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Effects of sulfur dioxide derivatives on expression of oncogenes and tumor suppressor genes in human bronchial epithelial cells

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ABSTRACT

Sulfur dioxide (SO_2) is a major air pollutant suspected to act as a promoter or co-carcinogen. The present study was designed to investigate whether SO_2 derivatives (bisulfite and sulfite) had effects on the expression of several proto-oncogenes and tumor suppressor genes in cultured human bronchial epithelial (BEP2D) cells. The mRNA and protein levels were measured by real-time RT-PCR and western blotting, respectively, following exposure to differing SO_2-derivative concentrations and exposure times. SO_2 derivatives caused mRNA and protein over-expression of c-fos, c-jun, and c-myc at all tested doses (0.001–2 mM). Over-expression of H-ras and p53 were observed in cells receiving the highest concentration (0.1–2 mM), as well as the under-expression of p16 and Rb. The over-expression of c-fos and c-jun was observed after 24 h recovery. The expression of c-myc and H-ras decreased to base line levels while the p53 expression decreased compared with control after 24 h recovery. The mRNA and protein expression of p16 and Rb remained at initial levels after 24 h recovery. The data support the hypothesis that SO_2 derivatives could cause the activation of proto-oncogenes and inactivation of tumor suppressor genes and SO_2 derivatives may play a role in the pathogenesis of SO_2-associated lung cancer.

1. Introduction

Increased combustion of fossil fuels in the last century is responsible for the progressive change in the atmospheric composition. The anthropogenic sulfur dioxide (SO_2) results from the combustion of sulfur-containing fossil fuels and the smelting of sulfur-containing ores, volcanoes and oceans are its major natural sources. Air pollution has both acute and chronic effects on human health, affecting a number of different systems and organs. It ranges from minor upper respiratory irritation to chronic respiratory and heart disease, or even lung cancer (Kampa and Castanas, 2008). SO_2 is a major air pollutant suspected to increase mortality from respiratory diseases in the general population (Lee et al., 2000; Shinkura et al., 1999) and to act as a promoter or co-carcinogen (Nisbet et al., 1984). Epidemiological studies in SO_2-exposed workers showed increased mortality from lung cancer (Lee et al., 2002).

The respiratory tract is a unique barrier to exposure of high levels of environmental pollutants. Bronchial epithelial cells are the progenitor cells for bronchogenic lung cancers (Mace et al., 1994). They are often exposed to airborne environmental pollutants such as SO_2. SO_2 is readily absorbed through the respiratory tract (99%) and subsequently dissociates to form its derivatives (bisulfite and sulfite, 1:3 M/M, in neutral fluid) (Shapiro, 1977). In addition to these, (bi)sulfite itself is added to a variety of foods, beverages, and drugs as a preservative.

Exposure to environmental pollutants may induce some or all of the multiple genetic changes involved in the development of lung cancer, such as deregulated expression of oncogenes and/or tumor suppressor genes through gene amplification, mutations, and epigenetic damage (Anderson and Spandidos, 1993). In the lung, changes of c-fos, c-jun, c-myc, H-ras, p53, p16, and Rb genes have been observed in preneoplastic as well as cancerous tissue (Milde-Langosch, 2005; Little et al., 1983; Takahashi et al., 1989). The proto-oncogenes, c-fos and c-jun, which code for a transcriptional activator in a form of dimer, can be upregulated as an early response to stimuli. Over-expression of c-jun in rat embryo fibroblasts co-transfected with c-Ha-ras give rise to immortalized cell lines that grow in soft agar and produce tumors in nude mice (Schutte et al., 1989). It has been observed that transcription and translation of c-fos and c-jun were increased after SO_2 inhalation (Qin and Meng, 2006).

Abbreviations: SO_2, sulfur dioxide; BEP2D, HPV-18 immortalized human bronchial epithelial cell line; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; AMV RT, avian myeloblostosis virus reverse transcriptase; dNTPs, deoxynucleoitide triphosphates; B(a)P, benzo(a)pyrene; CA, chromosomal aberrations; MN, micronuclei; SCE, sister chromatid exchanges.

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Most of studies focus on the mutation of those genes in the progress of lung cancer. However, our previous study provides evidence for SO_2 and its derivatives as weak gene mutagen because the chemical at high doses and at high cytotoxicity increases the mutant frequency (Meng and Zhang, 1999). The comparatively weak effect of SO_2 derivatives indicates that direct mutagenesis is not the predominant effect of SO_2 and its derivatives. So we are suspicious of that if the expression of some cancer-related genes were affected by SO_2 and its derivatives. So far few data are available on the exact relationship between SO_2 or its derivatives and the expression of cancer-related genes. Therefore, the effects of SO_2 derivatives on several oncogenes (c-fos, c-jun, c-myc, and H-ras) and tumor suppressor genes (Rb, p53, and p16) were investigated by using the HPV-18 immortalized human bronchial epithelial cell line (BEP2D), which is derived from normal human bronchial epithelial cells.

2. Materials and methods

2.1. Cell culture

The BEP2D cell line was kindly provided by Prof. Maoxiang Zhu (Beijing Institute of Radiation Medicine, China). Cells were cultured in 25 cm^2 cell culture flasks containing serum free LHC-8 medium (Biofluids Inc., USA) in an incubator (Model 3111, Thermo Forma, USA) at 37 °C with a humidified 5% CO_2. Cells were detached and passaged every 7 days using trypsin 0.20% and 0.02% EDTA-Na_2 in Dulbecco's Hanks Balanced Salt Solution (D-Hanks) containing 0.8% (w/v) NaCl, 0.012% $Na_2HPO_4 \cdot 12H_2O$, 0.04% KCl, 0.006% KH_2PO_4 and 5000 units/ml penicillin/streptomycin, without Ca^{2+}, Mg^{2+}. The fresh medium was changed every 2–3 days, and the cells were harvested and passaged for exposure to the test agents while in logarithmic growth phase.

2.2. Treatment protocol

Cells were divided randomly into six equal groups of three flasks: one control group incubated only in LHC-8 medium and five groups treated with 0.001, 0.01, 0.1, 1 and 2 mM SO_2 derivatives (bisulfite and sulfite, 1:3 M/M). The solution of SO_2 derivatives (10 mM) was freshly prepared before use by dissolving a 7.5 mM:2.5 mM mixture of Na_2SO_3 and $NaHSO_3$ into LHC-8 medium. Serial dilutions of needed SO_2 derivatives were applied to the BEP2D cell. After 4 h incubation, the cells were harvested for real-time RT-PCR and western blot.

In the recovery protocol, cells were exposed to 0.1 mM SO_2 derivatives for 4 h, and then the culture medium was removed and replaced with the fresh LHC-8 medium. The cells were harvested at 0, 0.5, 1, 4 and 24 h after SO_2 derivatives exposure and used for real-time RT-PCR and western blot.

2.3. MTT assay

Cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Briefly, experimental cells were seeded into 96-well plates at 8000 cells/well and grown to 80% confluence at 37 °C in 5% CO_2. The cells were treated with SO_2 derivatives at different concentrations and post-exposure times. Subsequently, 20 μl of MTT solution (5 mg/ml) per well was incubated with cells for 4 h. Then the wells were drained, cells were washed with PBS and 150 μl of DMSO was added to dissolve the intracellular crystalline formazan product for 10 min at room temperature. The cells in the control were performed as described above except for treatment with LHC-8 medium instead of SO_2 derivatives. Samples were read at 490 nm in a microplate reader (B–R Model 550). The results were expressed as a percentage of the absorbance of control cells.

2.4. RNA isolation and cDNA synthesis

Total RNA was isolated from small amounts (up to 10^6 cells per 25 cm^2) of cultured cells by using TRIzol Reagent (Invitrogen Life Technologies) according to the manufacturer's instructions. RNA quality was insured by 1% gel electrophoresis (28S/18S RNA) and spectrophotometric analysis (OD260/280). The OD260/OD280 ratio was in the range of 1.8–2.0. Total RNA was quantified by determination of optical density at 260 nm. First-strand cDNA was produced following a protocol of avian myeloblostosis virus reverse transcriptase (AMV RT, Promega, Madison, WI) with slight modification. In reverse transcription reaction system, 2 μg RNA, 1.05 μg oligo dT (18) primer, were contained in a 25 μl reaction volume. The mixture was incubated at 70 °C for 5 min, and 5 μl RT buffer, 5 μl of 10 mM of each deoxynucleoitide triphosphates (dNTPs), 40 U RNasin, 30 U AMV Reverse Transcriptase were added. The mixture was incubated at 42 °C for 60 min. The cDNA product was stored at −80 °C until use.

2.5. Real-time PCR amplification

Each 20 μl PCR reaction contained 1 μl cDNA, 2 μl PCR buffer, 3.5 mM $MgCl_2$, 0.2 mM of each dNTP, 500 nM each primer, 200 nM TaqMan probe and 1 U/20 μl *Taq* DNA polymerase. The primers and probes were designed by using DnaStar and Beacon Design software (Table 1). Each treatment had three samples and each PCR reaction carried out in duplicate. Reactions were run on a Rotor-Gene 3000 Real-Time Cycler (Corbett Research, Sydney, Australia). Cycling conditions were as follows: 3 min at 95 °C, 40 cycles of 20 s at 94 °C, 20 s at 55–62 °C, and 20 s at 72 °C. Fluorescence data were acquired at the 72 °C step.

2.6. mRNA relative quantification

In each PCR run, serial dilutions of known amounts of corresponding cDNA standard were included. To minimize inter-assay variability, the same dilution series of each gene-specific standard were run with samples in each experiment. The threshold cycle (C_t) was calculated by the Rotor-gene 6.0 software to indicate significant

Table 1
Primers and probes sequences used in real-time RT-PCR.

Gene	Accession No.	Sequences
GAPDH	NM_002046	
	Forward primer	5′-GGAAGGACTCATGACCACAGT-3′
	Reverse primer	5′-GCCATCACGCCACAGTTTC-3′
	TaqMan Probe	5′- FAM-TGCCATCACTGCCACCCAGAAGAC-TAMRA-3′
c-fos	NM_005252	
	Forward primer	5′-GCCGTCTCCAGTGCCAAC-3′
	Reverse primer	5′-AGATAACTGTTCCACCTTGCCC-3′
	TaqMan Probe	5′- FAM-CATTCCCACGGTCACTGCCATCTCG-TAMRA-3′
c-jun	NM_002228	
	Forward primer	5′-AGCCTGAAGCCGCACCTC-3′
	Reverse primer	5′-AGAACTCGGACCTCCTCACCTCGCC-3′
	TaqMan Probe	5′- FAM-CCGTTGCTGGACTGGATTATCA-TAMRA-3′
c-myc	NM_002467	
	Forward primer	5′-TCCATGAGGAGACACCGCC-3′
	Reverse primer	5′-GCTGTGAGGAGGTTTGCTGT-3′
	TaqMan Probe	5′- FAM-ACCACCAGCAGCGACTCTGAGGAGG-TAMRA-3′
H-ras	NM_004985	
	Forward primer	5′-GGGAACAAGTGTGACCTGGC-3′
	Reverse primer	5′-ACCAACGTGTAGAAGGCATCC-3′
	TaqMan Probe	5′- FAM-CGCACTGTGGAATCTCGGCAGGCTC-TAMRA-3′
p16	NM_000077	
	Forward primer	5′-CAGTAACCATGCCCGCATAGA-3′
	Reverse primer	5′-AAGTTTCCCGAGGTTTCTCAGA-3′
	TaqMan Probe	5′- FAM-CCTCAGACATCCCCGATTGAAAGAACC-TAMRA-3′
p53	NM_000546	
	Forward primer	5′-ATCTACAAGCAGTCACAGCACAT-3′
	Reverse primer	5′-GTGGTACAGTCAGAGCCAACC-3′
	TaqMan Probe	5′- FAM-AGGCGGCTCATAGGGCACCACCA-TAMRA-3′
Rb	NM_000321	
	Forward primer	5′-ATCTATATTTCACCCCTGAAGAGTC-3′
	Reverse primer	5′-TTCAGAAGGTCTGCCAACACCAACA-3′
	TaqMan Probe	5′- FAM-CGCTGTTACATACCATCTGATTTAT-TAMRA-3′

Table 2
Cell viability of different concentration of SO_2 derivatives exposure.

Concentration of SO_2 derivatives (mM)	Cell viability (%)
0	100.3 ± 3.1
0.001	96.7 ± 2.5
0.01	96.2 ± 3.0
0.1	94.7 ± 3.3
1	89.2 ± 4.6*
2	79.7 ± 3.1**

Data are expressed as means ± S.D. of three individual experiments. Statistical significances were analyzed using one-way ANOVA compared with the control.
* $P < 0.05$.
** $P < 0.01$.