Prostate cancer cell malignancy via modulation of HIF-1α pathway with isoflurane and propofol alone and in combination

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Background: Surgery is considered to be the first line treatment for solid tumours. Recently, retrospective studies reported that general anaesthesia was associated with worse long-term cancer-free survival when compared with regional anaesthesia. This has important clinical implications; however, the mechanisms underlying those observations remain unclear. We aim to investigate the effect of anaesthetics isoflurane and propofol on prostate cancer malignancy.

Methods: Prostate cancer (PC3) cell line was exposed to commonly used anaesthetic isoflurane and propofol. Malignant potential was assessed through evaluation of expression level of hypoxia-inducible factor-1α (HIF-1α) and its downstream effectors, cell proliferation and migration as well as development of chemoresistance.

Results: We demonstrated that isoflurane, at a clinically relevant concentration induced upregulation of HIF-1α and its downstream effectors in PC3 cell line. Consequently, cancer cell characteristics associated with malignancy were enhanced, with an increase of proliferation and migration, as well as development of chemoresistance. Inhibition of HIF-1α neosynthesis through upper pathway blocking by a PI-3K-Akt inhibitor or HIF-1α siRNA abolished isoflurane-induced effects. In contrast, the intravenous anaesthetic propofol inhibited HIF-1α activation induced by hypoxia or CoCl2. Propofol also prevented isoflurane-induced HIF-1α activation, and partially reduced cancer cell malignant activities.

Conclusions: Our findings suggest that modulation of HIF-1α activity by anaesthetics may affect cancer recurrence following surgery. If our data were to be extrapolated to the clinical setting, isoflurane but not propofol should be avoided for use in cancer surgery. Further work involving in vivo models and clinical trials is urgently needed to determine the optimal anaesthetic regimen for cancer patients.
Lin et al., 2011). A much-cited example is a retrospective study involving 225 patients with prostate cancer undergoing radical prostatectomy in which it was reported that general anaesthesia combined with epidural anaesthesia was associated with a 57% lower recurrence rate (Biki et al., 2008a) when compared with general anaesthesia alone. It is argued that general anaesthesia using inhalational agents is immunosuppressive, whereas the use of regional anaesthesia helps attenuating surgical stress and the associated neuroendocrine responses, perhaps accounting for the observed association between use of regional anaesthesia and a higher disease-free survival rate (Biki et al., 2008b; Welden et al., 2009; Gottschalk et al., 2010; Lin et al., 2011; de Oliveira et al., 2011; Gottschalk et al., 2012). However, other mechanisms beyond the immunosuppressive effect of general anaesthetics may also play an important contributory role in this process.

One such mechanism involves the action of anaesthetics on a specific cell signalling pathway. Inhaled anaesthetics such as xenon have been shown to upregulate transcription factors, for example, hypoxia-inducible factor-1α (HIF-1α), a phenomenon that appears to underpin the cytoprotective effects of these agents on organs experiencing hypoxic injury (Ma et al., 2009). Conversely, the intravenous anaesthetic, propofol, has been shown to have the opposite effect, suppressing HIF-1α protein synthesis in an oxygen tension-dependent manner (Takabuchi et al., 2004b).

Hypoxia induces crucial biological responses in tumour growth (Liao & Johnson, 2007). HIF-1α has been identified as a key regulator of this process, which activates a spectrum of downstream genes to promote cell proliferation (Terraone et al., 2010; Wang et al., 2011), angiogenesis (Ban et al., 2010; Eisinger-Mathason and Simon, 2010) and metastasis (Burrows et al., 2011). Cancer cells take advantage of the ensuing phenotypic responses, not only in promoting cell survival in the harsh tumour environment but also in gaining competitive advantage over neighbouring, healthy cells. High levels of HIF-1α within a tumour have been associated with poorer patient prognosis and it has been considered as a potential therapeutic target in cancer (Semenza, 2003b). It is therefore rational to predict that the further upregulation of HIF-1α by an anaesthetic would compound or even enhance its effect of promoting malignant behaviour in cancer cells, whereas the use of an agent such as propofol which even enhance its effect of promoting malignant behaviour in cancer cells, whereas the use of an agent such as propofol which

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**MATERIALS AND METHODS**

**Cell culture.** Human authenticated prostate adenocarcinoma PC3 cancer cell line, derived from an advanced androgen-independent bone-metastasised prostate cancer, were cultured in monolayer in 75-cm² tissue culture flasks (VWR, Leistershire, UK). They were maintained at 37°C in humidified air balanced with 5% CO₂ in RPMI 1640 medium (GIBCO, Invitrogen, Paisley, UK) supplemented with 10% heat-inactivated fetal calf serum (Thermo Scientific, Epsom, UK), 2 mM L-glutamine and 100 U ml⁻¹ penicillin-streptomycin (Invitrogen). Culture medium was replaced every 48 h.

**Isoflurane exposure.** Before gas exposure, PC3 cells were cultured at 1 × 10⁶ per ml density on 30-mm² Petri dishes (VWR, Leistershire, UK) and used 24 h later when they were 80% confluent. Cell media was replaced either with fresh RPMI 1640 medium supplemented with serum and then placed in 1.5-litre purpose-built airtight, temperature-controlled chambers equipped with inlet and outlet valves and an internal electric fan used to provide continuous delivery and mixture of gases. For cell cycle study, cell media was without serum as the cell cycle can be synchronized through serum deprivation (Gopinathan et al., 2014). The chamber was connected to calibrated flow meters and an in-line vaporiser used to deliver the desired composition (Datex gas monitor, Helsinki, Finland) of isoflurane (0.5–2.0%) (Abbott Laboratories, Maidenhead, UK) in 21% oxygen and 5% CO₂ balanced with nitrogen (BOC, Guildford, UK). The chamber was pre-flushed with the aforementioned gas mixture to ensure that a stable gas composition was achieved, and a closed system was established to prevent leakage. Gas treatment was given at the desired isoflurane concentration for 2 h at 37°C. The cells were then removed and the serum-free medium was replaced with full-culture medium and the Petri dishes were returned to a standard incubator containing humidified air and 5% CO₂ at 37°C for further analysis. Cells used as the naïve control group were placed in an identical gas chamber containing 21% oxygen and 5% CO₂ balanced with nitrogen at 37°C. Cells were analysed at different time points ranging from 0–24 h post gas exposure.

**Propofol treatment.** Two formulations of propofol were used, dissolved in either 10% intralipid (the formulation for clinical use, from Astra-Zeneca, London, UK) or in DMSO (Sigma-Aldrich, Dorset, UK). Before treatment, PC3 cells were cultured at the density of 1 × 10⁶ per ml on a 30-mm² Petri dishes, as described above.

In the case of propofol in intralipid formation, propofol was diluted with PC3 medium to a stock concentration of 0.4 mg ml⁻¹. On the day of the experiment, the stock solution of propofol was diluted with PC3 serum-free medium to the desired concentrations (0.5–10 μg ml⁻¹). For the intralipid control, 20% intralipid was diluted in cell medium to recreate the amount of intralipid in the highest 10 μg ml⁻¹ dose of propofol being used. In the case of propofol in DMSO, prior to incubating the cells, propofol was dissolved to clinically relevant concentrations (1–4 μg ml⁻¹) with PC3 serum-free medium. The DMSO control corresponded to the amount of DMSO (0.05%) in the 4 μg ml⁻¹ dose of propofol. Propofol-supplemented medium was then added to the cell cultures for 2 h. Cells were then washed with Dulbecco phosphate-buffered saline (DPBS; Gibco, Invitrogen) and replaced with normal PC3 cell culture medium. For exposure to both isoflurane and propofol, cells were treated with propofol (0–10 μg ml⁻¹ in either lipid or DMSO)-supplemented media and then exposed to 2% isoflurane for 2 h. Following exposure, propofol medium was replaced with full PC3 cell medium and cells were harvested 24 h following anaesthetic treatment.

**Cobalt chloride-mediated HIF-1α induction.** A 1-mM stock solution of cobalt chloride (CoCl₂) was prepared by dissolving CoCl₂ powder (Sigma-Aldrich, Dorset, UK) in dH₂O, this stock solution was then further diluted to a concentration of 100 μM with serum-free propofol (0–4 μg ml⁻¹ in intralipid)-supplemented PC3 medium. Before treatment, PC3 cells were cultured at a density of 1 × 10⁶ per ml on 30-mm² Petri dishes, as described above. Complete medium was then replaced with CoCl₂ and propofol-supplemented medium for 6 h. Cells were harvested immediately after exposure for western blotting.

**Hypoxic HIF-1α induction.** Cells were placed in a gas chamber as described earlier and exposed to a mixture of 1% oxygen balanced in nitrogen, and 5% CO₂ for 18 h at 37°C in the presence (1–4 μg ml⁻¹) or absence of propofol. The cells were then immediately harvested for western blotting.

**siRNA-mediated HIF-1α knockdown.** PC3 cells were transfected with high-quality human-specific HIF-1α siRNA (Qiagen, Sussex, UK):
sense strand 5'-GAAGAACUAUGACAAUAAAATT-3'; antisense strand 5'-UUUAUGUCUAUGUUCUCCT-3'). A scrambled non-sense siRNA (Qiagen) without specific gene-silencing activity was used as a negative control. Transfection was achieved using lipofectamine RNAi MAX (Invitrogen). PC3 cells were cultured at the density of 0.4 × 10^5 per ml and treated with siRNA targeting human HIF-1α or scrambled siRNA dissolved in siRNA suspension buffer supplement with lipofectamine which was administered to PC3 cells in a dose of 20 nM. Cells were incubated with siRNA for 6 h at 37°C in humidified air containing 5% CO₂, after which the cells were washed with DPBS, then serum-free medium was added followed by isofurane gas exposure and/or propofol exposure and in conjunction with the chemotherapeutic agent Docetaxel (see below).

Treatment with a chemotherapeutic agent. A typical anti-mitotic chemotherapeutic agent for prostate cancer, Docetaxel (DTX), was used in conjunction with isofurane and propofol treatments on PC3 cells. Twenty-four hours post isofurane exposure or propofol treatment with or without siRNA pre-treatment, DTX (Tocris, Bristol, UK) of 1-mM stock solution was diluted to a concentration of 0–100 μM with PC3 medium. Cell medium was then replaced with DTX-supplemented medium for 24 h.

P13K and MEK inhibition. PC3 cells were cultured at a density of 1 × 10^5 per ml in 30-mm² petri dishes and then treated with either a selective inhibitor of phosphatidylinositol 3 kinase (PI3K), LY294002 (Cell signaling, Hitchin, UK) or a selective inhibitor of mitogen-activated protein extracellular-regulated kinase (MAPK/ERK), U0126 (Cell signaling) for 16 h at 37°C in a humidified atmosphere containing 5% CO₂ prior to isofurane and/or propofol exposure. Both chemicals were dissolved in sterile DMSO to create a stock concentration of 10 mM. For the final solution, they were further diluted with serum-free culture medium to the final working concentration of 50 μM, with concentration of DMSO being 0.05%. Cells were harvested for western blotting immediately after anaesthetic exposure.

Western blotting. Control or treated PC3 cells described above were washed with ice-cold PBS buffer and lyed on ice by physical disruption using a cell scraper in 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA, 2 mM EGTA, 1% Triton, 2.5 mM Na₄P₂O₇, 1 mM beta-glycerophosphate, 1 mM Na₂VO₄, 1 μg ml⁻¹ leupeptin, 1 mM phenyl methanesulphonyl fluoride and 1 mM diithiothreitol cell lysis buffer (Cell Signalling). The samples were centrifuged at 13,000 rpm for 30 min at 4°C, and the supernatant was collected. Total protein concentration was quantified using the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA) and protein extracts were normalised for protein content to 40 μg per sample. Protein extracts were added to sodium dodecyl sulphate (SDS) sample buffer (Invitrogen) at 1:3 ratio, heated for 10 min at 95°C and then loaded on a NuPAGE 12% Bis-Tris gel (Invitrogen) for electrophoresis. Following electrophoresis, they were transferred to a polyvinylidenedifluoride (PVDF) membrane and blocked overnight in 5% non-fat powdered milk in 0.1% Tween 20/tris-buffered saline (TBST) at 4°C. The membranes were then probed with the following primary antibodies: mouse anti-HIF-1α (BD Biosciences, San Jose, CA, USA); rabbit anti-Bcl-2 (Abcam, Cambridge, UK); rabbit anti-HIF-1β (Cell Signalling); rabbit anti-phospho-Akt (Cell Signalling); mouse anti-phospho-ERK (Cell Signalling), in 5% non-fat dry milk in TBST overnight at 4°C, followed by HRP-conjugated anti-mouse or anti-rabbit secondary antibodies (Cell Signalling) for 1 h. The loading control was the constitutively expressed protein β-tubulin (Sigma-Aldrich, Dorset, UK). The blots were washed with TBST for 5 min three times followed by one wash with TBS and were visualised using the enhanced chemiluminescence (ECL) system (Santa Cruz, Dallas, Texas). The protein bands were captured with an image processor using the Syngene GeneSnap software (Syngene, Cambridge, UK) and the intensity of the bands corresponding to the protein expression level was measured using GeneTools software (Syngene, Cambridge, UK). Results were normalised to levels of the housekeeping protein β-tubulin and expressed as ratio of the control for data analysis.

Immunocytochemistry. PC3 cells were fixed in 4% paraformaldehyde for 10 min, rinsed in PBS (Sigma-Aldrich) two times for 5 min, permeabilised with 0.1% Triton X-100 in PBS (PBS-T) for 10 min and washed twice for 5 min. Blocking was carried out at room temperature for 1 h using 10% normal goat serum (Vector Laboratories, Peterborough, UK).

Cells were incubated overnight at 4°C in blocking solution containing one of the following antibodies: rabbit anti-HIF-1α (Novus Biologicals, Littleton, CO, USA), rabbit anti-VEGF (Abcam), mouse anti-Ki-67 (Dako, Cambridge, UK), mouse anti-cytochrome c (BD Biosciences, San Jose, CA, USA), mouse anti-cyclin D (Abcam), mouse anti-cyclin E (Abcam). Following three PBS rinses, cells were incubated for 2 h in PBS-T containing one of the following secondary antibodies: Rhodamine-conjugated donkey anti-rabbit IgG (Millipore, Oxford, UK), Fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG (Millipore) or FITC-conjugated anti-rabbit (Millipore), followed by final PBS rinses and coverslipping with Vectorshield fluorescence medium containing 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories). In the case of double labeling, the following combinations of antibodies were used for this study: HIF-1α/VEGF, HIF-1α/Ki-67, HIF-1α/cyclin D and HIF-1α/cyclin E. PC3 cells were incubated with the first primary antibody overnight, followed by the first secondary antibody, and then the second primary antibody and the second secondary antibody. Images of 10 high-power fields (HPFs) at ×20 magnification were obtained using an AxioCam digital camera (Zeiss, Welwyn Garden City, UK) mounted on an Olympus BX60 microscope (Olympus, Middlesex, UK) with Zeiss KS-300 software (Zeiss). Images were captured under identical exposure settings and final assessments were conducted under blinded conditions. In the case of HIF-1α, HIF-1α/VEGF, HIF-1α/kid67 and cytchrome-c staining, images were used for qualitative analysis. For Kid67 staining, eight representative fields were randomly selected and the mean pixel intensities for Kid67-positive cells were measured through ImageJ 1.35 software (NIH, Bethesda, MD, USA). Kid67-positive cells were then presented as a percentage of the total number of DAPI-positive cells.

Cell viability test (MTT Assay). The viability of cells was assessed by using a colorimetric 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Merck KGaA, Darmstadt, Germany) assay. MTT was diluted with minimum essential medium + Earle’s – L-glutamine × 1 (MEM) (GIBCO, Invitrogen) to a concentration of 0.5 mg ml⁻¹. Cells were then incubated with 0.5 mg ml⁻¹ of MTT for 2 h at 37°C in a humidified atmosphere containing 5% CO₂. The solution was then carefully aspirated out and 1 ml of DMSO, (Fisher Scientific, Leicestershire, UK) was added to dissolve the purple-coloured formazan particles and form a homogeneous purple colour. The samples from duplicate wells were transferred to a 96-well plate, and the absorbance of dissolved formazan crystals was spectrophotometrically assessed at a wavelength of 595 nm, using an MRx microplate reader (Dynex Technologies, Chantilly, VA, USA) and presented in arbitrary units. Viability was defined as the ratio of the optical density of formazan in the treated group against that in the control.

Scratch assay. The scratch assay (wound-healing assay) was performed for assessing tumour cell migration, as previously described (Liang et al, 2007). After cultured cells became confluent in 24-well plates, one artificial gap created by a sterile plastic
micropipette tip (1000 μl) to generate a uniform gap that was devoid of adherent cells. Gap closure was monitored with digital camera images taken in a phase-contrast microscope equipped with a digital camera (Olympus CK30, Tokyo, Japan). Images were analysed using ImageJ. The same scratch was analysed before and 24 h after treatment using ImageJ 1.35 software. Wound healing was quantified as the mean percentage of the remaining cell-free area compared with the area of the initial wound (Hu & Verkman, 2006).

**Enzyme-linked immunosorbent assay (ELISA).** Cultured cell medium with or without treatments was harvested for MMP-2 and MMP-9 measurements determined with ELISA kits (R&D Systems, Oxon, UK) following the manufacturer’s instructions.

**Statistical analysis.** All numerical data were expressed as mean ± s.d. Comparison between different treatments was analysed by one-way ANOVA followed by post hoc test using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). A *P*-value <0.05 was considered to be statistically significant.

## RESULTS

### Isoflurane induced persistent HIF-1α expression and translocation at clinically relevant concentrations.

In order to distinguish the effects of isoflurane exposure on HIF-1α protein levels, PC3 cells were exposed to 0.5–2% isoflurane for 2 h, and cells were harvested at 0, 2, 4, 8 and 24 h after gas exposure and examined using western blotting and immunocytochemistry. Isoflurane exposure induced a marked increase in HIF-1α protein level in a time-dependent (Figure 1A) and a dose-dependent manner (Figure 1B). Specifically, exposure to 2% isoflurane triggered significant upregulation of HIF-1α protein levels, which was observed from 4 h (3.33 ± 0.59 vs 1.00 of NC, *P* <0.001; Figure 1A) post gas exposure, maintained at 8 h (3.19 ± 0.62 vs 1.00 of NC, *P* <0.001; Figure 1A) and was maximally increased 24 h post exposure (3.74 ± 0.81 vs 1.00 of NC, *P* <0.001; Figure 1A). Furthermore, isoflurane-induced activation of HIF-1α expression was dose-dependent with significant increases after exposure with 1.5% isoflurane (2.11 ± 0.58 vs 1.00 of NC, *P* = 0.041; Figure 1B) and 2% isoflurane (3.20 ± 0.92 vs 1.00 of NC, *P* <0.001, Figure 1B). Moreover, it was observed that 24 h following isoflurane exposure there was a distinct translocation of HIF-1α from the cytoplasm into the nucleus (compared with the naïve control) where it initiates downstream gene expression as a transcriptional regulator (Figure 1C). It is important to note that protein levels of HIF-1α remained unchanged after isoflurane exposure both in the time course (*P* = 0.9672, Figure 1D) and the dose-response experiments (*P* = 0.6425, Figure 1E).

### Propofol inhibited activation of HIF-1α induced by various stimulants.

To test the effect of propofol on HIF-1α expression, co-intervention experiments were performed with clinically relevant concentrations of propofol (≤4 μg ml⁻¹) and HIF-1α inducers: hypoxia, CoCl₂ or isoflurane. Exposure times varied depending on the experiment performed. Cells were harvested either immediately after the co-intervention experiments (propofol and hypoxia or CoCl₂), or at different time points for propofol alone (0–24 h), or at 24 h post-anaesthetic exposure for propofol and isoflurane. In order to exclude any effects of the propofol vehicle, DMSO and intralipid controls were used in these experiments as well as the naïve control. Exposure to hypoxia significantly increased HIF-1α protein levels when compared with the naïve control (3.14 ± 0.50 vs 1.00 of naïve, *P* <0.01; Figure 2A); an effect that was abrogated following co-exposure to hypoxia and propofol in a dose-dependent manner (1.38 ± 0.64-fold of naïve for propofol at 2 μg ml⁻¹ and 1.16 ± 0.26-fold of naïve for propofol at 4 μg ml⁻¹ vs 3.14 ± 0.50-fold of naïve for isoflurane alone, *P* <0.01; Figure 2A). Similar results were observed for cells treated with both CoCl₂ and propofol. CoCl₂ was shown to induce HIF-1α activation (3.33 ± 0.41 vs 1.00 of naïve, *P* <0.01, Figure 2B) and propofol negated this effect (1.85 ± 0.30-fold of naïve for propofol at 1 μg ml⁻¹, 1.15 ± 0.30-fold of naïve for propofol at 2 μg ml⁻¹ and 0.87 ± 0.34-fold of naïve for propofol at 4 μg ml⁻¹ vs 3.33 ± 0.41-fold

Figure 1. Effect of isoflurane exposure on expression and translocation of HIF-1α in cultured human prostate cancer cells (PC3 cells). Cultured cells were exposed to 0.5–2% for 2 h and were harvested immediately or 2–24 h after exposure for western blotting. (A, B) A time- and concentration-dependent increase in HIF-1α expression after isoflurane exposure; (C) immunostaining further revealed isoflurane exposure promoted the translocation of HIF-1α from cytoplasm to nucleus where its downstream effects are initiated; (D, E) HIF-1α expression was unchanged after exposure to isoflurane. Results are expressed as mean ± s.d.; *P* <0.05; **P <0.001 vs naïve control. Bar = 25 μm.
of naïve for isoflurane alone, \( P < 0.01 \); Figure 2B). Most importantly, propofol abolished 2% isoflurane-induced HIF-1\( \alpha \) expression in a dose-dependent manner both when dissolved in lipid (1.95 ± 0.28-fold of naïve for propofol at 2 \( \mu \)g ml\(^{-1}\) \( P = 0.04 \), 0.89 ± 0.59-fold of naïve for propofol at 4 \( \mu \)g ml\(^{-1}\) and 0.82 ± 0.48-fold of naïve for propofol at 10 \( \mu \)g ml\(^{-1}\) vs 3.12 ± 0.24-fold of naïve for isoflurane alone, \( P < 0.01 \); Figure 2C) and when dissolved in DMSO (2.25 ± 0.38-fold of naïve for propofol at 1 \( \mu \)g ml\(^{-1}\) vs 3.01 ± 0.20-fold of naïve for isoflurane alone, \( P < 0.01 \); Figure 2D).

**Propofol and HIF-1\( \alpha \) siRNA inhibited isoflurane-induced VEGF upregulation and cell proliferation.** We next investigated whether isoflurane increased VEGF levels and cell proliferation in PC3 cells and, furthermore, whether HIF-1\( \alpha \) inhibition through siRNA treatment and propofol treatment abrogated these effects. PC3 cells (with or without siRNA pre-treatment) were either exposed to 2% isoflurane alone for 2 h or in combination with 4 \( \mu \)g ml\(^{-1}\) of propofol for 2 h. Cells were collected 24 h post-anaesthetic exposure and used to determine HIF-1\( \alpha \)/VEGF and HIF-1\( \alpha \)/Ki67 levels through immunocytochemistry and cell proliferation rates with an MTT assay. Propofol at 4 \( \mu \)g ml\(^{-1}\) effectively blocked isoflurane-induced HIF-1\( \alpha \) expression and the associated expression of VEGF (Figure 3A and C) and Ki67 (Figure 3B and D), which are well-established markers for angiogenesis and active cellular proliferation, respectively. A similar inhibitory effect was also observed with HIF-1\( \alpha \)-specific siRNA, which selectively blocked isoflurane-induced HIF-1\( \alpha \) overexpression (HIF-1\( \alpha \) level decreased from 2.47 ± 0.42 of isoflurane exposure vs 1 of NC to 0.74 ± 0.11 of isoflurane plus siRNA exposure vs 1 of NC, \( P < 0.001 \); Supplementary Figure 1). Both VEGF (Figure 3A) and Ki67 levels (Figure 3B) were reduced as a result of HIF-1\( \alpha \) inhibition.

Isoflurane promoted cell cycle progression and cell proliferation, which was inhibited by propofol and HIF-1\( \alpha \) siRNA. We next assessed the cell cycle progression and cell proliferation after exposure to isoflurane. Enhanced cyclin D and cyclin E expression in PC3 cells was found after being exposed to isoflurane, and co-localization of HIF-1\( \alpha \)-specific cyclin D or HIF-1\( \alpha \)-specific cyclin E was noted (Figure 4A–D); the treatments with either propofol or HIF-1\( \alpha \) siRNA abolished these effects (Figure 4A–D) when compared with that treated with isoflurane. Cell viability was decreased from 1.41 ± 0.12 (relative to control) with isoflurane exposure to 1.10 ± 0.20 with isoflurane plus propofol exposure (\( P = 0.012 \)) (Figure 4E).

**Isoflurane induced resistance to the chemotherapeutic agent docetaxel in PC3 cells, which could be reversed by propofol and HIF-1\( \alpha \)-specific siRNA.** The next step was to determine whether isoflurane and propofol had any effects on the efficacy of antimitotic chemotherapy drug commonly used in prostate cancer, DTx. To determine whether the efficacy of DTx was altered with changing HIF-1\( \alpha \) expression, PC3 cells were first exposed for 2 h to isoflurane, isoflurane plus propofol, or isoflurane in siRNA pre-treated cells followed by treatment with DTx for 24 h. Cells were harvested immediately after DTx treatment for immunocytochemistry and western blotting. It was observed that DTx was less effective in inhibiting active mitosis in isoflurane-exposed PC3 cells, evident by the 58.53 ± 7.32% of actively dividing cells (Ki67 positive) when compared with the DTx treatment alone (25.61 ± 6.71%, \( P < 0.001 \)), or to the cells that were treated with propofol plus isoflurane (17.96 ± 11.56%, \( P < 0.001 \)) (Figure 5A and B) and to the cells that were treated with isoflurane plus siRNA (27.52 ± 8.88%, \( P < 0.001 \); Figure 5A). This was further supported by an MTT assay, which showed that isoflurane-pre-treated PC3 cells had a higher proliferation rate after DTx challenge, when compared with non-pre-treated PC3 cells (0.36 ± 0.03-fold of naïve vs 0.13 ± 0.05-fold of naïve, \( P = 0.006 \); Figure 5B). With addition of propofol to isoflurane for the pre-treatment, the proliferation rate...
was decreased (0.18 ± 0.04-fold of naïve vs 0.36 ± 0.03-fold of naïve, P = 0.047). Furthermore, cytochrome c, a marker for cellular injury, was lower in the isoflurane and DTX co-treated cells when compared with DTX alone, isoflurane-propofol–DTX co-treated and isoflurane, siRNA and DTX groups (Figure 5C). This was coupled with overexpression of the anti-apoptotic Bcl-2 protein in cells with 6.8% isoflurane exposure and DTX treatment compared with cells with DTX treatment alone (2.54 ± 0.69-fold of naïve vs 0.45 ± 0.23-fold of naïve, P < 0.001; Figure 5D, left panel and 2.49 ± 0.50-fold of naïve vs 0.44 ± 0.19-fold of naïve, P < 0.001; Figure 5D, right panel). The overexpression of Bcl-2 was prevented with siRNA pre-treatment (2.54 ± 0.69-fold of naïve vs 0.25 ± 0.17-fold of naïve, P < 0.001; Figure 5D, left panel) and propofol pre-treatment (2.49 ± 0.50-fold of naïve vs 0.33 ± 0.12-fold of naïve, P < 0.001; Figure 5D, right panel).

**Phosphorylation of Akt, but not ERK, played an important role in the alteration of HIF-1α expression induced by isoflurane or propofol.** Collectively, these experiments established that isoflurane promotes cancer cell growth and survival in the face of various challenges via activation of HIF-1α and upregulation of anti-apoptotic protein Bcl-2. Next, we determined the molecular basis underlying isoflurane-induced upregulation of HIF-1α. We investigated whether the PI3K/Akt/mTOR pathway and MAPK/ERK pathways were involved in the isoflurane-induced upregulation and propofol-induced downregulation of HIF-1α. PC3 cells were treated with either a PI3K inhibitor (LY294002) or an MAPK/ERK (MEK) 1/2 inhibitor (U0126) overnight, then were exposed to either isoflurane or isoflurane and propofol for 2 h. Twenty-four hours later, the cells were harvested for western blotting and protein levels of HIF-1α, phosphorylated Akt (p-Akt) and phosphorylated ERK (p-ERK) were investigated. Phosphorylation of the kinase Akt, but not ERK 1/2, appear to play the dominant role in isoflurane-induced HIF-1α overexpression. LY294002 blocked HIF-1α overexpression showing a significant decrease in the inhibitor plus isoflurane group when compared with the isoflurane group alone (1.14 ± 0.38-fold of naïve vs 4.16 ± 1.02-fold of naïve, P < 0.001 Figure 6A), but no significant changes when compared with the group that had also received propofol (1.14 ± 0.38-fold of naïve vs 1.29 ± 0.61-fold of naïve, P = 1; Figure 6A). By contrast, the MEK 1/2 inhibitor, U0126, had no significant effect on HIF-1α expression with the inhibitor plus isoflurane group showing no significant difference to the isoflurane group alone (3.13 ± 0.67-fold of naïve vs 4.16 ± 1.02-fold of naïve, P = 0.443; Figure 6A), but did display a significantly higher amount of HIF-1α protein relative to the group that received isoflurane plus propofol (3.13 ± 0.67-fold of naïve vs 1.29 ± 0.61-fold of naïve, P = 0.035; Figure 6A). It was further shown that isoflurane and propofol had opposite effects on the phosphorylation of Akt kinase with isoflurane showing significantly higher amounts of p-Akt when compared to the naïve control (2.98 ± 0.57-fold of naïve P < 0.001; Figure 6B), an effect abolished by pre-treatment with LY294002 (2.98 ± 0.57-fold of naïve vs 1.29 ± 0.37, P = 0.002; Figure 6B) and co-intervention with propofol (2.98 ± 0.57-fold of naïve vs 1.68 ± 0.63, P = 0.02; Figure 6B). In terms of the phosphorylation of ERK, isoflurane alone showed an increasing trend compared to the naïve control but no significant change (1.71 ± 0.52-fold of naïve, P = 0.081; Figure 6C). However, there was a significant decrease in the propofol (0.95 ± 0.26-fold of naïve vs 1.71 ± 0.52, P = 0.048; Figure 6C) and inhibitor groups (0.79 ± 0.27-fold of naïve vs 1.71 ± 0.52, P = 0.013; Figure 6C) when compared to isoflurane...
Propofol exposure (4 µg/ml) reversed the effects of isoflurane exposure on phosphorylation of Akt as well as on the expression of its downstream genes including VEGF, a protein with hypoxia inducible factor-1α (HIF-1α) expression in an oxygen-independent manner. This, in turn, enhances cancer cell proliferation and migration together with the reinforcement of chemotherapy resistance. In contrast, propofol, the most commonly used intravenous anaesthetic, had no effect on HIF-1α expression in PC3 cells when used alone but it was capable of inhibiting chemical- or isoflurane-induced HIF-1α overexpression.

HIF-1α activation in cancer has been heavily implicated in disease progression. Elevated levels have been found in a variety of human cancers (Talks et al., 2000) and correlated with tumour growth, vascularisation, metastasis and hence poor prognosis (Semenza, 2012). As transcription factors directly govern the expression of hundreds of genes, HIFs orchestrate crucial steps in tumorigenesis including angiogenesis, metabolic reprogramming, proliferation, metastasis and invasion, thus proving an attractive target for cancer therapy (Semenza, 2010; Semenza, 2012). Intuitively, it follows that exposure to a stimulus that further boosts HIF-1α levels and its associated repertoire of downstream activities in cancer cells would add fuel to the flames.

Isoflurane exposure induced a more than three-fold rise in HIF-1α expression in PC3 cells when used alone but it was capable of inhibiting chemical- or isoflurane-induced HIF-1α overexpression.

In this study using cultured prostate cancer PC3 cells, we show that isoflurane, one of the most commonly used inhaled anaesthetics, activates HIF-1α expression in an oxygen-independent manner which, in turn, enhances cancer cell proliferation and migration together with the reinforcement of chemotherapy resistance. In contrast, propofol, the most commonly used intravenous anaesthetic, had no effect on HIF-1α expression in PC3 cells when used alone but it was capable of inhibiting chemical- or isoflurane-induced HIF-1α overexpression.

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a critical role in establishing neovascularization to boost a tumour’s supply of oxygen and nutrients, and Ki67, a nuclear protein required for cellular proliferation that is widely used as a clinical prognostic indicator. With the introduction of a HIF-1α-specific siRNA, the increased expression of these two proteins in response to isoflurane exposure was no longer seen. Considering the widespread use of volatile anaesthetics in clinical practice, it is rational to postulate that, during cancer surgery, cancer cells gain enhanced malignant transformation after exposure to isoflurane as a result of activation of HIF-1α. The current findings may tie up with the clinical observation that general anaesthesia was associated with increased risk of cancer recurrence in prostate cancer (Biki et al, 2008a).

One of the consequences of the rapid and uncontrolled proliferation that characterizes cancer cells is that the developing tumour soon outstrips its blood supply, leading to hypoxia that is most severe at the tumour’s inner core. Here, HIF-1α levels are driven upward as the oxygen-dependent reactions that permit proteasomal degradation are limited. But HIF-1α levels may also rise independently of oxygen via loss-of-function mutations to tumour suppressor genes, such as VHL and PTEN, or gain-of-function mutations to proto-oncogenes, or by activation of the PI3K/Akt/mTOR and MAPK/ERK pathways involved in neosynthesis of HIF-1α protein (Jiang et al, 2001). Various growth factors and cytokines including insulin, insulin-like growth factor, epidermal growth factor and interleukin-2 are known to activate these pathways and subsequently increase the rate of synthesis of epidermal growth factor and interleukin-2 is known to activate HIF-1α.

Figure 5. Propofol attenuated chemotherapy-resistance development induced by isoflurane exposure via inhibition of HIF-1α in human prostate cancer cells. (A) Exposure to 2% isoflurane induced resistance against docetaxel (an anti-mitotic chemotherapy medication), shown as positive staining for Ki67, a marker for active mitosis (mean ± s.d., n = 8; **P < 0.01 vs docetaxel; ##P < 0.01 vs isoflurane + docetaxel); (B) MTT assay also demonstrated that with isoflurane pre-treatment, cells had a higher proliferation rate, which was prevented with propofol (mean ± s.d., n = 6; **P < 0.01 vs docetaxel; #P < 0.05 vs isoflurane + docetaxel); (C) cytochrome-c release, as a marker of cellular injury caused by docetaxel treatment, was reduced in isoflurane-exposed cells, while treatment with propofol at 4 μg ml⁻¹ or HIF-1α-specific siRNA (20 nm) restored the sensitivity to Docetaxel treatment; (D) anti-apoptotic protein, Bcl-2 was also overexpressed following isoflurane exposure before chemotherapy challenge. All these changes in favour of cell survival were blocked by co-administration of propofol at 4 μg ml⁻¹ or HIF-1α-specific siRNA (20 nm) with isoflurane (n = 4; **P < 0.001 vs naive control; ##P < 0.001 vs isoflurane alone). Abbreviations: Doc = docetaxel; Iso = isoflurane; Pro = propofol. Bar = 50 μm.
afforded by isoflurane ultimately promote cancer cell survival and growth toward anti-cancer treatment resistance.

HIFs have also been linked with chemoresistance. HIF-1α regulates transcription of at least two genes, namely ABCB1 and ABCC2, belonging to the family of multi-specific drug efflux transporters that are responsible for the observed resistance to a widely used variety of cytotoxic agents such as taxanes, doxorubicin and Vinca alkaloids (Monti and Gariboldi, 2011). The HIF-regulated metabolic shift from oxidative to glycolytic mode and induction of carbonic anhydrase also hinders the delivery of these drugs to their intracellular targets (Greijer et al, 2005). We found that PC3 cells exposed to isoflurane showed significantly less response to the anti-mitotic chemotherapy agent, docetaxel, than naive controls, as evidenced by a higher proliferation rate, a higher proportion of cells seen to be in active mitosis, lower levels of the cell injury marker cytochrome c and overexpression of the anti-apoptotic protein Bcl-2. This resistance diminished when a HIF-1α-specific siRNA was introduced, supporting the expectation that this effect is mediated through isoflurane’s upregulation of HIF-1α.

Propofol is widely used in clinical practice, largely due to its advantageous pharmacokinetic profile. It has been reported that propofol reduced the invasive capability of human cancer cells by inhibiting the formation of actin stress fibres and focal adhesion in HeLa human cervix carcinoma cells and inhibited pulmonary metastasis in a murine model of osteosarcoma (Mammoto et al, 2002), and enhanced the activity of cytotoxic T cells in mice (Kushida et al, 2007) and humans (Ren et al, 2010). In another distinguished trial, serum from patients receiving propofol/paravertebral anaesthesia for breast cancer surgery inhibited proliferation of cultured breast cancer cell compared with serum from patients receiving sevoflurane/opioid anaesthesia-analgesia (Deegan et al, 2009). In addition, there are studies showing that propofol can negate the ‘bad’ or ‘good’ effects resulting from volatile anaesthetics exposure (Smul et al, 2010; Zhang et al, 2011) and that propofol suppresses HIF-1α protein synthesis (Takabuchi et al, 2004b) as well as its downstream effects (Yeh et al, 2011).

Our data demonstrate that propofol inhibits upregulation of HIF-1α by isoflurane as well as by copper chloride and hypoxia. Furthermore, it suppressed the rise of VEGF and Ki67 that had been observed after exposure to isoflurane and protected against isoflurane-induced resistance to docetaxel. These findings add further support to the growing evidence that propofol has properties against the malignant activities of cancer cells. Taken together with the experiments showing that propofol co-treatment reduces phosphorylation of Akt and suppresses upregulation of HIF-1α by known inducers, it may be that propofol’s inhibitory action on the PI3K pathway and HIF-1α underpins its observed anti-tumour effects. However, while there are data indicating that the inhibition of HIF-1α by propofol results from factors affecting its synthesis rather than its post-translational stabilisation (Takabuchi et al, 2004a; Tanaka et al, 2010), there is a relative paucity of detail on this subject which future work needs to elucidate. Interestingly, PC3 cells are known to have high p-Akt level due to loss of PTEN (Shukla et al, 2007). Our results show that isoflurane exposure induced even more p-Akt expression, which indicated that isofluorane could further enhance the tumor malignancy through increasing p-AKT to even higher expression level but this warrants further study.

Clinical studies comparing the effect of general anaesthesia vs the use of epidural anaesthesia on cancer recurrence have drawn different conclusions, and recent review articles have found the discrepancies too great to support a change in clinical practice at this time (Heaney and Buggy, 2012; Tavare et al, 2012). Our study demonstrates the importance of such studies not only...
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differentiating between general and epidural anaesthesia but also among the type of general anaesthetic agents used.

Despite conducting our experiments using anaesthetics, doses and exposure periods that are clinically appropriate, in vitro studies are clearly limited in their ability to model tumour responses or the delivery of the anaesthetics to different tissues in vivo. For example, while we have shown propofol to suppress isoflurane’s deleterious effects on cancer cell activity in vitro, we do not know whether the single dose given at induction in clinical practice is sufficient to protect against the far lengthier course of isoflurane administration or whether such protection could only be afforded by a sustained concentration of propofol over the course of the entire operation. Future work is needed to characterise the interaction between these two anaesthetics in a way that accurately reflects the timing and manner in which they are used clinically. This inevitably requires in vivo experiments or clinical trials to accurately account for the tumour’s activities within its host and replicate the factors involved during surgery. In addition, in clinical settings, patients’ conditions including anaesthetic regimens that are implemented during surgery are rather complex. Our augments would be much stronger if change of activation of signaling pathways is observed in xenograft tumours after being exposed to different anaesthetics, this certainly warrants further investigations in future studies. Simply, combined anaesthetics including opioids are essential for general anaesthesia. A previous elegant study showed that morphine-induced epidermal growth factor receptor phosphorylation leads to downstream MAPK/ERK and Akt phosphorylation and increased invasion in human lung cancer cultures (Fujioka et al, 2011). Morphine has also been reported in vitro and in vivo to promote cell proliferation and angiogenesis through cellular survival signals in human breast cancer cells (Gupta et al, 2002), whereas Fentanyl was found to activate HIF-1α in neuroblastoma cells (Daijo et al, 2011). Furthermore, the genetic and phenotypic heterogeneity of cancer as a disease makes it highly likely that different cancer types will respond to anaesthetics in different ways, making it important to conduct such experiments using a variety of tumour types which are ongoing in the authors’ laboratory.

The underlying molecular mechanism that isoflurane activates the PI3K/Akt pathway remains incompletely understood. It was demonstrated that isoflurane may activate the PI3K/Akt pathway by enhancing cytosolic calcium concentration via activation of inositol triphosphate receptors (Bickler and Fahlman, 2006; Bickler and Fahlman, 2010), with altered expression profile in genes regulating growth and survival. This observation is consistent with our studies. In addition, cytosolic calcium concentration has been demonstrated to be involved on cancer cell cycle progression and malignancy (Taylor et al, 2008). Furthermore, both isoflurane and propofol interact and activate GABA receptors (Garcia et al, 2010), but different from propofol, isoflurane inhibits NMDA receptor (Tsuda, 2010) and has been shown to significantly affect cytosolic calcium concentration (Xin et al, 2005). This may help to explain the notable difference between isoflurane and propofol on cancer cell growth and malignancy as observed in our studies. General anaesthetics have been increasingly shown to have dual effects of cytoprotection and cytotoxicity (Wei and Inan, 2013).
calcium has been shown to be involved; low dose for short duration is beneficial and promotes cell growth and proliferation, which induce endogenous cytoprotective mechanisms via moderate calcium release (Zuo, 2012; Wei and Inan, 2013). On the other hand, anaesthetics at high concentrations for prolonged duration is toxic to the cells and cause cell death via excessive and abnormal calcium release (Wei, 2011). Based on this, the association between general anaesthetics such as isoflurane and propofol, their duration and dose should be investigated in our future studies.

In conclusion, we have shown that isoflurane upregulates HIF-1α in prostate cancer cells via the PI3K/AKT/mTOR pathway, leading to concomitant increases in markers and mediators of angiogenesis, proliferation and chemoresistance. Meanwhile, the addition of propofol to isoflurane treatment suppresses all of those effects and appears to do so by antagonising phosphorylation of Akt. This study builds on the so far, primitive body of preclinical evidence that anaesthetics directly interfere with cancer cell biology and indicates that two of the most commonly used agents have disparate effects on HIF-1α and its associated actions promoting malignant progression. Clinical studies examining the impact of anaesthetics on cancer recurrence should now account for this in their trial design or data interpretation and future work should begin to characterise the effects of common combinations of anaesthetics in a variety of tumour models that replicate the operative period as accurately as possible.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

HH and DM designed studies, while conception of the work was from DM. Cancer line was from CB. HH, LLB and HZ performed experiments and analysed data. HRW and NJSP participated experiments. All authors wrote manuscript.

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