The Spontaneously Hypertensive Rat: An Experimental Model of Sulfur Dioxide–Induced Airways Disease

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Chronic obstructive pulmonary disease (COPD) is characterized by airway obstruction, inflammation, and mucus hypersecretion, features that are common in bronchitis, emphysema, and often asthma. However, current rodent models do not reflect this human disease. Because genetically predisposed spontaneously hypertensive (SH) rats display phenotypes such as systemic inflammation, hypercoagulation, oxidative stress, and suppressed immune function that are also apparent in COPD patients, we hypothesized that SH rat may offer a better model of experimental bronchitis. We, therefore, exposed SH and commonly used Sprague Dawley (SD) rats (male, 13- to 15-weeks old) to 0, 250, or 350 ppm sulfur dioxide (SO2), 5 h/day for 4 consecutive days to induce airway injury. SO2 caused dose-dependent changes in breathing parameters in both strains with SH rats being slightly more affected than SD rats. Increases in bronchoalveolar lavage fluid (BALF) total cells and neutrophilic inflammation were dose dependent and significantly greater in SH than in SD rats. The recovery was incomplete at 4 days following SO2 exposure in SH rats. Pulmonary protein leakage was modest in either strain, but lactate dehydrogenase and N-acetyl glucosaminidase activity were increased in BALF of SH rats. Airway pathology and morphometric evaluation of mucin demonstrated significantly greater impact of SO2 in SH than in SD rats. Baseline differences in lung gene expression pattern suggested marked immune dysregulation, oxidative stress, impairment of cell signaling, and fatty acid metabolism in SH rats. SO2 effects on these genes were more pronounced in SH than in SD rats. Thus, SO2 exposure in SH rats may yield a relevant experimental model of bronchitis.

Key Words: chronic obstructive pulmonary disease; bronchitis; spontaneously hypertensive rats; sulfur dioxide exposure; mucus hypersecretion; inflammation.

Chronic obstructive pulmonary disease (COPD) results primarily from cigarette smoking and is the fourth leading cause of mortality worldwide (Spurzem and Rennard, 2005). Although nearly 90% of COPD patients are smokers, only 10% of the smokers develop the disease, suggesting that genetic predisposition is a dominant factor (Siafakas and Tzortzaki, 2002). A variety of candidate genes have been associated with disease susceptibility. Gene variants of α-1 antitrypsin expression show high risk for damage to alveolar structure. However, bronchitis and COPD generally appear to have multigene predisposition (Siafakas and Tzortzaki, 2002). Smoker’s lung disease is characterized by chronic active inflammation, airway mucus hypersecretion, and emphysema (MacNee, 2005). It is only partially reversible upon cessation of smoking (Rennard, 2005). Animal models exposed to cigarette smoke and other chemicals have been studied to investigate disease pathogenesis and therapeutic interventions, however, with only limited success (Foronyi et al., 2006; Kodavanti et al., 1998, Kodavanti and Costa, 2001; Shapiro, 2000; Vlahos et al., 2006). Commonly used rat and mouse strains demonstrate only mild inflammation and mucus secretion in response to cigarette smoke (Guerrisimov et al., 2004) or sulfur dioxide (SO2) exposures (Kodavanti et al., 2000a; Lamb and Reid, 1968), and the corresponding injuries are rapidly reversible. It has been suggested that healthy laboratory rodents possess a remarkable ability to compensate and regenerate lung function and structure following an injury (Costa et al., 1983), which may underlie their relative insensitivity to experimental COPD.
These shortcomings have slowed the progress in therapeutic interventions, while the search continues for better experimental models of COPD.

Chronic bronchitis generally is a major attribute of COPD and is characterized by airway inflammation and mucus hypersecretion (Rennard, 2005). Experimental models of bronchitis have been pursued using long-term SO2 exposures in rats and dogs (Killingworth et al., 1996; Knauss et al., 1976; Lamb and Reid, 1968; Long et al., 1997, 1999; Shore et al., 1987, 1995). SO2 is an upper respiratory tract irritant and upon inhalation at high concentrations is known to induce neutrophilic inflammation and airway mucus hypersecretion. The mechanism of SO2-induced mucus secretion and inflammation seems to involve neurogenic substances such as NK-1 and substance P (Killingworth et al., 1996; Long et al., 1997). Involvement of oxidative stress also has been suggested (Gumuslu et al., 2001; Meng, 2003). Airways inflammation have been associated with chemokine expressions in mice (Meng et al., 2005) and increased airway hyperresponsiveness in Sprague Dawley (SD) rats (Shore et al., 1995). All these impairments are also apparent in human bronchitic patients (Grootendorst and Rabe, 2004; Spurzem and Rennard, 2005). Thus, these effects caused by SO2 in SD rats have been correlated with chronic bronchitis in humans, but the severity and persistence of these effects are not similar to those seen in human disease. It is likely that the mechanism by which SO2 induces bronchitis is different from causation of bronchitis in humans and that only limited injury is likely in healthy rat strains.

We hypothesized that as only a predisposed subgroup of humans acquire environmental disease, laboratory rodents likewise require unique genetic susceptibility to develop analogous experimental disease. Because the spontaneously hypertensive (SH) rats exhibit genetic risk of cardiac disease and phenotypic features of lung predisposition to injury (Kodavanti et al., 2000c), we hypothesized that these rats may be uniquely susceptible to develop a model of COPD with SO2. Although every genetic component that plays a role in the development of systemic hypertension has not been identified, SH rats are known to demonstrate phenotypes such as systemic oxidative stress (Kobayashi et al., 2005), inflammation (Schmid-Schonbein et al., 1991), hypercoagulation (Amagasa et al., 2005), immunosuppression (Khraibi et al., 1984), and borderline pulmonary hypertension (Aharinejad et al., 1996), all attributes also common in COPD (Sandford et al., 2002). The purpose of this study was to characterize and compare SO2-induced pulmonary and airway disease in SD and SH rats using physiological, pathological, and molecular measures. We compared SO2-induced pulmonary injury and inflammation, airway mucus production, airway and lung pathology, and lung tissue gene expression in SH as well as in commonly used SD rats. We report that SH rats demonstrate remarkable susceptibility to SO2-induced pulmonary inflammation and mucus hypersecretion that persists much longer when compared to SD rats. Differences in gene expression patterns in both of these strains provide mechanistic insight for greater susceptibility of SH than SD rats.

METHODS AND MATERIALS

Animals

Healthy, male, 12- to 15-weeks old, SD and SH (SHR/NCrHBR) rats were purchased from Charles River Laboratories, Raleigh, NC. All rats were maintained in an isolated animal room in an Association for Assessment and Accreditation of Laboratory Animal Care approved animal facility (21 ± 1°C, 50 ± 5% relative humidity, and 12-h light/dark cycle) for 1- to 2-week quarantine and nonexposure periods. The rats were housed in plastic cages with beta-chip bedding. All animals received standard (5001) Purina rat chow (Brentwood, MO) and water ad libitum except during the daily exposure periods of 5 h. The Environmental Protection Agency’s (EPA’s) Animal Care and Use Committee approved the protocol for the use of rats in these inhalation studies.

SO2 Exposures

Each rat strain was randomized by body weight into three groups and then further divided into two time points. In order to avoid acute ocular injury from SO2 in the whole-body exposures, we conducted exposures by nose-only inhalation. Concentrations of 200–500 ppm have been used previously in inducing experimental bronchitis in rodents and other laboratory animals (Knauss et al., 1976; Kodavanti et al., 2000b; Long et al., 1999; Shore et al., 1987). Rats were acclimatized once to nose-only tubes prior to their air or SO2 exposures. Rats were restrained in nose-only tubes, and then tubes were arranged in racks placed in stainless steel Hazelnut 2000 inhalation exposure chambers. Rats were then exposed to either filtered air or SO2 at 250 or 350 ppm, 5 h/day for 4 consecutive days. Anhydrous SO2 (99.8% purity, National Welders, Charlotte, NC) was delivered into the inlet of the chamber using a mass flow controller (Tylan FC-280, Mycrolis, Billerica, MA). The SO2 concentration in each chamber was monitored by an infrared gas analyzer (Micro 1a, ThermoElectron, Waltham, MA) (247 ± 15 ppm and 353 ± 11 ppm). Chamber relative humidity was 47–50%, temperature 23°C, and chamber airflow was ∼550 l/min.

Whole-Body Plethysmograph Data Acquisition and Analysis

Barometric plethysmography using a whole-body plethysmograph (WBP) system (Buxco Electronics, Inc, Sharon, CT) was employed to obtain data on breathing parameters prior to and after exposure. The use of this methodology permitted continuous monitoring of a number of ventilatory parameters, including breathing frequency (f), tidal volume (TV), minute ventilation (MV), peak expiratory flow (PEF), peak inspiratory flow (PIF), inspiratory time (TI), expiratory time (TE), pause (Pau), and enhanced pause (Penh). It should be noted that TV is not directly measured but represents a value calculated using the WBP system software. Since the system is calibrated over the normal physiological range, these values have been shown to accurately reflect direct measurements of this parameter (Tankersley et al., 1997).

Each of the four WBP chambers (Model PLY3213, Buxco Electronics, Inc., Sharon, CT) was calibrated prior to analysis. A bias flow regulator drew approximately 2 l/min of room air through each of the cylindrical chambers to prevent carbon dioxide buildup within the WBP chambers. Unrestrained, freely moving animals were placed in individual WBP and allowed for 1 min to settle down followed immediately by 5 min of data collection. Each animal was assessed several times prior to the start of first exposure and then at each time prior to the start and at the end of SO2 exposure. Assessment was also done at 1 and 4 days postexposure. Diurnal variation was apparent in the breathing parameters, and the values are given for only morning measurements. To prevent diurnal variability, preexposure analyses were conducted during the same time of day as the anticipated postexposure analyses. An analysis protocol
was designed to ensure that animals from each group were equally distributed among the WBP chambers. Data were collected for each parameter every 10 s and averaged over 1-min intervals using vendor-supplied software (BioSystem XA, Buxco Electronics, Inc., Sharon, CT). Automated breath-by-breath analyses were performed using a rejection algorithm to eliminate breaths that were outside a given range. Animals were taken out of the chambers after completion of the 6-min protocol.

Necropsy, Sample Collection, and Analysis

One or four days following the last exposure, rats were weighed and anesthetized with an overdose of sodium pentobarbital (50–100 mg/kg, ip). From the first group of rats, the trachea was cannulated and the lateral lung bronchus was tied. The right lung was lavaged with Ca\(^{++}/Mg\(^{++}\)-free phosphate-buffered saline (pH 7.4) with a volume equal to 35 ml/kg body weight (near total lung capacity) × 0.6 (right lung representing 60% of total lung mass). Three quick in-and-out washes were performed with the same buffer aliquot to enrich for protein and enzymes. This bronchoalveolar lavage fluid (BALF) was collected in tubes and kept on ice for further analysis. Lavaged lung lobes were quick-frozen in liquid nitrogen for RNA extraction. The left lung was inflated with 10% neutral formalin, at 20 cm water pressure for 30 min, and then tied and immersed in formalin for histology.

BALF Analysis for Determining Lung Injury

One aliquot of whole lavage fluid was used for determining total cell counts (Coulter Counter, Coulter, Inc, Miami, FL), and a second aliquot was centrifuged (Shandon 3 Cytospin, Shandon, Pittsburgh, PA) to prepare cell differential slides. The slides were dried at room temperature and stained with LeukoStat (Fisher Scientific Co, Pittsburgh, PA). Macrophages and neutrophils were counted using light microscopy (over 200 cells counted per slide). The remaining BALF was centrifuged at 1500 x g to remove cells, and the supernatant fluid was analyzed for markers of lung injury and inflammation. Cell-free BALF was analyzed for protein (µg/ml) content using Coomassie Plus Protein Assay Kit (Pierce, Rockford, IL) and BSA standards from Sigma-Aldrich Chemicals (St Louis, MO). BALF samples were analyzed for albumin content (µg/ml) using a commercially available kit (Diasorin, Stillwater, MN). Lung lavage fluid (LDF) (lactate dehydrogenase, LDH) activity (U/l) was determined using a Kit (#228) and standards from Sigma Chemical Co. N-Acetyl glucosaminidase (NAG) activity (U/l) was measured using a commercially available kit and standards from Roche Diagnostics (Indianapolis, IN). These assays were modified and adapted for use on the Hoffmann-La Roche Cobas FarA II clinical analyzer.

Histopathology and Mucin Quantification

Lung tissues were processed, embedded in paraffin, sectioned at 5 µm, and stained with hematoxylin and eosin (H&E) for pathological evaluation and semiquantitative scoring of lesions. Severity scores (1 = minimal, 2 = slight/ mild, 3 = moderate, 4 = moderately severe, and 5 = severe) were assigned for a variety of pathological indices. Mean severity was determined by adding severity scores of all animals within a group and then dividing the sum by the total number of animals. Additional serial sections from each lung were stained separately with alcian blue and periodic acid–Schiff (AB-PAS) to distinguish mucous-secreting cells on the basis of H&E-stained epithelial cells. Specifically, the percentage of airway epithelium with mucin was determined. Measurements were done by estimating the percentage of the epithelial layer that stained positive for AB-PAS (mucin) to the nearest 5%. This initial qualitative assessment permitted evaluation of patterns for different SO2 exposure levels. For quantitative analysis of epithelial mucousubstances, 10 airway fields at ×25 magnification of the main axial pathway between three and six generations of airway branching were examined for each animal. Images taken using AB-PAS–stained slides implemented a software program (Image J, version 1.240) and an image capture software system. Digital images in color were taken at an image setting of 32 bit to quantify the pixel volume of mucousubstances within the airway epithelial lining of each field. Also measured in the Image J and National Institute of Health Image was the basal lamina length within each field to express pixel area (mucosubstance) to linear length (basal lamina). These two measures were subsequently converted to mucosubstance volume/basal lamina surface area for each airway observed. Statistical analyses of these results were done using the computer program StatView 5.0. The ANOVA test with a p value of less than 0.05 (p < 0.05) was used to determine statistical significance of the results.

RNA Isolation

Total RNA was isolated from the apical lung lobes snap-frozen in liquid nitrogen. The frozen tissue was homogenized in TriReagent (Sigma), processed, and dissolved in 50 µl diethylpyrocarbonate-treated water. The quality and quantity of RNA were assessed by using an Agilent Bioanalyzer (Agilent 2100 Bioanalyzer, Agilent Technologies, Palo Alto, CA).

Microarray Analysis

Microarray data were collected at Expression Analysis, Inc. (http://www.expressionanalysis.com; Durham, NC) using the GeneChip Rat 230A Array (Affymetrix Inc., Santa Clara, CA) containing 15,923 probe sets with known genes including expressed sequence tags. After cRNA production, the quality and quantity of each sample were assessed using a 2100 BioAnalyzer (Agilent Technologies). The target cRNA was hybridized according to the “Affymetrix Technical Manual.” A brief description of the process for cRNA synthesis, hybridization, visualization, and quantification are described elsewhere (Gilmour et al., 2006).

Real-Time Quantitative PCR

RNA was DNase treated and one-step RT-PCR was carried out using “Platinum Quantitative RT-PCR ThermoScript One-Step System” (Invitrogen, Carlsbad, CA) following the protocol recommended for the kit. All reactions were run using 100 ng of total RNA. Reactions were multiplexed, that is, containing one pair of target gene primers and one pair of endogenous control gene (β-actin) primers in each sample well. Real-time PCR was conducted on an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA). RT-PCR conditions consisted of reverse transcription for 20 min at 58°C and inactivation of reverse transcriptase activity for 5 min at 95°C. There were 40 amplification cycles at 95°C for 15 s followed by 60°C for 60 s. Primers were Lux (Invitrogen, Carlsbad, CA) labeled by TAM (target genes) or by JOE (control gene, β-actin) and were purchased from Invitrogen. The sequences were (1) macrophage inflammatory protein-2 (MIP-2) 5′-CGGCC-AGAAATACACGTCGTC-3′ and 5′-CGGCCAGAATACACGTCGTC-3′, (2) TNF-α 5′-CGGCTGGCTACGCCTGGTGC-3′ and 5′-GGGCT- CCCTCTCATCGTC-3′, (3) heme oxygenase-1 (HO-1) 5′-CGTAAAGCC- GTCCTCAGCAGGT-3′ and 5′-CGCTAGAAGGTCACCCAGGTT-3′, and (4) β-actin 5′-CACACCCGAGCCTCATTTGAAGTG-3′ and 5′-TTGAA- ACACCCGGCATTTGAACCAA-3′. Data were analyzed using ABI Sequence Detection System software version 2.1. For each plate run, the cycle threshold (CT) was set to one in order of magnitude above background fluorescence, and CT values were obtained for each sample. In each case, CT of the target gene was normalized to changes in actin CT to account for variability in starting RNA amount. Expression for each exposure condition was quantified relative to expression of the corresponding saline control group.

Statistical Analysis

The data for breathing parameters were analyzed using repeated measure mix model ANOVA of SAS software program (SAS, Cary, NC). The BALF data were analyzed using a multivariate ANOVA model (SAS). The independent variables were strain (at two levels: SD and SH), exposure (at three levels: 0, 250, and 350), and day (at two levels: 1 and 4). In cases where the ANOVA assumptions were not satisfied, a transformation of the data was performed in order to meet these requirements. If the transformations failed to satisfy the assumptions, a distribution-free method was used for the analysis. Subsequent to the overall analysis, pairwise comparisons were performed as subsitest of this analysis. No adjustment was made to the level of significance.
due to multiple comparisons. PCR data (delta-cT values were considered as opposed to fold change over control) were analyzed by a two-way ANOVA with SO₂ exposure as one factor and rat strain as the other using SigmaStat software version 3.1 (SPSS, Inc, Chicago, IL). In the case of significant interaction, step-down ANOVAs were used to test for main effects with time. Pairwise comparisons between groups were made using Fisher’s least significant difference test. The accepted level of significance for all tests was \( p < 0.05 \).

**Analysis for gene chip data.** Affymetrix CEL data files were imported into R, an open source statistical scripting language (http://www.R-project.org) used in conjunction with the Bioconductor project (http://www.bioconductor.org) (Gentleman et al., 2004). Normalized values with RMA background correction, quantile normalization, and median polish were calculated with the R/bioconductor package AffylmGUI (Irizarry et al., 2003).

RMA normalized values were imported into the MultiExperimentViewer (TMEV4, The Institute of Genomic Research, http://www.TIGR.org) for statistical analysis (Irizarry et al., 2003; Saeed et al., 2003). Two-way ANOVA was used to identify differentially expressed genes by strain, exposure, or strain-exposure interaction at a \( p \) value of \(< 0.01\). Resulting gene lists were then filtered to remove nonannotatable probe sets.

A heat map was generated with the mean values of the four biological replicates for each strain and exposure normalized to the mean value of the SD air group. A hierarchical clustering analysis based upon average linkage and Euclidian distances was then used to cluster genes on the y-axis and strain/exposures on the x-axis using TMEV4.

The microarray data were deposited in the Gene Expression Omnibus Web site (http://www.ncbi.nlm.nih.gov/geo/) (accession number GSE4702).

## RESULTS

### Body Weight Changes and Breathing Parameters

Restraining rats in nose-only inhalation tubes for 5 h during acclimatization followed by a 4-day air exposure period caused nearly 5% weight loss in SH but not in SD rats. SO₂ exposure was associated with further weight loss in a concentration-dependent manner in both strains (Fig. 1). Unlike previously observed baseline differences in \( f \), MV, TV, PIF, and PEF between Wistar Kyoto (WKY) and SH rats (Kodavanti et al., 2005), there were no significant differences in these breathing parameters between air control SD and SH rats prior to restraining in tubes (Fig. 2). However, air-exposed control SD rats while restrained in the tubes did exhibit altered breathing parameters (a decrease in \( f \), MV, PIF, and PEF). Exposure to SO₂ in SD rats caused further decreases in \( f \) and MV. There were also increases in TV, TI, TE, RT, and PenH following SO₂ exposure in SD rats (Fig. 2). Because the air-exposed SH rats did not demonstrate significant changes in breathing parameters as a result of nose-only tube restraint, the changes induced by SO₂ exposure in these rats were more clearly distinguishable between control and SO₂ exposure. SH rats demonstrated marked and consistent decreases in \( f \), MV, and PIF while increases occurred in TV, TI, TE, RT, Pau, and PenH (Fig. 2). Some of these differences were found not to be significant when individual groups were considered using adjusted \( p \) values; however, they did demonstrate significant difference when considered collectively or with unadjusted \( p \) values. It is noteworthy that the changes in breathing parameters occurred in the same direction in both rat strains, although there were quantitative differences. The overall effect of SO₂ plus tube restraint in SD rats was comparable to the effect of SO₂ alone in SH rats.

### BALF Analysis of Lung Inflammation and Injury

These parameters were determined by analysis of BALF at 1 or 4 days following 4-day exposure to air or SO₂. Total lavageable cells increased significantly 1 day postexposure only in SH rats, while no increase occurred in SD rats (Fig. 3). The increase in cells persisted until 4 days postexposure. The increase in total cells was accounted partly by a small but concentration-dependent increase in alveolar macrophages (AMs) in SH rats at 1 day postexposure. However, AM returned to control 4 days postexposure. No changes occurred in SD rats. The increase in total cell number following SO₂ exposure was due to the increased \((-40\times)\) neutrophils in SH rats (Fig. 3). This increase persisted up to 4 day postexposure in rats exposed to 350 ppm SO₂. Unlike the increase in SH rats, SD rats demonstrated only a threefold increase in the neutrophils at the highest SO₂ concentration at 1 day but not at 4 day postexposure.

Since SO₂ effects are likely to occur primarily in the airways, the total lavageable protein and albumin, markers of alveolar protein leakage in the lung, did not increase significantly in SD or SH rats following 1 or 4 days after exposure (Fig. 4). However, LDH activity and NAG activity was moderately increased in BALF of SH rats suggesting cytotoxicity. This increase may have resulted from cytotoxicity in the airways with activation of resident macrophages as opposed to effects on deep lung parenchyma. SD rats did not demonstrate any SO₂-induced increases in LDH or NAG activity.

### Histological Evaluation and Mucus Quantification

A detailed histopathological analysis was done on respiratory tract tissues to determine the type and patterns of
FIG. 2. Comparative evaluation of breathing parameters in SD and SH rats prior to and during exposure to SO₂. All measurements were performed in the morning of each day using Buxco whole-body plethysmography. The measurements of day 1 (D1) show values prior to the start of SO₂ exposure. Rats were exposed to filtered air or SO₂ 5 h/day × 4 days. Values represent means of 17 SD or 17 SH rats for day 1 (D1) and five to eight rats per group thereafter. Note that Pause and PenH are unitless values. Standard errors are not placed on bars for improving clarity of data. The adjusted p values were as follows for comparisons: f: 0 ppm different from 250 and 350 ppm for SH rats at D3, D4, and D4 + 1; MV: 0 ppm different from 350 ppm for SH rats at D3, D4, and D4 + 1; TI: 0 ppm different from 350 ppm for SD and SH rats at D3 and D4 and for only SH at D4 + 1; PIF: 0 ppm different from 350 ppm for SH rats at D3, D4, and D4 + 1; and SD-0 ppm is different from SH-0 ppm at D4; all other comparisons were not significantly different when adjusted p values were considered.
pathological changes and mucus secretion in the airways of SD versus SH rats. Figure 5 demonstrates representative changes in the airways of SD rats (Fig. 5E) and SH rats (Fig. 5G) relative to air-exposed SD and SH rats (Fig. 5E). The presence of inflammatory cells within the airways of both the SD and SH rats was indicative of airway effects (Fig. 5).

Quantitative histological scoring of pathological lesions of the lung confirmed SO₂-induced bronchiolar epithelial cells hyperplasia, peribronchiolar inflammation, vascular mineralization, and increased goblet cells (Table 1). Morphological quantitative analysis of mucin volume in airways indicated marked and concentration-dependent increase in SH and SD rats following SO₂ exposure. The severity of increase was more pronounced in SH rats (Fig. 6).

**Gene Expression Analysis Using Real-Time PCR**

Real-time PCR was performed for pulmonary mRNA expression of MIP-2, TNF-α, and HO-1 in order to correlate inflammatory changes with expression of inflammatory cytokine genes. The expression of cytokine genes was less dramatically induced than the apparent neutrophilic inflammation in the lung (Fig. 7). Only a 3-fold increase in MIP-2 and 2.5-fold increase over control in TNF-α expression occurred in SH rats with no changes in SD rats. HO-1 expression was not significantly affected by SO₂ exposure in either strain.

**Gene Expression Analysis Using Microarray**

Lung RNA was analyzed to get further insight into the global gene expression profile with the goal to highlight rat strain differences in SO₂ effects. Expression changes associated with oxidative stress, acute phase response, immune modulation, cell signaling, protease/antiprotease balance, and inflammatory gene markers were analyzed. Among the oxidative stress genes, glutathione S-transferase expression in SH rats was higher than in SD rats, whereas glutathione peroxidase gene expression was lower (Fig. 8). Marked strain-related differences existed in the baseline expression of immune and inflammatory response genes. Many of which were upregulated several fold, while others were downregulated in SH rats relative to age-matched SD rats (Fig. 8). There were also marked strain differences in expression of several genes involved in cell signaling in which SH rats exhibited downregulation in most cases. A variety of differentially expressed genes could be placed in these categories: proteases/antiproteases, oxidases, cytochrome P450s, blood coagulation markers, mitochondrial function, fatty acid metabolism, and endothelial function.

A heat map of identified genes that showed significant interaction for SO₂ effects between SD and SH rats is given in Figure 8. Genes demonstrate interaction between rat strain and SO₂ effect were those that are primarily involved in inflammatory and immune modulation. Some of these genes also involved oxidative stress responsive protein markers and mitochondrial respiration. CD36 antigen, beta defensin,
hypoxia upregulated 1, and MIP-alpha receptor genes were among those which were downregulated in SH but upregulated in SD rats following SO2 exposure. However, myxovirus resistance 1, catechol-0-methyltransferase, cAMP responsive element binding protein-like 2, carbonic anhydrase 3, IL-1-beta, and glutathione S-transferase mu 5 genes were among those which were upregulated in SH and downregulated in SD rats following SO2 exposure.

**DISCUSSION**

Based on our earlier work with the SH rat, we hypothesized that this strain would exhibit greater responsiveness to irritant SO2 than other conventional laboratory strains and might reveal a better model for bronchitis in the rat. Our hypothesis centered on the fact that the SH rat demonstrates phenotypic risk factors of COPD, such as systemic inflammation and oxidative stress, hypercoagulation, suppressed immune function, and borderline pulmonary hypertension. Our earlier studies with irritant particles have shown that the SH rat to be more susceptible to lung injury and inflammation than healthy Wistar Kyoto rats (Kodavanti et al., 2000c, 2002). In this study, we examined SO2-induced airway inflammation, mucus hypersecretion, pathology, and gene expression patterns in the SH relative to SD rats, conventionally used to study bronchitis. Four consecutive days of 5-h SO2 exposures induced highly significant neutrophilic inflammation response and mucus production in the airways of the SH relative to that of the SD rats. The strain-related differences in inflammation could not be explained by SO2-induced changes in breathing parameters measured before or after exposure. The inflammatory response primarily occurred in the airways as no pulmonary protein leakage or alveolar pathology was noted in either strain. Four days after SO2 exposure, the inflammatory response persisted in SH rats but had subsided in SD rats. The mucus response similarly persisted. The slow recovery may reflect the extent of initial injury and inflammation caused by SO2 in SH rats. Gene expression patterns indicated significant strain-related differences in SO2 effect among key inflammatory/immune modulatory and oxidative stress response genes between strains following exposure. Our data suggest that SH rats may be a better strain than SD rats for development of an experimental model of SO2-induced bronchitis.

Since changes of breathing parameters can impact the deposition of SO2 in the airways and may influence the sensitivity of rats to develop toxicity, we wanted to determine if significant strain differences in breathing parameters were responsible for the difference in the strain sensitivity. It should be noted that the breathing parameters were not measured during actual exposure but were measured immediately after the exposure and next day with the assumption that the measurements at these times will reflect the changes that are likely during exposure. Restraining SD but not SH rats in nose-only inhalation tubes decreased f, PIF, and PEF but not TV and as a result decreased MV. However, because the values of breathing parameters were similar in SO2-exposed SD and SH rats despite differences in air-exposed controls, it is unlikely that differences in air-exposed controls, it is unlikely that differences in SO2 deposition accounted for greater effectiveness of SO2 in SH than in SD rats. There was no clear dose-response as both doses affected rats similarly in most parameters. Unlike the differences we observed between SH
and WKY rats in breathing parameters in an earlier study (Kodavanti et al., 2005) and those observed by other investigators (Debreczeni et al., 1989), SH rats did not differ significantly from SD rats in baseline values of breathing parameters. However, the effect of SO2 was more severe in SH than in SD rats. It is not clear at this time if the changes in breathing parameters relate to biological sensitivity to mucus cell proliferation.

Chronic active airway inflammation and mucus hypersecretion are two primary pathological features of human bronchitis and COPD (MacNee, 2005). Therefore, we wanted to thoroughly examine these changes qualitatively and quantitatively in both rat strains to demonstrate the severity and persistence of these features. Neutrophilic inflammation due to SO2 exposure has been noted in earlier studies using SD rats (Killingsworth et al., 1996; Kodavanti et al., 2000b), which is consistent with our present finding. However, the inflammatory response was mild and rapidly reversible upon termination of SO2 exposure with mucus production being more persistent than inflammation (Kodavanti et al., 2000a). We report here that SH rats mounted a remarkably greater inflammatory response with mucus production more persistent than that of SD rats. Although several laboratory species have been used in developing an SO2-induced bronchitis models (Killingsworth et al., 1996; Shore et al., 1987, 1995) and their limitations are well reported (Kodavanti et al., 2000a,b), no studies have compared more
than one strain in parallel to demonstrate differences in disease severity. The comparative evaluation of SD and SH rats indicate that the use of SH rats may offer more intense and persistent bronchitic symptoms reminiscent of the human disease. Further characterization of this model upon low concentration of SO2 exposure for a longer period of time is likely to increase persistency of inflammation.

Since SO2 is highly reactive and is hydrated very rapidly, it is converted to sulfite and bisulfite as soon as it comes in contact with airway lining fluid while triggering modifications in protein disulfide bonds (Petering and Shih, 1975). The exact mechanisms that trigger neutrophil migration and increased mucus secretion are unknown. C-fiber deactivation has been proposed to be involved as more severe SO2-induced inflammation was noted in capsaicin-pretreated rats (Long et al., 1997, 1999). It is not clear which mechanism may be responsible for exacerbated SO2-induced inflammation in SH rats. The evaluation of gene expression pattern may provide insights into mechanisms of exacerbated SO2 response in SH rats.

The present experimental design does not allow for identifying the genetic risk factors associated with greater susceptibility of SH rats; however, evaluating global gene expression profiles of strains in parallel may provide distinctive biological pathways or functions. The striking baseline differences in immune modulatory and inflammatory gene expression may reflect compromised immune function in SH rats (Khraibi et al., 1984). Immune deregulation may also be evident by deficiency in beta defensin and elevated expression of inflammatory cytokine mRNA expression. Gene expression analysis demonstrated reduced expression of beta defensin in the SH as opposed to SD rats in the present study. Genetic variants of human beta defensin have been associated with COPD (Matsushita et al., 2002). Also, increased expressions of cytokines, IL-1-beta, MIP-2 (a human analog of IL-8), and TNF-α, and iron-binding protein, transferrin, in inflammatory cells of

![FIG. 6.](image.png) Quantitative analysis of mucin levels in the airways of SD and SH rats following 4-day exposure to SO2. Percentage of airway epithelium containing mucin was determined by estimating the percentage of the epithelial layer that stained positive for AB-PAS (mucin) relative to the total epithelial layer area at 1 or 4 days following last SO2 exposure. Each group represents mean ± SE of five rats. *Indicates significant difference from time matched control, and **#** indicates significant strain difference at matching concentration.

![FIG. 7.](image.png) Differential effect of SO2 exposure in lung mRNA expression for inflammatory cytokines in SD and SH rats. Rats were exposed to filtered air or SO2 5 h/day × 4 days, and total lung RNA was analyzed by real-time quantitative PCR to determine mRNA expression for cytokines. Only control and 350 ppm SO2-exposed SD and SH rats at 1 day postexposure were evaluated. Values represent mean ± SE (four rats per group). *Indicates significant difference from time matched control, and **#** indicates significant strain difference.

### TABLE 1

<table>
<thead>
<tr>
<th>Pathology indices</th>
<th>SD rats</th>
<th>SH rats</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SO2, 0 ppm</td>
<td>SO2, 250 ppm</td>
</tr>
<tr>
<td>Macrophages, nonpigmented</td>
<td>1.3 ± 0.2</td>
<td>1.9 ± 0.2</td>
</tr>
<tr>
<td>Macrophages, pigmented</td>
<td>0.4 ± 0.2</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>Bronchiolar epithelial hyperplasia</td>
<td>0.8 ± 2.0</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>Peribronchiolar acute inflammation</td>
<td>0.0 ± 0.0</td>
<td>0.2 ± 0.2</td>
</tr>
<tr>
<td>Vascular mineralization</td>
<td>0.5 ± 0.3</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>Goblet cells</td>
<td>1.2 ± 0.1</td>
<td>1.5 ± 0.2</td>
</tr>
</tbody>
</table>

*Note.* The values indicate mean ± SE (n = 10 rats per group; five at 1 day and five at 4 day postexposure) of pathology score assigned to each rat following 5 h/day × 4-day SO2 exposure based on 1 = mild, 2 = moderate, 3 = marked, and 4 = severe. Note that based on H&E-stained slides evaluation, lesion severities at 1 and 4 day postexposure were not different, and therefore, the values for both time points were combined.
chronic bronchitic patients (Chung, 2001; Stites et al., 1998) are consistent with observed SO$_2$-induced expression changes in SH relative to SD rats. Exposure to SO$_2$ has been shown to induce inflammatory response in rats (Long et al., 1999), but systematic analysis of the role of cytokines has not been addressed in rat model of bronchitis. Thus, one can assume that some of the molecular changes leading to inflammation and mucus hypersecretion in SH rats after acute SO$_2$ exposure are common in susceptible humans who develop chronic bronchitis. However, it is likely that the pathogenesis of irreversible bronchitis resulting from years of cigarette smoke exposure may involve multiple component-specific cell injuries and complex interplay of injury and compensatory mechanisms.

SH rats harbor a deletion variant in the CD36 fatty acid transporter, an integral membrane glycoprotein expressed on the surface of cells, causing defective fatty acid metabolism, insulin resistance, and hypertension (Hajri et al., 2001; Pravenec and Kurtz, 2002; Pravenec et al., 2004). The nearly absent expression of the CD36 gene in SH but high expression in SD rats confirm this deficiency and validates the quality of data generated by the gene chip experiments in our study. This defect in CD36 has been shown to be associated with metabolic syndrome characteristics of hypertensive rats and is thought to contribute to cardiac hypertrophy (Hajri et al., 2001). However, it has been reported that transgene expression of CD36 in SH rats while ameliorating metabolic disturbances does not affect hypertension (Pravenec et al., 2003), again supporting the hypothesis that multigenetic defects are likely involved. The CD36 gene also has been shown to have a major role in immune function and is thought to be involved in inflammation associated with COPD (Pons et al., 2005). Although the complex functional roles of CD36 are still unclear, it is likely that increased susceptibility of SH rats to SO$_2$-induced inflammation may be in part due to their defect in CD36 and altered immune regulation and metabolic function.

Examples of genes that were differentially expressed between SH and SD rats include RT1 class I$\beta$, an MHC class I antigen involved in natural killer cell activity (Leong et al., 1999), cysteine-rich 61, involved in regulation of cell signaling (Lombet et al., 2003), and telomeric repeat binding factor 2...
critical in aging and cancer (Gu et al., 2005). It is not known why these genes may be downregulated in SH rats, but these might represent genetic markers involved in their greater susceptibility to environmental exposure. Clearly more investigation is needed.

Because SH rats begin their progressive hypertension at 12 weeks of age, their underlying sensitivity might reflect this change. The present study does not allow separation of the role of hypertension from other phenotypic and genetic factors. Drew et al. (1983) used DahlS rats (sensitive to salt-induced hypertension) and DahlR rats (resistant to salt-induced hypertension) and showed that the DahlR demonstrated a slight decrease in blood pressure following SO2 exposure (50 ppm), while DahlS rats demonstrated an increase. More studies will be required to investigate the contribution of hypertension. The purpose of this study was to investigate if SH rats demonstrated greater susceptibility than conventionally used SD rats and, if so, to validate the model at the physiological, pathological, and molecular levels. In SH rats, efforts of reducing hypertension also reduced underlying oxidative stress and vice versa (Lazaro et al., 2005; Rodriguez-Iturbe et al., 2003), suggesting that the hypertension phenotype is mechanistically linked to the presence of other ailments and may modify susceptibility to SO2.

Thus, careful experimentation will be required to determine the role of hypertension versus other risk factors in understanding their susceptibility to SO2 exposure.

Although biochemical, molecular, and morphological features of bronchitis and COPD are well categorized in patients (MacNee, 2005; Spurzem and Rennard, 2005), the pathogenic mechanisms by which reactive cigarette smoke components initiate the complex process of chronic active inflammation, airway remodeling, mucus hypersecretion, and alveolar destruction are less well understood. Matrix metalloproteinases, inactivation of protease inhibitors, and collagen and elastin degradation by-products have been shown to play a role in chronic activation of CD8 T lymphocytes, AMs, and neutrophils (Cawston et al., 2001; Postma and Timens, 2006). It is not understood why some individuals develop both alveolar as well as airway pathologies from cigarette smoke exposure as in COPD, whereas others develop primarily an airways disease as in bronchitis. It is likely that genetic and molecular differences between individuals determine the primary pulmonary target tissues affected by chronic cigarette smoking. Because high-level SO2 exposures, such as one used in the present study, primarily cause damage to airway epithelial cells and mucus hypersecretion without significant alveolar destruction, the mechanism by which SO2 cause bronchitis is likely to be different from that of tobacco smoke in humans and animals. Nevertheless, the SO2-induced bronchitis in SH rats may allow one to understand the role of airways disease and genetic susceptibility. Also, because SH rats demonstrate pathologies of persistent neutrophilic inflammation and mucus hypersecretion, they may provide a better model for therapeutic interventions. Based on the sensitivity of SH rats to SO2, we predict that experimental exposure of these rats to tobacco smoke may also yield a highly relevant animal model of bronchitis and COPD. Studies are in progress to characterize cigarette smoke-induced pulmonary disease in SH relative to healthy rats.

Bronchitis and COPD are chronic diseases that develop over several years of cigarette smoking (MacNee, 2005). Experimental animals are typically exposed to SO2 for 6–8 weeks to develop features of human bronchitis (Kodavanti et al., 2000b; Lamb and Reid, 1968; Shore et al., 1995). In this study, we exposed rats to SO2 for only 4 consecutive days because we initially wanted to determine relative susceptibility of two strains to acute exposures at concentrations that have been used previously in experimental bronchitis. Secondly, due to the sensitivity of SH rats to SO2 at concentrations used in the present study, we presumed that a longer duration exposure may induce severe compromise of pulmonary health. A follow-up study will involve long-term episodic exposures to SO2 at lower concentrations, which is hypothesized to provide longer lasting pulmonary inflammation and mucus hypersecretion than observed in this study.

In conclusion, we provide evidence that SH rats, which are genetically predisposed to hypertension and exhibit associated systemic risk factors also found in human COPD patients, are more susceptible to SO2-induced airway inflammation and mucus hypersecretion than conventionally used SD rats. Thus, the SH rat appears to be a more relevant experimental human bronchitis model than seen previously with other rats. Further, pulmonary gene expression profiling in SH and SD rats following air and SO2 exposure allowed identification of genetic markers which might be responsible for greater SO2 sensitivity. These genetic targets might also underlie human susceptibility to chronic bronchitis.

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