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ORIGINAL RESEARCH ARTICLE

Effects of sevoflurane and its metabolite hexafluoroisopropanol on hypoxia/reoxygenation-induced injury and mitochondrial bioenergetics in murine cardiomyocytes

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Abstract

Background: The volatile anaesthetic sevoflurane protects cardiac tissue from reoxygenation/reperfusion. Mitochondria play an essential role in conditioning. We aimed to investigate how sevoflurane and its primary metabolite hexafluoroisopropanol (HFIP) affect necrosis, apoptosis, and reactive oxygen species formation in cardiomyocytes upon hypoxia/reoxygenation injury. Moreover, we aimed to describe the similarities in the mode of action in a mitochondrial bioenergetics analysis.

Methods: Murine cardiomyocytes were exposed to hypoxia (0.2% O_2 for 6 h), followed by reoxygenation (air with 5% CO_2 for 2 h) in the presence or absence sevoflurane 2.2% or HFIP 4 mM. Lactate dehydrogenase (LDH) release (necrosis), caspase activation (apoptosis), reactive oxygen species, mitochondrial membrane potential, and mitochondrial function (Seahorse XF analyser) were measured.

Results: Hypoxia/reoxygenation increased cell death by 44% (+31 to +55%, $P<0.001$). Reoxygenation in the presence of sevoflurane 2.2% or HFIP 4 mM increased LDH release only by +18% (+6 to +30%) and 20% (+7 to +32%), respectively. Apoptosis and reactive oxygen species formation were attenuated by sevoflurane and HFIP. Mitochondrial bioenergetics analysis of the two substances was profoundly different. Sevoflurane did not influence oxygen consumption rate (OCR) or extracellular acidification rate (ECAR), whereas HFIP reduced OCR and increased ECAR, an effect similar to oligomycin, an adenosine triphosphate (ATP) synthase inhibitor. When blocking the metabolism of sevoflurane into HFIP, protective effects of sevoflurane – but not of HFIP – on LDH release and caspase were mitigated.

Conclusion: Together, our data suggest that sevoflurane metabolism into HFIP plays an essential role in cardiomyocyte postconditioning after hypoxia/reoxygenation injury.

Keywords: hexafluoroisopropanol; hypoxia/reoxygenation injury; mitochondrial bioenergy measurements; mitochondrial membrane potential depolarisation; oxygen consumption rate; postconditioning; sevoflurane; volatile anaesthetic protection

Hypoxia or ischaemia inhibit cellular adenosine triphosphate (ATP) production in mitochondria. The driving force for ion transport and, thus, also ATP production is the mitochondrial membrane potential (MMP).[1] Loss of MMP can lead to cellular and mitochondrial swelling and rupture, and promote cell death in prolonged hypoxia.[2] Reoxygenation often exacerbates cellular injury because of the formation of reactive oxygen species (ROS),[2] originating predominantly from mitochondria.[3]

Conditioning renders cells more tolerant to injurious events such as ischaemia or hypoxia, through biomolecular changes,

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such as protein kinase C (PKC) activation, nitric oxide (NO) release, and opening of the mitochondrial K_{ATP} channel (mitoK_{ATP}).[4–7] Conditioning research originates from ischaemic cardiac preconditioning. Short-term ischaemia before extended ischaemia decreases myocardial damage.[8] Years later, the concept of ischaemic and pharmacological postconditioning using volatile anaesthetics after cardiac ischaemia was reported.[9] Similar to other volatile anaesthetics, sevoflurane reduces myocardial damage[10] in ischaemia/reperfusion injury. On a molecular level, it reduces oxidative stress, attenuates mitochondrial damage[10] by activating signal transduction pathways, and minimises apoptosis by preventing phosphatidylinositol 3-kinase catalytic subunit type 3-mediated autophagy.[11] Volatile anaesthetic postconditioning and ischaemic postconditioning share common pathways such as activation of PKC[4,6] and the release of NO.[4,5] Activated PKC and NO induce an opening of the mitoK_{ATP}.[7] These and further findings suggest a central role of mitochondria in ischaemic and volatile anaesthetic preconditioning.

Recent studies have demonstrated that not only sevoflurane itself but also its metabolite hexafluoroisopropanol (HFIP) protect against tissue damage in sepsis models. The metabolism of sevoflurane by the cytochrome P450 2E1 enzyme (CYP2E1) produces free fluoride ions and HFIP. The role of HFIP in hypoxia/reoxygenation or ischaemia/reperfusion injury and its impact on mitochondria, has not yet been evaluated.

In the present work, we investigated the effects of sevoflurane and of a high concentration of its primary metabolite HFIP on apoptosis, necrosis, and mitochondrial metabolic profile of murine cardiomyocytes using an *in vitro* model of hypoxia/reoxygenation injury. We hypothesised that both substances protect from hypoxia/reoxygenation injury and have a similar effect on mitochondria by altering the mitochondrial respiratory chain.

Methods

Cell culture and hypoxia/reoxygenation experiment

The murine HL-1 cardiomyocyte cell line was a gift from W.C. Claycomb.[12] Cells were cultured in fibronectin/gelatine-coated flasks in Claycomb medium (Sigma-Merck, Buchs, Switzerland) supplemented with fetal bovine serum 10% (FBS; Gibco-Thermo Fisher Scientific, Bern, Switzerland), norepinephrine 100 μM (stock solution 10 mM; Sigma-Merck) dissolved in L-ascorbic acid 30 mM (Sigma-Merck), L-glutamine 2 mM (Sigma-Merck), penicillin 100 U ml^{-1}, and streptomycin 100 U ml^{-1} (Fisher Scientific, Reinach, Switzerland). Cells were grown until 95% confluent. Cells with direct cell–cell contact develop spontaneous and synchronised contractions. An automated cell counter (TC20; Bio-Rad, Freibourg, Switzerland) was used to confirm a constant cell concentration in all experiments. Before the experiments, cells were put on a starving medium containing 1% FBS overnight.

Cells were exposed to hypoxia (0.2% O_2) in a hypoxic workstation (Baker Ruskinn invivo2 400, Vienna, Austria) for 6 h in serum- and glucose-free Dulbecco's modified Eagle's medium (DMEM; Gibco-Thermo Fisher Scientific) at 37°C containing deoxyglucose 10 mM (Sigma-Merck) to inhibit glycolysis. Next, cells were reoxygenated for 2 h in air supplemented with 5% CO_2, with or without sevoflurane (2.2 vol%; AbbVie AG, Baar, Switzerland), with or without HFIP 4 mM (Acros Organics by Fisher Scientific) and with or without disulfiram (a CYP2E1 inhibitor; Sigma-Merck) 0.1 and 1 μM in DMEM supplemented with FBS 10% in an airtight chamber (Oxoid™ Anaerobic 3.5 L Jar; Thermo Fisher Scientific). We administered HFIP 4 mM to the cardiomyocytes, based on the work of Urner and colleagues.[13] Sevoflurane concentration was confirmed using an anaesthesia gas analyser (Dräger Medical, Germany).

Lactate dehydrogenase

The intracellular enzyme lactate dehydrogenase (LDH) is released in response to cell toxicity and quantifies cell death. HL-1 cells were plated in 96-well plates at 1.2×10^4 $well^{-1}$ for 3 days until 95% confluence, starved overnight in 1% FCS/DMEM, and exposed to hypoxia for 6 h followed by a 2-h reoxygenation with or without sevoflurane or HFIP and with or without disulfiram as described. Untreated cells exposed to normoxia for 8 h served as a control. The LDH release was determined in cell supernatants using an LDH detection kit (Promega, Dubendorf, Switzerland) following the manufacturer's protocol.

Apoptosis

Apoptosis was assessed by activation of caspases 3, 7, and 8 using a selective fluorogenic substrate, which measures the fluorogenic substrate's proteolytic cleavage (Ac-Asp-Glu-Val-Asp-AMC; Sigma-Merck). Fluorescence of the cleaved reporter group was measured at 360 nm/465 nm (excitation/emission) on a Tecan Infinite M200pro plate reader (Tecan, Zurich, Switzerland).

Reactive oxygen species

ROS generation, including superoxide, hydroxyl radical, singlet oxygen, and hydrogen peroxide, was measured using the ROS-Glo™ assay (Promega). After hypoxia (6 h) and reoxygenation (2 h), the level of hydrogen peroxide (H_2O_2) was measured using the ROS-Glo assay according to the manufacturer's protocol. Emitted light was measured (Tecan Infinite M200Pro). As a positive control, 3-morpholino-sydnonimine-*N*-ethyl-carbamide (Sin-1), which induces oxidative stress *in vitro*, was used in a final concentration of 200 μM (Molecular Probes, Thermo Fisher Scientific).

MMP measurement

Cardiomyocytes were plated at a density of 4×10^4 cells $well^{-1}$ in a black 24-well plate (lumox multiwell; Sarstedt, Numbrecht, Germany) and incubated with the MMP indicator tetramethyl rhodamine ethyl ester (TMRE, 500 nM; Abcam, Basel, Switzerland).

After three baseline measurements, three cycles at 0, 30, and 60 s (Tecan Infinite M200Pro with an injector module), the response to direct injection of sevoflurane (final concentration, 500 μM) and HFIP (final concentration, 4 mM), carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP; final concentration, 1 μM), or oligomycin (final concentration 1 μM). Signals were recorded for 20 min. FCCP, a mitochondrial uncoupler that eliminates the mitochondrial potential, and oligomycin, a mitochondrial ATP synthase inhibitor that leads to hyperpolarisation, were used as controls.

Direct injection of sevoflurane in its liquid state was used for these experiments. Overall, 1 MAC (minimum alveolar concentration; =2.2 vol%) corresponds to sevoflurane 0.5 mM in aqueous solutions.[14]

Analysis of mitochondrial bioenergetics

The XFp Extracellular Flux Analyzer (Seahorse; Bucher Biotec AG, Basel, Switzerland) allows real-time cellular oxygen consumption rate (OCR) monitoring and extracellular pH (extracellular acidification rate [ECAR]), which provides information about the mitochondrial energy state. The variables are determined using continuous oxygen concentration measurement and proton flux in the cell supernatant over time.

The effect of pharmacological agents on mitochondrial bioenergetics, that is OCR and ECAR, can be analysed. Moreover, basal respiration as a result of ATP turnover, uncoupled respiration linked to mitochondrial function, and ROS generation can be determined.

The following substances are components of the Seahorse mito stress assay: (1) oligomycin as an ATP synthase inhibitor; (2) FCCP, a mitochondrial protonophore/uncoupler; and (3) rotenone/antimycin-A, a complex I/III inhibitor.

For the experiment, the XFp cell culture miniplate was coated with fibronectin/gelatine for 45 min at 37°C, HL-1 were plated at a density of 2×10^4 cells well^{-1} and incubated for 24 h at 37°C in supplemented Claycomb medium (see above). For the experiment, Agilent Seahorse XF base assay medium was used. The assay medium was prepared by supplementing XF base medium (Seahorse; Bucher Biotec) with glucose 10 mM (Sigma-Merck), L-glutamine 2 mM, and sodium pyruvate 1 mM (adjusted pH to 7.4 with 0.1 N NaOH, 180 µl well^{-1} for (1) 1 h before the assay the plate was placed in a 37°C incubator with room air or (2) for 6 h in 2% O_2 without CO_2. Two wells on each plate did not contain cells and were used to correct for background noise.

A sevoflurane/medium stock solution was prepared with 1.3 µl sevoflurane (AbbVie AG) in 10 ml assay medium, stirred for 10 min in an airtight plastic vial to produce a relatively stable 1 mM solution.[14] After acquiring baseline measurements (OCR), the effects of the following drugs on mitochondrial

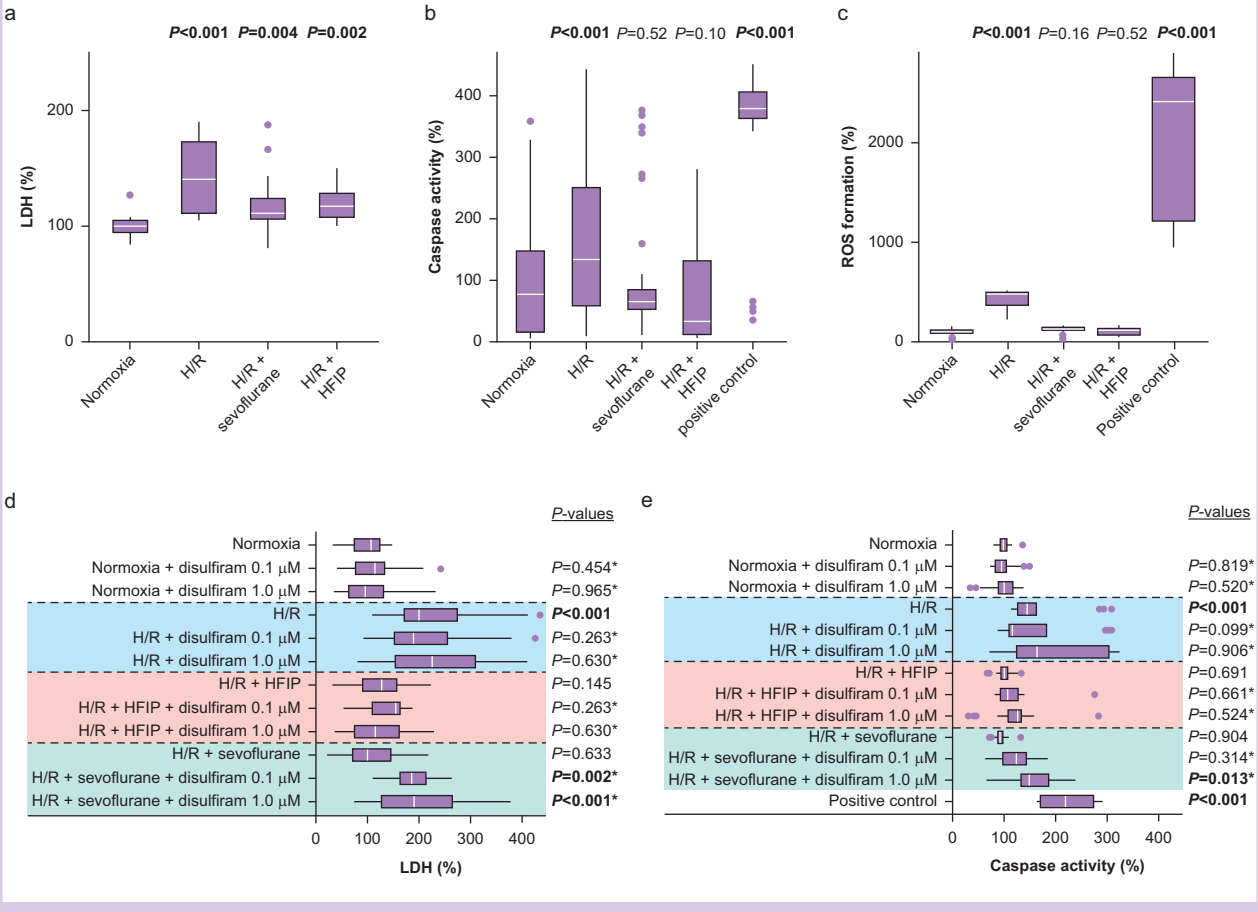

Fig 1. (a) Necrosis, (b) caspase activation, and (c) formation of reactive oxygen species (ROS) in cardiomyocytes exposed to 8 h normoxia (Nx) or 6 h hypoxia/2 h reoxygenation (Hx) in air with or without sevoflurane (Hx/Sevo; 1 minimal alveolar concentration [MAC], 2.2 vol%) or hexafluoroisopropanol (Hx/HFIP; HFIP, 4 mM). Necrosis experiments were repeated with the addition of disulfiram (DS; 0.1 and 1 µM) (c). DS is an inhibitor of the CYP 450 2E1 enzyme which metabolises the degradation of sevoflurane into HFIP and free fluoride. The effect of CYP 2E1 blocking on necrosis (d) and caspase activation (e) are described. Relative changes to normoxia are shown. Boxplots display medians and quartiles; whiskers represent 5% and 95% confidence intervals. For caspase activity and ROS production, a positive control (camptothecin and 3-morpholino-sydnonimine-*N*-ethyl-carbamide) were included. Datapoints (*N*) and independent experiments (*E*) for the figure: (a) n=48, E=5; (b) n=328, E=8; (c) n=100, E=4; (d) n=354, E=3; (e) n=288, E=3. LDH, lactate dehydrogenase.

bioenergetics were analysed by sequential automatic injection: sevoflurane 0.5 mM in aqueous solution (corresponding to 1 MAC),[14] HFIP 4 mM, or assay medium as a control (port A); oligomycin 1 μM (port B); FCCP 0.5 μM (port C); and rotenone/antimycin A mix 0.5 μM (port D). All OCR and ECAR measurements were normalised to total DNA content. All solutions were prepared fresh daily. The wells of the Seahorse plate were covered with mineral oil to minimise sevoflurane evaporation.

To determine how much of the respiration is used to synthesise ATP, oligomycin, an inhibitor of mitochondrial ATP synthase at complex V, was added. The decrease in OCR resulting from oligomycin is attributed to the oxygen consumption used to generate ATP. The oligomycin sensitive portion of respiration is termed the coupling efficiency.

Determination of maximum respiratory capacity. Next, we assessed the maximum respiratory capacity by adding an uncoupler, the proton ionophore FCCP. It uncouples the electron transfer along with electron transport chain (ETC) from the ATP synthesis in complex V and allows for unrestricted electron flux through the ETC, thus increasing OCR. The stimulated oxygen consumption is attributed to the maximal mitochondrial respiration rate.

Determination of non-mitochondrial oxygen consumption. Inhibition of complexes I and III by a rotenone/antimycin A mix allows us to determine how much oxygen is consumed outside of the mitochondria.

DNA quantitation fluorescence assay

The quantitation of DNA was measured at 360 nm/465 nm on a Tecan infinite M200Pro using the fluorescent dye bisbenzimide (Hoechst 33258, DNA quantitation kit; Sigma-Merck) according to the manufacturer's protocol.

Data presentation and statistical analyses

Effect estimates with 95% confidence intervals in response to different exposure conditions were calculated using multiple regression analyses. The number of total measurements (observations) and the number of individual experiments (clustering by assay) are indicated in the legends of figures and tables.

We used linear regression models to estimate the effect of reoxygenation with air, with or without sevoflurane, and HFIP on LDH release and caspase activity. Control experiments (normoxia, reoxygenation with air) were the reference category. Because of their variable distribution, the effects of reoxygenation with air, sevoflurane, or HFIP on the formation of ROS were reported using a negative binomial model.

MMPs were measured at baseline and then continuously recorded over time. We estimated the effect of different exposures, which took place at different time points during follow-up (injections of either HFIP, sevoflurane, oligomycin, or FCCP), on membrane potentials. To account for dependent measurements over time and between-experiment (and between-well) variability, we estimate the conditionally independent effects of multiple covariates (exposures in our experimental setup) on a time-varying continuous outcome using linear mixed-effects models with random slope and intercept.[15,16] Baseline measurements during the first 60 μs were considered the reference category. The same models were used to investigate the effect of HFIP, sevoflurane, oligomycin, FCCP, and rotenone/antimycin A on oxygen consumption and extracellular acidification rate. Baseline measurements during the first 20 min were considered the reference category. All analyses were conducted in R version 3.6.3. A *P*-value <0.05 was considered statistically significant.

Table 1 LDH assay, caspase activity, and reactive oxygen species (ROS) formation. Estimates with corresponding 95% confidence intervals (95% CI) of linear regression (LDH and caspase assays) and negative binomial regression (ROS formation) models. Cells were exposed to normoxia for 8 h (reference category) or to hypoxia 0.2% O_2 for 6 h and reoxygenation (reox) in air for 2 h. Reoxygenation occurred in the presence or absence of sevoflurane 2.2 vol% or HFIP 4 mM. Datapoints: *n*=48 from five independent experiments for LDH, *n*=328 from eight independent experiments for caspase, and *n*=100 from four independent experiments for ROS formation. LDH, lactate dehydrogenase; 95% CI, 95% confidence intervals; HFIP, hexafluoroisopropanol.

LDH	**Relative change in % (95% CI)**	***P*-value**
(Intercept)	100 (+91 to +109)	–
Hypoxia 6 h/2 h reox air	44 (+31 to +55)	<0.001
Hypoxia 6 h/2 h reox sevo 2.2 vol%	18 (+6 to +30)	0.004
Hypoxia 6 h/2 h reox HFIP 4 mM	20 (+7 to +32)	0.002
Caspase activity	Relative change in % (95% CI)	
(Intercept)	100 (+74 to +126)	–
Hypoxia 6 h/2 h reox air	65 (+30 to +99)	<0.001
Hypoxia 6 h/2 h reox sevo 2.2 vol%	−29 (−26 to +5)	0.520
Hypoxia 6 h/2 h reox HFIP 4 mM	−11 (−45 to +23)	0.100
Staurosporine (positive control)	258 (+212 to +304)	<0.001
ROS formation	Fold change in fluorescence (95% CI)	
(Intercept)	4.61 (+4.47 to +4.75)	–
Hypoxia 6 h/2 h reox air	1.46 (+1.26 to +1.67)	<0.001
Hypoxia 6 h/2 h reox sevo 2.2 vol%	0.15 (−0.06 to +0.36)	0.159
Hypoxia 6 h/2 h reox HFIP 4 mM	0.07 (−0.14 to +0.28)	0.519
SIN-1 (positive control)	3.00 (+2.77 to +3.23)	<0.001

Results

Sevoflurane and HFIP attenuate necrosis, apoptosis, and formation of ROS in hypoxia/reoxygenation injury

After exposure to hypoxia/reoxygenation, the total death rate (assessed by LDH release) of cells increased by (mean difference [95% confidence interval]) 44% (+31 to +55%; $P<0.001$) compared with normoxia. LDH release was less pronounced when reoxygenation was performed in the presence of sevoflurane or HFIP; LDH release increased by 18% (+6 to +30%) and 20% (+7 to +32%), respectively (Fig. 1a and Table 1; $P=0.004$ and $P=0.002$).

Caspase activity upon hypoxia/reoxygenation increased by 65% (+30 to +99%) compared with normoxia ($P<0.001$). In the presence of sevoflurane and HFIP, the increase was less pronounced; it was 29% (−26% to +5%) and 11% (−45% to +23%) compared with normoxic conditions (Fig. 1b and Table 1).

ROS formation after hypoxia/reoxygenation was 1.46 times higher (+1.26 to +1.67) than in normoxic control cells ($P<0.001$). When reoxygenation was conducted in the presence of sevoflurane or HFIP, ROS formation was similar to normoxic control cells (Fig. 1c and Table 1).

MMP depolarises in response to sevoflurane and HFIP exposure

After hypoxia/reoxygenation, an early depolarisation induced by sevoflurane and HFIP was observed (Table 2 and Supplementary Figure S1). The depolarisation pattern of the two drugs, however, was different. Sevoflurane induced a brief depolarisation followed, within 100 μs, repolarisation which in the case of 1 MAC, but not 2 MAC, sevoflurane, had an overshoot. HFIP, in contrast, triggered a sustained depolarisation (Supplementary Figure S1). This effect was more pronounced with higher concentrations. In control experiments, the depolarisation in response to FCCP was more pronounced when a higher concentration of FCCP was added, whereas oligomycin hyperpolarised the MMP.

In contrast to sevoflurane, HFIP reduces oxygen consumption and increases extracellular acidification

The effect of hypoxia for 6 h followed by reoxygenation in the presence of medium, sevoflurane, or HFIP on the mitochondrial metabolic state in HL-1 cardiomyocytes is illustrated in Fig. 2a and b. Mitochondrial respiration was monitored by assessing OCR and ECAR.

Sevoflurane did not impact OCR or ECAR. The values were similar to the baseline measurements. In contrast, HFIP reduced OCR by −40% (−51 to −30%) and increased ECAR by 262% (+238 to +286%). The pattern exerted by HFIP resembles that of the ATP synthase inhibitor oligomycin (Fig. 2a and b and Table 3). An overview of the experimental set-up and the readouts of the mito-stress assay is provided in Fig. 2c.

When sevoflurane metabolism to HFIP is blocked, more necrosis and apoptosis are observed

Compared with exposure to normoxia/air, caspase activity and LDH release increased by 109% (+76 to +142%) and by 124% (+94 to +154%) upon hypoxia/reoxygenation (Table 4). When reoxygenation was performed in the presence of sevoflurane or HFIP, caspase activity and LDH levels were similar to normoxic conditions.

The addition of disulfiram (a CYP 450 2E1 blocker which impairs the formation of HFIP from sevoflurane) at a concentration of 0.1 and 1.0 μM modified the effect of sevoflurane post-conditioning (Fig. 1d and e; Table 4). Although caspase remained unaffected with 0.1 μM, it increased by +58% (+13% to +104%; $P=0.013$) when disulfiram 1.0 μM was used. LDH release increased by 70% (+27% to +113%; $P=0.002$) and 95% (+57% to +132%; $P<0.001$) when reoxygenation was performed in the presence of disulfiram 0.1 and 1.0 μM.

LDH release and caspase activation were not affected by disulfiram in cells exposed to hypoxia and reoxygenation in the presence of HFIP.

Discussion

In this murine cardiomyocyte *in vitro* study, we found that sevoflurane and HFIP attenuated the cellular response to hypoxia/reoxygenation injury. In contrast to previous reports,[17,18] sevoflurane acted neither as a mitochondrial uncoupler nor as an inhibitor. Similar effects of the primary metabolite HFIP to oligomycin, a pharmacological inhibitor, were observed. However, oligomycin hyperpolarises the cellular membrane whereas HFIP depolarises it.

Table 2 Mitochondrial membrane potential (MMP) measurement. Estimates of relative change in fluorescence with corresponding 95% confidence intervals (95% CI) of a linear mixed model, accounting for dependent measurements over time and between-experiment variability. Cells were exposed hypoxia 0.2% O_2 for 6 h. Three baseline measurements during the first 60 μs were considered the reference category. After the third baseline measurement, either sevoflurane (0.5 or 1 mM corresponding to 1 or 2 MAC), HFIP (40 μM or 4 mM), or the pharmacological controls were injected into the medium of living cells and monitored during reoxygenation (reox) over 20 min. Datapoints: $n=377$ from five independent experiments. MAC, minimal alveolar concentration; FCCP, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone; TMRE, tetramethyl rhodamine ethyl ester.

	Relative change in fluorescence of TMRE Ex/Em 545 nm/580 nm (95% CI)	P-value
(Intercept)	100 (+100 to +102)	–
Hypoxia 6 h reox HFIP 40 μM	−4 (−6 to −2)	<0.001
Hypoxia 6 h reox HFIP 4 mM	−9 (−13 to −7)	<0.001
Hypoxia 6 h reox sevoflurane 1 MAC	−4 (−6 to 0)	<0.001
Hypoxia 6 h reox	−7 (−8 to −5)	0.001
Hypoxia 6 h reox oligomycin	8 (+6 to +13)	<0.001
Hypoxia 6 h reox FCCP	−7 (−9 to −6)	<0.001

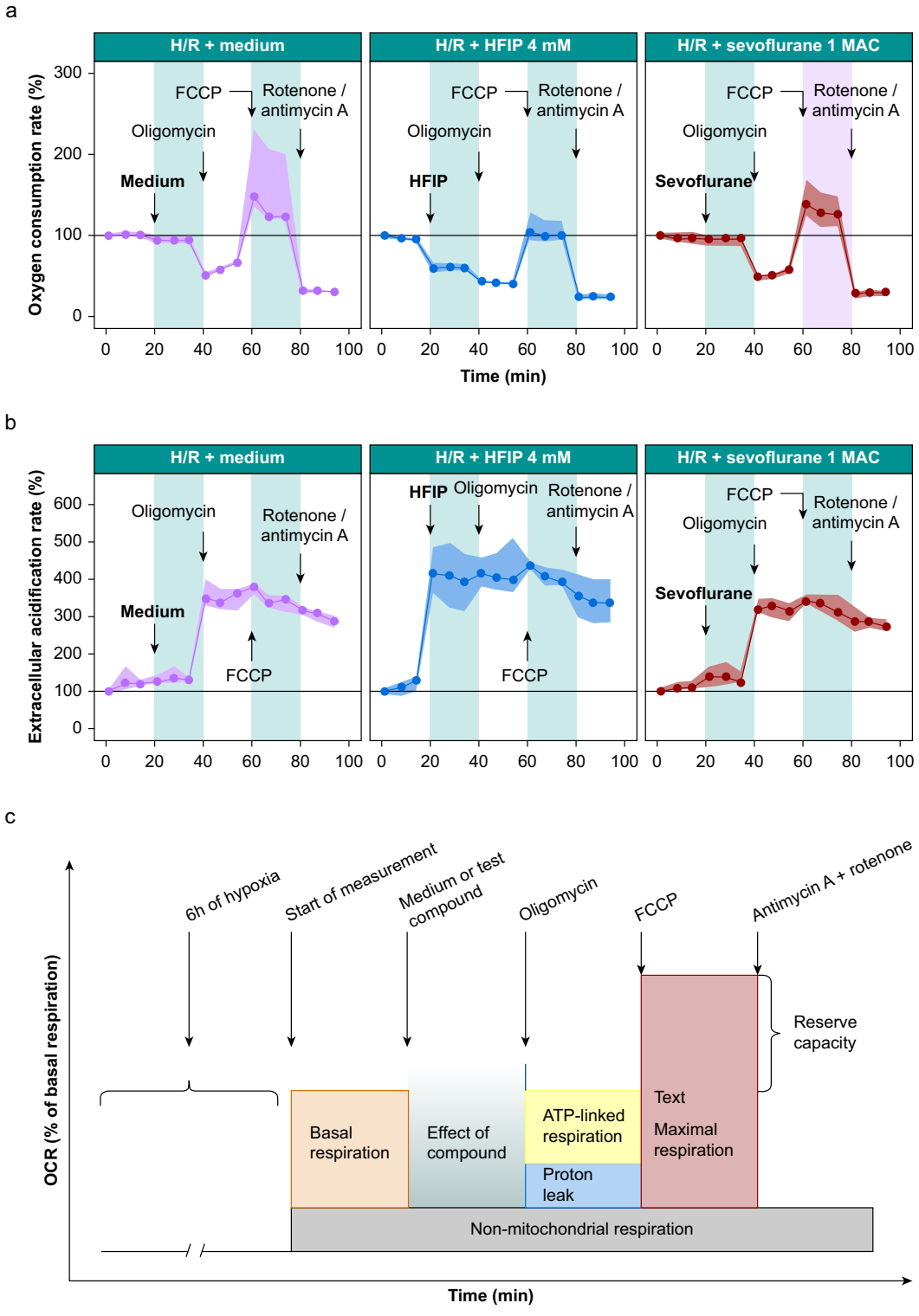

Fig 2. A representative example of the oxygen consumption rate (OCR) of medium control, sevoflurane, and HFIP (a) and the extracellular acidification rate (ECAR) (b), both in % of the baseline measurement. The experimental setup of the mito-stress assay and the corresponding readout parameters are displayed schematically depicted (c). Datapoints (*N*) and independent experiments (*E*) for the figure: (a and b) n=216, E=3. The shaded areas around parts (a) and (b) represent standard deviations/95% confidence intervals concerning the measurements. FCCP, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone; HFIP, hexafluoroisopropanol; MAC, minimum alveolar concentration; ATP, adenosine triphosphate.

Table 3 Mito-stress assay (mitochondrial bioenergy levels). Estimates with corresponding 95% confidence intervals (95% CI) of linear mixed model, accounting for dependent measurements over time. Cells were exposed to hypoxia 2% O_2. Baseline measurements during the first 20 min were considered the reference category. Sevoflurane (0.5 mM corresponding to 1 minimal alveolar concentration [MAC]), HFIP (4 mM), and the pharmacological controls were injected into the medium of living cells during reoxygenation (reox). Datapoints: n=216 of three independent experiments.

	Change in oxygen consumption rate (95% CI)	P-value	Change in extracellular acidification rate (95% CI)	P-value
(Intercept)	100 (95–105)	–	100 (+88 to +112)	–
Treatment effect				
Hypoxia 6 h reox medium	−4 (−14 to +6)	0.438	21 (−3 to +45)	0.082
Hypoxia 6 h reox HFIP 4 mM	−40 (−51 to −30)	<0.001	262 (+238 to +286)	<0.001
Hypoxia 6 h reox sevoflurane 1 MAC	−7 (−17 to +4)	0.211	27 (+3 to +51)	0.029
Mitochondrial uncoupling				
Hypoxia 6 h reox FCCP	66 (+56 to +76)	<0.001	206 (+182 to +230)	<0.001
Hypoxia 6 h reox HFIP 4 mM	6 (−4 to +17)	0.229	258 (+234 to +282)	<0.001
Hypoxia 6 h reox sevoflurane 1 MAC	39 (+29 to +49)	<0.001	203 (+179 to +227)	<0.001
Inhibition of ATP synthase				
Hypoxia 6 h reox oligomycin	−42 (−52 to −31)	<0.001	208 (+184 to +232)	<0.001
Hypoxia 6 h reox oligomycin +HFIP 4 mM	−56 (−66 to −45)	<0.001	269 (+245 to +293)	<0.001
Hypoxia 6 h reox oligomycin + sevoflurane 1 MAC	−49 (−60 to −39)	<0.001	192 (+168 to +216)	<0.001
Suppression of mitochondrial respiration				
Hypoxia 6 h reox rotenone/antimycin A	−69 (−79 to −58)	<0.001	165 (+141 to +188)	<0.001
Hypoxia 6 h reox rotenone/antimycin A + HFIP 4 mM	−76 (−86 to −65)	<0.001	202 (+178 to +226)	<0.001
Hypoxia 6 h reox rotenone/antimycin A + sevoflurane 1 MAC	−72 (−82 to −62)	<0.001	156 (+132 to +180)	<0.001

Why are sevoflurane and HFIP conditioning of interest?

Previous studies have reported protective effects of sevoflurane and other volatile anaesthetics after ischaemia/reperfusion injury and systemic inflammation.[19–23] Recently, protective effects of HFIP have been reported in sepsis and endotoxaemia *in vivo*[24,25] and *in vitro*.[13] The effect of HFIP on ischaemia/reperfusion injury has not been investigated. This may be of interest because indirect evidence suggests that HFIP, unlike sevoflurane, does not possess relevant anaesthetic potency. HFIP does not have the basic ether structure that is responsible for the hypnotic effects of volatile anaesthetics. Furthermore, HFIP can be detected up to 20 h after general anaesthesia, suggesting it does not act as a hypnotic. The present work adds to the current knowledge that HFIP, at a higher concentration than is achieved during sevoflurane anaesthesia, exerts pharmacological conditioning effects similar to sevoflurane in ischaemia/reperfusion or hypoxia/reoxygenation injury. The fact that disulfiram inhibits sevoflurane-mediated protection suggests that sevoflurane metabolism to HFIP is critical for mediating sevoflurane post-conditioning.

Dose considerations for HFIP conditioning

Approximately 2% of sevoflurane is metabolised to HFIP by liver cytochrome P450,[26] which can be detected 5 min after sevoflurane application and peaks after 60 min.[26,27] Eighty-five percent is rapidly converted into HFIP-glucuronide and eliminated via the kidneys.[26] Concentrations of HFIP in our experiments were similar to previous *in vitro*[28] and *in vivo*[24] studies but higher than those measured during sevoflurane anaesthesia.[26]

Role of mitochondria in conditioning

Ischaemia/reperfusion injury is associated with Ca^{2+} overload and increased ROS production. This leads to opening of the mitochondrial permeability transition pore (mPTP),[29] the main effector in the cell death pathway. A second key regulator of mitochondrial and cellular homeostasis is the mitoK_{ATP} channel.[30] Volatile anaesthetics appear to influence the mPTP[31] and mitoKATP.[7]

Effect of sevoflurane and HFIP conditioning on outcome variables in ischaemia/reperfusion injury

Various cardiac diseases, such as heart failure, myocardial infarction, and ischaemia/reperfusion injury, result from cardiomyocyte death by apoptosis and necrosis. LDH release by cells exposed to hypoxia/reoxygenation was mitigated by sevoflurane and HFIP. In line with previous work, apoptosis was attenuated by sevoflurane.[11] The work presented adds to the current knowledge that HFIP reduces apoptosis after hypoxia/reoxygenation.

Classification of sevoflurane and HFIP at the mitochondrial level in ischaemia/reperfusion injury

As mitochondria play a central role in cell death, but also in conditioning, the classification of sevoflurane and HFIP may be helpful for further projects. A mitochondrial uncoupler such as FCPP increases OCR and ECAR.[32] An ATP synthase inhibitor such as oligomycin decreases OCR and increases ECAR. When compared with these reference substances, sevoflurane acts neither as an uncoupler nor as an ATP synthase inhibitor, whereas HFIP acts as an inhibitor.

Table 4 Effect of disulfiram on LDH assay and caspase activity. Estimates with corresponding 95% confidence intervals (95% CI) of linear regression models investigating the effect of hypoxia, sevoflurane, HFIP, and disulfiram on LDH levels and caspase activity. Interaction terms for disulfiram have been calculated, to determine, if disulfiram is an effect modifier. Cells were exposed to normoxia for 8 h (reference category) or to hypoxia 0.2% O_2 for 6 h and reoxygenation (reox) in air for 2 h. Reoxygenation occurred in the presence or absence of sevoflurane 2.2 vol% or HFIP 4 mM with or without disulfiram 0.1 or 1 μM. Datapoints: n=354 from three independent experiments for LDH, n=266 from three independent experiments for caspase.

	Caspase activity		LDH	
	Relative change in % (95% CI)	P-value	Relative change in % (95% CI)	P-value
(Intercept)	100 (+45 to +154)	–	100 (+82 to +118)	–
Disulfiram, 0.1 μM	4 (−29 to +37)	0.819	9 (−15 to +32)	0.454
Disulfiram, 1.0 μM	−11 (−44 to +22)	0.520	1 (−21 to +22)	0.965
Hypoxia 6 h/2 h reox air	109 (+76 to +142)	<0.001	124 (94 to +154)	<0.001
Interaction with disulfiram, 0.1 μM	−39 (−83 to +6)	0.099	−23 (−64 to +17)	0.263
Interaction with disulfiram, 1.0 μM	2.8 (−43 to +49)	0.906	9 (−28 to +46)	0.630
Hypoxia/HFIP 4 mM	6.9 (−27 to +40)	0.691	21 (−7 to +50)	0.145
Interaction with disulfiram, 0.1 μM	10 (−35 to +55)	0.661	7 (−32 to +47)	0.714
Interaction with disulfiram, 1.0 μM	15 (−30 to 60)	0.524	−7 (−43 to +30)	0.728
Hypoxia/sevoflurane 2.2 vol%	−2 (−36 to +31)	0.904	7 (−22 to +37)	0.633
Interaction with disulfiram, 0.1 μM	24 (−21 to +69)	0.314	70 (+27 to +113)	0.002
Interaction with disulfiram, 1.0 μM	58 (+13 to +104)	0.013	95 (+57 to +132)	<0.001

The active transport of protons out of the inner membrane results in a net internal negative charge of −180 mV, the mitochondrial transmembrane potential. It is confirmed with positively charged dyes such as TMRE. Sevoflurane induced a short-term depolarisation followed by repolarisation. The reason why the lower sevoflurane concentration hyperpolarises the membrane is unclear. This may be an overshooting repolarisation. In the case of HFIP, a sustained depolarisation of the membrane was observed. This result was unexpected as our reference inhibitor, oligomycin, hyperpolarised the membrane. However, the literature reports mitochondrial complex I inhibitors, such as rotenone, depolarising the membrane.[33]

Most mitochondrial ATP synthase complex I and III inhibitors increase ROS formation.[34,35] However, mitochondrial complex I inhibitors are further divided into class A and B inhibitors. Class A inhibitors increase, whereas class B inhibitors decrease ROS production.[36]

Therefore, HFIP might be a mitochondrial complex I class B inhibitor.

Previous research has suggested that sevoflurane would act as a mitochondrial complex I inhibitor[16] or as a mild uncoupler.[17] We observed only the short-term effects of sevoflurane on OCR, ECAR, and MPP. Our measurements are surprising with short-term effects of sevoflurane on OCR, ECAR, and MPP. However, it is possible that the protective effect at the mitochondrial level is not achieved by sevoflurane but by its primary metabolite. Although sevoflurane is primarily metabolised in the liver (to HFIP), cardiomyocytes contain CYP450 enzymes, including CYP450 E1,[37] the predominant isoform for metabolising sevoflurane.[38] Our experiments with disulfiram, which inhibits the conversion of sevoflurane into HFIP, support this assumption.

Our study has several limitations. Mitochondria are only one of several cellular targets for volatile anaesthetic conditioning. The modulation of inflammatory mediators and cytokines mediate substantial effects.[39,40] We did not measure HFIP production in cardiomyocytes. We observed no postconditioning when a blocking agent known to inhibit the biotransformation of sevoflurane to HFIP was used. Another potential limitation is the use of disulfiram. Disulfiram activates the sarco-endoplasmatic reticulum calcium ATPase (SERCA).[41] Calcium is crucial for myocardial contractility and impacts on apoptosis. In these experiments, neither intracellular calcium levels nor SERCA activity have been determined. Moreover, the concentration of HFIP is based on previous studies but is higher than the concentration measured during sevoflurane anaesthesia, and the HFIP effect might therefore be overestimated. Regarding the data analysis, mixed-effects models with random slope and intercepts were used to address the variability between wells and between experiments. Nevertheless, despite using a very conservative and rigorous statistical analysis approach, we cannot fully exclude the presence of potential residual confounding in the analysis.

A strength of this study is the robust and consistent effect of sevoflurane and HFIP in this *in vitro* ischaemia/reperfusion model, which supports our conclusions. Further research is warranted to confirm whether our results can be observed in different species and after translation from *in vitro* to *in vivo* models.

In conclusion, our results suggest that sevoflurane and its primary metabolite HFIP can modify the response of cardiomyocytes to hypoxia/reoxygenation-induced injury. Metabolism of sevoflurane to HFIP, which appears to act as a mitochondrial class I type B inhibitor, might be crucial for mediating the effects of anaesthetic postconditioning in cardiomyocytes after hypoxia/reoxygenation injury.

Authors' contributions

Design of the study: BRZ, MU, BBS, MS
Conduct of experiments: BRZ
Data collection: BRZ, MS
Data analysis: MU, BBS, MS
Writing of the manuscript: BRZ, MU, MS
Provided funding: BBS
All authors were involved in revision of the manuscript and approval of the final version of the manuscript, and agreed to be accountable for all aspects of the manuscript.

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Declarations of interest

MS and BBS have received unrestricted research funds from Sedana Medical, Danderyd, Sweden, and from Roche Diagnostics International, Rotkreuz, Switzerland for an investigator initiated clinical trial (PI: MS). BBS and MS have submitted a patent to mitigate the negative effects of surgery and/or anaesthesia for patients using medical gases, particularly oxygen (O_2) and carbon dioxide (CO_2). BBS has received a research grant for an investigator initiated clinical trial from Baxter Healthcare Corporation, Deerfield IL, USA (PI: BBS). MU and BBS submitted US and EP patent applications for an injectable formulation for treatment and protection of patients having an inflammatory reaction or an ischaemia/reperfusion event. MU is supported by a Vanier Canada Graduate Scholarship from the Canadian Institutes of Health Research. BRZ declares that they have no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bjao.2022.100116.

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