Isoflurane suppresses the self-renewal of normal mouse neural stem cells in a p53-dependent manner by activating the Lkb1-p53-p21 signalling pathway

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Received February 17, 2015; Accepted September 11, 2015

DOI: 10.3892/mmr.2015.4387

Abstract. Isoflurane is widely used in anaesthesia for surgical operations. However, whether it elicits unwanted side effects, particularly in neuronal cells, remains to be fully elucidated. The Lkb1-p53-p21 signalling pathway is able to modulate neuronal self-renewal and proliferation. Furthermore, the suppression of Lkb1-dependent p21 induction leads to apoptosis. In the present study, whether Lkb1-p53-p21 signalling is involved in the response to isoflurane was investigated. A comparison of mouse primary, wild-type neural stem cells (WT NSCs) with the p53⁻/⁻ NSC cell line, NE-4C, revealed that isoflurane inhibited proliferation in a dose-, a time- and a p53-dependent manner. However, flow cytometric analysis revealed that the concentration of isoflurane which caused 50% inhibition (the IC₅₀ value) induced cell cycle arrest in WT NSCs. Furthermore, the protein expression levels of LKB1, p53 and p21 were increased, although those of nestin and survivin decreased, following treatment of WT NSCs with isoflurane. On the other hand, isoflurane induced the phosphorylation of Ser15 in p53 in WT NSCs, which was associated with p53 binding to the p21 promoter, and consequentially, the transcriptional activation of p21. All these events were abrogated in NE-4C cells. Taken together, the present study has demonstrated that isoflurane suppresses the self-renewal of normal mouse NSCs by activating the Lkb1-p53-p21 signalling pathway.

Introduction

Isoflurane is widely used in the clinic as a volatile anaesthetic. Previous studies revealed that preconditioning with isoflurane mimics the protective effects of early and delayed ischemic preconditioning in the brain (1-6). Isoflurane produced early preconditioning against spinal cord ischemic injury (6). However, whether isoflurane produces delayed preconditioning in neural stem cells (NSCs) remains to be fully elucidated. The serine/threonine kinase, LKB1 (also known as STK11), has assumed a prominent position among tumour suppressors (7-9). LKB1 is inactivated in individuals with Peutz-Jeghers syndrome, which is a familial cancer-prone disease (10). Best characterized as an upstream activator of the adenine monophosphate-activated protein kinase and the mammalian target of rapamycin pathways (10), LKB1 regulates cellular and organismal metabolism, cell polarity and a variety of other functions, ranging from proliferation and migration to senescence, apoptosis, the DNA damage response and differentiation (7,11). Zeng et al (12) reported that LKB1 physically associates with p53 in the nucleus and phosphorylates p53 at the amino acid residues Ser15 and Ser392. LKB1 is also recruited directly to the p21/WAF1 promoter and other p53-activated promoters, and thereby regulates the cell cycle. Furthermore, Liang et al (13) identified that knockdown of the endogenous LKB1 facilitated cell cycle progression through the G1 and S phases, which was, at least in part, due to a reduced activity of the p53 and p16 pathways (13). In addition, Liu et al (8) reported that the inactivation of LKB1 in the presence of active Ras facilitated metastasis, which was proposed to be mediated via the Src family kinase-dependent expansion of a CD24⁺ melanoma prometastatic tumour subpopulation (8). On the basis of these studies, the present study aimed to investigate whether the Lkb1-p53-p21/WAF1 axis has a regulatory role in the modulation of normal mouse NSC proliferation during isoflurane treatment.

Materials and methods

Isolation of the mouse NSCs. Mouse NSCs were isolated from the corpus striatum of the embryonic brain in a 13-day-old...
pregnant mouse. Isolation of the NSCs was performed as previously described (14). Briefly, the embryonic brain tissue was sectioned into small segments with a razor. The segments were digested with 0.125% trypsin-EDTA (Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 15 min. The resulting cell suspension was seeded in a six-well plate (Falcon, BD Biosciences, Franklin Lakes, NJ, USA) in Dulbecco’s modified Eagle’s medium (DMEM)/F12 (1:1) (Gibco, Thermofisher Scientific, Waltham, MA, USA), supplemented with 1% (v/v) B27® Supplement, 20 ng/ml epidermal growth factor, 20 ng/ml basic fibroblast growth factor, 5 μg/ml heparin, 1 μg/ml laminin, 100 U/ml penicillin and 0.3 mg/ml glutamine (all from Gibco), and incubated in a humidified tissue culture incubator containing 5% CO2 at 37°C. The mouse NSC (p53−) cell line, NE-4C, was kindly provided by the Stem Cell Bank, Chinese Academy of Sciences (Shanghai, China). The NE-4C cells were cultured in DMEM:F12 (1:1) medium, supplemented with 15% FBS, 1 mM sodium pyruvate, 2 mM l-glutamine, 0.1 mM non-essential amino acids and penicillin (25 U/ml)-streptomycin (925 mg/ml) (all from Gibco), prior to mixing. The cells were subsequently incubated in a humidified tissue culture incubator containing 5% CO2 at 37°C. The cells were cultured on an identical feeder until passage 2, prior to the subsequent experiments.

**RNA extraction and analysis by reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** The total RNA from each cell was isolated using TRIzol® reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the manufacturer’s instructions. The RNA samples were treated with DNase I (Sigma-Aldrich), quantified, and reverse-transcribed into cDNA using the ReverTra Ace-α First Strand cDNA Synthesis kit (ToyoBo Co., Ltd., Osaka, Japan). RT-qPCR was performed using a RealPlex4 real-time PCR detection system from Eppendorf Co., Ltd. (Hamburg, Germany), with SYBR-Green RealTime PCR Master mix (ToyoBo Co., Ltd.) used as the detection dye, RT-qPCR amplification was performed over 40 cycles, with denaturation at 95°C for 15 sec and annealing at 58°C for 30 sec. The target cDNA was quantified using the relative quantification method. A comparative threshold cycle (Ct) was used to determine gene expression relative to a control (calibrator), and the steady-state mRNA levels were reported as an n-fold difference relative to the calibrator. For each sample, the Ct values of the marker genes were normalized using the equation: ΔCt = Ct_genes - Ct_18S RNA. The relative expression levels were determined using the equation: ΔΔCt = ΔCt_sample_groups - ΔCt_control_group. The values used to plot the relative expression of markers were calculated using the expression 2−ΔΔCt. The mRNA levels were calibrated, based on the levels of 18S rRNA. The cDNA of each gene was amplified using the primers listed in Table I.

**Immunofluorescence staining.** The cultured cells were washed three times with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde (Sigma-Aldrich) for 30 min. For blocking of non-specific binding sites, the samples were incubated with phosphate-buffered saline (Sigma-Aldrich) containing 0.5% Triton X-100 (Sigma-Aldrich) and 5% calf serum (Gibco) for 40 min at 37°C. Subsequently, the cells were first incubated with a primary antibody (Table II) overnight at 4°C and then with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG antibody (1:200; Abcam, Cambridge, UK) and 5 μg/ml 4′,6-diamidino-2-phenylindole (Sigma-Aldrich) at 26°C for 30 min. Subsequently, the cells were thoroughly washed with TBS-T buffer and viewed under a fluorescence microscope (DMI3000; Leica, Allendale, NJ, USA).

**Co-immunoprecipitation assay.** For each experiment, the cells were seeded at 3x10⁶ cells/well in six-well plates, cultured until 85% confluence was reached, and subsequently lysed (500 μl per plate) in a modified cell lysis buffer [20 mM Tris/HCl, (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, sodium pyrophosphate, β-glycerophosphate, Na3VO4 and leupeptin; Beyotime Institute of Biotechnology, Shanghai, China], prior to western blot analysis and immunoprecipitation assay. Following lysis, each sample was collected to clear the lysate of the insoluble debris by centrifugation (Allegra 64R; Beckman Coulter, Miami, FL, USA) at 10,000 g for 10 min, prior to an incubation with 20 μg protein A-agarose beads (Beyotime Institute of Biotechnology) by agitation for 30 min at 4°C. Subsequently, the samples were centrifuged again at 10,000 g for 1 min and transferred to a fresh 1.5 ml tube. The antibodies (Table II) were incubated with the samples for 90 min prior to the re-addition of 20 μg protein A-agarose beads to capture the immune complexes. The pelleted beads were subsequently washed three times with 500 μl cell lysis buffer, dissolved in 4X SDS-PAGE sample loading buffer, and heated for 10 min at 95°C.

**Chromatin immunoprecipitation (ChIP) assays.** ChIP experiments were performed using the primary antibodies provided in Table II and normal rabbit immunoglobulin G (IgG; Upstate Biotechnology, Inc., Lake Placid, NY, USA) as a negative control. All the steps were performed as previously described (9,12). Briefly, the cells were fixed with 1% formaldehyde for 30 min at 37°C, and the reaction was subsequently quenched by the addition of 125 mM glycine for 10 min at room temperature to form DNA-protein cross-links. The products were sheared by sonication using 4–5 sets of 10–sec pulses on wet ice with a Cole-Parmer High Intensity Ultrasonic Sonicator (50-watt model; Cole-Parme rInstrument Co., San Diego, CA, USA), equipped with a 2-mm tip and set to 30% of the maximum power, which produced the appropriate 200–800 bp DNA fragments. Subsequently, the samples were incubated with antibodies at 4°C overnight. The PCR amplification experiment was performed under the following conditions: 33 cycles run by denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec. Changes in the binding were expressed as the relative fold enrichment, following the subtraction of the matched IgG negative control.

**Western blot analysis.** The total protein extracts from each treatment group were resolved by 12% SDS-PAGE at 20°C over 120 min and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Following blocking, the PVDF membranes were washed four times for 15 min with TBS-T buffer at room temperature, and subsequently incubated with the primary antibodies listed in Table II. Following extensive washing, the membranes were...
incubated with secondary peroxidase-linked goat anti-rabbit IgG (1:1,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for 1 h. Following a further four washing steps (15 min each) with TBS-T buffer at room temperature, the immunoreactivity was visualized by enhanced chemiluminescence (ECL) using the ECL kit of Pierce Biotechnology, Inc. (Rockford, IL, USA). The membranes were subsequently exposed to Kodak XAR-5 films (Sigma-Aldrich). GAPDH was used as a loading control.

Flow cytometric (FCM) analysis of the cell cycle by propidium iodide (PI) staining. Each group of cells was seeded at 3x10^5 cells/well in six-well plates and cultured until 85% confluence was reached. The cells were washed three times with PBS, prior to collection by centrifugation (Allegra X-22R, Beckman Coulter, Miami, FL, USA) at 1,000 x g for 5 min. The cell pellets were subsequently resuspended in 1 ml PBS, fixed in 70% ice-cold ethanol and kept in a freezer for >48 h. Prior to flow cytometric analysis, the fixed cells were centrifuged at 1,000 x g for 5 min, washed twice with PBS, and resuspended in PI staining solution (Sigma-Aldrich), containing 50 µl/ml PI and 250 µg/ml RNase A (Sigma-Aldrich). The cell suspension, which was protected from the light, was incubated for 30 min at 4°C and analysed by fluorescence-activated cell sorting (FACS) using a BD FACSAria cell sorter (BD Biosciences, Franklin Lakes, NJ, USA). A total of 20,000 events were acquired for analysis using CellQuest software version 2.0 (BD Biosciences).

Methyl thiazolyl tetrazolium (MTT) assay for cell proliferation. Each group of cells was seeded at 2x10^3 cells/well in six-well plates, and cultured in DMEM supplemented with 10% FBS at 37°C with 5% CO₂ until 85% confluence was reached. The MTT reagent (5 mg/ml; Sigma-Aldrich) was added to the maintenance cell medium containing different concentrations of isoflurane (0, 0.125, 0.25 and 0.5 mM) for different time periods (0, 12, 24 and 48 h), and incubated at 37°C for an additional 4 h. The reaction was terminated by the addition of 150 µl dimethylsulfoxide (DMSO; Sigma-Aldrich) per well, and the cells were lysed for 15 min. The absorbance was measured at 490 nm using an enzyme-linked immunosorbent assay reader (Model 680; Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis. The data in each experiment are shown as the mean ± standard error of the mean where applicable; differences were evaluated using the Student’s t-test. GraphPad Prism version 5.00 (GraphPad Software Inc, La Jolla, CA, USA) was used for statistical analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

Isoflurane suppresses proliferation of WT NSCs by inducing cell cycle arrest. The results of the MTT assay indicated that isoflurane significantly inhibited the proliferation of
WT NSCs (P<0.01, n=3) in a dose-dependent manner (Fig. 1). Furthermore, following treatment of the WT NSCs with the concentration of isoflurane which caused 50% inhibition (IC\textsubscript{50} = 0.246 mM), cell proliferation was significantly inhibited at all time points (P<0.01, n=3) (Fig. 1). The complete inhibition of proliferation induced by isoflurane in WT NSCs appears to occur during the G0/G1 phase, as determined from the FACS profile (Fig. 1).

\textbf{Discussion}

Following mammalian nerve cell injury, there is a spontaneous induction of NSC migration to sites of nerve injury, followed by appropriate repair of the damaged tissue to restore the original neuronal function (14). Therefore, NSCs have an important role in the repair of nerve injury and in nerve function.
As a well-established inhalational anesthetic, isoflurane is generally considered to induce ischemic tolerance in the myocardium, cerebrum and spinal cord (1-6), however, the effects of isoflurane on NSCs have not been extensively studied. In the present study, a dose-dependent inhibition of mouse neural cell proliferation was observed. These results suggested that this may contribute to certain of the side effects of isoflurane. The additional experiments gave further credence to the hypothesis that the Lkb1-p53-p21 signalling pathway exerts a role in this response. A number of reports have indicated that this pathway is important in the regulation of cell proliferation (7-13). In response to specific

Fig. 2. Isoflurane activates Lkb1-p21 signalling in WT NSCs, although not in NE-4C (p53⁻) cells. (A) RT-qPCR analysis reveals that the mRNA expression levels of Lkb1, p53 and p21 in WT NSCs treated with an IC₅₀ concentration of isoflurane were significantly higher compared with that in untreated cells (**P<0.01; *P<0.05 compared with the non-treated group; n=3). (B) RT-qPCR analysis indicates that the mRNA expression levels of p53 and p21 in isoflurane-treated NE-4C (p53⁻) cells were unchanged compared with the untreated cells (**P<0.01; *P<0.05; #P>0.05 compared with the non-treated group; n=3). (C) Western blotting indicates that the protein expression levels of Lkb1 and p21 in isoflurane-treated WT NSCs were significantly increased compared with untreated cells (**P<0.01; *P<0.05; #P>0.05 compared with the non-treated group; n=3). (D) Western blotting indicates that only the protein expression level of Lkb1 was increased in isoflurane-treated NE-4C (p53⁻) cells (**P<0.01; *P<0.05 compared with the non-treated group; n=3). GAPDH was used as a loading control. RT-qPCR, reverse transcription-quantitative polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; WT NSCs, wild-type neural stem cells.

Fig. 3. Immunofluorescence analysis of the Lkb1-p21 signalling pathway in WT NSCs. The protein expression levels of LKB1, p53-Ser-pho and p21 in isoflurane-treated WT NSCs were significantly increased compared with the untreated cells (scale bar, 50 µm). FITC, fluorescein isothiocyanate; DAPI, 4',6-diamidino-2-phenylindole; WT NSCs, wild-type neural stem cells; p53-Ser-pho, Ser15-phosphorylated p53.
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stimuli, LKB1 phosphorylates the p53 transcription factor at specific sites (9,12), which increases the affinity of p53 for its target promoters, including that of the p21 gene, thereby increasing target gene expression (9,12). The expression of p21 elicits cell cycle arrest, which, in certain contexts, may be followed by apoptosis (9,12). The present study revealed that the expression of Lkb1, p53 and p21 increased in WT NSCs, although not in the NE-4C (p53<sup>-/-</sup>) cells, following isoflurane treatment. Furthermore, isoflurane promoted p53 Ser15 phosphorylation and increased the binding of p53 to the p21 promoter.

Taken together, the results suggest that the Lkb1-p53-p21 signalling pathway is important in the response of NSCs to isoflurane. Further studies are required to determine whether...
this is of clinical relevance, and whether the manipulation of p53 activation may help to attenuate the side effects of isoflurane.

Acknowledgements

This study was supported by grants from the National Natural Science Foundation of China (no. 81202811) and a project funded by the China Postdoctoral Science Foundation (nos. 2014M550250 and 2015T80455) and the Shanghai Municipal Health Bureau Fund (no. 20124320).

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