of different causes (2–5), including bleomycin-induced fibrosis (1). The mechanism for the suppression of fibrosis by CO is not known. To identify individual genes or pathways responsible for the effects of CO, we performed gene profiling of murine fibroblasts exposed to transforming growth factor (TGF)- β_1 alone or TGF- β_1 and CO for 24 hours. Among the functional categories of genes, those related to the ubiquitin proteasome pathway and to skeletal and muscular system development were most highly differentially expressed in the microarray. Genes in this second category are associated with the myofibroblast phenotype, and are known to be highly induced in fibroblasts by TGF- β_1 (6). Fibroblasts expressing proteins involved in cell contraction, in particular α -smooth muscle actin (α -SMA), are responsible for the generation of contractile force and extracellular matrix deposition associated with wound healing (7).

Of the individual genes that were most highly differentially expressed, 3 of the top 10 most statistically significant genes belonged to the family of small proline-rich (Sprr) proteins. All

CLINICAL RELEVANCE

Although carbon monoxide (CO) has been shown to confer protection in the setting of lung fibrosis, the mechanism by which it does this remains poorly understood. This study points to potential mechanisms by which CO may inhibit fibrogenesis and introduces a new candidate molecule that may be involved in the pathogenesis of fibrosis: small proline-rich protein-1.

three of these genes were modestly induced by TGF- β_1 , but were greatly upregulated by the addition of CO. Members of this family of genes was originally identified as markers of terminal squamous cell differentiation (8), acting as substrates of transglutaminase in the formation of the crosslinked envelope (9). Sprr genes comprise a four-member family (Sprr1–4) contained within a 170-kb region located on chromosome 1q21 (10). Given their biological annotation as mediators of keratinization, Sprr genes did not appear in any of the functional groupings of genes that were differentially expressed in the microarray. To date, there is no known function of Sprr family genes in fibroblasts or in the progression of fibrosis. However, Sprr genes have been identified as being among the most highly upregulated genes in the setting of injury or inflammation in the lung, intestine, biliary tree, skin, arterial intima, cardiomyocytes, and neurons (11–13). In the lung, cigarette smoke and vitamin A have been shown to induce epithelial expression of Sprr1a, and allergic inflammation in ovalbumin and aspergillus models has been shown to increase Sprr2a, Sprr2b, and Sprr1a expression (14). In these models of allergic inflammation, Sprr2 expression was shown to be increased in a broad spectrum of lung epithelial cells, as well as infiltrating mononuclear cells. The function of these proteins in the lung remains unknown. Recent reports suggest that Sprr1a provides protection against cardiomyocyte death in the setting of ischemia-reperfusion injury (12), and that Sprr2a promotes wound healing through enhanced epithelial cell migration (15). These reports suggest possible functions for Sprr proteins in the lung, but these have yet to be investigated.

MATERIALS AND METHODS

Animals

Male C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME). All animals were housed in accordance with guidelines from the American Association for Laboratory Animal Care and Research Protocols, and were approved by the Animal Care and Use Committees of Pittsburgh University School of Medicine. Bleomycin (Bristol-Myers Squibb, Princeton, NJ) was administered intratracheally as previously described (1) at a dose of 0.075 units/mouse in 50 μ l sterile saline.

CO Exposures

Mice or cells were exposed to compressed air with CO at a concentration of 250 ppm. CO exposure of mice began immediately after

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treatment with bleomycin, and exposure of cells began immediately after stimulation with TGF- β_1 . For cell culture experiments, the atmosphere included 5% CO₂ for adjustment of pH. Details of CO administration have been previously described (3).

Cell Culture Experiments

Mouse lung fibroblasts were isolated from C57Bl/6 mice and were maintained and grown in Dulbecco's modified Eagle's medium containing 10% FBS and 1% pen-strep. TGF- β_1 (R&D Systems, Minneapolis, MN) was reconstituted in sterile 4 mM HCL containing 1% BSA. Tricarbonyldichlororuthenium(II) dimer (CORM3) (Sigma, St. Louis, MO), PD098059, and SB203580 (Calbiochem, San Diego, CA) were dissolved in DMSO; control cells were treated with an equivalent volume of DMSO alone.

Microscopic Analysis of α -SMA Expression

At 7 days after the administration of bleomycin with or without the addition of CO, lungs were harvested and inflation fixed with 2% paraformaldehyde in PBS and immersed in the same fixative for 1 hour. The lungs were then placed in 30% sucrose in PBS overnight, and flash frozen in liquid nitrogen-cooled isopentane. Lung cryosections (5 μ m) were cut and washed sequentially in PBS, then in PBS containing 0.5% BSA and 0.15% glycine. The sections were washed and incubated in a 1:1,000 dilution of monoclonal cy3-conjugated anti-mouse α -SMA antibody (American Research Products, Inc., Belmont, MA), with anti-mouse vimentin (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-mouse pro-surfactant protein C (Chemicon, Atlanta, GA) for 1 hour. After three washes in BSA, the sections were stained with a 1:3,000 dilution of cy3-conjugated AffinPure Goat Anti-Rabbit IgG (H+L; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 1 hour, washed and stained with Hoechst dye (Sigma) for 30 seconds, and mounted in Gelvatol (Monsanto, St. Louis, MO). Images were collected with an Olympus Provis microscope (Olympus, Tokyo, Japan) as well as an Olympus Fvoview scanning confocal microscope.

Cell Extracts and Western Blot Analysis

Cellular protein extracts were collected and electrophoresed under denaturing conditions as previously described (1). α -SMA and Sprr1a were detected using rabbit polyclonal antibodies according to the manufacturer's suggestions (Santa Cruz Biotechnology and Abcam [Cambridge, MA], respectively). The imaging and analysis of the blots were performed as previously described (1).

α -SMA Promoter Constructs and Luciferase Assay

The α -SMA construct was kindly provided by Dr. S.H. Phan (Department of Pathology, University of Michigan Medical School, Ann Arbor, MI). Details have been previously published (16, 17). Transfection of fibroblasts was performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. pRL-TK control vector (Promega, Madison, WI) was used to normalize transfection efficiencies. Luciferase activity was assessed using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. The construct was tested in triplicate, and the data shown represent three to four independent experiments.

DNA Microarray Hybridization and Analysis

Fibroblasts treated with TGF- β_1 alone for 24 hours were compared with fibroblasts treated with both TGF- β_1 and CO 250 ppm for 24 hours. Control groups included untreated fibroblasts and cells exposed to CO alone. Extracted RNA was used to generate labeled cRNA and then hybridized to Codelink mouse 20K arrays that were scanned and normalized as previously described (18). Statistical analyses were performed using Scoregene as previously described (19). For data mining and visualization, we used Genomica, Ingenuity Pathways Analysis (Ingenuity Systems, Redwood City, CA) (20) and Spotfire Decision Site 8.0 (Spotfire Inc., Göteborg, Sweden). To identify individual genes that best distinguish between TGF- β_1 -treated and CO plus TGF- β_1 -treated cells, we used the threshold number of misclassification (TNoM) score (21), which counts the number of classification errors that occur between compared groups for each gene of the dataset, as well as the Student's *t* test and significance analysis of microarrays (SAM) (22).

Statistical Analysis

Experiments were repeated at least three times, and statistical evaluation was performed using an unpaired Student's *t* test. In all cases, two-tailed *P* values less than 0.05 were considered statistically significant.

RESULTS

Expression Profiling in Response to TGF- β_1 and CO

To examine the effect of CO on fibroblast behavior in the setting of TGF- β_1 stimulation, gene expression was compared between fibroblasts stimulated with TGF- β_1 alone and those treated with both TGF- β_1 and CO for a period of 24 hours. The most significantly enriched canonical pathway was the protein ubiquitination pathway, and the most significantly enriched functional categories included skeletal and muscular system development and function, cell migration, and cell death. Although most of the statistically significant genes were included in the functional categories listed above, strikingly, three of the most affected genes belonged to the small proline-rich family of proteins (Sprr1a, Sprr2a, and Sprr3). This protein family is not included in any of the functional categories or canonical pathways listed, as the gene ontology (GO) annotation biological process for these genes includes epidermis development and keratinization. These genes are therefore discussed separately from the brief description of each functional group of genes provided subsequently here.

The Protein Ubiquitination Pathway

The ubiquitin proteasome pathway, which is responsible for degradation of the majority of intracellular proteins, had the lowest log *P* value in the microarray analysis. Ubiquitin, which marks proteins for degradation, is affected by CO treatment, as are components of the 26S proteasome. All three enzymatic components required to link chains of ubiquitin to proteins destined for degradation (E1 [ubiquitin-activating enzyme], E2 [ubiquitin-carrier or conjugating proteins], E3 [ubiquitin-protein ligase]) have members demonstrating decreased transcription in response to CO. There is little known about the effects of CO on protein degradation. A recent study examining the effect of CO on endothelial cell apoptosis reported inclusion bodies with ubiquitin-decorated material, indicating altered proteolytic processing in response to CO (23). The functional consequence of this effect is unknown.

Genes Related to Skeletal and Muscular System Development and Function

With the exception of EGR1 (5, 24), the genes in this functional category were not previously known to be regulated by CO, although the majority are known to be TGF- β_1 responsive. For example, cytoskeletal genes involved in cell contraction were upregulated by TGF- β_1 , as might be expected during transition to a myofibroblast phenotype (ACTA2, TAGLN, ACTG2, TNNT3); expression of these genes was inhibited by CO. Similarly, the expressions of genes known to regulate myogenesis changed in response to both TGF- β_1 and CO, including RRAS2, WNT10B, USMG1, and DACH2. One of the genes in this category most profoundly suppressed by TGF- β_1 was the transcriptional regulator, heat shock factor (HSF)-4 (10-fold decrease, *P* = 0.00001); the addition of CO antagonized the effect of TGF- β_1 , partially restoring the expression of HSF4. We confirmed these findings by real-time PCR (shown in Figure 1). Known HSF4 target genes, fibroblast growth factor 7 and heat shock proteins 68 were also differentially expressed in our array in response to TGF- β_1 and CO. This finding is notable as

Figure 1. Small proline-rich (Sprr) family proteins are highly up-regulated in fibroblasts by the addition of carbon monoxide (CO) to transforming growth factor (TGF)-β₁ stimulation. (A) The scatter plot shows Sprr family genes highlighted in red (Sprr1a is shown in green). The relative expression of Sprr1a, Sprr2a, and Sprr3 mRNA in response to TGF-β₁ treatment and TGF-β₁ plus CO treatment are shown by microarray analysis (B) and real-time PCR (C). (D) Expression of heat shock factor 4 (HSF4) mRNA in response to TGF-β₁ treatment assessed by microarray analysis and real-time PCR.

the first report of regulation of HSF4 by TGF-β₁ and as a potential link between the heat shock response and fibrosis. HSF4 is expressed primarily in the adult brain and lung, and has been implicated, along with TGF-β₁, in cataract formation (25).

Genes Related to Cell Movement

There is little known about the effects of CO on cell movement, although recent reports suggest that CO is capable of down-regulating leukocyte adhesion and migration (26, 27). The genes in this category overlap with the functional classification for tumor cell invasion, and the majority of these were previously known to be TGF-β₁ responsive. In addition to regulating tumor invasion, TGF-β₁ is thought to provide a stimulus for fibroblast migration in the setting of wound healing (28, 29). None of the genes in this functional classification was known to be responsive to CO, with the exception of EGR1, as described above. The genes affected by CO contribute to cell migration by a variety of mechanisms, such as cytoskeletal organization (e.g., ZYX, TAGLN, CRK, and NEDD9), proteolysis (e.g., SERPINA3, PLAUR, MMP9, and MMP13), cell adhesion (CD97), and transcriptional regulation (RUNX3 and EGR1).

Genes Related to Cell Death

Both TGF-β₁ and CO have previously been implicated in regulation of apoptosis (30–34). Although genes in this functional category regulate apoptosis by a variety of mechanisms, this grouping includes p53 homolog p63 and a number of genes in the p53 network (WFOX, EGR1, HSPA1B, and POLB). p53 Tumor suppressor is a transcription factor that coordinates cellular

responses to DNA damage and stress, initiating cell cycle arrest or triggering apoptosis. CO has previously been shown to induce growth arrest via modulation of expression of the p53 target gene, p21 (1, 35, 36), and the antiapoptotic action of CO in vascular smooth muscle cells has previously been associated with suppression of p53 expression (37). Our current findings support a role for p53 in CO-mediated suppression of apoptosis.

Small Proline-Rich Proteins

Of the individual genes that were most responsive to CO, three with the most statistically significantly up-regulated genes in response to CO belong to the family of Sprr proteins (Figure 1A). Sprr proteins were originally identified as markers for the terminal squamous cell differentiation and protein precursors of mammalian cornified envelope (9). This family of proteins is also expressed in nonsquamous cells, although their biological function in these tissues is not known. Several studies have shown that expression of Sprr1a and -2a is increased in response to cellular stress (11, 12, 38). We confirmed by real-time PCR that Sprr1a, Sprr2a, and Sprr3 mRNA are increased in fibroblasts stimulated concomitantly with TGF-β₁ and CO (Figure 1C).

Effect of CO on α-SMA Expression *In Vitro* and *In Vivo*

Given that genes related to skeletal and muscular system development were differentially expressed in the microarray experiment, we examined the effect of CO on fibroblast expression of α-SMA. Fibroblasts express α-SMA in response to TGF-β₁ stimulation, and this change is associated with

Figure 2. CO suppresses α -smooth muscle actin (α -SMA) expression *in vitro* and *in vivo*. Fibroblasts treated with TGF- β_1 (5 ng/ml) were subsequently exposed to CO (250 ppm) or control incubator air. (A) Cells were analyzed by Western blotting for α -SMA expression at the time points indicated. (B) Effect of varying doses of TGF- β_1 . (C) The effect of CO on TGF- β_1 -induced activation of the α -SMA promoter was examined using a rat wild-type promoter construct with a luciferase reporter. * $P < 0.05$ compared with control; # $P < 0.05$ compared with TGF- β_1 -treated. (D) The same suppressive effect of CO occurs *in vivo*; bleomycin-treated mice were exposed to inhaled CO (250 ppm) or room air for 1 week, and lungs were harvested for α -SMA staining. Image A is of untreated lung, image B is of bleomycin-treated lung, and image C is of bleomycin and CO-treated lung. Red stain represents α -SMA expression; immunofluorescence is quantitated in the accompanying graph (bleomycin-treated animals, $n = 4-9$; untreated control animals, $n = 1$). Quantitation excluded staining in airways and blood vessels to exclude smooth muscle cells. * $P < 0.05$ compared with bleomycin-treated wild type. (E) Costaining of α -SMA and cell markers vimentin and pro-pro-surfactant protein C. The majority of costaining (yellow) occurs in α -SMA and vimentin-stained cells shown in the lower image.

a profibrotic myofibroblast phenotype (39). We hypothesized that CO might inhibit lung fibrosis in part by suppressing the development of myofibroblasts from fibroblasts. To examine this question *in vitro*, cultured fibroblasts were stimulated with TGF- β_1 (5 ng/ml) and then exposed to CO or control air. Cell lysate was harvested after 6, 24, and 48 hours for Western blot analysis of α -SMA expression. As illustrated in Figure 2A, TGF- β_1 induced a time-dependent increase in α -SMA expression, and this was suppressed by treatment with CO. The same experiment was repeated using a TGF- β_1 dose range of 0.5–5 ng/ml (Figure 2B); CO suppressed α -SMA expression throughout this dose range. CO had a similar effect on TGF- β_1 -induced α -SMA promoter activity, as shown in Figure 2C. To test whether the suppressive effect of CO on α -SMA expression occurs *in vivo*, bleomycin-treated mice were exposed to inhaled CO (250 ppm) or room air for 1 week, and lungs were harvested for α -SMA staining. As shown in Figure 2D (image A), mice

that were untreated expressed little lung parenchymal α -SMA. After bleomycin treatment, α -SMA staining in the lung increased as expected (image B). Addition of inhaled CO to bleomycin treatment resulted in less α -SMA staining than bleomycin alone (image C). Quantitation of immunofluorescence for each group is shown in the Table 1. To address the question of which cell type was expressing α -SMA, costaining was performed using vimentin, which is primarily expressed in fibroblasts, and pro-surfactant protein C, which labels type-II pneumocytes. The greatest amount of costaining occurred with α -SMA and vimentin (Figure 2E).

Effect of CO on Sprr1a Expression *In Vitro* and *In Vivo*

As Sprr1a was the most significantly differentially expressed gene in the microarray experiment, and, as this molecule had previously been shown to confer protection against cellular stress in a manner similar to CO (12), we chose to focus on this

TABLE 1. FOUR HIGHEST-SCORING CATEGORIES OF GENES GROUPED ACCORDING TO BIOLOGICAL FUNCTION

Functional Category	Range <i>P</i> Values	Log <i>P</i> Value	Genes
Ubiquitin proteasome pathway	1×10^{-4} to 2×10^{-2}	-4.0	AMFR, BIRCH4, PSMB3, PSMB5, PSMB8, PSMD1, PSMD3, PSMD8, PSMD14, SMURF1, TCEB1, UBD, UBE2A, UBE2J2, USP1, USP5, USP26, USP38, USP9X, VDP, VHL
Skeletal and muscular system development and function	1×10^{-4} to 4×10^{-2}	-3.8	MMP9, MMP13, SMURF1, ANKH, MEOX1, PHC1, RPS6KA3, TP73L, SOX2, FHL2, ACTA2, TNNT2, TNNT3, TMOD4, WNT10B, EGR1, PLAUR, HSF4, PDLIM7, SPNB2, DACH2, RRAS2, USMG1, MTM1, TAGLN, ACTG2
Cell movement	2×10^{-4} to 4×10^{-2}	-3.7	PLAUR, TAGLN, CD97, MMP9, CRK, EGR1, ANGPTL4, APBB2, BDNF, CXCL13, DUSP1, FHL2, NEDD9, PENK, PPBP, SERPINA3, SEMA3C, ZYX, SH2D2A, CXCR6, PRKD1, TP73L, ALOX5AP
Cell death	2×10^{-4} to 4×10^{-2}	-3.6	MMP9, MMP13, BDNF, PPBP, PLAUR, TUB, RUNX3, ATP1A1, MME, HSPA1B, EGR1, WWOX, TRP63, STC2, AKT3, POLB, TLR3, HDAC2

member of the *Sprr* family in our further studies. We confirmed by Western blotting that *Sprr1a* protein is increased in fibroblasts exposed to TGF- β_1 , and that the increase is further enhanced by CO exposure (Figure 3A). In mice treated with intratracheal bleomycin and inhaled CO, the expression of

Sprr1a mRNA was significantly increased after 7 days (Figure 3B). The maximal expression of *Sprr1a* protein in whole-lung homogenate occurred at 7 days after bleomycin treatment, and persisted until 14 days (Figure 3C); the increase at 14 days was enhanced by inhaled CO (Figure 3D). The effect of CO on both α -SMA and *Sprr1a* expression *in vitro* was dose dependent (Figure 4A). The effect of exogenously administered CO gas was also recapitulated through use of a soluble CO donor molecule, CORM3 (Figure 4B).

The Effect of CO on α -SMA and *Sprr1a* Expression Depends upon Signaling Via Mitogen-Activated Protein Kinase Pathways

Sprr1a gene activation has previously been shown to depend upon extracellular signal-regulated kinase (ERK) activation downstream of gp130 (12). Because CO has been shown, in some systems, to activate Stat3 (2, 40), which is also downstream of gp130, we tested the effect of combined TGF- β_1 and CO stimulation on both ERK and Stat3 activation in fibroblasts. As shown in Figure 5A, TGF- β_1 stimulation led to a small increase in ERK phosphorylation at 6 hours; this effect was greatly enhanced by the addition of CO. Stat3 phosphorylation, on the other hand, was not increased by CO (Figure 5B). The mitogen-activated protein kinase kinase 1 (MEK1) inhibitor, PD098059, increased α -SMA expression in response to TGF- β_1 and diminished the inhibitory effect of CO. Inhibition of the ERK pathway with PD098059 also abrogated the effect of CO on *Sprr1a* expression, in this case preventing the enhanced expression of *Sprr1a* (Figure 5C). Given previously reported involvement of the p38 pathway in CO signaling (35, 41, 42), we also tested the effect of chemical inhibition of the p38 pathway with SB203580. Interestingly, SB203580 led to a complete abrogation of α -SMA expression (Figure 5D). SB203580 inhibited *Sprr1a* expression in a manner similar to PD098059.

DISCUSSION

Although the myofibroblast is thought to play a central role in the development of pulmonary fibrosis (39, 43–45), factors affecting the emergence of this fibroblast phenotype are still poorly understood. We have previously shown that CO inhibits the development of lung fibrosis in a murine bleomycin model (1), and we postulated that this effect of CO may be due to its ability to prevent the emergence of myofibroblasts in the lung. We used microarray to screen for genes or pathways that might contribute to the mechanism of action of CO, and made some expected and unexpected observations. The inhibition by CO of expression of genes related to skeletal and muscular system

Figure 3. TGF- β_1 and CO increase *Sprr1a* expression *in vitro* and *in vivo*. (A) Western blot of mouse lung fibroblasts stimulated with TGF- β_1 (2 ng/ml) and exposed to CO or control incubator air. (B) *Sprr1a* mRNA expression increases in whole-lung homogenate in response to bleomycin and CO (7 d). (C) *Sprr1a* protein expression is increased in whole-lung homogenate 7 and 14 days after bleomycin exposure. (D) *Sprr1a* protein expression is further increased in whole-lung homogenate of lungs of mice exposed to CO.

Figure 4. CO and the CO donor molecule, CORM3, alter Sprr1a and α -SMA expression in a dose-dependent fashion. (A) Treatment of fibroblasts *in vitro* with CO results in a dose-dependent suppression of α -SMA expression and increase in Sprr1a expression. (B) Treatment with the CO donor molecule, CORM3, has a similar effect.

development and function conformed to our hypothesis that CO was capable of modulating myofibroblast differentiation (8). The decrease in expression of α -SMA with CO treatment in bleomycin-treated mouse lung or TGF- β_1 -treated fibroblasts correlates with the decrease in collagen synthesis observed with CO treatment (1). Myofibroblasts are important sources of collagen production (39), and it is therefore reasonable to postulate that CO may affect matrix deposition by inhibiting myofibroblast activation.

An unexpected finding was that, among the individual genes most affected by CO treatment in conjunction with TGF- β_1 , the Sprr family proteins were strikingly up-regulated. These genes were not included in any of the functional groupings of genes, and they have no known function in fibrosis. Sprr proteins were originally identified as markers of terminal squamous cell differentiation (8). Although no role has been identified for Sprr proteins in fibroblasts, recent studies have shown that Sprr genes are induced in response to stress injury (12, 13, 46). When we looked for them in the AsthmaMap database (47), we found that Sprr1 and Sprr2 were both changed in animal models of asthma. Finkelman and colleagues (38) demonstrated in a model of murine asthma that IL-13 reproducibly induced the expression of three Sprr family members in the lung. In a manner similar to CO, up-regulation of Sprr proteins appears to contribute to cell protection (12). The protective effects of heme oxygenase and its catabolic byproduct, CO, have been well documented in a number of disease models, including transplantation, ischemia-reperfusion, vascular injury, and acute lung injury (2, 4, 36). Part of the mechanism for the protection afforded by CO includes inhibition of apoptosis, which can be modulated via the MAP kinase pathways (33, 34, 41, 48, 49), and there is evidence to suggest that Sprr proteins similarly inhibit apoptosis. Sprr proteins are substrates of transglutaminase in the formation of the crosslinked envelope (50), and transglutaminase appears to be involved in stabilizing apoptotic cells (51–53). Furthermore, ectopic expression of Sprr1a has been shown to prevent the disruption of myofibrils in cardiomyocytes, suggesting that stabilization of the actin cytoskeleton may contribute to this protective effect (12). Our microarray data indicate that one of the most differentially expressed categories of genes in response to CO treatment is

Figure 5. Effect of extracellular signal-regulated kinase (ERK) and p38 inhibition on Sprr1a and α -SMA expression in the setting of CO treatment. (A) Fibroblasts were stimulated with TGF- β_1 with or without CO, and cell lysate was analyzed by Western blotting for ERK phosphorylation. CO exposure enhanced ERK phosphorylation at 3- and 6-hour time points. (B) Stat3 phosphorylation was not significantly affected by CO exposure. (C) Inhibition of mitogen-activated protein/ERK1 kinase led to a loss of the effect of CO on both Sprr1a and α -SMA expression. (D) p38 inhibition decreased both Sprr1a and α -SMA expression.

that related to cell death; most of these are down-regulated, suggesting that CO may be antiapoptotic in this setting.

We have shown, in our model of fibroblast stimulation with TGF- β_1 , that the addition of CO significantly increases ERK pathway activation. The ERK pathway appears to play a role in the effect of CO on both α -SMA expression and Sprr1a expression, as shown in the experiments using the MEK1 inhibitor, PD098059. The ERK pathway inhibitor, PD098059, significantly inhibited Sprr1a induction in response to TGF- β_1 and CO. This finding is consistent with previous studies in cardiomyocytes and human conducting airway epithelial cells, where PD098059 also blocked Sprr1 gene induction (12, 54). The effect of ERK pathway activation on α -SMA expression in fibroblasts is less well established. Absence of focal adhesion kinase-1 (FAK) and decreased ERK phosphorylation has been associated with increased α -SMA and stress fiber expression. On the other hand, incubation of rat kidney fibroblasts with TGF- β_1 , followed by PD98059 and connective tissue growth factor has been shown to abolish the increase in α -SMA attributable to connective tissue growth factor (55). Our findings suggest that loss of ERK activity enhances TGF- β_1 -induced α -SMA expression and prevents inhibition by CO. This implicates ERK signaling in CO effects on both α -SMA and Sprr1a expression. The involvement of the p38 pathway appears

to be less clear. Inhibition of p38 had a similar effect on Sprr1a expression as inhibition of ERK activity; this is in keeping with the multiple reports of Sprr1a induction under conditions of stress (12, 11, 14, 50). However, the effect on α -SMA expression was different for ERK and p38 inhibition; whereas ERK inhibition reversed the suppressive effect of CO and enhanced α -SMA expression, p38 inhibition potentially decreased α -SMA expression under all conditions. Although relatively little is known about the relationship between p38 and α -SMA expression, several recent reports do implicate the p38 pathway in myofibroblast differentiation. For instance, the neuronal adaptor protein, Unc119, has been shown to signal via p38 pathway to regulate myofibroblast differentiation (56), and renal tubular cells treated with p38 inhibitor express less α -SMA in response to TGF- β 1 (57). The mechanism for this is not known, although p38 has been shown to alter nuclear trafficking of myocardin-related transcription factor, a serum response factor cofactor involved in α -SMA transcription, during the process of epithelial-mesenchymal transition (58).

In summary, these results provide the first evidence of a role for CO in the emergence of myofibroblasts, providing a potential mechanism for the antifibrotic effects of CO observed in a bleomycin model of lung fibrosis. These studies further demonstrate induction of expression of Sprr family proteins by TGF- β 1 and CO *in vitro* and *in vivo*. Although the role of Sprr proteins in fibrosis remains to be defined, future studies should focus upon the role of Sprr1a in epithelial cells, where it may confer protection against cell death. We have shown that activation of the ERK signaling pathway is required for both of these effects of CO, lending new insight into the mechanism by which CO opposes fibrogenesis.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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