Retinoic Acid–related Orphan Receptor- α Is Induced in the Setting of DNA Damage and Promotes Pulmonary Emphysema

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Rationale: The discovery that retinoic acid–related orphan receptor (Rora)- α is highly expressed in lungs of patients with COPD led us to hypothesize that Rora may contribute to the pathogenesis of emphysema.

Objectives: To determine the role of Rora in smoke-induced emphysema.

Methods: Cigarette smoke extract *in vitro* and elastase or cigarette smoke exposure *in vivo* were used to model smoke-related cell stress and airspace enlargement. Lung tissue from patients undergoing lung transplantation was examined for markers of DNA damage and Rora expression.

Measurements and Main Results: Rora expression was induced by cigarette smoke in mice and in cell culture. Gene expression profiling of Rora-null mice exposed to cigarette smoke demonstrated enrichment for genes involved in DNA repair. Rora expression increased and Rora translocated to the nucleus after DNA damage. Inhibition of ataxia telangiectasia mutated decreased the induction of Rora. Gene silencing of Rora attenuated apoptotic cell death in response to cigarette smoke extract, whereas overexpression of Rora enhanced apoptosis. Rora-deficient mice were protected from elastase and cigarette smoke induced airspace enlargement. Finally, lungs of patients with COPD showed evidence of increased DNA damage even in the absence of active smoking.

Conclusions: Taken together, these findings suggest that DNA damage may contribute to the pathogenesis of emphysema, and that Rora has a previously unrecognized role in cellular responses to genotoxicity. These findings provide a potential link between emphysema and features of premature ageing, including enhanced susceptibility to lung cancer.

Keywords: chronic obstructive pulmonary disease; p53; retinoic acid–related orphan receptor- α ; senescence; apoptosis

Retinoic acid–related orphan receptor- α (Rora) is a nuclear hormone receptor named for its sequence homology with the retinoic acid receptor; its designation as “orphan” indicates that

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Senescence has been implicated in the development of chronic obstructive pulmonary disease, but the mechanism and factors contributing to individual susceptibility are poorly understood.

What This Study Adds to the Field

This study identifies a new molecule, retinoic acid–related orphan receptor- α , which is responsive to DNA damage and capable of regulating cell death. *In vivo* experimental models indicate that mice are protected against airspace enlargement when retinoic acid–related orphan receptor- α is absent. This study also offers evidence of increased DNA damage and activation of a DNA damage response network in patients with COPD relative to smoking control subjects, providing a new perspective for thinking about the pathogenesis of emphysema.

its natural ligand is unknown. We discovered that Rora expression is robustly increased in lungs of patients with chronic obstructive pulmonary disease (COPD), and this led us to examine whether Rora contributes to the pathogenesis of this disease. Little is known about the role of Rora in the lung, although its absence had been reported to lead paradoxically to increased LPS-induced lung inflammation (1) and decreased allergic inflammation (2). Outside the lung, Rora has been implicated in processes as diverse as osteogenic differentiation (3), circadian rhythm (4), apolipoprotein transcription (5), and Purkinje cell development (6). Gene expression profiling in Rora-null mice led us to the hypothesis that this nuclear receptor is involved in responses to DNA damage.

Although the contribution of DNA damage to COPD has not been examined in detail, there are theoretical reasons to believe that genotoxicity could be involved in the pathogenesis of emphysema. Compromised DNA damage repair is known to result in accelerated ageing, as occurs in progeroid syndromes (7). Individuals with these syndromes may manifest skin wrinkles, osteoporosis, neoplasms, atherosclerosis, increased levels of circulating cytokines, and type II diabetes (8, 9), all of which have also been associated (with varying degrees of rigor) with COPD or CT evidence of emphysema independent of age and cumulative pack-years smoked (10–17). Smokers sustain higher levels of DNA damage than nonsmokers, up to 10⁵ lesions per cell per day (18). The fact that many smokers do not develop lung disease suggests that these lesions are generally effectively repaired. It is possible, however, that some smokers are either

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susceptible to DNA damage or have lower intrinsic DNA repair capacity, leading to persistent damage and loss of normal lung architecture. The recent identification of XRCC5 (X-ray repair complementing defective repair in Chinese hamster cells 5) as a COPD susceptibility gene lends credence to this speculation (19). The product of XRCC5 is an ATP-dependent DNA helicase involved in DNA double-strand break repair and telomere maintenance (19, 20). Impaired function of XRCC5 could lead to accumulation of nuclear double-strand breaks. This in turn would be expected to trigger increased activity of the p53 tumor-suppressor pathway, a critical component of the DNA damage response that has been linked to cellular and tissue senescence (21). Caramori and coworkers (22) recently reported a selective decrease in the level of XRCC5 (also called Ku86) in the bronchiolar epithelium of patients with COPD. The authors of this manuscript speculate that patients with COPD have ineffective DNA repair, possibly caused by loss of XRCC5 expression.

Several other lines of evidence support a hypothesis that repair of DNA strand breaks could be impaired in patients with COPD. For instance, p53 is increased in type II pneumocytes of smokers with COPD relative to smokers without COPD (23), and patients with COPD have shortened telomeres in peripheral blood compared with control subjects, independent of smoking history (24). This may be caused by dysregulated antioxidant defense mechanisms (24) or repeated cycles of lymphocyte activation and proliferation (25), but a third possibility is that these individuals have impaired ability to repair double-strand DNA breaks resulting in premature telomere shortening (26). Additional evidence implicating features of senescence in COPD, including nucleic acid oxidation and expression of senescence-associated cyclin kinase inhibitors (27–31), was recently summarized in an excellent review by Tudor and colleagues (32).

In this manuscript, we examine the expression of Rora in human COPD and a mouse model of cigarette smoke-induced emphysema. We demonstrate that Rora is responsive to DNA damage and involved in cell fate decisions after injury, and that Rora-deficient mice are protected against airspace enlargement. These findings are translated back to human disease by showing that patients with COPD have evidence of unrepaired DNA damage and activation of DNA damage response networks. Some of the results of these studies have been previously reported in the form of an abstract (33).

METHODS

Animal Models

Animals were housed in accordance with guidelines from the American Association for Laboratory Animal Care. Staggerer mutant mice (sg) are commercially available (Jackson Laboratories, Bar Harbor, ME). Mice were exposed to total body cigarette smoke using a smoking machine (Model TE-10; Teague Enterprises, Woodland, CA) as previously described (34). Details of lung morphometry can be found in the online supplement.

Cell Culture, Cigarette Smoke Extract Treatment, and Cell Viability Assay

Cells were maintained in Dulbecco's modified Eagle medium containing 10% fetal bovine serum and antibiotics. Preparation of cigarette smoke extract (CSE) from Kentucky 3R4F research-reference filtered cigarettes (The Tobacco Research Institute, University of Kentucky, Lexington, KY) has previously been described (34). Cell viability was determined by the conventional 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay, as described previously (35). KU-55933 (Tocris Bioscience, Ellisville, MO) was used at a concentration of 10 μ M.

Microarray Analysis

All analyses were performed with three independent samples in each group. Total RNA isolated from lung homogenate was hybridized to a mouse genome microarray (430 2.0; Affymetrix, Santa Clara, CA). The microarrays were washed, developed, and scanned using standard protocols. Raw data were analyzed using commercially available (Gene Chip Operating Software ver. 1.4; Affymetrix), pathway analysis tool (Ingenuity Systems, Redwood, CA), and free (BRB ArrayTools [www.nci.nih.gov/brb-arraytools.html/]) software packages. Detailed protocols for sample preparation, microarray hybridization, and analysis of the microarray data have previously been published (36).

Statistical Analysis

Results are expressed as means $\pm$ SD; differences in measured variables between experimental and control group were assessed by using the Student *t* test or analysis of variance and Bonferroni correction where appropriate. Statistically significant difference was accepted at *P* less than 0.05.

Human Specimens

Human tissues came from a variety of sources; all samples were deidentified. Details of lung tissue obtained from thoracic surgical cases have been published previously (34). Additional samples of Global Initiative for Chronic Obstructive Lung Disease (GOLD) stages 0–4 lung for the DNA damage assay were obtained from the National Heart, Lung, and Blood Institute-sponsored Lung Tissue Research Consortium. Details regarding collection of these tissues can be found at <http://www.ltrcpublic.com> and in the online supplement. Guidelines of the GOLD were used for classifying disease severity in COPD.

RESULTS

Rora Expression Is Increased in Lungs of Patients with COPD and in Experimental Smoke Exposure

As shown in Figure 1A, Rora protein expression is uniformly and robustly increased in explanted lung tissue of patients with advanced COPD when compared with unused donor lungs obtained during cadaveric multiorgan harvests. Details of tissue procurement have been previously published (37). All of the patients with COPD had stage IV disease according to the GOLD classification and had ceased smoking for at least 6 months at the time of transplantation. Two of the deidentified control lung donors were nonsmokers; the remaining two had 5 and 20 pack-year histories of smoking. We went on to examine a separate larger group of lung samples from patients with known disease severity and smoking status. Details of these samples have previously been published (38). The smoking history of the GOLD 0 and GOLD 2 group averaged 50 pack-years compared with 56 pack-years for the GOLD 3–4 group. All control patients in this analysis were never-smokers. The densitometry of Western blots in Figure 1B shows that Rora expression is higher in smokers with obstructive lung disease than in non-smokers or smokers without obstruction. We then tested whether Rora expression could be induced *in vitro* using CSE. Figures 1C and 1D demonstrate that Rora expression is induced in epithelial (Beas-2B) and fibroblast (MRC-5) cell lines by CSE. Rora expression can also be induced in lungs of mice by exposure to cigarette smoke. Mice (C57Bl/6) placed in a whole-body cigarette smoke exposure chamber 5 days per week exhibit increased lung expression of Rora protein after 4 and 6 months. Although inter-individual variability in expression can be appreciated, expression continues to increase with time (Figure 1E). Figure 1F shows Rora immunostaining of lungs of mice after a 4-month cigarette smoke exposure. Expression is diffuse but seems most intense in airway epithelium. We also assessed expression of two known Rora transcriptional targets, lipoprotein lipase and the

Figure 1. (A) Western blot demonstrating increased expression of retinoic acid–related orphan receptor- α (Rora) in whole-lung homogenate of patients with severe chronic obstructive pulmonary disease (COPD) (former smokers) compared with rejected transplant donor lungs. (B) Densitometry of Western blots (normalized to β -actin) showing expression of Rora by Global Initiative for Chronic Obstructive Lung Disease classification; one-way analysis of variance $P < 0.001$; * $P < 0.05$ for individual t tests relative to nonsmoking control subjects and to G0. A total of six control lungs and 22 COPD lungs were analyzed. (C) Immunoblots demonstrating increased expression of Rora in response to cigarette smoke extract (CSE) in epithelial and fibroblast cell lines over time and (D) in epithelial cell line with increasing CSE dose. (E) Increased Rora protein expression in whole mouse (C57Bl/6) lung homogenate after smoke exposure for 4 and 6 months. Three replicates of cell culture experiments and two replicates of the *in vivo* experiment were performed. (F) Rora immunostaining of mouse lung exposed to smoke 4 months versus control; DM = digital magnification of 40 $\times$ immunohistochemistry stain; six lungs in each group were examined. (G) Expression of known Rora target genes is increased in whole mouse lung after 12 weeks smoke exposure assessed by quantitative (real time) reverse transcriptase polymerase chain reaction, * $P < 0.05$ for individual t tests relative to sham smoked control subjects; seven to eight mice were examined in each group (in duplicate).

nuclear receptor NR1D2 (39, 40), by real-time reverse transcriptase polymerase chain reaction in whole-mouse lung homogenate after smoke exposure. As shown in Figure 1G, both transcripts increased in response to 3 months of smoke exposure.

Figure 2A shows that the most intense Rora expression in mouse lung exposed to smoke for 4 months or COPD lung is in type II cells (surfactant protein C–expressing cells). Figure 2B shows cut-outs for von Willebrand factor or PECAM1 expressing endothelial cells, CD14 expressing macrophages, and aquaporin 5 or surfactant protein C expressing type I or type II epithelial cells in COPD lung. Rora is expressed in each of these cell types to varying degrees. Expression is primarily cytoplasmic, although some nuclear expression can be appreciated in types I and II epithelial cells.

Rora Is Responsive to DNA Damage

To investigate the function of Rora in the lung, we used the staggerer mutant mouse (sg), which lacks an exon encoding part of the ligand binding domain, generating a Rora-truncated protein (Rora[sg]). This sg mutation acts identically to a null allele (41). We exposed Rora^{sg/sg} and littermate control subjects to cigarette smoke 5 days per week for 4 months and performed gene expression profiling using whole-lung homogenate ($n = 4$ per group). Initial analysis of the data was performed using the Significance Analysis of Microarray program (42). A total of 106 known genes passed the false discovery rate of less than 5% correction for multiple testing; 700 genes achieved a P value cut-off of 0.01. This second larger group was analyzed using Ingenuity software (Ingenuity Systems, Redwood City, CA). The top functional category differentially regulated in Rora^{sg/sg} mice was “Cell Cycle, DNA Replication, Recombination, and Repair.” The genes from this category are listed in Table 1. Additionally, a number of other known p53 target genes, such as Cdkn1a, Bmp7, Tgfa, AFP, ANLN, and p53 itself were among the most differentially expressed genes in the array, further suggesting a link between Rora and the DNA damage response. Given these results, we tested the response of Rora to classical DNA damaging

agents. Mitomycin C is a potent genotoxic agent that causes interstrand cross-linking and double-strand DNA breaks (43). Figure 3A demonstrates increased phosphorylated aggregates of the histone protein H2AX, a marker of DNA damage, after incubation of Beas-2B cells with 2.5 μ g/ml mitomycin C for 24 hours. In this setting of DNA damage, Rora can be seen translocating to the nucleus. The Western blot in Figure 3B shows that treatment with mitomycin C results in higher total intracellular levels of Rora; ultraviolet light exposure and bleomycin treatment had similar effects (data not shown).

Ataxia telangiectasia mutated (ATM) is a serine-threonine protein kinase that is activated by DNA double-strand breaks. It phosphorylates several key proteins that initiate activation of the DNA damage checkpoint, including H2AX. Because of phenotypic similarities between ATM^{−/−} mice and Rora^{sg/sg} mice (Purkinje cell and thymus development abnormalities, defects in metabolism, osteoporosis, circulating lipid, and cholesterol abnormalities), we hypothesized that Rora expression might be regulated by ATM activation. Figure 3C demonstrates that inhibition of ATM by the specific chemical inhibitor KU-55933 (44) (10 μ M) partially abrogated H2AX phosphorylation and the increase in Rora expression in response to CSE.

Hypothesizing that Rora may play a role in DNA damage response, we overexpressed Rora in Beas-2B cells and assessed intracellular levels of p53 and phosphorylated H2AX. As shown in Figure 3D, cells transfected with Rora cDNA showed increased levels of p53 and γ -H2AX compared with control plasmid transfected cells. Figure 3E shows that lungs of Rora^{sg/sg} mice have increased levels of DNA damage than wild-type (WT) mice, further implying that Rora may have a role in cellular responses to DNA damage.

Loss of Rora Expression Protects against Apoptotic Cell Death

To address the functional consequences of altered cellular expression of Rora, we examined the effect of loss or gain of function on CSE-induced cell death. Apoptosis is thought to play

Figure 2. Immunofluorescent staining of mouse tissue exposed to cigarette smoke for 4 months and human chronic obstructive pulmonary disease (COPD) Global Initiative for Chronic Obstructive Lung Disease stage 4. (A) The most intense staining for retinoic acid-related orphan receptor- α (Rora) is in type 2 pneumocytes expressing surfactant protein C (SPC), although other cell types show increased Rora expression. (B) Costaining with cell markers for endothelial cells (von Willebrand factor [vWF] and PECAM-1), macrophages (CD14), type II pneumocytes (SPC), and type I pneumocytes (AQP5). Some intranuclear staining for Rora can be appreciated in types I and II pneumocytes in COPD lung, but expression is primarily cytoplasmic.

a role in the pathogenesis of emphysema (45), and one important trigger of apoptosis is DNA damage and activation of p53 (46). We therefore postulated that Rora may play a role in cell fate decisions in response to cell stress or DNA damage. As shown in Figure 4A, caspase 9 and caspase 3 cleavage in fibroblasts increased in response to CSE treatment, but this increase was attenuated in Rora^{sg/sg} fibroblasts. The same effect was observed in epithelial cells (Beas-2B), where treatment with RORA siRNA led to decreased caspase 9 cleavage in response to CSE and, conversely, transfection with increasing concentrations of RORA cDNA potentiated caspase 9 cleavage in response to CSE (Figure 4B). These findings correlated with increased viability of Rora^{sg/sg} cells in the presence of CSE and decreased viability of Beas-2B cells overexpressing RORA in the presence of CSE (Figures 4C and 4D). Interestingly, although overexpression of RORA in absence of CSE made little difference to cell viability over 24 hours, at later time-points cell viability was significantly reduced relative to mock-transfected cells (Figure 4E), indicating that increased expression of Rora alone is sufficient to induce cell death.

Expression of p53 Target Genes Is Decreased in Rora-Null Cells, but p53 Is Not Required for Induction of DNA Damage Pathways or Cell Death by Rora

Given the findings of our microarray, we tested whether p53-mediated transcriptional activity is affected in Rora^{sg/sg} lung

TABLE 1. LIST OF GENES FROM STATISTICALLY ENRICHED FUNCTIONAL CATEGORY “CELL CYCLE, DNA REPLICATION, RECOMBINATION, AND REPAIR”

Gene Symbol	Expression in Array	Q value	P Value
ABCD1	Increased	0.14	0.002
ANAPC2	Increased	0.22	0.007
ANLN	Increased	0.03	2.00×10^{-6}
AP2A2	Increased	0.14	0.005
ATAD2	Increased	0.10	0.002
CDKN1A	Increased	0.07	0.002
CLCA2	Increased	0.05	0.001
DBF4	Increased	0.12	0.002
DLEU2	Increased	0.17	0.009
DNMT3B	Increased	0.40	0.001
E2F4	Increased	0.12	0.009
GDF15	Increased	0.12	0.006
ITGB1BP3	Increased	0.09	0.007
NEK6	Increased	0.05	0.0007
PHGDH	Increased	0.06	0.0003
PLK4	Increased	0.10	0.002
PPP5C	Increased	0.17	0.002
RECQL	Increased	0.12	0.003
SMC2	Increased	0.18	0.007
TFDP2	Increased	0	1.00×10^{-5}
UBE2C	Increased	0.07	0.0003
BUB3	Decreased	0.07	0.0005
CABIN1	Decreased	0.12	0.003
DNMT3L	Decreased	0.08	0.004
EIF2AK1	Decreased	0.12	0.008
GRIA3	Decreased	0.07	0.0002
MDM4	Decreased	0.10	0.002
NEK2	Decreased	0.20	0.008
SP4	Decreased	0.11	0.007

fibroblasts. As shown in Figure 5A, mRNA levels of two p53 target genes, Bax and Dram, were significantly lower in Rora^{sg/sg} cells than in WT. This corresponded also with lower protein levels (Figure 5B). When Rora was overexpressed in p53^{-/-} fibroblasts, however, γ -H2AX and p-ATM were still induced (Figure 5C). Interestingly, baseline expression of Rora was higher in p53^{-/-} fibroblasts as were levels of γ -H2AX and p-ATM, implying that defective DNA repair in these cells may be inducing higher levels of Rora expression. Overexpression of Rora led to the same extent of cell death in p53^{-/-} fibroblasts as WT fibroblasts, which suggests that Rora is able to kill cells in a non-p53-dependent fashion.

Loss of Rora Protects against Cigarette Smoke-induced Airspace Enlargement in Mice

To determine whether Rora would affect the development of emphysema, mice were exposed to cigarette smoke for 6 months. Because Rora^{sg/sg} mice had been reported to have increased susceptibility to LPS-induced lung inflammation (although decreased allergen-induced inflammation) (1, 2) we measured a panel of 21 chemokines and cytokines in whole-lung homogenate of mice exposed to smoke at 2 weeks and 4 months time-points using Luminex Corp (Austin, TX) technology. After adjustment for multiple tests, only four analytes achieved a statistically significant ($P < 0.0025$) difference between smoking and control conditions. These were tumor necrosis factor- α , IL-9, IL-12, and IL-13, all decreased after 4 months of smoke exposure compared with baseline values. There were no significant differences in expression of inflammatory mediators between the Rora^{sg/sg} mice and control mice, indicating that inflammation is unlikely to account for divergent outcomes in Rora^{sg/sg} versus WT mice (Figure 6A). We assessed differences in airspace enlargement after 6 months of smoke exposure. As shown in Figures 6B and 6C, the mean linear intercept increased by 13% in WT mice, whereas in

Figure 3. (A and B) Mitomycin C causes DNA damage as shown by phosphorylation of H2AX, seen as green fluorescent puncta (A) and by Western blot (B). The increase in γ -H2AX is associated with increasing intracellular levels of retinoic acid-related orphan receptor- α (Rora) and movement into the nucleus. (C) Inhibition of ataxia telangiectasia mutated activity using the chemical inhibitor KU-55933 (10 μ M) results in lower levels of γ -H2AX and Rora in response to treatment with cigarette smoke extract (CSE). (D) Overexpression of Rora in Beas-2B cells leads to increased p53 expression and increased phosphorylation of H2AX. (E) DNA lesions per 10 kb genomic DNA isolated from whole-lung homogenate of wild-type (WT) and Rora^{sg/sg} mice. Results were replicated twice.

Rora^{sg/sg} mice there was no increase in mean linear intercept. Lung volumes were estimated from lung sections by application of Cavalieri's principle, as previously described (47). There were no statistically significant differences among volumes assessed (see Figure E3 in the online supplement). Levels of γ -H2AX were also assessed in whole-lung homogenate. Baseline γ -H2AX expression in Rora mice was significantly higher than in WT mice, as would be expected from the higher level of DNA damage present in lungs from these mice. Smoking led to an increase in γ -H2AX expression that did not achieve statistical significance in either WT or Rora^{sg/sg} mice.

DNA Damage Is Increased in COPD

Because we have shown that Rora is responsive to DNA damage and that its expression is increased in lungs of patients with COPD, we tested whether COPD is associated with unrepaired

pulmonary DNA lesions. We measured the number of DNA lesions in lung tissue of patients with various GOLD stages of COPD and control tissues using a quantitative polymerase chain reaction technique previously validated in humans and mice (48, 49). Samples were divided according to available information regarding smoking status and severity of obstruction. The samples from GOLD stage IV patients were collected at the time of transplantation or lung volume reduction surgery and therefore these individuals had documented abstinence from cigarettes for a minimum of 6 months. GOLD stages 0 and 2 categories include current and former smokers. Control samples were taken from lungs of never-smokers. As shown in Figure 7A, individuals with documented obstruction had significantly more DNA lesions than smokers or nonsmokers without obstruction, and the number of lesions increased with disease severity. We also examined γ -H2AX expression, which is thought to correlate with increasing numbers of DNA double-strand

Figure 4. (A) Immunoblots demonstrating decreased caspase 3 and caspase 9 cleavage in retinoic acid-related orphan receptor- α (Rora)^{sg/sg} fibroblasts compared with wild-type (WT) control fibroblasts in response to cigarette smoke extract (CSE) treatment. (B) Inhibition of Rora expression in the Beas-2B epithelial cell line using siRNA leads to decreased caspase 9 cleavage in response to CSE, whereas overexpression of RORA by cDNA transfection enhances caspase 9 cleavage in response to CSE. (C) Cell viability is higher in Rora^{sg/sg} than control cells 24 hour after treatment with CSE (0–45%). (D) Transiently transfected Beas-2B cells overexpressing Rora and treated with CSE have lower survival relative to control transfected cells treated with CSE. (E) Rora overexpression alone decreases cell viability at late time-points (2 and 3 d) compared with control transfected cells. * P < 0.05 relative to control, ** P < 0.005 relative to control. At least three independent experiments were conducted for each condition.

Figure 5. (A and B) mRNA and protein levels of two p53 target genes, Bax and Dram, are significantly lower in retinoic acid-related orphan receptor- α ($Rora$)^{sg/sq} cells than in wild-type (WT) cells. (C) Rora overexpression in $p53^{-/-}$ fibroblasts led to increased γ -H2AX and p-ataxia telangiectasia mutated (ATM) expression as in WT fibroblasts. (D) Overexpression of Rora led to the same extent of cell death in $p53^{-/-}$ fibroblasts as WT fibroblasts. * $P < 0.05$ relative to WT control; ** $P < 0.005$ relative to WT. At least three independent experiments were conducted for each condition. CSE = cigarette smoke extract.

breaks, and p53 expression. As shown in Figures 7B and 7C, both were elevated in COPD tissue relative to control and expression increased with worsening severity. This is consistent with previously published data showing increased p53 protein levels in lungs of smokers with COPD compared with lungs of smokers without COPD (23, 50).

DISCUSSION

The discovery of increased Rora protein expression in lungs of patients with COPD led to the identification of Rora as a molecule responsive to DNA damage and capable of determining cell

fate. The findings here show that Rora expression is induced by cigarette smoke in mice and in cell culture, and that Rora-deficient mice have dysregulated expression of genes involved in DNA repair. DNA damage leads to increased Rora expression and movement into the nucleus, and chemical inhibition of ATM diminishes the rise in Rora expression after DNA damage. Overexpression of Rora is sufficient to induce cell death, indicating that Rora plays an active and not merely permissive role in determining cell fate. Rora-deficient mice are protected from elastase- and cigarette smoke-induced emphysema, and this is associated with lower levels of apoptosis *in vivo*. Finally, lungs of patients with COPD show evidence of increased DNA damage

Figure 6. (A) Change in cytokine levels in whole-lung homogenate of smoke-exposed mice. Rora = retinoic acid-related orphan receptor- α ; TNF = tumor necrosis factor; WT = wild-type. (B) Representative images of lungs from $Rora^{sg/sq}$ and WT smoke-exposed mice. (C) Quantitation of airspace enlargement (mean linear intercept) of smoke-exposed mice; * $P < 0.05$ relative to untreated, ** $P < 0.005$ relative to WT treated. (D) Western blot showing increased γ -H2AX expression in whole mouse lung homogenate in $Rora^{sg/sq}$ control subjects and in response to cigarette smoke. (E) Densitometry of the blot shown in D; * $P < 0.05$ relative to WT control.

Figure 7. (A) DNA lesions per 10 kb genomic DNA isolated from whole-lung homogenate of nonsmokers, current smokers without airway obstruction (G0), mild-to-moderate airway obstruction (G2), and severe airway obstruction (G4). One-way analysis of variance, $P < 0.0001$; $*P < 0.05$ compared with nonsmoking control subjects; $**P < 0.05$ compared with nonsmoking control subjects and G0. The assay was replicated three times using the same samples. (B) Western blot showing increased γ -H2AX in whole-lung homogenate of patients with mild-to-moderate (G2) and severe (G4) chronic obstructive pulmonary disease. (C) Western blot showing increased p53 in whole-lung homogenate of patients with mild-to-moderate (G2) and severe (G4) chronic obstructive pulmonary disease.

even in the absence of active smoking. Taken together, these findings suggest that DNA damage may contribute to the pathogenesis of emphysema, and that Rora has a previously unrecognized role in cellular responses to genotoxicity.

The finding that DNA damage is increased in lungs of patients with COPD in the absence of ongoing smoking does not tell us whether these individuals have increased susceptibility to damage or reduced DNA damage repair capacity. Persistent inflammation or oxidant-antioxidant imbalance after smoking cessation could create an environment promoting ongoing injury to DNA. However, the recent identification of XRCC5 as a COPD susceptibility gene (19) and the demonstration of reduced expression of XRCC5 in COPD lung (22) raise the intriguing possibility that smokers who develop COPD have impaired capacity to repair DNA damage. Individuals with subtle defects in DNA repair may be unable to manage the degree of DNA damage caused by cigarette smoking, which is estimated to increase DNA lesions by at least one order of magnitude (18). Regardless of the etiology of increased DNA damage in COPD, it is plausible to speculate that it contributes to disease pathogenesis. Accumulation of unrepaired or erroneously repaired strand breaks would be expected to lead to activation of p53 and related pathways; this is consistent with increased H2AX phosphorylation, p53 expression, and Rora expression in COPD lung. DNA damage and increased p53 activity have been strongly linked with premature ageing (51), and

an excess of DNA strand breaks would contribute to higher rates of tumorigenesis observed in patients with COPD (15–17).

Implication of Rora in the genotoxic response may help to explain phenomena not directly related to the development of emphysema. For example, the ataxia and Purkinje cell abnormalities seen in Rora^{sg/sg} mice are common features of an impaired DNA damage response (52). Rora could also provide a clue to the relationship between the genotoxic stress response and circadian rhythm, because Rora is known to participate in regulation of circadian rhythm (4), and we have observed circadian variation in expression of Rora in mouse lung (unpublished data). Additionally, our data suggest involvement of Rora in a tumor-suppressor pathway, which correlates with reports of decreased Rora expression in tumors and tumor cell lines (53, 54).

In conclusion, we have identified Rora as a novel participant in cell fate decisions in response to DNA damage, and we have shown that the absence of Rora can protect against airspace enlargement in animal models of emphysema. These findings contribute a new aspect to the understanding of the pathogenesis of COPD and open novel avenues for exploring the susceptibility of individual smokers. Further definition of the role of Rora in cell death and its relationship to canonical DNA repair networks will have implications for the understanding of human diseases beyond COPD.

Author disclosures are available with the text of this article at www.atsjournals.org.

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