Reactivity of mouse alveolar macrophages to cigarette smoke is strain dependent

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Vecchio D, Arezzini B, Pecorelli A, Valacchi G, Martorana PA, Gardi C. Reactivity of mouse alveolar macrophages to cigarette smoke is strain dependent. Am J Physiol Lung Cell Mol Physiol 298: L704–L713, 2010. First published February 12, 2010; doi:10.1152/ajplung.00013.2009.—Cigarette smoke (CS) is a main risk factor in chronic obstructive pulmonary disease (COPD), but only 20% of smokers develop COPD, suggesting genetic predisposition. Animal studies have shown that C57BL/6J mice are sensitive to CS and develop emphysema, whereas Institute of Cancer Research (ICR) mice are not. To investigate the potential factors responsible for the different susceptibility of ICR and C57BL/6J mice to CS, we evaluated in alveolar macrophages (AMs) isolated from these strains of mice the possible mechanisms involved in the inflammatory and oxidative responses induced by CS. Lactate dehydrogenase (LDH) release revealed that C57BL/6J AMs were more susceptible to CS extract (CSE) toxicity than ICR. Differences were observed in inflammatory and oxidative response after CSE exposure. Proinflammatory cytokines and matrix metalloproteinases (MMPs) were increased in C57BL/6J but not ICR AMs. Control C57BL/6J AMs showed a higher baseline production of reactive oxygen species (ROS) and H2O2 with lower baseline levels of GSH, nuclear factor erythroid 2-related factor 2 (Nrf2), heme oxygenase-1 (HO-1), and glutathione peroxidase (GPX2). This was associated with reduced histone deacetylase-2 (HDAC2) expression, activation of NF-κB, and higher basal levels of TNF-α and IL-6. CSE induced a decrease in HDAC2 protein levels in both C57BL/6J and ICR AMs; however, the level of HDAC2 was significantly lower in C57BL/6 than in ICR AMs. Furthermore, CSE enhanced NF-κB-dependent cytokine release only in C57BL/6J AMs. We suggest that an imbalance in oxidative stress decreases HDAC2 levels and facilitates NF-κB binding, resulting in a proinflammatory response in C57BL/6J but not in ICR AMs. These results could contribute in understanding the different susceptibility to CS of these strains of mice.

chronic obstructive pulmonary disease; glutathione; histone deacetylase-2; mouse strains; nuclear factor-κB

Among all the inflammatory cells, alveolar macrophages (AMs) play a pivotal role in the pathogenesis of COPD. In fact, macrophages are the most abundant cell type recovered from bronchoalveolar lavage fluid (BALF) of patients with COPD (51), and macrophage number in BALF correlates with disease severity (20). Similarly, AM numbers are greatly elevated in BALF and lung tissues of animal models of emphysema induced by CS exposure (12, 27, 57). In addition, in patients with emphysema, macrophages are localized at sites of alveolar wall destruction (14). Macrophages are the source of many proinflammatory mediators such as TNF-α, leukotriene B4, reactive oxygen species (ROS), and chemokines such as IL-8 and IL-6 (4). Additionally, macrophages release a number of matrix metalloproteinases (MMPs) with potential degrading activity on lung matrix, such as MMP-2, -9, and -12, and the production of these proteases has been found to be elevated in patients with COPD (13, 45).

CS-induced oxidative stress plays an important role in lung inflammation occurring in COPD. CS contains >1017 oxidants/free radicals and 4,700 chemical compounds, and many of these are relatively long-lived and can generate ROS and hydrogen peroxide (H2O2; Refs. 32, 55). GSH is the most important intracellular antioxidant thiol involved in the redox defense against oxidative stress. GSH redox homeostasis is critical to protect the lung from oxidative injury and for the transcriptional regulation of many proinflammatory genes (38, 39).

Moreover, nuclear factor erythroid 2-related factor 2 (Nrf2) is a central transcription factor that regulates the antioxidant defense system and acts as a modifier of several lung diseases that involve oxidative stress and inflammation (25). Nrf2 mediates transcriptional regulation of various antioxidant genes, including glutathione peroxidase (GPX2) and heme oxygenase-1 (HO-1), by binding to the antioxidant responsive element present in the promoters of these genes (16, 54). A significant decline in Nrf2-regulated antioxidant defenses and a greater degree of oxidative damage were observed in lung tissues of patients with COPD compared with those without COPD (25).

At the molecular level, increased oxidative stress has been implicated in stimulation of the inflammatory response through the activation of transcription factors, such as NF-κB, chromatin remodeling, and gene expression of inflammatory mediators (29, 36, 40). NF-κB is critical for the transcription of proinflammatory genes and is known to possess an intrinsic histone acetyl transferase (HAT) activity (35). Gene expression is regulated, at least in part, by acetylation of core histone through the action of coactivators with intrinsic HAT activity. Conversely, histone deacetylases (HDAC) and other corepres-
ors mediate proinflammatory gene repression. In COPD, there is no change in HAT activity but a great reduction of HDAC activity. In particular, HDAC2 is reduced in peripheral lung and in AMs of patients with COPD, leading to an increased inflammatory cytokine expression (21).

In the present study, we assessed the responses of murine AMs after CS extract (CSE) exposure. The macrophages were isolated in BALF from two strains of mice, either susceptible (C57BL/6J) or resistant [Institute of Cancer Research (ICR)] to CS (7).

C57BL/6J mice respond to acute smoke exposure with a significant increase in BALF macrophages that are also activated. In an acute study in C57BL/6J mice, CS caused a significant and reversible decrease in total lung antioxidant capacity and significant changes in GSH, ascorbic acid, protein thiols, and 8-epi-PGF2α in BALF (8). C57BL/6J mice chronically exposed (7 mo) to CS developed emphysema, and their lung elastin content was significantly decreased. On the other hand, ICR mice, acutely exposed to CS, significantly increased the antioxidant capacity in their BALF, and when chronically exposed they did not develop emphysema and other lung changes (7).

Since mice of an inbred strain are genetically identical, lung cells (and particularly macrophages) from mice of different strains may represent an ideal model for investigating genetically different inflammatory and oxidative responses to CS. This may be of particular interest since also in humans the individual susceptibility to CS and the magnitude of the CS-mediated inflammation are generally accepted to be genetically mediated. The present study reports the results of the different susceptibility of C57BL/6J and ICR AMs to CSE and suggests a possible mechanism involved in their responses.

MATERIALS AND METHODS

Materials. Chemicals and reagents were from Sigma-Aldrich (St. Louis, MO) unless otherwise specified. Commercial cigarettes yielding 8 mg of tar and 0.7 mg of nicotine were used in this study. Male mice 6 wk old of the strains C57BL/6J and ICR (Harlan, Udine, Italy) were used. All animal experimentation was approved by the Local Ethical Committee of the University of Siena (Siena, Italy).

Preparation of CSE. CSE was prepared as described by Aoshiba and coworkers (1). The smoke was generated from one cigarette by drawing consecutive puffs into a 20-ml plastic syringe, with a stopcock connected through one port to a glass bottle containing 10 ml of RPMI 1640. A 20-ml puff drawn in 1 s was obtained at 10-s intervals, and each puff was held for 3 s and bubbled through RPMI 1640 in 5 s. CSE was drawn into the syringe through a 0.22-µm pore size filter (Sartorius, Göttingen, Germany) rated to remove the tar phase of CS. The CSE solution was freshly prepared and used within 3 min after incubation medium samples were analyzed for TNF-α, macrophage inflammatory protein-2 (MIP-2), keratinocyte-derived chemokine (KC), IL-6, and IL-10 using the Bio-Plex Protein Array System (Bio-Rad, Milan, Italy) according to the manufacturer’s instructions. The assay permits the simultaneous cytometric quantitation of multiple cytokines with minimal sample volume. The levels of cytokines were expressed as picograms per milliliter.

MMP detection. Semiquantitative measurement of MMP activity was performed using zymography (33). Samples were loaded into 10% polyacrylamide gels incorporating as substrate 0.1% (wt/vol) either gelatin (for MMP-2 and MMP-9 detection) or casein (for MMP-12 detection). Proteins were subjected to electrophoresis at 125 V for 90 min, and the gels then washed three times in 20 mM Tris-HCl, pH 7.8, 2.5% (vol/vol) Triton X-100 for 15 min. The gels were then washed twice in Tris-HCl, pH 7.8, containing 1% (vol/vol) Triton X-100, 10 mM CaCl2, 5 µM ZnCl2, and incubated for 18 h at 37°C. Gels were fixed by washing in 50% methanol, 7% acetic acid and stained with 0.05% Coomassie blue in 50% methanol, 7% acetic acid. Bands of lysis (enzyme activity) were visualized by washing in 20% methanol, 7% acetic acid. Quantitative evaluation of the gelatinolytic or caseinolytic activity was performed by scanning the gel using a Bio-Rad densitometer.

Measurement of ROS and H2O2. Oxidative stress in terms of ROS and H2O2 was determined in AMs from C57BL/6J and ICR. AMs were suspended in RPMI 1640 medium plus 10% FBS and seeded in 96-well plates (4 × 104 cells/well) and allowed to adhere for 2 h before the experiments. Levels of ROS were measured in untreated and CSE-treated cells using the 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA) probe according to the method of O’Toole and coworkers (30) with slight modifications. Before the experiments, AMs were incubated in media containing 1% FBS, washed, and then loaded with 1 µM H2DCFDA in PBS for 1 h at 37°C. After washing, the cells were returned to PBS in the presence or absence of 5% CSE, and changes in fluorescence [excitation (Ex.) 490 nm; emission (Em.) 535 nm] were measured by a PerkinElmer Applied Biosystems (Milano, Italy) fluorescence plate reader. Rate of ROS production was determined over a 40-min period.

H2O2 production was measured according to the method of Mo-hanty and coworkers (28), by monitoring the horseradish peroxidase (HRP)-catalyzed oxidation of the probe N-acetyl-3,7-dihydroxyphenoxazine (A6550; Molecular Probes, Eugene, OR), which becomes highly fluorescent only after oxidation by H2O2. After adhesion, AMs were washed twice with PBS, and the medium was replaced by Krebs-Ringer phosphate buffer (200 µl/well), pH 7.4, containing 20 mM HEPES, 130 mM NaCl, 1.2 mM Na phosphaté, 5 mM KCl, 2 mM CaCl2, 1.2 mM MgSO4, and 1 g/l glucose, with or without 5% CSE. The probe A6550 and HRP were added at final concentration of 50 µM and 1 U/ml, respectively, and fluorescence was read by a PerkinElmer Applied Biosystems fluorescence plate reader (Ex. 560 nm; Em. 642 nm).

Measurement of intracellular GSH levels. Cells were seeded at a density of 5 × 104/well in 96-well culture plates (unless otherwise indicated) at 37°C in 5% CO2. After 2 h, the cells were treated with CSE solutions for the following experiments.

Lactate dehydrogenase release. Preliminary experiments were carried out to assess the range of CSE concentrations nontoxic for AMs by incubating the cells with CSE at various concentrations for 24 h. CSE toxicity was assessed by measuring the release of lactate dehydrogenase (LDH) activity in culture media using the LDH-P kit (Dasit, Milano, Italy). Cell viability was also assessed by trypan blue exclusion test.

Cytokine assay. After 18 h of treatment with 5% CSE, cell-free incubation medium samples were analyzed for TNF-α, macrophage inflammatory protein-2 (MIP-2), keratinocyte-derived chemokine (KC), IL-6, and IL-10 using the Bio-Plex Protein Array System (Bio-Rad, Milan, Italy) according to the manufacturer’s instructions. The assay permits the simultaneous cytometric quantitation of multiple cytokines with minimal sample volume. The levels of cytokines were expressed as picograms per milliliter.

Measurement of intracellular GSH levels. Cells were seeded at a density of 5 × 104/well in 96-well culture plates and treated with 5% CSE in medium containing 1% FBS for 18 h. GSH levels were measured by the DTNB-glutathione disulfide reductase recycling method described by Tietze (52) with slight modifications (41).
Briefly, the cells were washed in cold PBS and then sonicated in 0.1 M phosphate buffer containing 5 mM EDTA, 0.1% (vol/vol) Triton X-100, and 0.6% (wt/vol) sultosalicylic acid. After centrifugation, the supernatant was incubated with 0.2 mg/ml DTNB and 1.67 U/ml glutathione reductase in phosphate buffer-EDTA for 30 s. Then, 0.2 mg/ml β-NADPH was added, and the rate of DTNB reduction was spectrophotometrically measured at 405 nm. The concentration of GSH in the supernatant was determined by comparison with the rate of DTNB reduction by known concentrations of GSH.

Quantitative real-time PCR. mRNA levels of genes of interest were evaluated by quantitative real-time PCR on total RNA isolated from AMs using SYBR Green (PerkinElmer Applied Biosystems) and ABI PRISM 7700 Sequence Detector (PerkinElmer Applied Biosystems). Primers were synthesized using Primer Express version 1.0 software (PerkinElmer Applied Biosystems) and according to the published cDNA sequences for Nrf2, HO-1, GPX2, and (PerkinElmer Applied Biosystems) and evaluated by quantitative real-time PCR on total RNA isolated from AMs using SYBR Green (PerkinElmer Applied Biosystems) and ABI PRISM 7700 detection system. The mRNA level analysis was done in triplicate, and the values were normalized to β-actin.

Immunoblot analysis of Nrf2, HO-1, and GPX2 in AMs. Immunoblot analysis of Nrf2, HO-1, and GPX2 was performed in AMs treated for 24 h with 5% CSE. Whole cell extracts were prepared by lysing the AMs in RIPA buffer (20 mM Tris·HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 1% Triton X-100, 0.1% SDS, and 1% sodium deoxycholate) containing a protease inhibitor cocktail and 1 mM PMSF incubated on ice for 10 min and sonicated. Cell lysates were resolved on SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane (Amersham Biosciences, Amersham, United Kingdom). Immunoblots were performed using primary antibodies for Nrf2, HO-1, and GPX2 (Santa Cruz Biotechnology, Santa Cruz, CA) as previously described (25). We used β-actin as the loading control. Immunodetected proteins were visualized using ECL kit (Amersham Biosciences).

Western blotting for HDAC2 protein. Total cellular proteins were extracted from AMs by freeze-thawing samples in lysis buffer (50 mM Tris·HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate) with a protease inhibitor cocktail and 1 mM PMSF, as previously described (22). Isolated soluble proteins (20 μg) were electrophoresed on 10% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane (Amersham Biosciences). Immunoblotting was performed using goat polyclonal anti-HDAC2 antibody (1:1,000; Santa Cruz Biotechnology) at 4°C overnight. After washing, the levels of HDAC2 protein were determined with HRP-conjugated rabbit anti-goat antibody (1:20,000), and immunodetected proteins were visualized with ECL assay kit (Amersham Biosciences). The level of loading was controlled by determining β-actin expression.

Assay of total HDAC activity. HDAC activity was measured with an HDAC fluorometric cellular activity assay (AK503; Biomol). AMs were seeded at a density of 2.5 × 10⁵/well in 1/2 volume 96-well plates and treated for 4 h with 5% CSE in medium containing 1% FBS and 200 μM HDAC Fluor-de-Lys substrate. The procedure determines the total amount of deacetylated substrate in a single well by adding developer in cell lysis buffer directly to cells. The fluorescence was read in a fluorometer (Ex. 380 nm; Em. 460 nm). HeLa cell nuclear extract was used as a positive control. Standard curve was performed using the known amount of the Fluor-de-Lys deacetylated standard included in the kit.

Effect of HDAC inhibition on cytokine release. The role of HDAC on cytokine release was tested by using the HDAC inhibitor trichostatin-A (TSA). AMs (3 × 10⁴/well) were incubated with 5% CSE or TSA (100 ng/ml) individually or in combination for 18 h. The levels of TNF-α and MIP-2 were measured by sandwich ELISA (Duoset; R&D Systems Europe, Abingdon, United Kingdom) according to the instructions of the manufacturer.

NF-κB activation. The level of NF-κB activation was determined using a commercially available p65 plate assay (TransAM; Active Motif, Rixensart, Belgium). AMs were incubated either in presence or absence of 5% CSE for 2 h. Total protein was extracted from AMs by adding lysis buffer supplemented with 1 M dithiothreitol and protease inhibitor cocktail and processed according to the manufacturer’s instructions. Results were expressed as absorbance per milligram of total protein.

Statistical analysis. Results were expressed as means ± SD. Statistical analysis of significance was calculated using one-way ANOVA followed by Tukey post hoc test for multigroup comparisons. The value of P < 0.05 was considered as significant.

RESULTS

Effect of CSE on LDH release from AMs. Toxicity of various CSE concentrations was assessed by means of LDH release in AMs from ICR and C57BL/6J mice. The results are shown in Fig. 1. After 24 h, a significant LDH release was observed in C57BL/6J AMs starting at the 10% concentration onward. On the other hand, in ICR AMs, a significant release was found only at the higher CSE concentration of 25% (Fig. 1). Thus, in the following experiments, AMs were exposed only to the nontoxic 5% CSE concentration.

Cytokine release from AMs after CSE exposure. CSE exposure (18 h) induced a significant release of MIP-2 and its isoform KC in C57BL/6J AMs (Fig. 2, A and B). In addition, there was a fivefold increase in TNF-α and a threefold increase in IL-6 only in C57BL/6J AMs. The baseline release of both of these cytokines was significantly higher in C57BL/6J AMs (Fig. 2, C and D). No changes were seen in ICR AMs after CSE exposure (Fig. 2). These results demonstrate a different inflammatory response by AMs from the two strains of mice and are consistent with a higher susceptibility of C57BL/6J mice to CS exposure. The anti-inflammatory cytokine IL-10...
was significantly reduced in AMs of both strains following CSE exposure (Fig. 2).

**MMP activity in AMs after CSE exposure.** Since MMPs play a prominent role in smoking-induced emphysema, conditioned media from AMs were characterized by gelatin and casein zymography. Figure 3A shows that AMs produced two bands of gelatinolytic activity at 72 and 92 kDa. These data are consistent with the presence of MMP-2 and MMP-9 activity in the macrophage media. In particular, a significant increase in MMP-9 (+65%) and MMP-2 (+61%) was observed in C57BL/6J cells after 18 h of CSE treatment. No significant changes were observed for both MMPs in ICR AMs after CSE exposure (Fig. 3B).

CS exposure in mice is associated with MMP-12 expression in macrophages and development of emphysema (18, 47). Under our experimental conditions, a band corresponding to MMP-12 (22 kDa) appeared on the casein zymogram (Fig. 4A), and this was again induced by CSE only in AMs of the C57BL/6J (Fig. 4B).

Fig. 2. Effect of CSE on the release of cytokines by mouse AMs. The cells were exposed to CSE for 18 h, and cytokine release was evaluated by Bio-Plex Cytokine Assay. Macrophage inflammatory protein-2 (MIP-2; A), keratinocyte-derived chemokine (KC; B), TNF-α (C), and IL-6 (D) release were significantly increased only in CSE-treated C57BL/6J AMs (black bar). In addition, a greater release of TNF-α (C) and IL-6 (D) was also observed in control C57BL/6J AMs compared with ICR AMs (white bar). E: IL-10 release decreased in AMs of both strains. Data are means ± SD of 3 experiments. *P < 0.05 compared with respective control; **P < 0.05 vs. respective ICR group.

Fig. 3. Effect of CSE on matrix metalloproteinase (MMP) activity. A: the activity of MMP-9 and MMP-2 was evaluated by zymography in culture media of mouse AMs. The cells were exposed for 18 h to CSE. B: in C57BL/6J AMs (black bar), a significant increase in MMP-9 and MMP-2 was observed. The results are expressed as relative density (arbitrary units) of the bands. Data are means ± SD of 3 experiments. *P < 0.05 vs. respective controls; **P < 0.05 vs. respective ICR group (white bar).

Fig. 4. Effect of CSE on metalloelastase activity. A: MMP-12 activity was evaluated by zymography in culture media of mouse AMs. The cells were exposed for 18 h to CSE. B: in C57BL/6J AMs (black bar), a significant increase in MMP-12 was observed. The results are expressed as relative density (arbitrary units) of the bands. Data are means ± SD of 3 experiments. *P < 0.05 vs. respective control; **P < 0.05 vs. respective ICR group (white bar).
CSE-induced oxidative stress in AMs. To elucidate the oxidative stress level induced by CSE in AMs from the two strains of mice, the levels of ROS and H2O2 were evaluated. Our data show that, under basal conditions, ROS generation was significantly higher in C57BL/6J AMs than in ICR (Fig. 5A). After CSE treatment, ROS levels were further increased in the AMs of both strains with a more important response in C57BL/6J.

As shown in Fig. 5B, similar to the ROS results (Fig. 5A), basal H2O2 production was significantly higher in C57BL/6J AMs, and also, in this case, CSE exposure increased H2O2 production in both strains (~4-fold) compared with respective controls. The levels of H2O2 in C57BL/6J AMs were significantly higher than in ICR AMs (Fig. 5B).

These findings suggest that the different CSE-mediated inflammatory response observed between ICR and C57BL/6J AMs could be mediated by a higher intracellular oxidative stress generation.

Effects of CSE on GSH levels of AMs. The antioxidant GSH plays a critical role in the lung antioxidant defenses. Of interest, control C57BL/6J AMs had significantly lower basal intracellular levels of GSH than ICR AMs (Fig. 6). CSE treatment induced a significant decrease of GSH levels in ICR AMs after 18 h. On the other hand, CSE induced only a small and not statistically significant decrease of the GSH levels in C57BL/6J AMs (Fig. 6).

Effects of CSE on Nrf2, GPX2, and HO-1 mRNA levels. The results depicted in Fig. 7A demonstrate that Nrf2 mRNA levels were lower in AMs isolated from C57BL/6J than in the ones from ICR, and, after CSE treatment, there was a significant increase in both strains. With regard to GPX2, the basal mRNA levels did not show any significant difference between the two strains. CSE exposure increased the expression of GPX2 gene in both strains, and this increase was more evident in C57BL/6J AMs (Fig. 7B). The same trend was observed for HO-1 mRNA levels (Fig. 7C).

Effect of CSE on Nrf2, HO-1, and GPX2 protein expression in mouse AMs. Immunoblot analysis shows that, in C57BL/6J AMs, basal expression of Nrf2 (Fig. 8A), and of the related proteins HO-1 (Fig. 8B) and GPX2 (Fig. 8C), was lower than in ICR AMs. After CSE exposure, a significant increase of Nrf2 and HO-1 levels was observed in both strains. However, absolute protein values were significantly higher in ICR AMs. After CSE exposure, GPX2 levels (Fig. 8C) were increased in ICR but not in C57BL/6J AMs. These results demonstrate a different response in antioxidant protein expression by AMs from the two strains of mice.

Levels of HDAC in AMs after CSE exposure. C57BL/6J AMs showed a basal expression of HDAC2 significantly lower than ICR AMs (Fig. 9). CSE treatment significantly decreased HDAC2 protein levels in both strains; however, the HDAC2 levels in the C57BL/6J AMs were markedly lower than in ICR AMs. This is in partial agreement with results obtained in the experiment on total HDAC activity (Fig. 10). The basal total activity of HDAC in AMs from C57BL/6J mice was significantly lower compared with that of ICR but did not change after CSE exposure. On the other hand, CSE treatment significantly decreased HDAC activity in ICR AMs (Fig. 10).

Effect of HDAC inhibition on cytokine release. Additional experiments were carried out in the presence of TSA, a potent inhibitor of HDAC, to examine the role of HDAC in the different CSE-mediated inflammatory response observed between the two strains of mice. In particular, TNF-α and MIP-2 release was assayed after CSE and/or TSA treatment. Basal release of TNF-α was higher in C57BL/6J AMs compared with ICR (Fig. 11A). Pretreatment of control AMs with TSA did not

Fig. 5. CSE-induced oxidative stress in AMs. AMs were treated with or without CSE, and reactive oxygen species (ROS) and H2O2 production was evaluated. A: ROS levels were measured using the probe 2’,7’-dichlorodihydrofluorescein diacetate (H2DCFDA). Rate of ROS production was determined over a 40-min period [excitation (Ex.) 490 nm; emission (Em.) 535 nm]. B: H2O2 production was evaluated by monitoring the horseradish peroxidase (HRP)-catalyzed oxidation of the fluorescent probe A6550 over 5 min (Ex. 560 nm; Em. 642 nm). Baseline levels of both markers of oxidative stress were higher in C57BL/6J AMs (black bar) than in ICR AMs (white bar). CSE exposure for 18 h decreased of GSH levels in ICR AMs (white bar). Data are means ± SD of 3 experiments. *P < 0.05 vs. respective control; **P < 0.05 vs. respective ICR group.

Fig. 6. Intracellular GSH levels in AMs before and after CSE treatment. C57BL/6J AMs (black bar) show reduced baseline levels of GSH compared with ICR AMs (white bar). CSE exposure for 18 h decreased of GSH levels in ICR AMs (white bar). Data are means ± SD of 3 experiments. *P < 0.05 vs. respective control; **P < 0.05 vs. respective ICR group.
Effect of CSE on NF-κB activation. NF-κB is activated in cells challenged with CSE and other inflammatory insults, and it is involved in the transcriptional activation of proinflammatory genes (55, 56). However, no data are available on a potentially different activation of NF-κB in AMs from susceptible and resistant mouse strains after CS exposure. NF-κB activation, measured by the levels of p65-DNA binding, was evaluated in AMs isolated from the two strains of mice. Untreated AMs from C57BL/6J mice showed a markedly higher NFκB activation with respect to ICR AMs (Fig. 12). CSE caused a significant increase in NF-κB activation in C57BL/6J AMs (black bar) or ICR (white bar) was evaluated by real-time PCR. The isolated mRNA samples were analyzed using the specific primers and compared with β-actin (housekeeping gene) levels. Data are expressed as means ± SD of 3 experiments. *P < 0.05 vs. respective control; **P < 0.05 vs. respective ICR group.

**DISCUSSION**

The pathogenesis of COPD has been studied in various murine models of CS exposure. The effects of CS in the mouse are believed to be strain-dependent, since inbred mouse strains can be either susceptible or resistant to the CS-induced lesions (7, 17, 57).

In this regard, it has been previously reported that ICR mice are resistant to the effects of smoke exposure, whereas C57BL/6J are susceptible and respond with the development of emphysema (7). However, the cellular mechanisms responsible for the different susceptibility of these mouse strains to CS are still unknown.

In the present investigation, AMs isolated from C57BL/6J and ICR mice showed significantly different CSE-mediated inflammatory responses. C57BL/6J AMs showed a higher susceptibility to CSE toxicity than AMs from ICR. Furthermore, CSE exposure resulted in a different quantitative as well as qualitative release of proinflammatory mediators in the two mouse strains. In fact, after CSE exposure, C57BL/6J AMs released a significant amount of chemoattractants (MIP-2 and KC), proinflammatory cytokines (TNF-α and IL-6), and MMPs (MMP-12, MMP-9, and MMP-2), whereas this was not noticed in ICR AMs. These results are consistent with the data obtained in vivo by Yao and coworkers (57). The increase in TNF-α in C57BL/6J AMs is of interest. TNF-α is a major proinflammatory cytokine that enhances macrophage and neutrophil oxidant burst, MMP production, and stimulation of cytokine release (10). TNF-α upregulates the release of IL-6, a cytokine that causes hepatic production of acute phase proteins, and it is an important mediator of pathophysiological reactions in the lung (19). In addition, TNF-α activates macrophages to produce inflammatory cell chemoattractants, such as IL-8 (the human homolog of murine MIP-2 and KC; Ref. 10).

Our data show that TNF-α baseline levels in C57BL/6J AMs were significantly higher than in ICR AMs and increased fivefold after CSE. Of note is that this increase was accompanied by an enhanced level of MMP-12, MIP-2, and KC. In a recent study performed in C57BL/6J mice acutely exposed to CS, it has been proposed that MMP-12 functions also as a TNF-α-converting enzyme. Under the effect of MMP-12, the active form of TNF-α is released from the proform and induces the liberation from AMs of chemoattractants such as MIP-2 and KC. This evokes an influx of inflammatory cells and, in particular, of neutrophils that release elastase, leading to matrix breakdown and emphysema (11).

The increased release of MMP-12, TNF-α, and chemoattractants observed in this study in C57BL/6J AMs (but not ICR AMs) is consistent with previous in vivo works (10, 11, 18), where the development of emphysema in C57BL/6J mice has been studied.

In addition, we have shown that MIP-2 and MPP-9 were increased in C57BL/6J after CSE treatment. The prodomain of MMP-9 can be cleaved by MMP-2, and both of these proteinases degrade elastin (3, 46). Thus both have the potential of inducing emphysema. Examination of macrophages isolated from smokers indicates that CS increases the production of MMP-9 in these cells (44), and MMP-9 (together MMP-12) accounts for most of the macrophage-derived elastase activity in smokers (47). The release of MMP-9, an NF-κB-dependent protease, is under the influence of several mediators and cytokines, in particular of TNF-α (24). In fact, MMP-9 can increase TNF-α levels by cleaving the membrane-bound form...
of this cytokine (15), and, on the other hand, TNF-α can upregulate MMP-9 production.

MMP-9 is also decreased by IL-10 (24), and, in our study, the reduced level of this anti-inflammatory cytokine after CSE exposure can also account for the increase of this MMP.

An imbalance between oxidants and antioxidants plays an important role in CS-induced airway diseases (37, 38). CS contains high concentrations of ROS, which lead to the activation of many intracellular pathways and cause an inflammatory cascade. Our data show that C57BL/6J AMs had basal levels of intracellular ROS and H2O2 significantly higher than ICR AMs. CSE exposure increased in both of the strains ROS and H2O2 production, which was more evident in C57BL/6J. Additionally, C57BL/6J AMs showed lower baseline levels of GSH compared with ICR, although CSE exposure did not further decrease GSH levels in C57BL/6J AMs.

It is then likely that the imbalance between oxidants and antioxidants renders C57BL/6J AMs more susceptible to CSE and mediates the upregulation of inflammatory mediators. Such an imbalance has been shown to occur in C57BL/6J mice also in vivo, where the decreased level of GSH was associated with the increased level of lipid peroxidation in susceptible mouse strains (57).

Oxidative stress may also contribute to the lung antioxidant defenses by activating Nrf2, which is involved in the transcriptional regulation of many antioxidant genes, including glutamate cysteine ligase, thereby regulating GSH levels (39). In vitro studies demonstrated increased levels of ROS and diminished GSH levels in Nrf2−/− cells compared with Nrf2+/+ cells (43). Furthermore, Nrf2 regulates several other antioxidant genes such as GPX2 and HO-1 (25, 42, 49).

Among inbred mouse strains, the C57BL/6J strain showed lower basal Nrf2 levels compared with strains of mice resistant to oxidative stress (9). In the present study, we show that AMs from C57BL/6J mice had a basal expression of Nrf2 mRNA significantly lower than ICR AMs. This is consistent with previously reported in vivo data (9, 50). Surprisingly, after
CSE treatment, C57BL/6J AMs showed a high expression of Nrf2 mRNA similar to that of ICR AMs. Also, after CSE, GPX2 and HO-1 genes actually had a higher expression in C57BL/6J than in ICR AMs. On the other hand, the basal expression of Nrf2 protein and its target proteins, HO-1 and GPX2, was significantly lower in C57BL/6J than in ICR AMs. CSE treatment significantly increased Nrf2, HO-1, and GPX2 protein levels in ICR AMs. Similarly, CSE also increased Nrf2 and HO-1 (but not GPX2) protein levels in C57BL/6J; however, their values were markedly lower than in ICR AMs. Thus there is a partial discrepancy between the mRNA and protein data with regard to these antioxidants. However, it is well-known that mRNA levels do not always parallel protein levels (25, 53). In fact, also in humans, it has been shown that Nrf2 protein does not correlate with Nrf2 mRNA level (25). The present results suggest that the low expression of Nrf2 and its target proteins in AMs of C57BL/6J mice may impair the antioxidant defense system against CS.

Oxidative stress can also regulate histone modifications, such as acetylation, methylation, and phosphorylation, resulting in uncontrolled transcription of some inflammatory genes. Changes in transcription depend on chromatin remodeling and the relative activity of HATs and HDACs (40). HDAC2 expression and activity were found to be decreased in patients with COPD (5) as well as in macrophage cultures treated with CSE (55). The reasons for HDAC reduction are not yet completely understood, but there is increasing evidence that it may be secondary to oxidative stress (29, 40). Previous studies suggested that HDAC enzyme is redox regulated, and elevation of intracellular GSH restored HDAC activity (55). Interestingly, in the present study, we found that untreated AMs from C57BL/6J showed HDAC2 basal protein expression and total HDAC basal activity significantly lower than in ICR AMs. CSE treatment significantly decreased HDAC2 levels in both strains, whereas the absolute levels were markedly lower in C57BL/6J AMs. This is consistent with previously reported data showing that in vivo HDAC2 levels decreased in lungs of C57BL/6J but not resistant mice in response to CS exposure (57).

However, the data on total HDAC activity show no difference between the strains after CSE, and also the treatment with TSA (a potent HDAC inhibitor) failed to increase cytokine release in control and CSE-treated AMs from both strains, indicating that total HDAC activity may not be considered here as a key factor. The reason for this discrepancy remains to be determined. It is known that CSE decreases HDAC activity, which is associated with decreased levels of proteins belonging to class I/II HDACs, and each of these isoforms may be subjected to different levels of posttranslational modifications (55). This may explain the difference between the data on total HDAC activity and HDAC2 protein expression. On the other hand, the downregulation of HDAC2 in C57BL/6J AMs is of interest since it may be a relevant model for the drop of HDAC2 observed in macrophages of patients with COPD and cigarette smokers, which is associated with steroid resistance (22, 55).

On the basis of these data, we hypothesize that, in control C57BL/6J AMs, the increase in oxidative stress coupled with a decrease in antioxidant GSH, Nrf2, HO-1, and GPX2 levels may result in a chain of events consisting of low HDAC2 levels, activation of NF-κB, and enhanced expression of inflammatory mediators (such as TNF-α and IL-6). This certainly is not the case in control ICR AMs, with significantly lower oxidative stress, higher levels of antioxidants and HDAC2, and lower levels of NF-κB activation and cytokine expression.

Treatment with CSE resulted in AMs of both strains in oxidative stress associated with an increase of antioxidant defenses, but these increases were significantly higher in ICR AMs. On the other hand, CSE treatment induced a much greater decrease in HDAC2 levels in C57BL/6J AMs and only in AMs from this strain caused an activation of NF-κB. Thus it may be concluded that, in C57BL/6J AMs, the high basal levels of HDAC2 observed in macrophages of patients with COPD and cigarette smokers, which is associated with steroid resistance (22, 55), may result in a chain of events consisting of low HDAC2 levels, activation of NF-κB, and enhanced expression of inflammatory mediators (such as TNF-α and IL-6). This certainly is not the case in control ICR AMs, with significantly lower oxidative stress, higher levels of antioxidants and HDAC2, and lower levels of NF-κB activation and cytokine expression.

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level of inflammatory cytokines and proteases as well as their increased production following CSE may be mainly accounted for by the enhanced NF-κB activation. In fact, markedly higher levels of NF-κB activation were observed in C57BL/6J AMs under basal condition. After treatment with CSE, these levels were further significantly augmented only in C57BL/6 AMs. NF-κB is an important transcription factor that has been shown to be activated by oxidative stress (36). This factor is critical for the transcription of proinflammatory genes, such as TNF-α, IL-8, IL-6, and MMPs. Exposure to lipid peroxidation products or depletion of GSH causes rapid degradation of the IkB complex, a critical step for NF-κB activation (40). The present results are consistent with in vivo studies showing increased levels of RelA/p65 and NF-κB DNA-binding activity in lungs of C57BL/6J and other susceptible strains in response to CS exposure (57). Therefore, it can be postulated that NF-κB could be one of the genetic determinants that contribute to the increased susceptibility of C57BL/6J mice to CS.

In conclusion, all these results taken together demonstrate a different qualitative and quantitative response to CSE by AMs from C57BL/6J and ICR mice. Although these results were obtained in isolated AMs after a single treatment with CSE, they could contribute to the understanding of the different susceptibility to CS observed in vivo in these strains of mice after chronic exposure.

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DISCLOSURES

No conflicts of interest are declared by the author(s).

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