SIRT1, an antiinflammatory and antiaging protein, is decreased in lungs of patients with chronic obstructive pulmonary disease

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ONLINE DATA SUPPLEMENT

METHODS

Preparation of cigarette smoke extract (CSE)

Research grade cigarettes (1R3F) were obtained from the Kentucky Tobacco Research and Development Center at the University of Kentucky (Lexington, KY). The composition of 1R3F/cigarettes was: total particulate matter: 17.1 mg, tar: 15 mg, and nicotine: 1.16 mg. CSE (10%) was prepared by bubbling smoke from one cigarette into 10 ml of culture media supplemented with 1% FBS at a rate of one cigarette/ 2 minutes as described previously (E1,E2), using a modification of the method described earlier by Carp and Janoff (E3). The pH of the CSE was adjusted to 7.4, and was sterile filtered through a 0.45 μm filter (25 mm Acrodisc; Pall Corporation, Ann Arbor, MI). CSE preparation was standardized by measuring the absorbance (OD 0.72 ± 0.02) at a wavelength of 320 nm. The pattern of absorbance (spectrogram) observed at λ_{320} showed a very little variation between different preparations of CSE. CSE was freshly prepared for each experiment and diluted with culture media supplemented with 1% FBS immediately before use.

Extraction of nuclear protein

MonoMac6 cells or human lung homogenates were washed with ice-cold PBS, resuspended/homogenized in buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 0.5 mM PMSF) and allowed to swell on ice for 15 min. 10% Nonidet P-40 was added to the tubes, vigorously vortexed for 15 sec and centrifuged to collect the supernatant containing cytosolic proteins. The pelleted nuclei were resuspended in buffer B (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM PMSF) and kept on ice for 30 min. After vortex for 20 sec, the cell lysates were centrifuged, and supernatants containing the nuclear proteins were collected.

Western blot analysis

Equal amount of (30 μg) nuclear proteins from each group were resolved by electrophoresis on 7.5% sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gels and electro-blotted onto nitrocellulose membrane (Amersham, Arlington Heights, IL). The nitrocellulose membrane was blocked with 5% nonfat dry milk for 1 hr at room temperature, and incubated with the primary antibody at 4°C for overnight (1:1,000 dilutions in 5% BSA). After being washed with phosphate-buffered saline containing 0.05% TWEEN-20, the membrane incubated with respective secondary antibody (1:10,000 dilution in 5% BSA for 1 hr at room temperature) linked to horseradish peroxidase (Dako, Santa Barbara, CA, USA). Proteins were detected by enhanced chemiluminescence method (Jackson Immunology Research, West Grove, PA), and were quantified using the image processing and analysis software, ImageJ (NIH software). Protein levels were expressed as percent of controls. For normalization, we used levels of the housekeeping protein β-actin.

Immunohistochemistry

Buffered formalin (10%) fixed paraffin embedded lung sections (3-µm thick) of non-smokers, smokers and COPD patients were deparaffinized using xylene and rehydrated in a graded ethanol series. Heat-induced antigen retrieval was performed in a microwave oven before immunohistochemical staining. After cooling and in running tap water, endogenous peroxidase activity was blocked by incubating in 3% hydrogen peroxide. To avoid the non-specific background, blocking was done with 5% BSA-PBS solution for 1 hr at room temperature. For the detection of SIRT1 protein, the slides were incubated with polyclonal rabbit anti-SIRT1 (1:100 dilution) at 4°C for over night in a humidified chamber. The signal conversion was carried out with avidin-biotin-peroxidase complex (ABC) method, as described by Toyokuni (E4) followed by hematoxylin counter staining. The assessment of immunostaining intensity was performed semi-quantitatively and in a blinded fashion. For the detection of NF-κB RelA/p65 protein, the slides were incubated with rabbit polyclonal anti-RelA/p65 (1:100 dilution) at 4°C for over night in a humidified chamber. Subsequent incubations with FITC conjugated anti-rabbit secondary antibody for 20 min in dark, the slides were rinsed in PBS and mounted with Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA). Labeled tissues were viewed and photographed with a Nikon Eclipse TE2000-S phase-contrast microscope with fluorescence optics and a Nikon COOLPIX 5400 camera.

Immunocytochemistry

MonoMac6 cells were treated with CSE and washed with ice-cold phosphate buffered saline and fixed with 4% paraformaldehyde in PBS. To detect the nuclear protein (SIRT1), the cells were permeabilized with 0.1% Triton X-100 and blocked with 10% goat serum for 1 hr at

room temperature. The immunostaining was performed using polyclonal rabbit anti-SIRT1 followed by the avidin-biotin-peroxidase complex (ABC) method and counterstained with hematoxylin, as described by Toyokuni (E4).

Immunoprecipitation and immunoblotting

After the extraction of nuclear proteins from MonoMac6 cells and human lung homogenates, SIRT1 antibody (1:80 dilution; Abcam) was added to 100 µg of nuclear protein in a final volume of 400 µl of RIPA buffer and incubated for 1 hr. Protein-A/G agarose beads (20 µl) (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 40 µl of lysis buffer. For Western blots, 100 µg of the immunoprecipitated SIRT1 agarose bead suspension were added to 10 µl of 5x sample buffer, boiled, and resolved by SDS-PAGE as described above. To determine the post-translational modification of SIRT1, blots were probed with anti-4-hydroxy-2-nonenal antibody, stripped, and reprobed with anti-3-nitrotyrosine antibody.

Reverse transcriptase polymerase chain reaction

After treatments, total RNA was isolated from MonoMac6 cells using RNeasy kit (Qiagen, Valencia, CA, USA). Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using oligo(dT) primers and superscript reverse transcriptase (Invitrogen Life Sciences) following the manufacturer's recommendations. The PCR conditions for the house keeping gene GAPDH were 20 thermal cycles of 94°C for 45 s, 60°C for 45 s, and 72°C for 90 s, followed by final extension for 10 min at 72°C. SIRT1 was subjected to 35 thermal cycles of

95°C for 30s, 55°C for 30s, 72°C for 30 sec followed by an extension at 72°C for 10 min. The primer pairs were as follows (forward and reverse, respectively): hSIRT1 (Integrated DNA technologies (IDT), IA, USA., 5'-TCA GTG TCA TGG TTC CTT TGC-3' and Up: Rev: 5'-AAT CTG CTC CTT TGC CAC TCT-3' (Product size 200 bp), and GAPDH, 5'-AGTGTAGCCCAGGATGCCCTT-3' and 5'-GCCAAGGTCATCCATGACAAC-3'. Amplified products were resolved by 1.5% agarose gel electrophoresis, stained with ethidium bromide, visualized and scanned by a white/UV transilluminator and quantified by densitometry.

Transfection of siRNA

Predesigned SIRT1 siRNA duplex (sense sequence: GAUUGGGUACCGAGAUAUU, antisense sequence: 5′-PAAAGUAUAUGGACCUAUCCUU), which is not homologous to other isoforms, was used to knock-down human SIRT1. siCONTROL non-targeting scrambled siRNA (5′-UAGCGACUAAACACAUCAAUU-3′) was used as a negative control. Human MonoMac6 cells were transfected with SIRT1 siRNA (L-003540-00) or non-target scrambled siRNA (D-001810-01) using DharmaFECT2 transfection reagent (T-2002-01) according to manufacturer's (Dharmacon, Lafayette, CO, USA) instructions. Briefly, 100 nM siRNA was mixed with the transfection reagent and incubated for 20 min at room temperature. The mixture was added to the 0.2 X 10^6 cells in the 12-well plate and incubated at 37 °C. At 36-48 hr after transfection, the cells were washed and used for the treatments.

Transfection of SIRT1 and SIRT1-H363Y

MonoMac6 cells were transfected with SIRT1 and SIRT1 deacetylase defective mutant or deacetylase lacking mutant-SIRT1-H363Y plasmids (both obtained from Addgene, Cambridge,

MA) using the commercially available calcium phosphate transfection kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Briefly, MonoMac6 cells were seeded at 0.2 X 10^6 cells/well in 12-well plate and were transfected with 20 μg of SIRT1 and SIRT1-H363Y constructs using the calcium phosphate transfection method. Two days after transfection, cells were incubated in a absence or presence of CSE (0.5%) for 4 h. The cell free culture medium was collected at the end of the experiment for IL-8 assay.

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