

Sirtuin regulates cigarette smoke-induced proinflammatory mediator release via RelA/p65 NF- κ B in macrophages in vitro and in rat lungs in vivo: implications for chronic inflammation and aging

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Submitted 10 August 2006; accepted in final form 7 October 2006

Yang S-R, Wright J, Bauter M, Seweryniak K, Kode A, Rahman I. Sirtuin regulates cigarette smoke-induced proinflammatory mediator release via RelA/p65 NF- κ B in macrophages in vitro and in rat lungs in vivo: implications for chronic inflammation and aging. *Am J Physiol Lung Cell Mol Physiol* 292: L567–L576, 2007. First published October 13, 2006; doi:10.1152/ajplung.00308.2006.—The silent information regulator 2 (Sir2) family of proteins (sirtuins or SIRT1s), which belong to class III histone/protein deacetylases, have been implicated in calorie restriction, aging, and inflammation. We hypothesized that cigarette smoke-mediated proinflammatory cytokine release is regulated by SIRT1 by its interaction with NF- κ B in a monocyte-macrophage cell line (MonoMac6) and in inflammatory cells of rat lungs. Cigarette smoke extract (CSE) exposure to MonoMac6 cells caused dose- and time-dependent decreases in SIRT1 activity and levels, which was concomitant to increased NF- κ B-dependent proinflammatory mediator release. Similar decrements in SIRT1 were also observed in inflammatory cells in the lungs of rats exposed to cigarette smoke as well as with increased levels of several NF- κ B-dependent proinflammatory mediators in bronchoalveolar lavage fluid and in lungs. Sirtinol, an inhibitor of SIRT1, augmented, whereas resveratrol, an activator of SIRT1, inhibited CSE-mediated proinflammatory cytokine release. CSE-mediated inhibition of SIRT1 was associated with increased NF- κ B levels. Furthermore, we showed that SIRT1 interacts with the RelA/p65 subunit of NF- κ B, which was disrupted by cigarette smoke, leading to increased acetylation RelA/p65 in MonoMac6 cells. Thus our data show that SIRT1 regulates cigarette smoke-mediated proinflammatory mediator release via NF- κ B, implicating a role of SIRT1 in sustained inflammation and aging of the lungs.

oxidants; resveratrol; MonoMac6 cells; chronic obstructive pulmonary disease; histone; acetylation; deacetylase

SIRTUINS (SIRT1s) belong to class III histone/protein deacetylases (HDACs) and are members of the silent information regulator 2 (Sir2) family. They are widely distributed in all the phyla of life and are implicated in aging, cell cycle regulation, apoptosis, metabolism, and inflammation (12, 16, 31, 32). Members of this evolutionary family of deacetylases include five homologues in yeast (γ Sir2 and Hst1-4) and seven in humans (SIRT1-7) (14). Unlike class I and II HDACs, which consume a water molecule for direct hydrolysis of the acetyl group, sirtuins require NAD⁺ as a cosubstrate for the deacetylation reaction. The stoichiometry between NAD⁺ and the substrate (acetylated protein) is 1:1 and forms the deacetylated product 2-*O*-acetyl-ADP-ribose, cleaving nicotinamide and

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deacetylated protein. It has been suggested that Sir2 (human analog is SIRT1) HDAC directly binds to one or more constituent in the chromatin complex, resulting in structural reorganization, and therefore has the ability to establish silent chromatin domains (15). However, its role in the regulation of expression and release of proinflammatory cytokines in response to environmental stresses is not known. This is especially important in light of a recent study showing that Sirt1 regulates NF- κ B by yet unknown mechanisms (36).

Cigarette smoke contains an estimated 10^{15–17} oxidants/free radicals and ~4,700 different chemical compounds, including reactive aldehydes and quinones, per puff (10). It has been long known that tobacco smoking is associated with premature aging of the lungs because of its direct attack with inhaled cigarette smoke-derived oxidants and free radicals (5, 17, 35). We have recently shown that cigarette smoke activates NF- κ B and proinflammatory cytokine release in human primary small airway epithelial cells and macrophages and in rat lungs (18, 19). However, the mechanism underlying cigarette smoke-mediated proinflammatory cytokine release is unknown.

Macrophages are derived from monocytes and are suggested to be the main orchestrators of the chronic inflammatory responses seen in patients with chronic obstructive pulmonary disease (27). Macrophages from patients with chronic obstructive pulmonary disease release increased levels of proinflammatory cytokines compared with nonsmoking control subjects (1). In view of the inhibitory effects of SIRT1 on NF- κ B and the activation of the NF- κ B by cigarette smoke, we therefore hypothesized that cigarette smoke-mediated proinflammatory cytokine release in macrophages is due to alterations in sirtuin levels and disruption of the sirtuin deacetylase-NF- κ B complex interaction in macrophages. To test our hypothesis, we investigated the effect of cigarette smoke on sirtuin levels and its interaction with NF- κ B-RelA/p65 complex as well as post-translational modification of RelA/p65 by cigarette smoke in a macrophage cell line (MonoMac6) and in inflammatory cells (predominantly macrophages) of rat lungs. In addition, the role of sirtuin in regulation of cigarette smoke-mediated proinflammatory cytokines (IL-8 and TNF- α release) in MonoMac6 cells was also investigated.

MATERIALS AND METHODS

Unless otherwise stated, all biochemical reagents used in this study were purchased from Sigma (St. Louis, MO).

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Materials. Penicillin, streptomycin, and RPMI 1640 were obtained from Life Technologies (Gaithersburg, MD). FBS was obtained from HyClone Laboratories (Logan, UT). Resveratrol (FR104-0100) was obtained from Biomol (Plymouth Meeting, PA). Sirtinol (566320) was purchased from Calbiochem (La Jolla, CA). Rabbit polyclonal antibodies to SIRT1 (Ab7343) and SIRT2 (Ab10659) were procured from Abcam (Cambridge, CA). Rabbit polyclonal antibodies to I κ B- α (sc-371) and NF- κ B-RelA/p65 (sc-372) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Acetylated-lysine mouse monoclonal antibody (9681) was obtained from Cell Signaling Technology (Beverly, MA).

MonoMac6 cell culture. The human monocyte-macrophage cell line (mature monocytes-macrophages) MonoMac6, which was established from peripheral blood of a patient with monoclastic leukemia (21, 37), were grown in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 100 μ g/ml penicillin, 100 U/ml streptomycin, 1% nonessential amino acids, 1 mM sodium pyruvate, 1 μ g/ml human holo-transferrin, and 1 mM oxaloacetic acid. These cells do not require PMA to differentiate into the macrophages, thus avoiding any stress to the cells. The cells were cultured at 37°C in a humidified atmosphere containing 7.5% CO₂.

Preparation of aqueous cigarette smoke extract. Research grade cigarettes (1R3F) were obtained from the Kentucky Tobacco Research and Development Center at the University of Kentucky (Lexington, KY). The total particulate matter (TPM) content of 1R3F was 17.1 mg/cigarette, tar (15 mg/cigarette) and nicotine (1.16 mg/cigarette). A 10% cigarette smoke extract (CSE) was prepared by bubbling smoke from one cigarette into 10 ml of culture medium supplemented with 1% FBS at a rate of one cigarette per minute as described previously (18, 22, 35), using a modification of the method described by Carp and Janoff (6). The pH of the CSE was adjusted to 7.4 and was sterile filtered through a 0.45- μ m filter (25-mm Acrodisc; Pall, Ann Arbor, MI). The CSE preparation was standardized by monitoring the absorbance at 320 nm (optical density of 0.74 \pm 0.05). The spectral variations observed between different CSE preparations at 320-nm wavelength were found to be within the acceptable limits. CSE was freshly prepared for each experiment and diluted with culture medium containing 1% FBS immediately before use. Control medium was prepared by bubbling air through 10 ml of culture medium supplemented with 1% FBS, adjusting pH to 7.4, and sterile filtered as described for 10% CSE.

Treatments. MonoMac6 cells were seeded at a density of 1.5 \times 10⁶ cells/well (total final volume = 2 ml), grown to ~80–90% confluency in six-well plates containing RPMI 1640 medium with 10% FBS, washed in Ca²⁺- and Mg²⁺-free PBS, and then exposed to the treatments in media containing 1% serum. All treatments were performed in duplicate. The cells were treated with CSE (1, 2.5, and 5%) for 1, 4, and 24 h at 37°C with 7.5% CO₂. At the end of treatment, the cells were washed with cold, sterile Ca²⁺- and Mg²⁺-free PBS and were lysed before being used to assay SIRT1 and SIRT2 levels and SIRT activity. Culture media from these cells were collected and stored at -80°C until analyzed for IL-8 and TNF- α release.

To investigate the effect of sirtinol (sirtuin inhibitor) and resveratrol (sirtuin activator), MonoMac6 cells were seeded as described above and treated with resveratrol (1 and 5 μ M), sirtinol (10 μ M), and CSE (1%) alone or in combination with CSE (1%) for 24 h at 37°C with 7.5% CO₂. Cell culture media were used for IL-8 and TNF- α release and cell lysate for sirtuin deacetylase activity.

Cigarette smoke exposure to rats. Male Sprague-Dawley rats (280 \pm 2.5 g; Jackson Laboratory, Bar Harbor, ME) were housed in the Inhalation Core Facility at the University of Rochester. Two animals were housed per cage containing two nest packs filled with grade 6 saw dust. Rats were placed in individual compartments of a wire cage, which was placed inside a closed plastic box connected to the smoke source. The smoke was generated from 2R4F research cigarettes containing 11.7 mg/cigarette of tar or TPM and 0.76 mg/cigarette of nicotine (University of Kentucky, Louisville, KY).

Exposure was performed according to the Federal Trade Commission protocol (1 puff/min of 2-s duration and 35-ml volume) in a Baumgartner-Jaeger CSM2082i cigarette smoking machine (CH Technologies, Westwood, NJ). Main-stream cigarette smoke was diluted with filtered air and directed into the exposure chamber. The smoke exposure (TPM per cubic meter of air) was monitored in real time with a MicroDust Pro aerosol monitor (Casella CEL, Bedford, UK) and verified daily by gravimetric sampling. The smoke concentration was set at a nominal value of 300 mg/m³ TPM by adjusting the flow rate of the dilution air. Sham control animals were exposed to air only in the same manner in the same duration of time. To ensure a consistent exposure across exposed animals, cotinine levels were measured. Blood carboxyhemoglobin and carbon monoxide levels were measured immediately after the animals were removed from the chambers. Rats were killed 2 and 24 h after the last exposure by an intraperitoneal injection of 50 mg/kg (body wt) pentobarbital sodium. The University of Rochester Institutional Committee of Animal Research approved all experiments described in this study (protocol 2005-215).

Lung lavage and process of bronchoalveolar lavage fluid. The lungs were lavaged via a butterfly cannula inserted into the trachea; the thymus, heart, and lung were removed en bloc; and the right lobe was tied off and then instilled with 5 \times 3-ml aliquots of saline. First and second aliquots were combined and centrifuged, and the cell-free supernatants were frozen for later analyses. Other aliquots were combined, and the supernatant was discarded after centrifuge. After all aliquots were combined, the bronchoalveolar lavage (BAL) fluid inflammatory cell pellet was resuspended in saline, and the total cell number was determined with a hemocytometer. Differential cell counts (minimum of 500 cells/slide) were performed on Cytospin-prepared slides (Thermo Shandon, Pittsburgh, PA) stained with Diff-Quik (Dade Behring, Newark, DE).

Sirtuin deacetylase activity. Sirtuin activity was measured by a SIRT1 fluorometric kit (AK-555, Biomol). The assay involved the use of the SIRT1 fluorometric substrate (Fluor de Lys-SIRT1 substrate-developer combination, 0.2 U/ μ l), which comprised amino acids 379–382 of human p53 (Arg-His-Lys-Lys AC), and incubation with human recombinant SIRT1 and the cosubstrate NAD⁺. Deacetylation of SIRT1 sensitizes the substrate, and treatment with the developer produces a fluorophore, which can be analyzed with a fluorescence plate reader at 460 nm. Resveratrol (200 μ M) was used as a positive control, and 1 mM nicotinamide and 200 μ M suramin sodium served as negative controls. A standard calibration curve was prepared with a known amount of the deacetylated standard included in the kit.

Western blot analysis. MonoMac6 cells were exposed to CSE, and cellular nuclear proteins were extracted from treated MonoMac6 cells with 10% Nonidet P-40 (Igepal CA-630) lysis buffer supplemented with a protease inhibitor cocktail (leupeptin, aprotinin, pepstatin, and PMSF). For SIRT1 and SIRT2 assays, 20 μ g of isolated nuclear proteins (obtained from MonoMac6 cells and rat lung BAL cells) were subjected to electrophoresis on 7.5% PAGE gels and then transferred onto nitrocellulose membranes (Amersham, Arlington Heights, IL) by electroblotting technique. The nitrocellulose membrane was blocked with 10% nonfat dry milk and subsequently incubated with rabbit polyclonal anti-human, anti-SIRT1 (1:1,000), SIRT2 (1:1,000), NF- κ B p65 (1:1,000), and acetylated-lysine mouse monoclonal antibody (1:1,000) separately with 5% nonfat dry milk. After three washing steps (5 min each), protein levels were detected with the use of goat anti-rabbit or anti-mouse antibody (1:20,000 dilution in 2.5% nonfat dry milk in TBS for 1 h) linked to horseradish peroxidase (Dako, Santa Barbara, CA); bound complexes were detected with the use of the enhanced chemiluminescence method (Jackson Immunology Research, West Grove, PA).

SIRT1 immunoprecipitation and its interaction with RelA/p65. MonoMac6 cells were treated with CSE (1, 2.5, and 5%) for 1 h at 37°C, nuclear fraction was isolated, and SIRT1 was immunoprecipitated: SIRT1 antibody (1:80 dilution; Abcam) was added to 100 μ g of

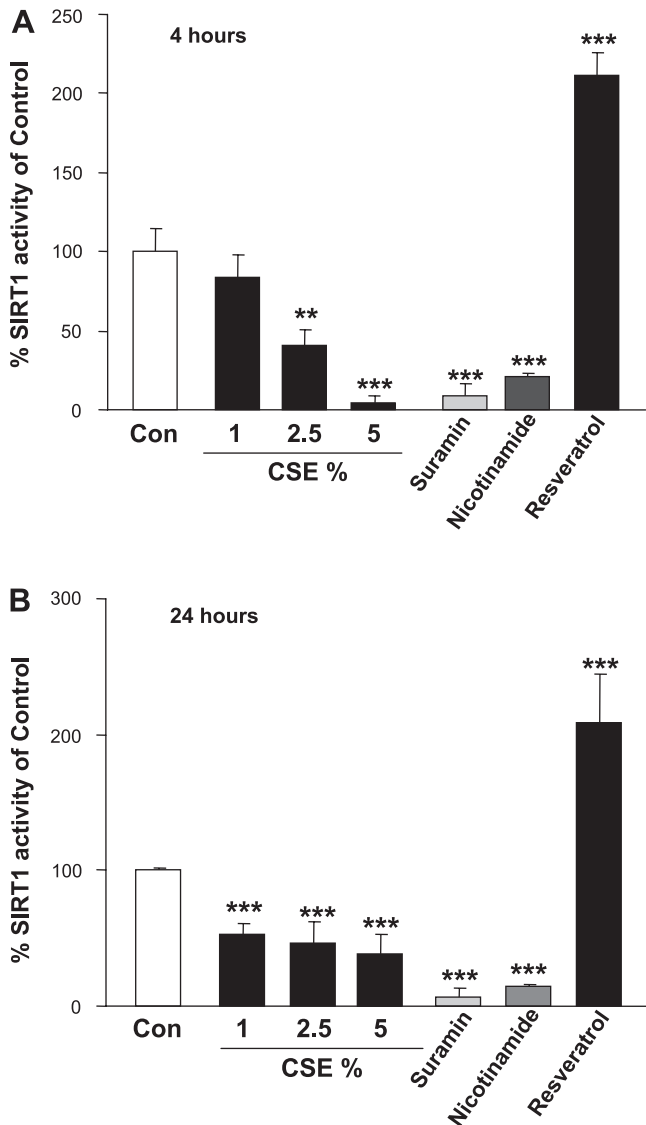


Fig. 1. Cigarette smoke extract (CSE) exposure significantly decreased sirtuin 1 (SIRT1) activity at 4 (A) and 24 h (B) in MonoMac6 cells. SIRT1 activity was measured with a SIRT1 fluorometric kit (Biomol) at 460 nm with SIRT1 fluorometric substrate (0.2 U/ μ l) and cosubstrate NAD⁺. Resveratrol (200 μ M) was used as a positive control, and nicotinamide (1 mM) and suramin (200 μ M) were used as negative controls. A standard curve was prepared with known amounts of the deacetylated standard included in the kit. ** P < 0.01 and *** P < 0.001 compared with control (Con) values.

protein in a final volume of 400 μ l and incubated for 1 h. Protein-A/G agarose beads (20 μ l) (Santa Cruz) were added to each sample and left overnight at 4°C on a rocker. The samples were then centrifuged at 13,000 rpm at 4°C for 5 min. The supernatant was discarded, and the beads were washed three times and then resuspended in 400 μ l of lysis buffer. For Western blots, 100 μ g of the immunoprecipitated SIRT1 agarose bead suspension were added to 20 μ l of 5 \times sample buffer, boiled, and resolved by SDS-PAGE as described above. Negative alone (beads only) was used as negative control. To demonstrate the interaction of SIRT1 protein with RelA/p65 subunit of NF- κ B, 50 μ g of immunoprecipitated SIRT1 were blotted against RelA/p65 subunit of NF- κ B. Similarly, Western blotting against anti-acetylsine (no. 9681; Cell Signaling Technology) was performed on immunoprecipitated RelA/p65 to determine the acetylation of RelA/p65.

ELISA for IL-8 and TNF- α . The culture medium was collected after treatment and centrifuged at 2,500 rpm for 5 min to pellet the cells. The supernatant was then removed and stored at -20°C before analysis. IL-8 and TNF- α levels in the supernatants were determined by ELISA from the respective duo-antibody kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Analyses of proinflammatory mediators in BAL fluid and in lung homogenate. The levels of cytokine-induced neutrophil chemoattractant (CINC)-1 (DY515), IL-6 (DY506), and TNF- α (DY510) in BAL fluid and/or lung homogenate were measured by ELISA duo-set from R&D Systems. Monocyte chemoattractant protein-1 (MCP-1) in BAL fluid was measured with a rat ELISA kit (no. 900-077; Assay Designs, Ann Arbor, MI), and rat GRO β /MIP-2 in BAL fluid and/or lung homogenate was measured with a separate ELISA kit (no. RRF428CK; Antigenix America, Huntington Station, NY). To confirm the sensitivity and detection limit of various proinflammatory cytokines measured by ELISA, the Luminex assay was performed in rat BAL fluid (240 μ l) for various proinflammatory mediators using the sensitive rat Multi-Analyte Profile (version 1.6) screening by Luminex (Rules Based Medicine, comarketed with Charles River Laboratories, Austin, TX). In addition, a small piece of left lung (100 mg) was homogenized in Tris buffer as described elsewhere (30), and

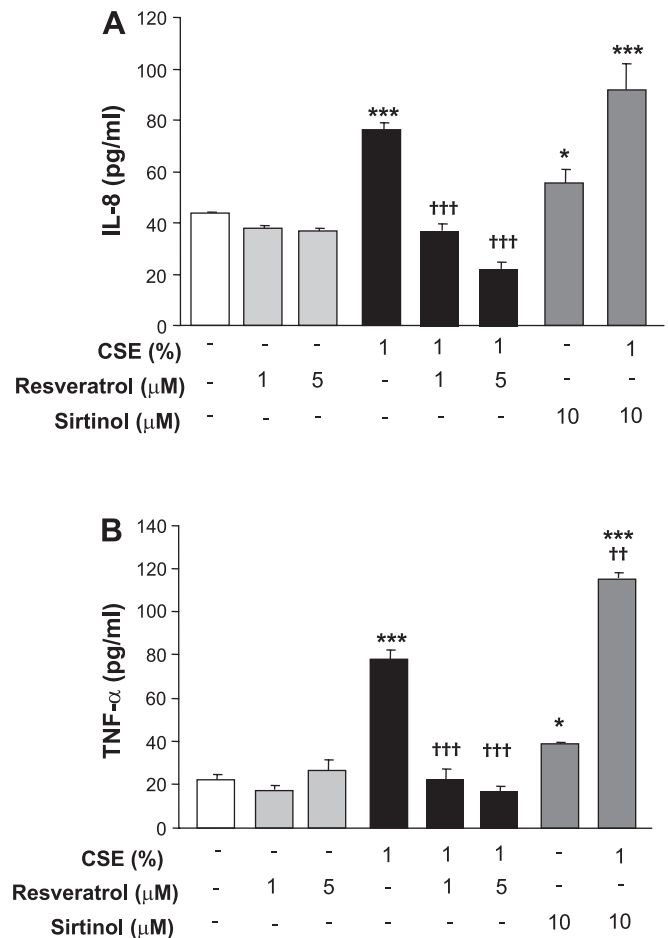


Fig. 2. Pharmacological regulation of sirtinol inhibited SIRT1 and activated CSE-mediated IL-8 (A) and TNF- α (B) release in MonoMac6 cells. MonoMac6 cells were pretreated with SIRT1 activator resveratrol (1 and 5 μ M) and SIRT1 inhibitor sirtinol (10 μ M) for 2 h, washed, and then incubated with freshly prepared CSE (1%) for 24 h; IL-8 and TNF- α were measured by ELISA. The results are presented as an average of 3 separate experiments performed in triplicate. * P < 0.05; ** P < 0.01; *** P < 0.001 compared with control values; †† P < 0.01; ††† P < 0.001 compared with CSE (1%).

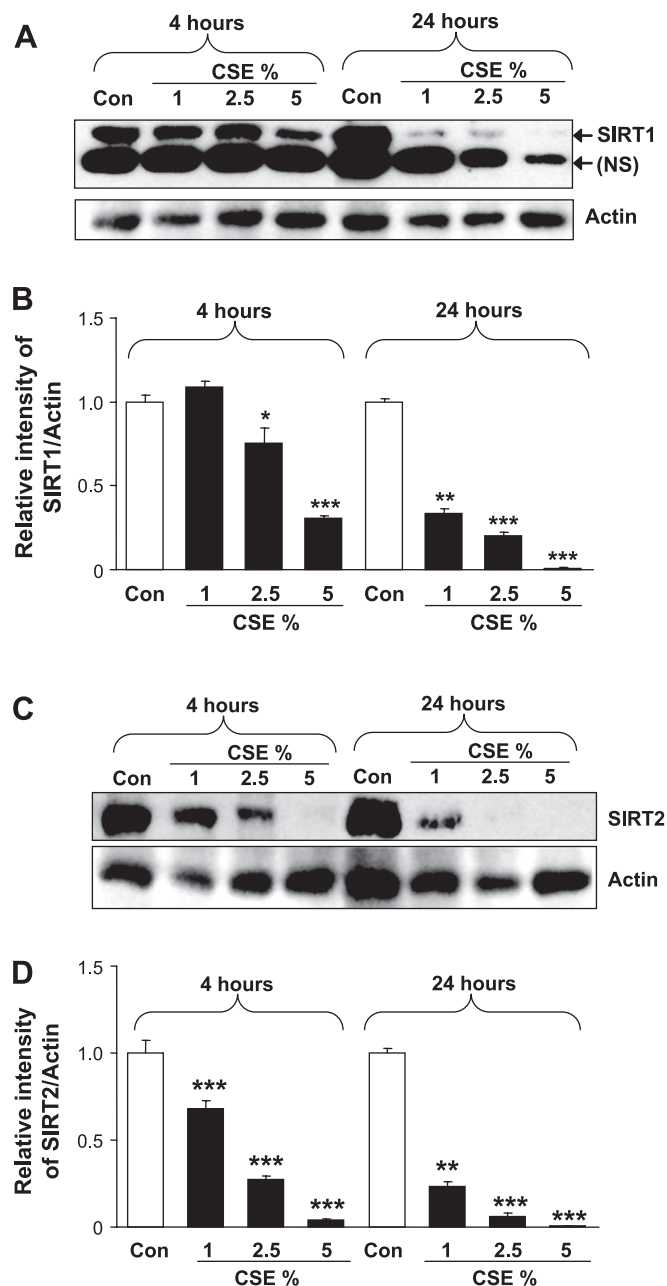


Fig. 3. CSE decreased the levels of SIRT1 (A and B) and SIRT2 (C and D) proteins at 4 and 24 h (B and D) in MonoMac6 cells. Western blots of soluble nuclear proteins extracted from treated MonoMac6 cells were electrophoresed on 7.5% PAGE gels and electroblotted onto nitrocellulose membranes. SIRT1 band size was same as predicted (81.3 kDa). In B and D, relative densities (% of control) are shown of SIRT1 and SIRT2 in MonoMac6 cells at 4 and 24 h. NS, nonspecific bands. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with control values.

80–100 μ l of clear supernatant were used for TNF- α , IL-6 and MIP-2 assays.

Protein assay. Protein levels were measured by using the bicinchoninic acid kit (Pierce, Rockford, IL). Protein standards were obtained by diluting a stock solution of BSA. Linear regression was used to determine the actual protein concentration of the samples.

Statistical analysis. Results are shown as means \pm SE of three experiments. Statistical analysis of significance was calculated by one-way ANOVA followed by Tukey's post hoc test for multigroup comparisons, using StatView. Significance is as indicated.

RESULTS

Cigarette smoke decreases sirtuin activity in MonoMac6 cells. In this study, we determined whether SIRT1 is regulated by cigarette smoke in MonoMac6 cells. CSE (1, 2.5, and 5%) dose dependently and significantly decreased SIRT1 activity after 4 and 24 h ($P < 0.001$). Sirtuin inhibitors suramin and nicotinamide abolished sirtuin activity, whereas resveratrol, a SIRT1 activator, significantly increased sirtuin activity ($P < 0.001$) (Fig. 1).

Pharmacological manipulation of SIRT1 regulates cigarette smoke-induced IL-8 and TNF- α release in MonoMac6 cells. We further studied whether cigarette smoke-mediated reduction in SIRT1 was associated with induction of IL-8 and TNF- α and whether pharmacological manipulation of SIRT1 can regulate CSE-induced proinflammatory cytokines in MonoMac6 cells. We pretreated MonoMac6 cells with resveratrol (SIRT1 activator) and sirtinol (SIRT1 inhibitor) and studied the CSE-induced cytokine release. Resveratrol activated SIRT1 levels and significantly inhibited CSE-mediated release of IL-8 (Fig. 2A) and TNF- α (Fig. 2B) ($P < 0.001$). This suggests that upregulation of SIRT1 inhibited CSE-mediated increase in IL-8 and TNF- α release. We further examined the effects of sirtinol (SIRT1 inhibitor) on cigarette smoke-mediated

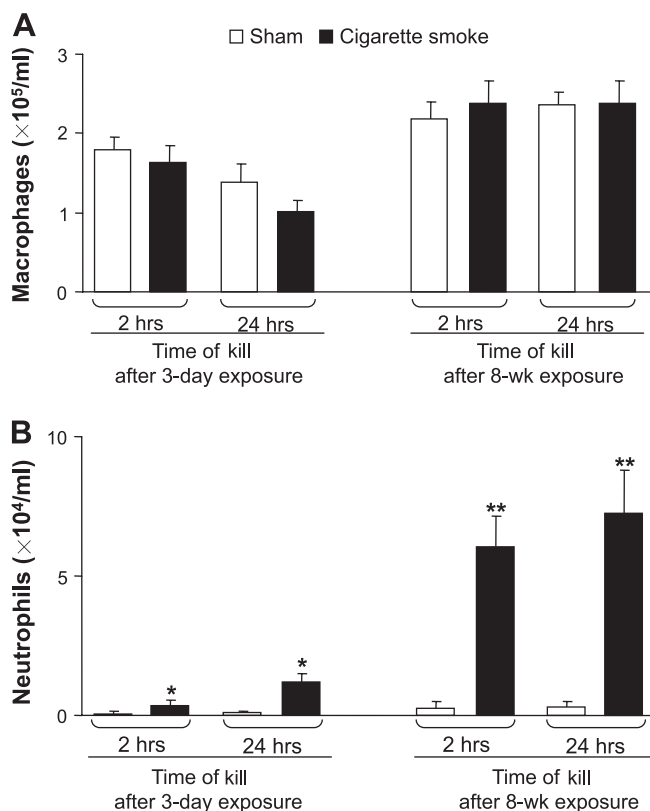


Fig. 4. Cigarette smoke exposure caused neutrophil influx in bronchoalveolar lavage (BAL) fluid of rat lungs. Rats were exposed to filtered air (sham) or cigarette smoke as described in MATERIALS AND METHODS for sham and cigarette smoke-exposed groups. Differential counts were performed on BAL cells for the number of macrophages (A) and the number of neutrophils (B). Animals were exposed to cigarette smoke at a concentration of 300 total particulate matter (TPM) mg/m³ for 3 days or 8 wk and killed at 2 and 24 h after last exposure. Results shown are for $n = 7$ rats/group. * $P < 0.05$ and ** $P < 0.01$ compared with air group.

ated IL-8 and TNF- α release in MonoMac6 cells. Interestingly, sirtinol treatment alone induced increased IL-8 release ($P < 0.05$), and this effect was augmented ($P < 0.01$) when the cells were treated with sirtinol and CSE, suggesting that inhibition of SIRT1 leads to induction of IL-8 and TNF- α release in response to CSE in MonoMac6 cells.

Cigarette smoke decreases sirtuin levels in MonoMac6 cells. We further investigated whether the CSE-mediated decrease in sirtuin activity was associated with decreased sirtuin levels. It was found that the levels of SIRT1 and SIRT2 were significantly decreased in response to CSE (1, 2.5, and 5%) after 4 and 24 h of treatment compared with the controls (Fig. 3).

Cigarette smoke causes influx of inflammatory cells in rat lungs. Rats were exposed to cigarette smoke at 300 TPM mg/cm³ for 1 h, twice a day for 3 days (acute exposure) or 8 wk (subchronic exposure), and then killed at 2 and 24 h after the last exposure. Diff-Quik staining showed 98% macrophages out of total BAL cells in the sham group (data not shown). Cigarette smoke exposure induced the number of neutrophils significantly after acute and subchronic exposure; however, the number of macrophages was not affected by cigarette smoke

exposure (Fig. 4). The levels of plasma cotinine, the major metabolite of nicotine, were 41.48 ± 1.7 and 75.0 ± 1.1 pg/ml ($n = 7$, $P < 0.001$) against nondetectable levels measured in the air group.

Cigarette smoke induces proinflammatory cytokine release in BAL fluid and lung. We measured the levels of proinflammatory mediators in BAL fluid in response to cigarette smoke exposure to rats after 3 days or 8 wk of cigarette smoke exposure (killed at 2 and 24 h after last exposure). We found that a variety of NF- κ B-dependent proinflammatory mediators were increased, as measured by Luminex-based assay (data not shown). In particular, the levels of MCP-1, granulocyte macrophage-colony stimulating factor, granulocyte chemotactic protein-2, factor VII, macrophage-derived chemokine, and MIP-2 were elevated approximately twofold after 3 days or 8 wk of cigarette smoke exposure compared with air-exposed animals. A lesser degree of changes of proinflammatory mediators was found in BAL fluid of rats exposed to the lower concentration of cigarette smoke (300 TPM mg/m³) at 3 days. In addition, the levels of CINC-1 (a neutrophil chemoattractant equivalent of human IL-8), MCP-1, and MIP-2 were significantly increased as measured by ELISA in BAL fluid after

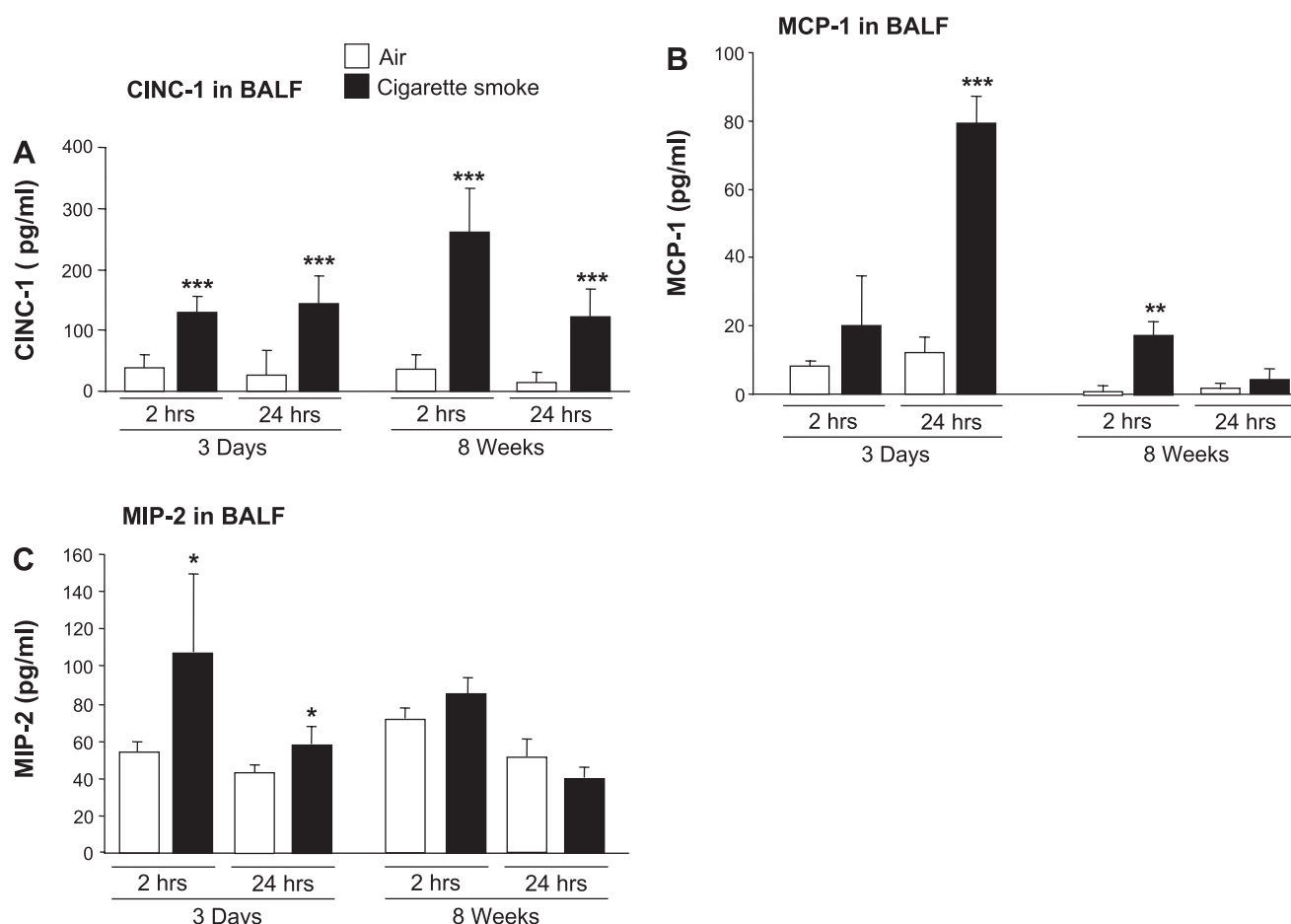


Fig. 5. Cigarette smoke increased the levels of NF- κ B-dependent proinflammatory chemokines in BAL fluid (BALF). Air- (white bars) and cigarette smoke-exposed rats (black bars) were exposed to filtered air or cigarette smoke, respectively. Levels of BALF cytokines cytokine-induced neutrophil chemoattractant 1 (CINC-1; A), monocyte chemoattractant protein-1 (MCP-1; B), and major intrinsic protein-2 (MIP-2; C) were increased, as measured by commercially available ELISA kits (described in MATERIALS AND METHODS). Animals were exposed to cigarette smoke at a concentration of 300 TPM mg/m³ for 3 days or 8 wk and killed at 2 and 24 h post-last exposure. Results shown are from a representative experiment with 7 rats/group. Significant difference from air group: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

3 days; however, only CINC-1 and MCP-1 were increased after 8 wk of cigarette smoke exposure (Fig. 5). The levels of TNF- α were not detected in BAL fluid with the ELISA method after either 3 days or 8 wk of cigarette smoke exposure compared with air-exposed animals. However, the levels of TNF- α , IL-6, and MIP-2 were significantly increased in lung homogenates both after 3 days and 8 wk of cigarette smoke exposure (Fig. 6).

Cigarette smoke decreases sirtuin levels in inflammatory cells obtained from rat lungs. To determine whether levels of sirtuin were associated with inflammatory cells obtained in vivo, BAL cells from rats were analyzed for levels of SIRT1 and SIRT2 by Western blot analysis. The levels of SIRT1 and SIRT2 were significantly decreased in response to cigarette smoke exposure after both 3 days and 8 wk of exposure when killed at 2 and 24 h after last exposure compared with air (sham)-exposed group (Fig. 7).

Cigarette smoke-mediated reduction of SIRT1 was associated with increased activation and acetylation of RelA/p65. We further examined whether SIRT1 protein is physically associated with RelA/p65 of NF- κ B. With the use of immunoprecipitation of SIRT1 and Western blotting of RelA/p65, NF- κ B increased significantly in nuclear extracts of MonoMac6 cells treated with CSE (Fig. 8). This suggested that SIRT1 interacted with RelA/p65 subunit of NF- κ B in the nucleus.

It is known that phosphorylation and acetylation of RelA/p65 play key roles in NF- κ B transactivation (29), and lysine moiety of NF- κ B is involved in several posttranslational modifications (13). We determined whether the CSE-mediated reduction of SIRT1 deacetylase has any role on NF- κ B activation/acetylation and in transcriptional activation of proinflammatory cytokines. MonoMac6 cells were treated with CSE (1, 2.5, and 5%) for 1 h, and whole cell lysates were prepared and immunoprecipitated with anti-RelA/p65 antibody and then analyzed by Western blot using anti-acetyl-lysine antibody. CSE (1, 2.5, and 5%) rapidly caused the acetylation of RelA/p65 (Fig. 9), indicating that CSE induces acetylation of RelA/p65-NF- κ B. This suggests that SIRT1-interacted with RelA/p65 and that SIRT1 modification corresponded with RelA/p65 activation and acetylation in response to cigarette smoke, leading to increased proinflammatory cytokine release. Acetylation of RelA/p65 at lysine residues (particularly at 310)

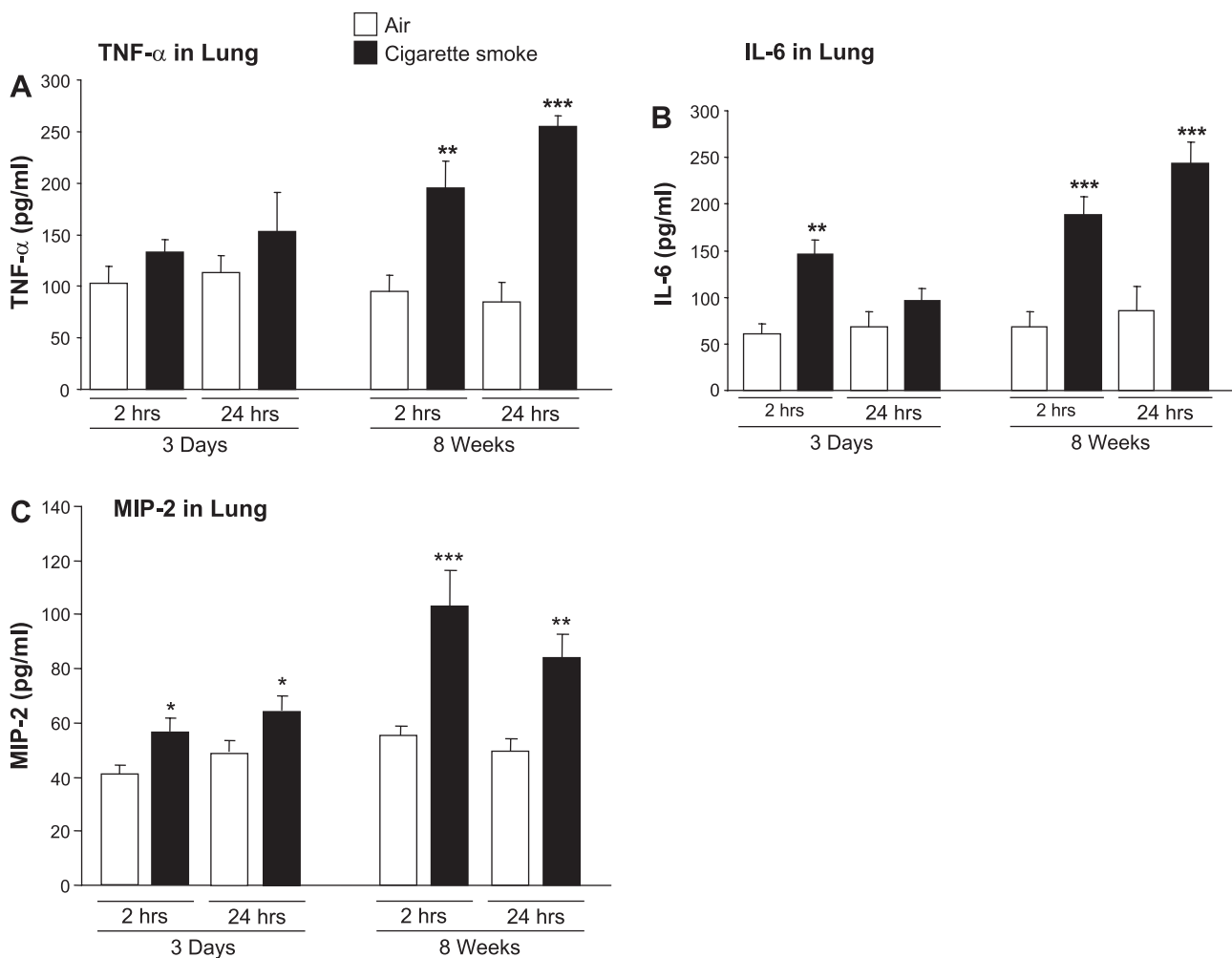


Fig. 6. Cigarette smoke increased the levels of NF- κ B-dependent proinflammatory chemokines in lung homogenate. Air- (white bars) and cigarette smoke-exposed (black bars) rats were exposed to filtered air or cigarette smoke, respectively. Levels of lung homogenate cytokines TNF- α (A), IL-6 (B), and MIP-2 (C) were increased as measured by commercially available ELISA kits (described in MATERIALS AND METHODS). Animals were exposed to cigarette smoke at a concentration of 300 TPM mg/m³ for 3 days or 8 wk and killed at 2 and 24 h postlast exposure. Results are from a representative experiment with 7 rats/group. Significantly different from air group: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

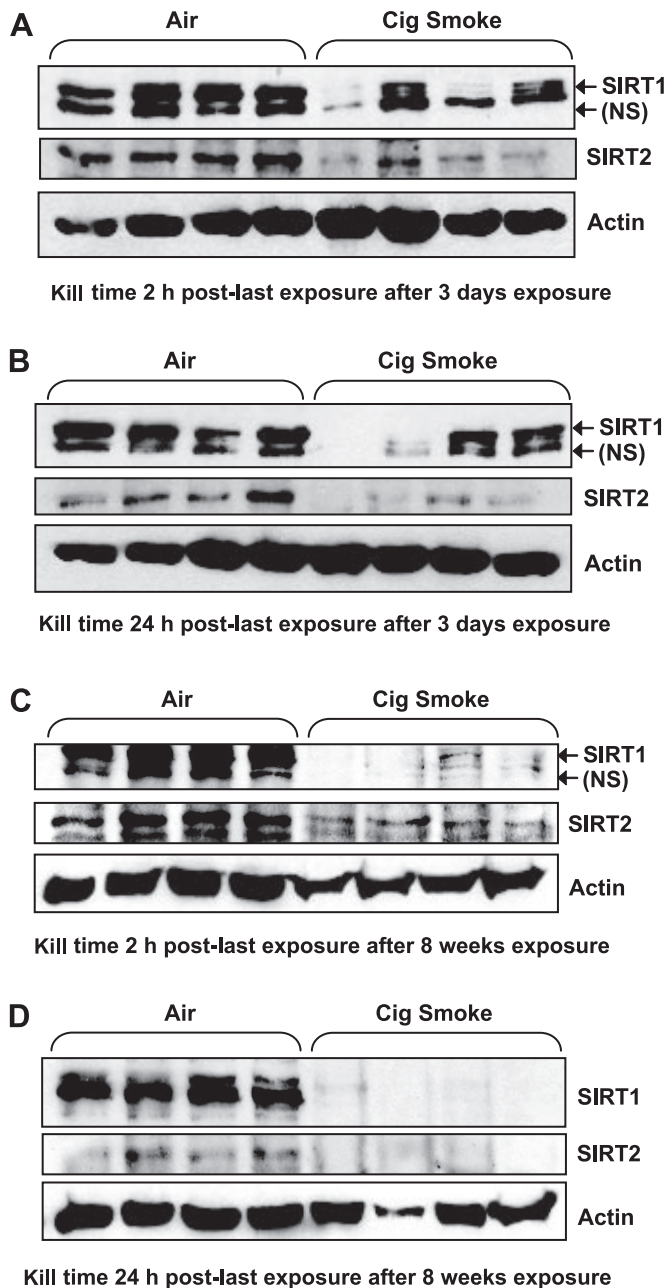


Fig. 7. In inflammatory cells of rat lungs, cigarette smoke (300 TPM mg/cm³) exposure decreased the levels of SIRT1 and SIRT2 proteins at 2 and 24 h (kill time) post-last exposure after 3 days and 8 wk of exposures compared with that shown in the sham-exposed group. *A*: 2 h post-last exposure kill time after 3-day exposure. *B*: 24 h post-last exposure kill time after 3-day exposure. *C*: 2 h post-last exposure kill time after 8-wk exposure. *D*: 24 h post-last exposure kill time after 8-wk exposure. Western blots of soluble nuclear proteins extracted from BAL cells were electrophoresed on 7.5% PAGE gels and electroblotted onto nitrocellulose membranes. According to the antibody supplier, the predicted molecular mass of SIRT1 was 81.3 kDa, but we found SIRT1 ran slower (molecular mass slightly higher than 100 kDa).

may lead to prolonged transcription of proinflammatory cytokine release.

DISCUSSION

Lung macrophages are considered to be an important component in perpetuating the inflammatory responses of cigarette

smoke (27). Previously, we have shown that cigarette smoke induces proinflammatory cytokine release via activation of NF- κ B in the monocyte-macrophage cell line (35). In this study, we showed that cigarette smoke exposure to MonoMac6 cells decreased sirtuin levels (involved in deacetylation process) associated with increased activation of RelA/p65 subunit of NF- κ B and increased proinflammatory cytokine release. Western immunoblot analyses indicated decreased levels of SIRT1 and SIRT2 in both MonoMac6 and rat lung inflammatory cells in response to cigarette smoke exposure. We also demonstrated CSE induced dose- and time-dependent decreases in SIRT1 activity in MonoMac6 cells. This decrease coincided with increased release of TNF- α and IL-8, suggesting that CSE decreases sirtuin levels and induces proinflammatory cytokine release. McBurney et al. (20) reported that SIRT1-deficient mice have severe phenotype changes such as small size, delay in eyelid opening, cardiac defect, and sterility; in addition, lung was consistently affected in the SIRT1 mutants. Increased neutrophil infiltration in the lungs of these mice after chronic pulmonary infection led to pneumonitis, pulmonary edema, and right ventricular hypertrophy (20). Thus it is possible that cigarette smoke-mediated reduction of SIRT1 is responsible for neutrophil influx and inflammatory response seen in rat lungs. In this study, we show that cigarette smoke exposure induced neutrophilic influx, which was associated

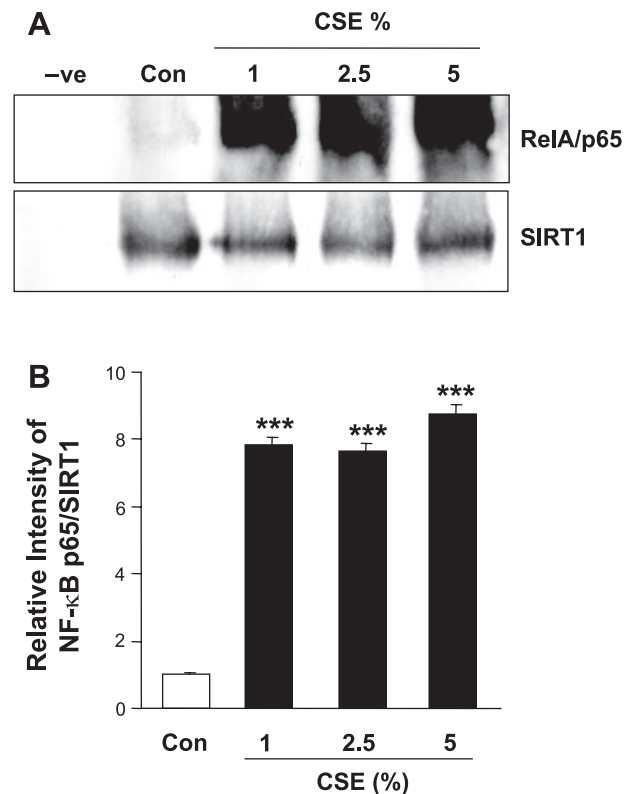


Fig. 8. CSE-mediated decrease in SIRT1 levels was associated with increased levels of RelA/p65 of NF- κ B in MonoMac6 cells. SIRT1 was immunoprecipitated from MonoMac6 cells treated with CSE (1, 2.5, and 5%) for 1 h. Levels of RelA/p65 were analyzed by immunoblotting. *A*: CSE (1, 2.5, and 5%) significantly increased RelA/p65 NF- κ B levels in the sirtuin-RelA/p65 complex. Equal amount (50 μ g) of immunoprecipitated SIRT1 protein was used for Western blots. *B*: for negative control (Con), only bead was used. Results in *B* are expressed as relative intensity of SIRT1 protein bands. ****P* < 0.001 compared with control values.

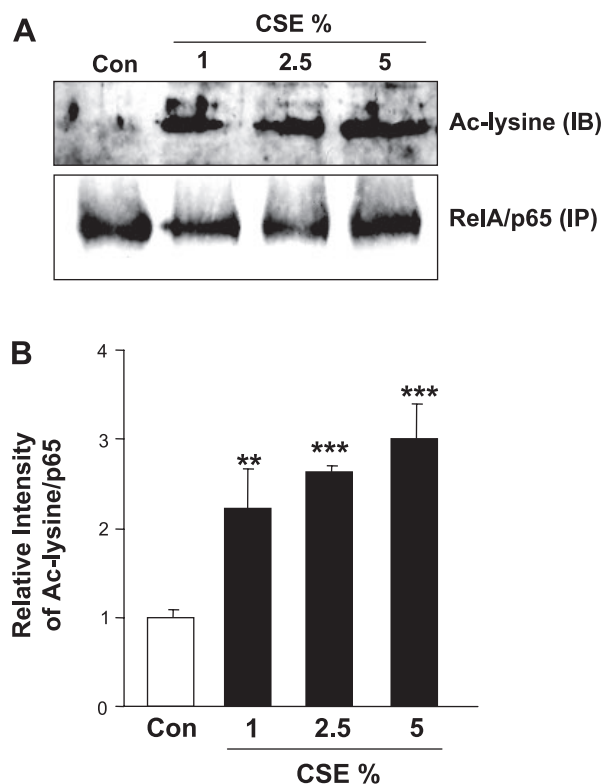


Fig. 9. Cigarette smoke-mediated activation of RelA/p65 was associated with increased acetylation of RelA/p65 of NF- κ B protein in MonoMac6 cells. The acetylation of RelA/p65 NF- κ B proteins was increased in response to CSE (1, 2.5, and 5%) exposure at 1 h. Whole cell extracts were immunoprecipitated (IP) with anti-RelA/p65 antibody, and acetylation was examined by Western blot analysis using anti-acetyl-lysine antibody. A: Western blots of soluble proteins extracted from treated MonoMac6 cells electrophoresed on 7.5% PAGE gels and electroblotted onto nitrocellulose membranes. IB, immunoblot. B: relative density (% of control) of RelA-to-p65 acetylation ratio in MonoMac6 cells after 1 h of CSE treatment. ** $P < 0.01$ and *** $P < 0.001$ compared with control values.

with increased levels of various NF- κ B-dependent proinflammatory mediators, including CINC-1, MIP-2, granulocyte macrophage-colony stimulating factor, granulocyte chemotactic protein-2, macrophage-derived chemokine, MCP-1, IL-6, and TNF- α , in BAL fluid and lung homogenates of rats. The role of SIRT1 in regulation of other NF- κ B-dependent mediators such as matrix metalloproteinases (MMP-1, MMP-9, and MMP-12) and factor VII is also possible because these mediators are NF- κ B-dependent, and the levels are increased in BAL fluid of rats exposed to cigarette smoke.

We further studied the effect of pharmacological regulation of sirtuin on CSE-induced IL-8 release in MonoMac6 cells. We observed that resveratrol activated sirtuin and inhibited against the CSE-mediated reduction of SIRT1 and SIRT2 levels and SIRT1 activity in MonoMac6 cells. Resveratrol is known to lower the K_m of SIRT1 for both the acetylated substrate and NAD⁺, thereby simulating SIRT1 activity (3, 15). Although how CSE mediates the reduction of SIRT1 and SIRT2 is not presently understood, it may however be possible that cigarette smoke-derived oxidants lower the K_m of SIRT, alter the NAD⁺/NADH ratio and induce oxidative modification of SIRT1 protein (Fig. 10). We observed that resveratrol significantly inhibited CSE-induced cytokine (IL-8 and TNF- α) re-

lease and increased SIRT1 activity in MonoMac6 cells. In contrast, sirtinol (a sirtuin inhibitor) significantly augmented the CSE-induced IL-8 and TNF- α release compared with the controls. Sirtinol competes for coenzyme binding in the active site, and therefore it inhibits sirtuin-deacetylase activity (15). Although the mechanism of resveratrol-mediated restoration of SIRT1 and SIRT2 is presently not known, it is however noteworthy that resveratrol can modulate reactive oxygen species production, NAD⁺/NADH ratio, and NF- κ B signaling pathways, which may be directly involved in the SIRT1 and SIRT2 protective effects of resveratrol (25). It is known that resveratrol activates SIRT1, but not other SIRT2 homologues, and it is proposed that resveratrol binds to SIRT1 and induces a conformational change near the coumarin group of bound 7-amino-4-methylcoumarin (p53-AMC peptide) (3). The protein conformational-altering ability of resveratrol was further confirmed by Borra et al. (3), who reported that resveratrol may serve as a mimic to endogenous regulators, which could alter SIRT1 structure and function, perhaps yielding an apparent "upregulated" SIRT1. The cytokine release-inhibiting ability of resveratrol gains further credence from the observation of Shen et al. (28) who reported that resveratrol could inhibit PMA-induced IL-8 protein production and PGE₂ production in human monocytic cells (28). Because SIRT1 is reported to harbor no substrate selectivity on its own (2), we provide herein the first evidence that CSE-mediated IL-8 and TNF- α release was associated with a reduction in SIRT1 activity, and this contention is underpinned by our further observation that resveratrol could increase SIRT1 activity and inhibit IL-8 release from the MonoMac6 and inflammatory cells from the rat lungs. Earlier reports have demonstrated that pharmacological manipulation of SIRT1 deacetylase blocked the upregula-

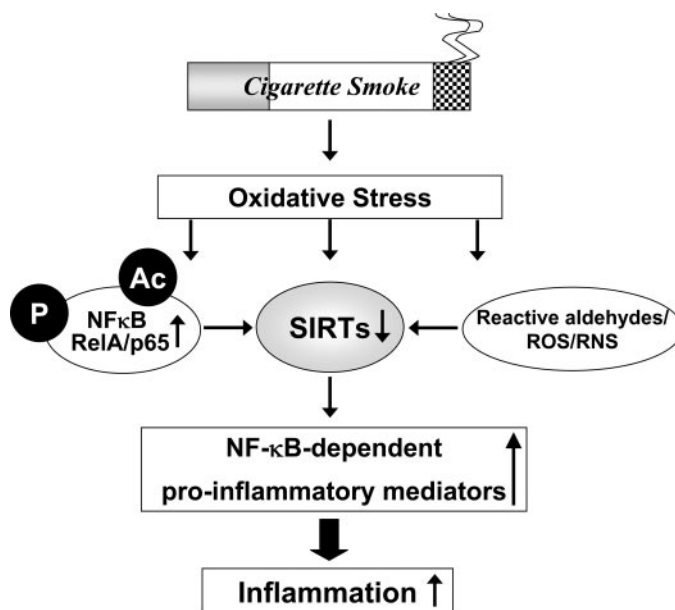


Fig. 10. Hypothesized mechanism of cigarette smoke-induced proinflammatory mediator release via sirtuin-NF- κ B RelA/p65 interaction. Cigarette smoke decreased sirtuin levels and caused posttranslational modification by reactive aldehydes/reactive oxygen species/RNS, thereby disrupting SIRT1-RelA/p65 complex via phosphorylation (P) and acetylation (Ac) of RelA/p65. This leads to increased release of proinflammatory mediators. RNS, reactive nitrogen species.

tion of NF- κ B signaling by A β -(1–42) (7) and TNF- α (36), lending further support to our contention. The decrease in SIRT1 levels due to CSE may partially be explained on the basis of the oxidative/nitrosative/aldehydes alterations CSE may have inflicted on the SIRT1 proteins. Such oxidative/nitrosative modifications may in turn render SIRT1 ineffective for interaction with other signaling components. Similarly, the covalent modification of other redox-sensitive transcription factors has been recently shown (reviewed in Ref. 23). In light of our data, it can thus be construed that sirtuin is a redox-sensitive protein, which is prone to alterations by cigarette smoke-derived oxidants and/or free radicals. However, further studies using genetic approaches will shed more knowledge about the role of this protein in lung injury and inflammation, particularly in emphysema.

Recent reports from our laboratory have revealed that HDAC proteins undergo posttranslational modification in response to cigarette smoke-mediated oxidative stress (19, 35), which render the HDACs inactive leading to activation of NF- κ B and induction of proinflammatory cytokines. Our data show for the first time that CSE-mediated NF- κ B activation was associated with increased acetylation of RelA/p65. It is likely that, once RelA/p65 is acetylated and SIRT1 modified (loss of deacetylase activity), NF- κ B will persistently be activated in response to CSE exposure. Recently, Yeung et al. (36) demonstrated that SIRT1 physically interacts with the RelA/p65 subunit of NF- κ B and inhibits transcription by deacetylating RelA/p65 at lysine 310, suggesting acetylated Lys310 might form a platform for the binding of a bromodomain-containing protein that is required for the full transcriptional activity of RelA/p65 (11, 24). In support of this, Chen, Greene, and colleagues (8, 9) recently demonstrated that acetylation at Lys310 is required for the full transactivation function of RelA/p65. Our data showing RelA/p65 interaction with SIRT1 and the disruption of this complex and NF- κ B activation by exposure to CSE may explain the sustained proinflammatory cytokine release (Fig. 10). However, it still remains to be determined whether CSE acetylates Lys310 moiety of Rel/p65 and whether SIRT1 deacetylates this specific Lys residue or other subunits of NF- κ B such as p50, RelB, and c-Rel. In support of SIRT1 involvement in NF- κ B-dependent-regulated gene transcription, Borra et al. (3) reported that SIRT1 physically interacted with and repressed p300 transactivation. In addition, there are reports that SIRT1 represses acetylation of various transcription factors (p53, RelA/p65, FOXO3/4) (4, 34). Thus our data showing reduced levels of SIRT1 by CSE would have an impact on activation and acetylation of RelA/p65 NF- κ B.

Our data regarding cigarette smoke-mediated reduction in sirtuin (anti-aging protein) and augmented lung inflammatory response have special implications in lung senescence, as the role of senescence has been recently implicated in emphysema (26, 33). Thus it is tempting to speculate that cigarette smoke-induced oxidative stress triggers inflammation and senescence of lung cells via SIRT1 and NF- κ B axis.

In summary, our data show for the first time that cigarette smoke decreased SIRT1 levels and activity by oxidant-mediated mechanisms. Such an oxidative modification promotes SIRT1 disruption through acetylation of RelA/p65, resulting in increased levels of proinflammatory cytokines in MonoMac6 cells as well as in rat lungs. A pharmacological activator (resveratrol) activated

SIRT1 activity and inhibited CSE-mediated proinflammatory cytokine release, whereas sirtinol (SIRT inhibitor) augmented CSE-mediated cytokine release, suggesting that SIRT1 regulates CSE-mediated induction of IL-8 and TNF- α release in MonoMac6 cells. Overall, our study provides novel data on an important molecular mechanism by which SIRT1 regulates cigarette smoke-driven NF- κ B-dependent proinflammatory cytokine release in macrophages in vitro and in rat lungs in vivo.

ACKNOWLEDGMENTS

We thank Dr. Indika Edirisinghe for preparing the first draft of this manuscript, Dr. Saibal Biswas for proof-reading the manuscript, and Drs. Hongwei Yao and Saravanan Rajendrasozhan for technical assistance. We are grateful to Dr. Michael McBurney, Ottawa Health Research Institute, for technical information about sirtuins.

GRANTS

This study was supported by National Institute of Environmental Health Sciences Environmental Health Sciences Center (EHSC) Grant ES-01247 and an EHSC pilot project fund.

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