Emulsified isoflurane treatment inhibits the cell cycle and respiration of human bronchial epithelial 16HBE cells in a p53-independent manner

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Abstract. Emulsified isoflurane (EIso), as a result of its rapid anesthetic induction, recovery and convenience, is widely used as a novel intravenous general anesthetic. Treatment with EIso can reduce injuries caused by ischemia/reperfusion (I/R) to organs, including the heart, lung and liver, without knowing understanding the molecular mechanism. The present study hypothesized that treatment with EIso can affect the physiological processes of human lung bronchial epithelial cells (16HBE) prior to I/R. To test this hypothesis, the present study first constructed stable p53 knockdown and synthesis of cytochrome c oxidase $(SCO)_2$ knockdown 16HBE cells. The above cells were subsequently treated with EIso at a concentration of 0.1 and 0.2% for 24 h. The relevant concentration of fat emulsion was used as a negative control. The expression levels of p53, p21, SCO_1, SCO_2 and Tp53-induced glycolysis and apoptosis regulator (TIGAR) were detected by reverse transcription-quantitative polymerase chain reaction and western blotting. Subsequently, the cell proliferation, respiration and glycolysis were investigated. The results revealed that EIso treatment significantly decreased the transcription of TIGAR, SCO_1 and SCO_2, and increased the transcription of p21, which are all p53 target genes, in a p53-independent manner. The cell cycle was inhibited by arresting cells at the G0/G1 phase. Respiration was reduced, which caused a decrease in oxygen consumption and the accumulation of lactate and reactive oxygen species. Taken together, EIso treatment inhibited the proliferation and respiration, and promoted glycolysis in 16HBE cells. This regulatory pathway may represent a protective mechanism of EIso treatment by inhibiting cell growth and decreasing the oxygen consumption from I/R.

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Introduction

Emulsified isoflurane (EIso), which has clear advantages in its convenience, low environmental pollution and tissue toxicity, is widely used as a novel intravenous general anesthetic (1,2). Compared with isoflurane (Iso) inhalation, intravenous administration of EIso makes it easier to control the depth of anesthesia and eliminates the requirement for specific ventilatory circuits. In 1997, a previous study demonstrated that Iso pre-treatment reduced myocardial infarct size in an animal model, indicating pre-treatment as a promising approach for limiting ischemia/reperfusion (I/R) (3). Recently, more focus was put into researching the molecular mechanism of Iso protection from I/R.

Similar with Iso, pre-treatment with 8% EIso demonstrated a myocardial protective effect on I/R injury in rabbits, indicating that, despite the difference in form, both Iso and EIso effectively protected against myocardial ischemia (4). In the brain and heart, injury caused by prolonged anoxia and ischemia was decreased by the pre-treatment with Iso (5,6). In the lung, pre-treatment with EIso was revealed to reduce lung injury induced by hepatic I/R by decreasing the expression of tumor necrosis factor (TNF)-α and downregulation of intercellular adhesion molecule (ICAM)-1 (7). It was also found that pre-treatment with EIso caused cardioprotection against myocardial I/R injury in rats by inhibiting apoptosis and stabilizing mitochondria (8,9).

At present, it is widely accepted that I/R injury is caused by reactive oxygen species (ROS) accumulation, pH normalization and $[Ca^{2+}]$ rise, which leads to mitochondrial destabilization and creates an ideal scenario for mitochondrial permeability transition pore opening (10). Iso pre-treatment has been reported as one of the most effective strategies by attenuating ROS level upregulated by I/R, without a clear understanding of its molecular mechanism. Notably, downregulation of tumor

suppressor protein p53 by Iso pre-treatment indicates the association of Iso pre-treatment with networks controlled by p53.

Tumor suppressor p53 is well-known for its role in regulating apoptosis and the cell cycle in response to stress signals. However, novel roles of p53 in regulating the respiratory chain by transcriptionally regulating its downstream genes has attracted recent attention. Previously, it was reported that p53 regulates mitochondrial respiration by directly targeting to its downstream target gene and inducing the synthesis of cytochrome *c* oxidase $(SCO)_2$ (11). SCO_2, which is transcriptionally regulated by p53, serves a key role in maintaining mitochondrial respiration. Downregulation of SCO_2 restored the impaired aerobic respiration, indicating the regulatory role of p53 in mitochondrial respiration in a SCO_2-dependent manner (11). It has been previously showed that p53/SCO_2 signaling is activated by ROS generation to promote mitochondrial oxygen consumption, resulting in stabilization of the mitochondrial membrane (12).

As one of the numerous p53 target genes in an unstressed condition, the cyclin-dependent kinase inhibitor, p21, mediates the p53-dependent cell cycle G0/G1 phase arrest in response to a variety of stress stimuli (13). Its role in regulating the cell cycle was revealed to have protective effects to the kidney against I/R injury in mice, suggesting that p21 may confer tolerance to I/R injury (14). However, the association between p53 and p21 under EIso treatment remains to be elucidated.

In the present study, the effects of EIso treatment on physiological processes of 16HBE cells, and the regulatory role of p53 on cell proliferation and respiration were investigated. The present study also investigated whether EIso treatment regulates ROS level by affecting the regulatory activity of p53 on its downstream genes associated with mitochondrial respiration.

Materials and methods

Cells culture and antibodies. Human lung bronchial epithelial cells (16HBE) were stably transfected with short hairpin (sh) RNA constructs targeted against p53 mRNA (16HBE-$p53^{KD}$), SCO_2 mRNA (16HBE-SCO_2^{KD}) or scramble control (16HBE-SC). Untreated cells constituted non-transfected 16HBE cells, and mock cells constituted transfected 16HBE cells treated with fat emulsion. All cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin, and were cultured in an atmosphere of 5% CO_2 at 37˚C.

Rabbit anti-actin (cat. no. ab179467), rabbit anti-p53 (cat. no. ab179477), rabbit anti-p21 (ab7960), mouse anti-SCO_1 (cat. no. ab88658), rabbit anti-SCO_2 (cat. no. ab115877) and rabbit anti-Tp53-induced glycolysis and apoptosis regulator (TIGAR; cat. no. ab37910) primary antibodies were all purchased from Abcam (Cambridge, MA, USA) and were used at a dilution of 1:1,000. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG H&L (ab97040) and donkey anti-rabbit IgG H&L (ab7083) secondary antibodies were also purchased from Abcam and were diluted to 1:5,000.

Preparation of EIso. The 8% EIso (v/v) was manufactured by Huarui Pharmacy, Ltd. (Wuxi, China), according to the procedures described previously (15,16). Briefly, liquid Iso was mixed with 30% Intralipid® (fat emulsion injection; Sino-Swed Pharmaceutical Corp, Ltd., Beijing, China) at the final concentration of 8% (v/v) in an ampoule. The sealed ampoule containing mixture of liquid isoflurane and Intralipid® was agitated vigorously on a vortex for 30 min until Iso was solubilized into lipid emulsion. The stability of 8% EIso was at least 6 months at room temperature.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The expression levels of certain genes were measured by RT-qPCR. The total RNA was extracted from the cultured cells using TRIzol reagent (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Complete extraction of RNA was identified by running a MOPS denaturing agarose gel. The first-strand cDNA was synthesized using random hexamer and the AMV reverse transcriptase (Thermo Fisher Scientific, Inc.). For qPCR, 0.1 μl cDNA was used as a template in a 20 μl reaction volume containing 20 pmol of each primer and 10 μl SSO Fast EvaGreen Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA) on an Applied Biosystems® 7500 Real-Time PCR System (Thermo Fisher Scientific, Inc.). After 3 min initial melting at 95˚C, the mixture was amplified for a total of 40 cycles with a two-step cycle process that began with melting at 95˚C for 30 sec, and annealing and extension at 60˚C for 1 min. The nucleotide sequences of the PCR primers were as follows: β-actin, forward, 5'-CGCAAAGACCTGTATGCCAA-3' and reverse, 5'-CACACAGAGTACTTGCGCTC-3'; p53, forward, 5'-TGGCCATCTACAAGCAGTCA-3' and reverse, 5'-GGTACAGTCAGAGCCAACCT-3'; p21, forward, 5'-GGGCTGGGAGTAGTTGTCTT-3' and reverse, 5'-ATTGTGGGAGGAGCTGTGAA-3'; SCO_1, forward, 5'-ATTGCCCTGATGTCTGTCCA-3' and reverse, 5'-CTCTTCTCTCGTGCCAGTCA-3'; SCO_2, forward, 5'-TCTTCATCACTGTGGACCCC-3' and reverse, 5'-TTGGGGCCTGCATTGTAGTA-3'; TIGAR, forward, 5'-CTGGACCAGGTGAAAATGCG-3' and reverse, 5'-ACTGGCTGCTAATCCTGGAA-3' (Tsingke Biological Technology, Beijing, China).

Western blot analysis. Total protein was extracted using ProteoPrep® Total Extraction Sample kit (cat. no. PROT-TOT; Sigma-Aldrich, St. Louis, MO, USA) and the concentration was determined using the QuantiPro™ BCA Assay kit (cat. no. QPBCA; Sigma-Aldrich). The total proteins from the target cells were mixed with the equal quantities of 2X sodium dodecyl sulfate (SDS) sample buffer [10 mM Tris-HCl (pH 6.8), 0.05% SDS and 0.01% Bromophenol Blue] and boiled at 100˚C for 10 min. The proteins (50 μg) were separated by 10% SDS-polyacrylamide gel electrophoresis and were subsequently transferred onto nitrocellulose membranes. The membranes were blocked for 1 h at room temperature with blocking solution [5% bovine serum albumen in phosphate-buffered saline (PBS) with Tween-20]. The membranes were then incubated overnight at 4˚C with the primary antibodies, followed by washing three times with PBS containing 0.3% Tween-20 and incubation with the HRP-conjugated secondary antibodies for 1 h at room temperature. Immunoreactive proteins were visualized using enhanced chemiluminescence detection (Pierce, Rockford, IL,

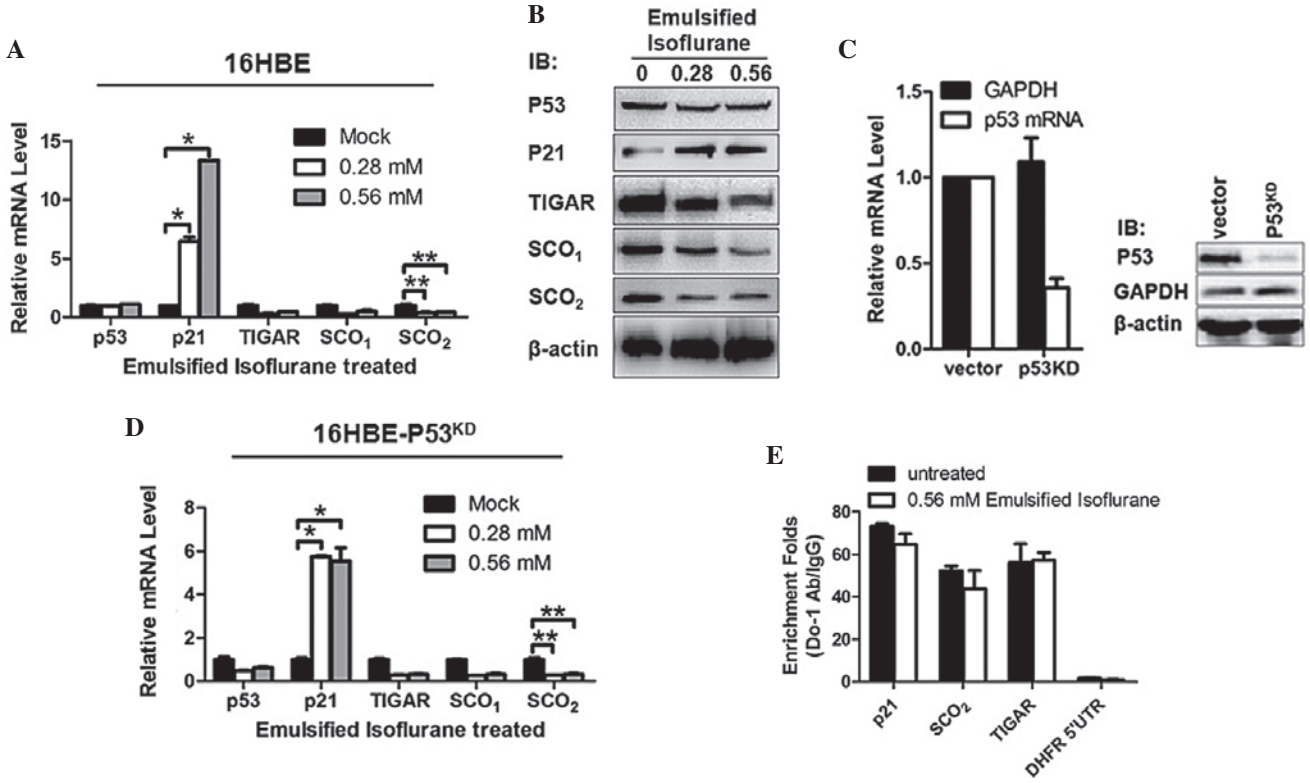

Figure 1. EIso treatment transcriptionally increased the expression of p21 and decreased the expression levels of TIGAR, SCO_1 and SCO_2. (A) RT-qPCR was performed to assess the transcriptional expression levels of p53, p21, TIGAR, SCO_1 and SCO_2 in EIso treated 16HBE cells. (B) Western blotting was performed to detect the protein expression levels of p53, P21, TIGAR, SCO_1 and SCO_2 in EIso treated 16HBE cells. β-actin was used as a loading control. (C) The relative mRNA and protein expression levels of p53 were determined in 16HBE cells stably transfected with short hairpin RNA targeted to p53 mRNA. GAPDH and β-actin were used as a loading controls for normalization. (D) The transcriptional levels of p53, p21, TIGAR, SCO_1 and SCO_2 were assessed in EIso treated 16HBE-$p53^{KD}$ cells. (E) Chromatin immunoprecipitation-qPCR analysis was performed to identify the binding of p53 to the promoter region of p21, SCO_2 and TIGAR. The data are presented as the mean ± standard deviation ($^{*}P<0.05$ and $^{**}P<0.01$ compared with the mock/untreated cells). EIso, emulsified isoflurane; KD, knockdown; RT, reverse transcription; qPCR, quantitative polymerase chain reaction; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Ig, immunoglobulin; SCO, synthesis of cytochrome c oxidase; TIGAR, Tp53-induced glycolysis and apoptosis regulator.

USA). Quantification of the band intensities was performed using the ChemiDoc MP system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with Image Lab 5 software.

Cell phase percentage assay. The cell cycle phase percentage was analyzed by flow cytometry (17). Trypsinized cells were washed with PBS and fixed in 75% ethanol. The cells were subsequently incubated with 100 μg/ml RNase at 37˚C for 30 min and stained with 50 μg/ml propidium iodide for 10 min at room temperature. The cells were analyzed on a FACScan flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) with FCS Express 5 software (De Novo Software, Glendale, CA, USA).

EdU incorporation assay. EdU is a thymidine analogue used to label proliferating cells, which can incorporate into replicating DNA when the cells are dividing (18). The cells were assessed using Cell-LightTM EdU DNA cell Proliferation kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Each assay was performed three times.

Oxygen consumption. Treated or untreated cells were trypsinized and resuspended at 10^6 cells/ml in DMEM. The oxygen consumption was measured in a 1 ml volume stirred, sealed chamber using a Clark-type oxygen micro-electrode (Unisense, Aarhus, Denmark) at 37˚C (11).

Production of lactate. Treated or untreated cells were trypsinized and washed with PBS twice. A total of 10^6 cells were resuspended in 100 μl ice-cold PBS, containing 3 mg/ml glucose and 0, 0.28 or 0.56 mM EIso. Lactate production was measured at 37˚C for 30 min using the Lactate reagent kit (Trinity Biotech Plc., Co Wicklow, Ireland) on a microplate reader (Synergy 2; BioTek Instruments, Inc., Winooski, VT, USA), according to the manufacturer's protocol.

Measurement of intracellular ROS. To identify the intracellular ROS levels, treated or untreated cells were harvested following any relevant treatment after incubation with DMEM containing 1% FBS and 100 μM DCFDA (freshly prepared) for 30 min in dark. The cells were washed twice with PBS and treated with either dimethyl sulfoxide (0.1%) alone or grape seed extract (GSE; 100 μg/ml) in PBS in the dark. The increase in fluorescence was measured at an excitation wavelength of 485 nm and emission wavelength of 538 nm using a fluorescent plate reader (Synergy 2). The background fluorescence of GSE (100 μg/ml) in the absence of DCFDA was also adjusted.

Statistical analysis. Statistical analyses were performed using JMP 9 software (http://www.jmp.com/en_gb/home.html). Data were analyzed by the Student's t-test and Fisher's exact

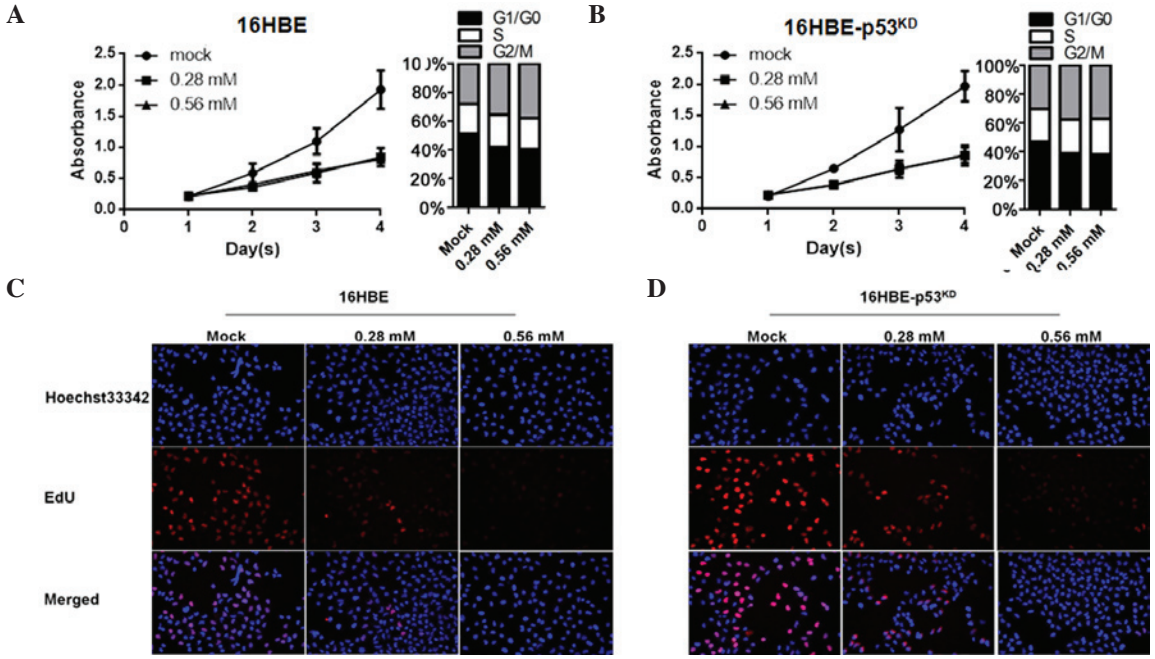

Figure 2. EIso treatment inhibited the proliferation of 16HBE cells. A CCK-8 analysis of the proliferation of (A) 16HBE and (B) 16HBE-p53^{KD} cells following pre-treatment with EIso was performed. EdU staining of (C) 16HBE and (D) 16HBE-p53^{KD} cells pretreated with EIso was performed to further assess cell proliferation. EIso, emulsified isoflurane; CCK, cell counting kit; KD, knock down; EdU, 5-ethynyl-2'-deoxyuridine.

test. P<0.05 was considered to indicate a statistically significant difference.

Results

EIso increases the mRNA expression levels of p21 and decreases the mRNA expression levels of TIGAR, SCO_1 and SCO_2 in a p53-independent manner in 16HBE cells. To investigate the effects of EIso treatment on gene expression in 16HBE cells, 0.28 or 0.56 mM EIso or mock were added into medium. The mRNA expression levels of p53 and its downstream genes were detected by RT-qPCR. The results revealed that, without disturbance of the p53 mRNA level, p21 mRNA was upregulated significantly and the mRNA expression levels of TIGAR, SCO_1 and SCO_2 were significantly decreased, indicating that changes in mRNA expression levels were irrelevant to p53's transcriptional regulation (Fig. 1A). Western blot analysis of these proteins further confirmed the downregulation by EIso treatment (Fig. 1B). Knockdown of p53 mRNA expression caused no changes in the effects of EIso treatment on these gene expression levels, further confirming that these processes were p53-independent (Fig. 1C and D). For further confirmation that the p53 DNA binding activity was not affected by EIso treatment, ChIP was performed and the quantity of responsive DNA element bound by p53 of each gene were quantified by qPCR. Consistently, p53 DNA binding activities at TIGAR, SCO_1, and SCO_2 were not inhibited by EIso treatment (Fig. 1E).

EIso treatment inhibits the cell cycle and thus inhibits cell proliferation by upregulating p21. The expression of p21 serves an important role in cell cycle arrest by inhibiting cyclin-dependent kinase activities. Considering the effect of p21 on cell cycle arrest, the present study next investigated the effect of EIso on the cell cycle by upregulating p21. Compared with the mock group, EIso-treated 16HBE cells exhibited a reduction in cell proliferation by cell cycle inhibition at the G0/G1 stage (Fig. 2A). Consistent with previous results (Fig. 1D and E), no change occurred in 16HBE cells with reduced levels of p53 (Fig. 2B). The EdU incorporation assay was performed to detect whether EIso treatment influenced the number of proliferating cells. The results demonstrated that the number of EdU-positive cells in the EIso-treated group was reduced compared with the mock group (Fig. 2C and D).

EIso treatment inhibits respiration, promotes glycolysis and sensitizes cells to oxidative damage. As one of the essential proteins for the assembly of the mitochondrial cytochrome *c* oxidase, SCO_2 serves a critical role in the metabolic centre of eukaryotic oxygen consumption (19,20). Since SCO_2 levels were reduced by EIso treatment, the present study assessed the oxygen consumption and lactate production in EIso-treated s16HBE cells. To create a stable, respiration-inhibited cell line, shRNA targeting SCO_2 mRNA was transfected into 16HBE cells (16HBE-SCO_2^{KD}). The RT-qPCR and semi-quantitative western blotting were performed to confirm the disruption of SCO_2 expression (data not shown). In 16HBE and 16HBE-p53^{KD}, EIso treatment exhibited a marked reduction in oxygen consumption compared with the mock goup. However, this effect was abolished by SCO_2 knockdown in 16HBE-SCO_2^{KD} cells, indicating that the effect of EIso treatment was functioning by a cessation of oxidative phosphorylation and a compensatory increase in lactate production (Fig. 3A and B). 16HBE and 16HBE-P53KD, but not 16HBE-SCO2KD cells, demonstrated a bioenergetic balance on respiration and glycolysis, as evidenced by a negative association between oxygen consumption and lactate production (Fig. 3A and B). These results suggested that the sensitivity of EIso treated 16HBE cells to oxygen was caused by generation of ROS. To further examine this possibility,

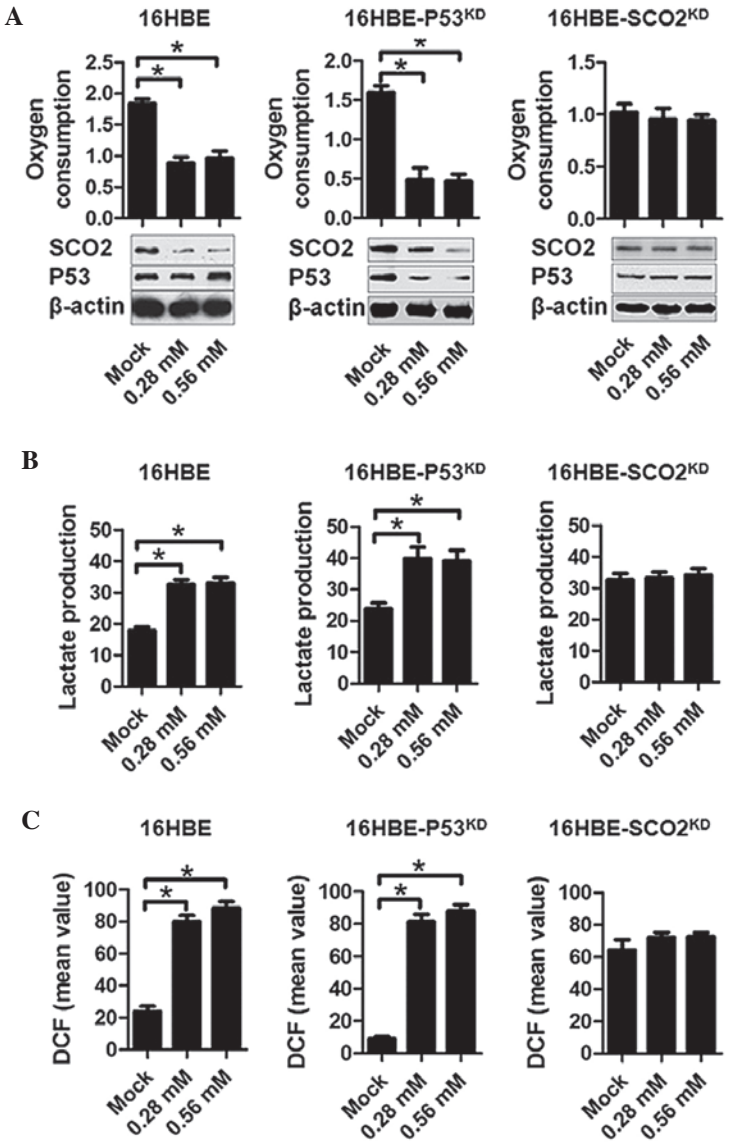

Figure 3. EIso treatment reduced oxygen consumption by downregulating the expression of SCO_2. (A) Oxygen consumption, (B) lactate production and (C) accumulation of reactive oxygen species were assessed following pretreatment with EIso (0.28 or 0.56 mM). *P<0.05 vs. the mock group. EIso, emulsified isoflurane; KD, knockdown; DCF, dichlorofluorescein; SCO, synthesis of cytochrome c oxidase.

intracellular ROS levels were measured using the hydrogen peroxide-sensitive dye dichlorofluorescein (DCF). Compared with the mock group, 16HBE and 16HBE-$p53^{KD}$ cells exhibited notably increased levels of ROS, as indicated by DCF staining intensity. No detectable change was observed in the 16HBE-SCO_2^{KD} cells (Fig. 3C).

Discussion

Ischemia followed by reperfusion injury causes a number of clinical disorders to the lung, including lung dysfunction syndrome and failure. This process may cause I/R injury directly to the lung and indirectly by causing remote organ injury, including gut and liver I/R injury (21). The activation of inflammatory reactions may occur via various signaling pathways that culminate in the activation of nuclear factor (NF)-κB and upregulation of TNF-α and ICAM-1 (22). It is reported that 1.5 h hepatic ischemia followed by 4 h reperfusion caused irreversible lung damage, with a significant increasing trend of NF-κB translocation, and an increase in TNF-α, and ICAM-1 transcriptional levels in the lung tissue (7).

EIso has been focussed on due to its effects on eliminating the requirement for specific ventilator circuits, and providing rapid anesthetic induction and recovery (2). Additionally, EIso pre-treatment has been found to attenuate oxidative stress and prevent I/R injury. Wang *et al* (23) reported that EIso pre-treatment protects isolated rat Kupffer cells against I/R induced injury by decreasing the production of ROS. It is also been reported that EIso pre-treatment caused a significant effect on attenuating inflammation and oxidative-caused damage (24). Notably, EIso pre-treatment markedly decreased I/R-induced lung injury in rats, which prompted the present study to investigate whether it is the same in human lung cells.

The results of the present study revealed that EIso pre-treatment markedly attenuated the mRNA and protein expression levels of TIGAR, SCO_1 and SCO_2, and stimulated the expression of p21, which all are the direct downstream target genes of p53 in 16HBE cells. As a result of the downregulation of p21,

the cell cycle was arrested at G0/G1 phase and cell proliferation was significantly inhibited compared with the untreated group. Considering the important roles of TIGAR, SCO_1 and SCO_2 in respiration, downregulation of these proteins prompted the present study to detect the respiration-associated processes. Consistently, EIso treatment decreased the oxygen consumption in 16HBE cells and promoted the production of lactate and the levels of ROS. To the best of our knowledge, this is the first study providing evidence treatment of 16HBE cells with EIso inhibits cell proliferation by arresting the cell cycle and inhibits respiration by transcriptionally downregulating respiration chain-associated genes, including TIGAR, SCO_1 and SCO_2 in a p53-independent manner.

ROS accumulate during ischemia and increase rapidly in the process of reperfusion. The existence of ROS causes membrane lipid peroxidation, changes in protein structure or function, and irreversible oxidative damage to the genome (25-27). The findings that overexpression of antioxidant enzymes at or prior to reperfusion contributes to significant protection from I/R injury in numerous models (28), indicating the damages of EIso treatment to treated cells via the inhibition of respiration. In the present study, it was found that EIso treatment increased ROS accumulation in 16HBE cells by downregulation respiration-associated genes, including TIGAR, SCO_1 and SCO_2. Whether the accumulated ROS causes DNA damage or not following treatment with EIso in different oxygen concentrations was subsequently investigated, and revealed that in high oxygen concentration >20% EIso treatment promoted cell apoptosis (data not shown). This result indicated that the protective effect of EIso treatment in 16HBE cells may be dependent on low oxygen concentration.

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