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Effects of perfluorohexane vapor in the treatment of experimental lung injury☆

Jörg U. Bleyl[a,*], Axel R. Heller[a], Manuel Heintz[a], Matthias Schlemmer[a], Roland Koch[b], Marcelo Gama de Abreu[a], Matthias Hübler[a], Peter M. Spieth[a,c,d,e], Thea Koch[a]

[a] *Department of Anesthesiology and Intensive Care Medicine, University Hospital Carl Gustav Carus, Dresden, Germany*
[b] *Institute of Medical Informatics and Biometrics, Technical University Dresden, Fetscherstraße 74, 01307 Dresden, Germany*
[c] *Department of Anesthesia and Interdepartmental Division of Critical Care, University of Toronto, Keenan Research Center, Li Ka Shing Knowledge Institute, St. Michael's Hospital, 30 Bond Street, M5B 1W8, Toronto, ON, Canada*
[d] *Department of Physiology and Interdepartmental Division of Critical Care, University of Toronto, Keenan Research Center, Li Ka Shing Knowledge Institute, St. Michael's Hospital, 30 Bond Street, M5B 1W8, Toronto, ON, Canada*
[e] *Department of Medicine and Interdepartmental Division of Critical Care, University of Toronto, Keenan Research Center, Li Ka Shing Knowledge Institute, St. Michael's Hospital, 30 Bond Street, M5B 1W8, Toronto, ON, Canada*

1. Introduction

Liquid ventilation with Perfluorocarbons (PFC) have been widely used for the treatment of experimental and clinical acute respiratory distress syndrome (ARDS) [1–6]. Despite promising results from early experimental and clinical trials [1–4] liquid ventilation failed in a recent phase III clinical trial [5]. Therefore, less invasive modes of PFC application have been developed whereby PFC was either vaporized [7] or aerosolized [8,9]. The inhalation of therapeutic agent for the treatment of ARDS is a well-established mode of application. Surfactant, prostacyclin and nitrous oxide have been successfully applied and were of therapeutic benefit in small single center studies [10,11]. In larger randomized trials these effects were less persuasive [12]. Several mechanisms of action have been suggested to contribute to the beneficial effects of inhaled PFC in the treatment of ARDS such as improved oxygen delivery and reduction of pulmonary surface tension [7,8,13]. In vivo studies with inhaled PFC documented a reduction in alveolar edema, vascular leakage, and inflammatory response [14–16]. In vitro studies using liquid PFC indicated further anti-inflammatory effects in alveolar macrophages [17] with a decrease in reactive oxygen species [18] and suggested

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* Corresponding author. Tel.: +49 351 4582785; fax: +49 351 4584336.
E-mail address: bleyl@rcs.urz.tu-dresden.de (J.U. Bleyl).

that PFC may protect lung epithelial cells from neutrophil-mediated injury [19]. Due to their state of aggregation, inhaled PFCs may reduce the risks and adverse effects seen with total and partial liquid ventilation while exerting the beneficial effects of PFCs.

The aim of this study was to examine possible therapeutic effects of perfluorohexane (PFH) vapor and their dose-dependency on pulmonary function and lipid mediators in the isolated perfused and ventilated rabbit lung.

2. Material and methods

2.1. Animal preparation

The study protocol was approved by the Institutional Animal Care Committee and the Government of the State of Saxony, Germany and conformed to the National Institutes of Health guidelines for animal use.

Female rabbits ($n = 23$) weighing 2.8 ± 0.5 kg were anesthetized using ketamine, 50 mg/kg (CuraMED, Karlsruhe, Germany) and xylazine hydrochloride, 4 mg/kg (Bayer, Leverkusen, Germany) via the auricular vein. Subsequently all animals received 1000 U/kg heparin (Liquemin, Hoffman-La Roche, Grenzach-Wyhlen, Germany) intravenously. Following a tracheostomy, animals were ventilated with room air using a Servo 900C ventilator (Siemens-Elema, Solna, Sweden) (tidal volume: 8 ml/kg; respiratory frequency: 25 per min; positive end-expiratory pressure: 3 cm H_2O; inspiratory to expiratory ratio: 1:2).

After opening the thorax via a median sternotomy, heart and lungs were carefully prepared. Subsequently a perfusion catheter was placed in the pulmonary artery. The trachea, the lungs and the heart were removed en bloc from the thoracic cavity and the heart separated from the lungs. The trachea-lung organ bloc was than hung on a weight transducer (Hottinger Baldwin, Meßtechnik, Darmstadt, Germany) in a temperature controlled (37 °C) and humidified chamber.

Following the cannulation of the pulmonary artery the lungs were ventilated with 4% CO_2 in air while the ventilator setting remained unchanged. Initially lungs were perfused in an open circuit to remove remaining blood cells and cellular debris from the vascular bed with a Krebs-Henseleit solution (KHS) using low flow rates. After exchanging the perfusion fluid via two separate perfusion circuits at 10 and 20 min after the beginning of extracorporal circulation lungs were perfused in a closed recirculation system containing 200 ml KHS. The temperature of the perfusate was maintained at 37 °C and the pH between 7.35 and 7.45. Flow rates were successively increased to 150 ml/min (calibrated roller pump, Masterflex L/S, Cole–Parmer, Mfg. Barnant, Barrington, IL). After a steady state period of 30 min only lungs having a homogeneous perfusion, a constant airway pressure and a stable lung weight were included in this study.

2.2. Vaporization of perfluorohexane (PFH)

All experiments were performed using PFH ($CF_3(CF_2)_4CF_3$) (ABCR, Karlsruhe, Germany), whose physical and chemical characteristics have been described before [8,9]. PFH was vaporized using a modified vaporizer (Isoflurane Vaporizers 952, Siemens-Elema, Solna, Sweden) for Servo 900C ventilators. Concentrations of PFH were measured continuously by infrared spectroscopy (Iria, Dräger, Lübeck, Germany) and adjusted if necessary. The concentrations of 4.5 vol.% and 18 vol.% PFH applied in this study present the minimum and maximum concentration that can be delivered using this experimental set-up.

2.3. Experimental protocol

After an initial stabilization period of 30 min, 23 lung preparations were randomly assigned to two groups:

2.3.1. Uninjured lungs

- PFH-sham group (PFH-sham): Uninjured lungs ($n = 5$) received 18 vol.% PFH for a study period of 60 min and were followed up for another 60 min. Perfusate samples were taken before the beginning (t_{base}), and at 5 (t_5), 15 (t_{15}), 30 (t_{30}), 60 (t_{60}), 90 (t_{90}), and 120 (t_{120}) minutes after the beginning of PFH application.

2.3.2. Injured lungs

- PFH treatment group (PFH-Tx): Lungs ($n = 12$) were injured by adding calcium ionophore A23187 (Sigma, Deisenhofen, Germany) with a concentration of 1 μM to the perfusion fluid. After an increase of the mean pulmonary artery pressure of 25% from baseline (t_{injury}), lungs were treated with either 4.5 vol.% PFH (4.5 vol.% PFH-Tx; $n = 6$) or 18 vol.% PFH (18 vol.% PFH-Tx; $n = 6$) for 60 min followed by a 60 min observation period.
- Control group (Control): Lungs ($n = 6$) were equally injured by adding calcium ionophore A23187 at a concentration of 1 μM to the perfusion fluid but remained untreated. After an increase of the mean pulmonary artery pressure of 25% from baseline (t_{injury}), lungs were followed up for 120 min.

2.3.3. Interruption criteria

Experiments were terminated before the end of follow up period (t_{120}) when the increase in lung weight exceeded 40 g as ventilation was than almost impossible due to pulmonary edema.

2.3.4. Functional measurements

Mean pulmonary artery pressure (mPAP), lung weight (weight), and peak inspiratory pressure (P_{max}) were measured and recorded in 1 min intervals using a component monitoring system (CMS, Philipps, Böblingen, Germany). MPAP was assessed using an Ohmeda pressure transducer (GE Healthcare, Freiburg, Germany). Because of the constant perfusion flow, alterations of perfusion pressure directly reflected changes of pulmonary vascular resistance. P_{max} was measured by an integrated piezoresistive transducer within the Servo 900C ventilator. A complete set of measurements including perfusate samples was taken before induction of lung injury (t_{base}), at lung injury (t_{injury}) as well as 15 (t_{15}), 30 (t_{30}), 60 (t_{60}), 90 (t_{90}) and 120 (t_{120}) minutes after t_{injury}.

2.3.5. Measurement of lipid mediators

Perfusate samples were taken from the catheter that collects the effluent from the pulmonary veins to determine thromboxane A_2 (TXA_2), prostacyclin (PGI_2) and leukotriene B_4 (LTB_4) formation. TXA_2 and PGI_2 were measured by analyzing the concentration of their stable metabolites thromboxane B_2 (TXB_2) and 6-keto-prostaglandin $F_{1\alpha}$ ($PGF_{1\alpha}$) using the ELISA technique (Assay Designs, Ann Arbor, MI). Samples were drawn into 2 ml syringes containing 10 μg diclofenac (ASTA Medica AWD, Frankfurt, Germany) to stop the formation of TXA_2 and PGI_2. They were immediately centrifuged at 14 000 rpm and the supernatant subsequently frozen for later analysis. In addition LTB_4 concentrations were analyzed using the RIA technique which was performed according to the supplier's instructions (Amersham, Little Chalfont, England).

Table 1
Data of 18 vol.% pfc-sham.

	Baseline	5 min	15 min	30 min	60 min	90 min	120 min
mPAP (mmHg)	9 ± 0.9	9 ± 0.8	9 ± 0.6	10 ± 1.1	11 ± 0.9	10 ± 1.1	11 ± 0.8
Weight gain (Δg)	0	0	0	0 ± 0.23	0 ± 0.3	0.2 ± 0.3	1.4 ± 0.21
P_{max} (cm H_2O)	9 ± 0.9	8 ± 0.5	9 ± 0.7	10 ± 1	10 ± 0.5	11 ± 0.5	12 ± 0.4
Thromboxane B_2 (pmol/ml)	49 ± 9	51 ± 12	43 ± 14	62 ± 12	54 ± 10	67 ± 11	56 ± 14
Prostaglandin $F_{1\alpha}$ (pmol/ml)	250 ± 25	210 ± 35	220 ± 43	250 ± 37	220 ± 42	280 ± 45	300 ± 35
Leukotriene B_4 (pmol/ml)	12 ± 2	10 ± 3	14 ± 2	16 ± 2	22 ± 3	18 ± 4	25 ± 3

mPAP: mean pulmonary artery pressure; weight gain: gain of lung weight; P_{max}: peak inspiratory pressure. Values are given as mean ±SEM.

2.3.6. Statistical analysis

Data were analyzed in co-operation with the Institute of Medical Informatics and Biometrics of the Technical University Dresden. The frequent meeting of the early termination criterion (weight gain: >40 g) resulted in variable durations of the experiments. For a correct statistical analysis Kaplan–Meier and the Log Rank Test were used. Changes from t_{injury} had to be defined for all studied parameters and their occurrence searched. Comparisons of lipid mediator concentrations between the study groups were tested using the Mann–Whitney-U-Test and Bonferroni post-hoc analysis. Data are presented as mean ± standard error of the means (SEM). Significance was accepted at $p < 0.05$.

3. Results

3.1. Uninjured lungs

After the steady state period lungs ($n = 5$) received 18 vol.% PFH over 60 min. Subsequently they were followed up for another hour. The defined termination criterion for the experiments (weight gain of >40) was not reached by any of the lungs. All lungs could therefore be followed over the complete study time. mPAP, lung weight and P_{max} did not change significantly either during or after the application of 18 vol.% PFH. In addition TXB_2, $PGF_{1\alpha}$ and LTB_4 levels remained unchanged in following the presence of PFH (Table 1).

3.2. Injured lungs

The addition of calcium ionophore A23187 to the perfusate led to an increase of mean pulmonary artery pressure (mPAP) by 25% in all lungs. At t_{injury} there were no significant differences in mPAP, lung weight (weight), peak inspiratory pressure (P_{max}) and mediator levels between the three study groups.

3.2.1. Control lungs

The early termination criterion (weight gain > 40 g) was reached by all control lungs before the end of the observation period. An increase of mean pulmonary artery pressure (mPAP) > 10 mmHg as well as >20 mmHg from t_{injury} was observed in all control lungs.

An increase in lung weight by >10 g and >20 g could be observed in all control lungs.

Peak inspiratory pressure (P_{max}) increased by >5 cm H_2O and >10 cm H_2O in all lungs in the control.

Since just two lungs of the control group reached the criteria to terminate the experiment at t_{112} and t_{118} shortly before the end of the study period their mediator levels at that time are shown at t_{120}. In the control lung injury was accompanied by an increase of thromboxane B_2 (TXB_2) level in the perfusate. Differences in 6-keto-prostaglandin $F_{1\alpha}$ ($PGF_{1\alpha}$) levels between the control and 4.5 vol.% PFH-Tx could be demonstrated at the end of the treatment interval t_{60}, while differences between the control and the 18 vol.% PFH-Tx were only seen at t_{120}. Control lungs were associated with highest Leukotriene B_4 (LTB_4) levels. Significant differences between the control and the 4.5 vol.% PFH-Tx were seen as early as 15 min after the start of treatment (Fig. 7).

3.2.2. PFH treatment

In none of the PFH-Tx-groups a weight gain >40 g was observed and all experiments continued to the end of the observation period (t_{120}) ($p < 0.05$) (Fig. 1). mPAP did not increase >10 mmHg in the 4.5 vol.% PFH ($p < 0.05$) and only in two lungs of the 18 vol.% PFH ($p < 0.05$). An increase of mPAP > 20 mmHg could not be observed in the 4.5 vol.% PFH-Tx ($p < 0.05$) group and only seen in one lung of the 18 vol.% PFH-Tx ($p < 0.05$) group. Differences between both PFH-Tx-groups were not noted (Fig. 2).

None of the 4.5 vol.% PFH-Tx ($p < 0.05$) and only two lungs in the 18 vol.% PFH-Tx ($p < 0.05$) were associated with a weight gain by >10 g and >20 g (Fig. 3).

A rise in peak inspiratory pressure by >5 cm H_2O was observed in two lungs in the 18 vol.% PFH-Tx ($p < 0.05$) and three lungs in the 4.5 vol.% PFH-Tx (ns). One lung of the 4.5 vol.% PFH-Tx ($p < 0.05$) and none of the lungs in the 18 vol.% PFH-Tx ($p < 0.05$) showed an increase of inspiratory pressure >10 cm H_2O. Differences between both PFH-Tx-groups were not seen (Figs. 4 and 5).

In both PFH groups TXB_2 levels did not increase over time and differences between the two PFH-Tx were not noted. Significant differences in $PGF_{1\alpha}$ levels between both PFH-Tx-groups could be observed from t_{60} onwads (Fig. 6). LTB_4 levels did not differ significantly among the 18 vol.% PFH-Tx and the control or the 4.5 vol.% PFH-Tx during the entire study period.

4. Discussion

The main findings of this study could be summarized as follows: 1) Inflammatory lung injury was attenuated by the application of

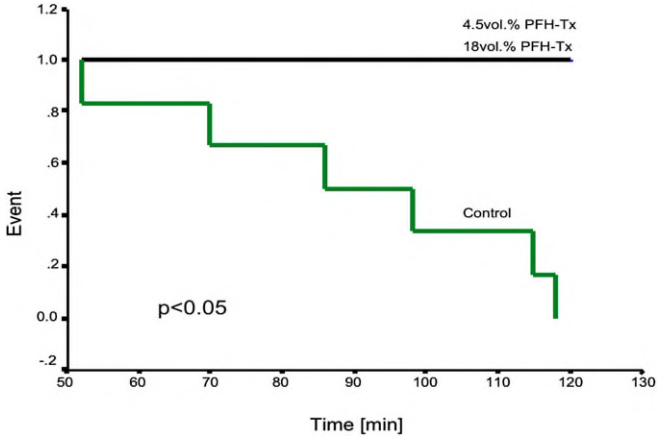

Fig. 1. Kaplan–Meier analysis demonstrating the time before either fulfilling the termination criteria (weight gain > 40 g) or reaching the end of the observation period (120 min after induction of lung injury). None of the lungs in control ($n = 6$) reached the end of the observation period. In contrast all lungs in the 4.5 vol.% PFH-Tx-group ($n = 6$) and 18 vol.% PFH-Tx-group reached the end of the observation period ($p < 0.05$).

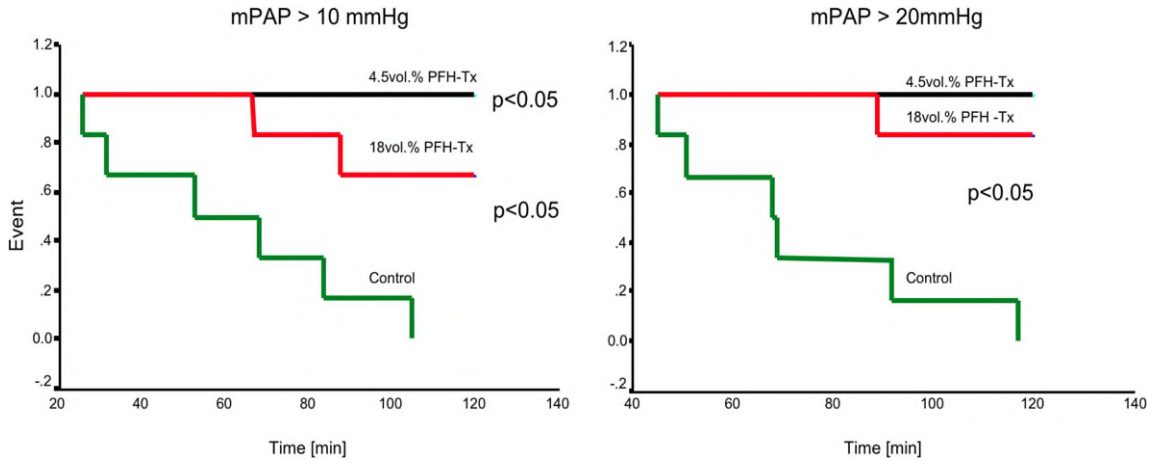

Fig. 2. Increase of mean pulmonary artery pressure (mPAP) >10 mmHg and 20 mmHg: Significant differences were observed between the control and both PFH-Tx-groups for an increase of mPAP of >10 mmHg and >20 mmHg. Different levels of significance could be demonstrated between the PFH-Tx-groups and the control. Differences between both PFH-Tx-groups could not be demonstrated.

PFH vapor 2) Therapeutic effects were more pronounced with a concentration of 4.5 vol.% PFH.

Before discussing these findings, some methodological issues should be addressed. The isolated lung model is a standardised experimental model and has been used in the past to study effects of new therapeutic agents for ARDS on pulmonary circulation and edema formation. Despite its artificial nature, this model offers the unique possibility to selectively study effects on pulmonary vascular resistance, edema formation, and mediator release avoiding interferences from the systemic circulation and other organ systems [20,21]. A23187 is an established stimulus to provoke acute lung injury in the model of isolated perfused and ventilated rabbit lung causing pulmonary vasoconstriction and vascular leakage [20].

Treatment of A23187-induced lung injury with 4.5 vol.%- and 18 vol.%-PFH attenuated the rise in mPAP, lung weight and P_{max}. The marked therapeutic effects of PFH were reflected in significant longer running time of the experiments in both treatment groups compared to the control group where all lungs fulfilled the termination criterion (weight gain > 40 g) before the end of the observation time (t_{120}). This difference in running time made statistical analysis rather cumbersome. Changes from t_{injury} had to be defined for all studied parameters, their occurrence searched for, and analyzed using Kaplan–Meier Analysis and the Log Rank Test.

Significant differences for an increase of mPAP as well as for a gain in lung weight and for an increase in P_{max} could be observed between both PFH-Tx and the Control. Dose dependant effects with different level of significance could be demonstrated between both PFH-Tx and the Control. More pronounced therapeutic effects on mPAP and lung weight were achieved with the lower dose of 4.5 vol.%PFH. Therapeutic effects on P_{max} were less marked with 4.5 vol.% PFH. However Gama de Abreu et al. reported that the application of 5 vol.% PFH permitted stabilization of the lung at lower PEEP levels during the open lung approach and was associated with better histological scores when compared to 10 vol.% PFH and PLV [15].

A23187 is a potent activator of arachidonic acid synthesis leading to an influx of calcium into the cytoplasm with a resulting in the synthesis and liberation of lipid mediators. Therapeutic effects of PFH on the metabolites of arachidonic acid, thromboxane A_2 (TXA_2) prostacyclin (PGI_2) and leukotriene B_4 (LTB_4) were therefore studied in the presence and absence of PFH. TXA_2 has been shown to be the predominant mediator for the increase in arachidonic acid induced pulmonary vascular resistance and bronchial tone in ARDS [22]. In contrast PGI_2 mediates pulmonary vascular vasodilatation [23]. The arachidonic acid induced increase in pulmonary vascular permeability is primarily caused by leukotrienes (LTB) especially LTB_4 [21]. PFH-therapy was associated with

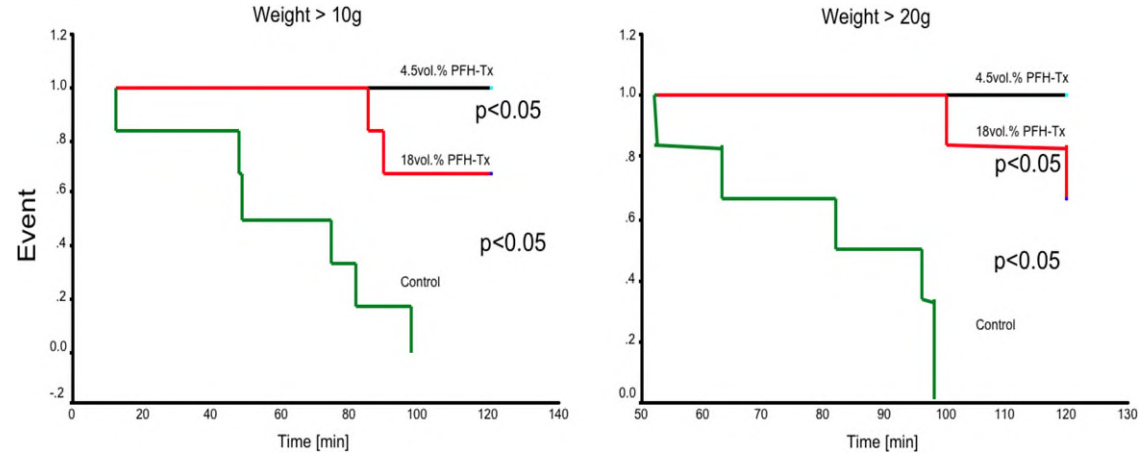

Fig. 3. Changes of lung weight by >10 g and >20 g: Significant changes in lung weight could be seen between the Control and both PFH-Tx-groups. These differences were $p < 0.05$ for an increase of >10 g and >20 g in the 18 vol.% PFH-Tx-group. More pronounced therapeutic effects could be seen in the 4.5 vol.% PFH-Tx-group with a $p < 0.05$ for an increase in lung weight by >10 g and >20 g. Differences between both PFH-Tx-groups were not encountered.

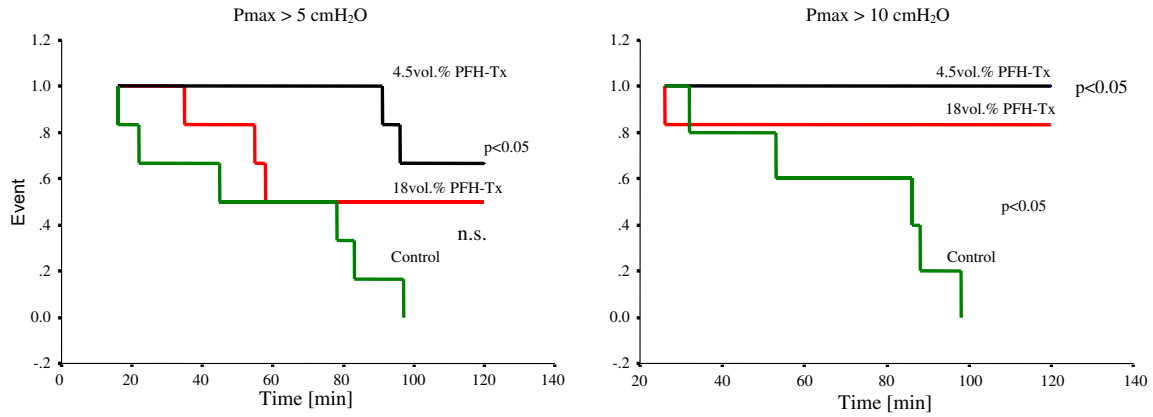

Fig. 4. Increase of peak inspiratory pressure (P_{max}) >5 cm H_2O and >10 cm H_2O: The effects of 4.5 vol.% PFH on P_{max} were less pronounced. For an increase of >5 cm H_2O no statistical differences was noted compared to the control. However a significance was observed for the 18 vol.% PFH-Tx-group with a $p < 0.05$. Different level of significance were as well encountered for an increase in P_{max} of >10 cm H_2O between PFH-Tx-groups and the control. Differences between both PFH-Tx-groups were not observed.

almost unchanged TXB_2 levels in both PFH-Tx-groups. This corresponds well with the observed therapeutic effects on pulmonary vascular and bronchial tone where the increase of mPAP and of P_{max} during and following PFH application was strongly attenuated. The attenuation of mPAP increase respective attenuated rise of pulmonary vascular resistance could be responsible for the delayed and dampened development of pulmonary edema in both PFH groups. In addition the lack of pulmonary edema formation especially in the 4.5 vol.% PFH-Tx correlates well with low LTB_4 levels and the maintenance of pulmonary vascular integrity. Overall the more pronounced therapeutic effects of 4.5 vol.% PFH-Tx on mPAP and lung weight were reflected in lower levels of TXB_2, $PGF_{1\alpha}$, and LTB_4 which were in part significant when compared to the 18 vol.% PFH-Tx. The ability of PFC to interfere with the formation of lipid mediator seems to be one important mechanism of its anti-inflammatory action. A reduction of lipid mediator synthesis has been reported in the past with application of vaporized and liquid PFC [24]. More pronounced effects were observed with PFCs of a high vapor pressure such as PFH [25]. While the mode of action remains unclear at present, several indirect and direct cytoprotective mechanisms have been proposed.

Woods et al. were able to show that direct contact of PFC with the endothelium was not necessary to attenuate an inflammatory reaction [26]. They speculated that diffusion of PFC from the alveolar space into the adjacent pulmonary vascular endothelial layer could modulate neutrophil adhesion and thereby reduce the rate of infiltration of activated neutrophils. As our experimental set-up was using a cellfree perfusion fluid neutrophil adhesion seems unlikely to be the central cause for the observed modulation of mediator release at first sight. However even after extensive rinsing of the pulmonary vasculature before steady state, a considerable number of endothelium adherent leukocytes are thought to remain within the pulmonary bed [20]. These may be considered as the physiological marginal pool and may contribute to the induced inflammatory reaction [20]. The diffusion of PFH to the endothelium with the resulting modulation of neutrophil adhesion may therefore be an important contributing factor for the observed effects. This hypothesis is supported by reports that adhesion molecule expression on pulmonary vasculature is directly inhibited on mRNA level by aerosolized and liquid PFC [27].

Obraztsov et al. could demonstrate the integration of PFC in the lipid layer of cell membranes [28]. Driving force for the uptake of

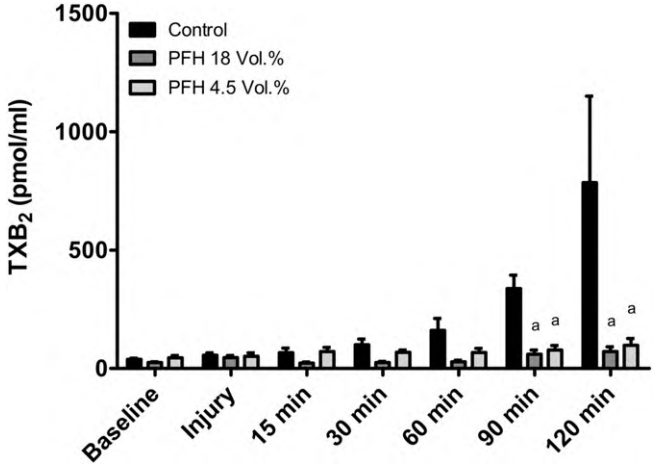

Fig. 5. Thromboxane B_2 levels (TXB_2) of the control and the 4.5 vol.% PFH and 18 vol.% PFH at baseline, lung injury and 15, 30, 60, 90 and 120 min after induction of lung injury. Significant differences ($p < 0.05$) between both PFH-Tx-groups and the Control group where seen at 90 and 120 min after induction of lung injury. Significant differences between both PFH-Tx were however not encountered. a) $p < 0.05$ vs. control.

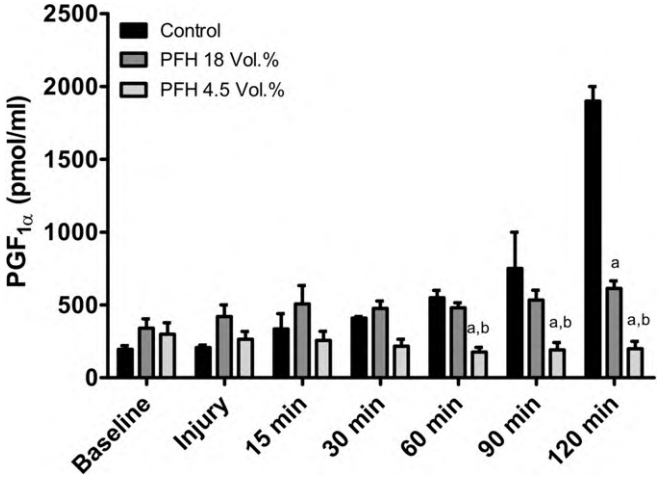

Fig. 6. 6-keto-Prostaglandin $F_{1\alpha}$ level ($PGF_{1\alpha}$) of the control and the 4.5 vol.% PFH and 18 vol.% PFH at baseline, lung injury and 15, 30, 60, 90 and 120 min after induction of lung injury. Significant differences between the control and both PFH-Tx-groups as well as between both PFH-Tx-groups could be demonstrated. a) $p < 0.05$ vs. control; b) $p < 0.05$ vs. 18 vol.% PFH-Tx-group.

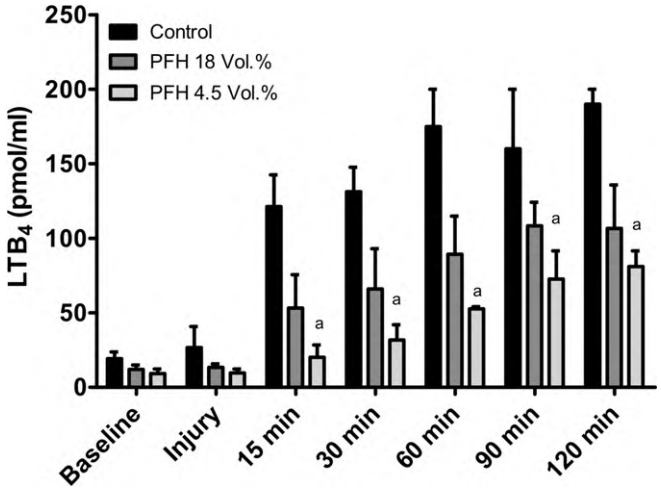

Fig. 7. Leukotriene B_4 level (LTB_4) of the control and the 4.5 vol.% PFH and 18 vol.% PFH at baseline, lung injury and 15, 30, 60, 90 and 120 min after induction of lung injury. Significant differences were only encountered between the control and the 4.5 vol.% PFH-Tx-group. a) $p < 0.05$ vs. control.

PFC seems to be their lipid solubility and the vapor pressure [29]. The integration of PFC into endothelial cell membrane or into inflammatory cell membrane could lead to their stabilization. This may affect cell membrane protein responses and have a generalized protective or attenuating effect on a variety of membrane-mediated cell responses to stress or activation [28]. The application of A23178 is associated with a transmembranous influx of calcium (Ca^{2+}) and an increase of cytoplasmic Ca^{2+} with activation of arachidonic acid liberating phospolipase A and lipoxygenase [30]. By stabilization of cell membrane and membrane-mediated cell responses PFH might therefore interfere directly with and attenuated the A23187-induced activation of arachidonic acid synthesis. While the exact mechanism remains unclear at present our data support the hypothesis that the application of vaporized PFH is accompanied with a modulation of the inflammatory reaction.

The application of 18 vol.% PFH vapor did not lead to changes in mPAP, lung weight and P_{max} in uninjured lungs during or after the application period. This was reflected in the almost unaltered release of lipid mediators. Therefore, the application of PFC in general and 18 vol.% PFH in particular seems not to be associated with any adverse effects in healthy lungs.

In summary, calcium ionophore A231873 mediated lung injury was attenuated by treatment with 4.5 vol.%- and 18 vol.%-PFH vapor in an experimental model of isolated perfused and ventilated rabbit lung. More pronounced therapeutic effects were achieved with 4.5 vol.% PFH. The mechanism of PFH vapor in the treatment of inflammatory-mediated increase in pulmonary arterial pressure, pulmonary edema and peak inspiratory pressure may rely in part on the modulation of lipid mediator formation.

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