■ LABORATORY INVESTIGATIONS

Perfluorohexane Attenuates Proinflammatory and Procoagulatory Response of Activated Monocytes and Alveolar Macrophages

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Background: A number of studies have demonstrated the effectiveness of liquid ventilation with perfluorocarbons in improving pulmonary function in acute respiratory distress syndrome. Although it is known that perfluorocarbon-associated gas exchange facilitates lung mechanics and oxygenation, the complete mechanism by which perfluorocarbons exert their beneficial effects in acute lung injury still remains unclear. Possibly, an influence of perfluorocarbons on proinflammatory and procoagulant features of monocytic cells present in the alveolar space, such as alveolar macrophages (AMs), may be involved. Therefore, we examined in an *in vitro* model the effects of perfluorocarbon on both activated mononuclear blood cells (MBCs) and AMs by monitoring the expression of interleukin (IL)-1β, tumor necrosis factor (TNF)α, and tissue factor (TF).

Methods: Mononuclear blood cells, obtained from peripheral blood of healthy volunteers, or AMs from diagnostic bronchoalveolar lavage were stimulated by incubation with lipopolysaccharide in the presence of different amounts of perfluorohexane, which was devoid of cytotoxicity.

Results: Using both video-enhanced contrast and electron microscopy, the authors observed that perfluorohexane droplets were phagocytosed by activated monocytes as well as by *in vitro*–cultured AMs within 1–3 h. After lipopolysaccharide stimulation of monocytes or AMs, we observed a down-regulation of TF mRNA and a significant inhibition ($P < 0.05$) of cellular TF antigen by perfluorohexane. In addition, the concentration of both IL-1β and TNFα in the supernatant of lipopolysaccharide-stimulated MBC was significantly decreased ($P < 0.01$) by perfluorohexane compared with controls without perfluorohexane. By preincubation of lipopolysaccharide-containing medium with perfluorohexane, the authors could exclude that the inhibitory effect of perfluorohexane was caused by binding or sequestering limited amounts of lipopolysaccharide.

Conclusion: Taken together, our results demonstrate an interference of perfluorohexane with the expression of the procoagulant protein TF on monocytes and AMs as well as with the release of proinflammatory cytokines by MBCs. These effects may contribute to the protective role of liquid ventilation with perfluorocarbons in injuries associated with local activation of inflammatory processes.

IN recent years, numerous experimental studies were performed to evaluate new therapeutic strategies in the treatment of acute lung injury and acute respiratory distress syndrome (ARDS). The inhalation of nitrous oxide, the intratracheal application of surfactant preparations, or the high-frequency ventilation have shown beneficial effects.[1] Promising results were also gained by partial liquid ventilation with perfluorocarbons, a class of biochemical inert liquids with low surface tension and high oxygen as well as carbon dioxide–carrying capacity. Thus, an improvement in oxygenation, lung mechanics, and pulmonary shunt was demonstrated in both experimental models of lung injury[2,3] and clinical studies of newborns and adults with ARDS.[4-6]

A considerable problem occurring during liquid ventilation, however, is an elevation of airway pressure immediately after instillation of perfluorocarbon, resulting in an increased incidence of barotrauma with the development of pneumo- and liquothoraces.[5,6] The application of vaporized perfluorohexane, a recently established new therapeutic approach, led to an improvement of oxygenation and lung mechanics with significantly reduced airway pressures in an ovine model of acute lung injury. This new method seems to combine the beneficial effects of perfluorocarbons on gas exchange and lung mechanics without the untoward side effects, *e.g.*, the increased risk of barotrauma.

Until now, the mechanisms of action by which liquid ventilation with perfluorocarbons exerts its effects in acute lung injury have been poorly understood. However, it is supposed that physical properties of perfluorocarbons contribute to the observed clinical improvement of lung function. Furthermore, a decrease of inflammatory infiltrate together with a reduction of corresponding tissue damage was observed in a number of animal studies after partial liquid ventilation with perfluorocarbons.[2,8,9] It was assumed that this observation may have been caused by anti-inflammatory effects of perfluorocarbons. This presumption was underlined by several studies in which a direct anti-inflammatory effect on cells involved in the pathogenesis of ARDS has been demonstrated.[10-13]

Increased levels of proinflammatory cytokines such as

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interleukin (IL)-1β and tumor necrosis factor (TNF)α have been shown in bronchoalveolar lavage of patients with ARDS and seem to be correlated with their poor outcome.[14-16] The release of these cytokines by resident or immigrated pulmonary mononuclear cells is supposed to play an important pathophysiologic role in the development and prolongation of lung injury,[17] especially by chemoattractant effects on neutrophils.[18,19] To elucidate the assumed interaction of perfluorohexane with phagocytic cells, we analyzed in an *in vitro* model whether perfluorohexane affects the release of IL-1β and TNFα by both peripheral mononuclear blood cells (MBCs) and isolated alveolar macrophages (AMs) after stimulation. In addition, the expression of tissue factor (TF) by stimulated monocytes or AMs on both RNA and protein level was determined. TF, a transmembrane protein that functions as the primary cellular initiator of blood coagulation, is described to be a proinflammatory marker of cellular activation of monocytes/macrophages.[20]

In the present study we examined whether the expression or release of proinflammatory and procoagulant mediators was influenced by perfluorohexane in *ex vivo* stimulated phagocytic cells.

Material and Methods

Perfluorohexane

Perfluorohexane [$CF_3(CF_2)_4CF_3$; ABCR, Karlsruhe, Germany] with a purity of 99% was used for *in vitro* studies on MBCs and AMs. Perfluorohexane is a clear, radiolucent, colorless liquid with a molecular weight of 338 g/mol, a boiling point of 57°C, a vapor pressure (at 20°C) of 177 mmHg, a density of 1.672 g/ml, a viscosity of 0.66 cP, and a surface tension of 11.4 dyne/cm^3. Its oxygen-carrying capacity is 57 ml O_2/100 ml perfluorohexane.

Isolation of Mononuclear Blood Cells and Alveolar Macrophages

Mononuclear blood cells were prepared from venous citrated blood of healthy volunteers by density centrifugation on Ficoll-Paque as previously described.[21] After washing, cells were resuspended in RPMI 1640 medium (Sigma, Deisenhofen, Germany) supplemented with 5% fetal calf serum yielding a concentration of 1×10^6 cells/ml. Mean cell viability was 98% as assessed by trypan blue exclusion.

Alveolar macrophages were obtained from diagnostic bronchoalveolar lavages of patients with chronic inflammatory lung disease. Cells were washed twice in 0.9% saline and resuspended in RPMI medium with 5% fetal calf serum at a concentration of 0.25×10^6 cells/ml. The percentage of macrophages was determined by flow cytometry (FACScan, Becton Dickinson, Heidelberg, Germany) after staining the cells selectively with CD14 antibodies.[22]

Preparation of Mononuclear Blood Cells and Alveolar Macrophages for Cytokine and Tissue Factor Analysis

Mononuclear blood cells were stimulated by addition of commercially available *Escherichia coli*–derived lipopolysaccharide (*E. coli* serotype 055:B5, Sigma). Perfluorohexane was added to the cell suspension to a final volume concentration [vol/vol] of 5, 10, and 30%. After vortexing, cells were incubated for 6 or 24 h at 37°C and 5% CO_2. As perfluorohexane is not miscible with the medium, cells were exposed to perfluorohexane both with and without shaking. After centrifugation, 600 μl of perfluorohexane-free supernatants were removed and stored frozen at -20°C for IL-1β and TNFα analysis. The pelleted MBCs were resuspended in the remaining supernatants (containing perfluorohexane) and stored frozen at -20°C until use. For the determination of TF antigen, cells were disrupted by two cycles of freezing and thawing followed by solubilization of TF in 100 μl of lysis buffer (50 mM TrisHCl, 100 mM NaCl, 1% [vol/vol] Triton X-100, pH 7.6) for 20 min (final volume, 0.5 ml). All controls were treated similarly except that the respective amount of perfluorohexane [vol/vol] was added after incubation immediately before removal of the cell-free supernatant.

The kinetics of cytokine secretion and TF expression of lipopolysaccharide-stimulated cells were examined at 6, 12, 24, 48, and 72 h of incubation in the presence (concentration [vol/vol] 30%) or absence (controls) of perfluorohexane. The interaction of perfluorohexane with AMs was analyzed after the same experimental procedure as described for MBCs. TNFα secretion and TF expression were examined after incubation of AMs with perfluorohexane at a concentration of 30% [vol/vol] for 6 h.

Evaluation of Lipopolysaccharide Binding by Perfluorohexane

The possible interference of perfluorohexane with lipopolysaccharide was determined by means of endotoxin ability to stimulate TNFα and TF generation after preincubation of lipopolysaccharide with perfluorohexane. RPMI medium (2 ml) containing lipopolysaccharide (10 μg/ml) was preincubated with 100, 200, or 600 μl perfluorohexane for 90 min both with and without shaking. After incubation, samples were centrifuged (275g, 10 min) to layer out perfluorohexane. For controls, RPMI medium was spiked with lipopolysaccharide (concentrations ranging from 10 pg/ml to 100 μg/ml). MBCs (1×10^6) were resuspended in 1 ml of either perfluorohexane-free RPMI supernatant or RPMI controls for stimulation. After incubation for 6 h at 37 °C, cell suspensions were centrifuged at 275g for 10 min. TNFα and TF were analyzed by enzyme-linked immunosorbent assay (ELISA) in the supernatant and cell lysate, respectively.

Cytokine and Tissue Factor Enzyme-linked Immunosorbent Assay

Cytokine release (IL-1β and TNFα) was assayed by means of commercially available ELISA kits (Genzyme/R&D Systems, Wiesbaden, Germany) according to the manufacturer instructions. All samples were determined in duplicate and measured using an automated multichannel Titertek MS2 microplate reader (ICN, Eschwege, Germany).

Tissue factor antigen was determined using a sandwich-type ELISA with two monoclonal antibodies as described previously.[23] Briefly, microtiter plates (Maxisorp, NUNC, Wiesbaden, Germany) coated with purified anti-TF monoclonal antibody VIC7 (2.5 μg/ml) were incubated with cell lysates diluted 1:1 in sample buffer (50 mM TrisHCl, 100 mM NaCl, 0.2% [vol/vol] Triton X-100, 1% [wt/vol] bovine serum albumin, pH 7.6) for 2 h at 37°C. Twofold serial dilutions of standard recombinant TF (American Diagnostica Inc., Greenwich, CT) in sample buffer were added as a reference standard. After incubation with peroxidase-labeled anti-TF monoclonal antibody IIID8 (90 min at 37°C) and subsequent substrate reaction with 3,3′5,5′-tetramethylbenzidine (K & P Laboratories, Gaithersburg, MD) for 20 min, the absorbance was measured at 450 nm with the multichannel photometer. TF values were expressed as nanograms TF per 10^6 cells.

Analysis of Tissue Factor mRNA by Reverse-transcription Polymerase Chain Reaction

Mononuclear blood cells were stimulated with lipopolysaccharide (10 μg/ml) in the presence of perfluorohexane (concentration [vol/vol] 10%) for 2 h at 37°C. After thorough shaking, perfluorohexane was allowed to settle down to the bottom. The aqueous layer containing the MBC suspension was collected and centrifuged at 275 g for 10 min at 4°C. Cell pellets were immediately frozen at −80°C in RNAse free plastic tubes until use in polymerase chain reaction (PCR). Controls consisted of both nonstimulated and lipopolysaccharide-stimulated MBCs incubated as described in the absence of perfluorohexane. Cellular mRNA was isolated using the Micro Fast Track kit (Invitrogen, Carlsbad, CA), and cDNA was synthesized using the cDNA-Cycle kit (Invitrogen). PCR was performed in a marker mix containing 1 × PCR buffer (Perkin-Elmer/Applied Biosystems, Foster City, CA), 2 mM of each deoxynucleotide triphosphate (Stratagene, LaJolla, CA), 2 μM of TF-specific 5' and 3' primers,[24] 0.5 U AmpliTaq Gold DNA polymerase (Perkin-Elmer/Applied Biosystems), and 2 μl cDNA in a total volume of 50 μl. The amplification profile consisted of denaturation at 94°C for 1 min, annealing at 60°C for 30 s, and primer extension at 72°C for 1 min in a 44-cycle reaction. Twenty microliters of each reaction mixture was electophoresed in 4–12% TBE Page (Novex, Offenbach, Germany), stained with SYBR Green (Biozym

Diagnostica GmbH, Hess. Oldendorf, Germany), and analyzed with the Fluor-S MultiImager (BioRad, München, Germany) using Multi-Analyst PC Software (BioRad).

Video-enhanced Contrast Microscopy. Lipopolysaccharide-stimulated MBCs (10 μg/ml) were incubated with perfluorohexane (concentration [vol/vol] 10%) for 1 h. Thereafter, cells were allowed to adhere to small glass plates (diameter, 16 mm; thickness, 150–170 μm) for a further 30 min. Plates were attached to a perfusion chamber,[25] and microscopy was conducted using an inverse microscope (Leica DMIRB, Bensheim, Germany) with differential interference contrast optics. This device was video-enhanced with a CCD-camera (Optronics DEI 470, Goleta, CA), yielding a total magnification of 100 × 1.0 × 2.5 (15.000 pixel/μm). Frames were transferred to an Apple Macintosh 7100/66AV (Apple, Cupertino, CA) and processed with the public domain NIH image program developed at the National Institutes of Health (Bethesda, MD; available *via* the Internet).

Electron Microscopy

Mononuclear blood cells were stimulated with lipopolysaccharide (10 μg/ml) and incubated with or without perfluorohexane (concentration [vol/vol] 10%) for 4 h at 37°C. After several washes with phosphate-buffered saline and centrifugation (275g for 10 min), cell pellets were incubated with 100 μl of recalcified human plasma at 37°C until clot formation was reached. The fibrin clots including the cells were fixed in 0.1 M sodium cacodylate buffer (pH 7.4) containing 3% [vol/vol] glutaraldehyde for 1 h at room temperature. After washing in 0.1 M cacodylate buffer, clots were fixed with 0.1 M cacodylate buffer containing 1% osmic acid (pH 7.4) for 1 h at 4°C. Dehydration was performed with acetone and aceton–vestopal ([vol/vol] 2:1 and 1:1 at room temperature, 1:2 overnight at 4°C). Thereafter, vestopal was prepared with hardener ([vol/vol] 1%) and accelerator ([vol/vol] 1%) for 3 h at 4°C and 1 h at room temperature. Vestopal-filled gelatin capsules were polymerized onto the glass slides containing the cells for 48 h at 60°C. Ultrathin sections were mounted on nickel grids. All buffers, fixatives, and embedding materials for electron microscopy were purchased from Serva (Heidelberg, Germany).

Data Analysis

Data are expressed as mean ± SD. ELISA results are presented as percentage of perfluorohexane-exposed cells compared with nonexposed control cells. The statistical association between data of perfluorohexane-incubated samples and corresponding controls was evaluated with the paired Student t test. The dependency of the observed effect from the perfluorohexane doses was analyzed using the Kruskal-Wallis test. All calculations were performed using the StatView 5.0 statistical pack-
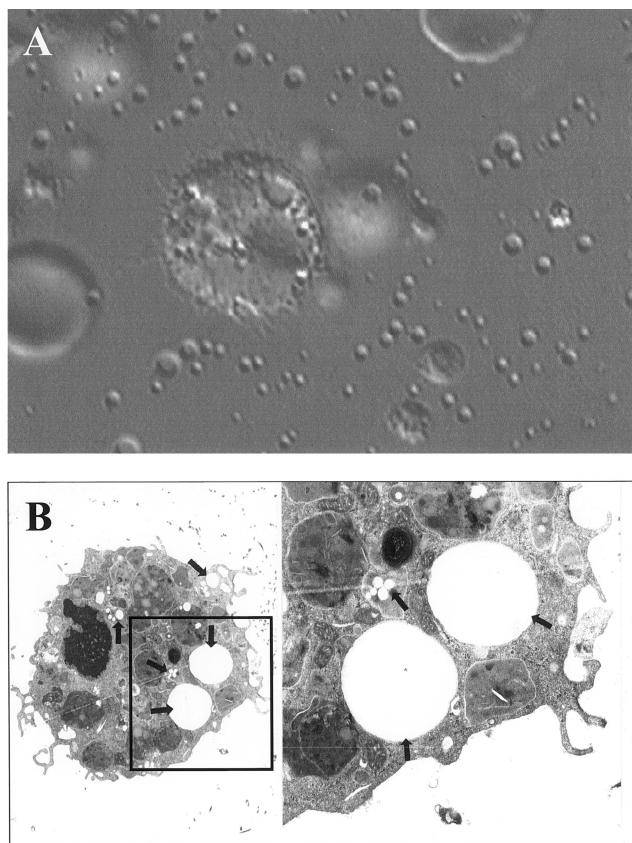
Fig. 1. Phagocytosis of perfluorohexane droplets by lipopolysaccharide-stimulated monocytes–macrophages. (*A*) Detection of perfluorohexane droplets in close contact to monocytes by video-enhanced contrast microscopy after 1 h of stimulation with lipopolysaccharide (10 μg/ml) in the presence of perfluorohexane ([vol/vol] 10%). (*B*) Ultrastructural detection of phagocytosed perfluorohexane droplets. Arrows indicate circular vacuoles of extracted perfluorohexane droplets.

age (SAS Institute Inc., Cary, NC). Statistical values of *P* less than 0.05 were considered significant.

Results

Interaction of Monocytes–Macrophages with Perfluorohexane and Cell Viability

Cellular interactions of activated monocytes as well as *in vitro*–cultured AMs with perfluorohexane were investigated by video-enhanced microscopy and in more detail by electron microscopy. An image of lipopolysaccharide-stimulated monocytes surrounded by multiple perfluorohexane droplets of various sizes is presented in figure 1A. The activated monocyte has close contact with the perfluorohexane particles, but whether they are adherent to the cell membrane or already incorporated into the cytoplasm cannot be reliably decided using video-enhanced microscopy. However, the perfluorohexane-free halo around the cell membrane suggests that phagocytosis had occurred. Final evidence of the intracellular location of perfluorohexane was obtained

by ultrastructural detection of phagocytosed perfluorohexane droplets using electron microscopy. The vacuolated circular structures in stimulated monocytes represent perfluorohexane particles within the cytoplasm, extracted during the fixation process (fig. 1B). Similar results were observed with *in vitro*–cultured AMs (data not shown).

The mean viability of MBCs after preparation and resuspension of cells was 98%. More than 90% of cells remained vital during incubation for 6 or 24 h in the presence of lipopolysaccharide and perfluorohexane as assessed by trypan blue exclusion. There was no significant difference in cell viability between samples with MBCs exposed to perfluorohexane and controls. These results could be confirmed when cell viability was determined by flow cytometric analysis measuring propidium iodide staining (data not shown).

Effect of Perfluorohexane on Tissue Factor mRNA and Protein Expression

To study the effect of perfluorohexane on monocytes–macrophages, we first investigated *in vitro* the influence of perfluorohexane on lipopolysaccharide-mediated TF induction at both the transcriptional and protein levels. After maximum stimulation of MBCs by lipopolysaccharide (10 μg/ml) in the presence of perfluorohexane, we observed a distinct down-regulation of TF mRNA transcription as shown in figure 2A, which is representative for three independent reverse-transcription PCR analyses.

To analyze the perfluorohexane effect on TF expression in dependence of lipopolysaccharide concentration, we stimulated the cells with increasing concentrations of lipopolysaccharide (final concentrations ranging from 100 pg/ml to 10 μg/ml) for 6 h at 37°C. We observed a substantial inhibition of cellular TF antigen content over the entire lipopolysaccharide concentration range tested (fig. 2B). The kinetics (n = 3) of TF protein expression after maximum stimulation of MBCs with lipopolysaccharide (10 μg/ml) is shown in figure 2C. Compared with controls without perfluorohexane, TF antigen expression on perfluorohexane-exposed cells was maximally decreased at 6 h after lipopolysaccharide stimulation, when the highest TF antigen concentrations in MBC lysates were detected.[22] These data illustrate that perfluorohexane attenuated TF expression without altering the time course. For all doses of perfluorohexane tested, the determination of TF antigen expression at 6 h after lipopolysaccharide stimulation revealed a significant decreased percentage of cellular TF content to 85–87% compared with controls without perfluorohexane (n = 17; fig. 2D). On the other hand, we did not observe any TF induction in nonstimulated cells by perfluorohexane alone (data not shown).

Fig. 2. Down-regulation of tissue factor (TF) messenger RNA transcription and protein expression in lipopolysaccharide (LPS)-stimulated mononuclear blood cells (MBCs) in the presence of perfluorohexane (PFH). (*A*) Detection of tissue factor messenger RNA by reverse-transcription polymerase chain reaction in MBCs after 2 h of stimulation with lipopolysaccharide (10 μg/ml). (*B*) Perfluorohexane effect on TF expression of MBCs in dependence of increasing concentrations of lipopolysaccharide compared with control samples without perfluorohexane. Note the substantial inhibition of cellular TF antigen content over the entire lipopolysaccharide concentration range tested. (*C*) Kinetics of TF expression in MBCs after lipopolysaccharide stimulation (10 μg/ml) in the presence of 30% perfluorohexane [vol/vol] (mean ± SD of three independent experiments). Note the significant decrease of cellular TF content after 6 h, when maximum levels of TF expression in monocytes were observed. (*D*) Inhibition of TF expression in MBCs after 6 h of lipopolysaccharide stimulation (10 μg/ml) in the presence of increasing amounts of perfluorohexane. Results are presented as mean percentage of TF content of control cells without perfluorohexane (± SD) from 17 independent experiments (paired *t* test, *$P < 0.05$, **$P < 0.001$). GAPDH = glyceraldehyd-3-phosphate dehydrogenase.

Effect of Perfluorohexane on Tumor Necrosis Factor α and Interleukin 1β Levels

The influence of perfluorohexane on the secretion of proinflammatory cytokines was studied by determination of TNFα and IL-1β levels in supernatants of lipopolysaccharide-stimulated MBCs. We observed a substantial inhibition of TNFα and IL-1β induction over a broad lipopolysaccharide concentration range (incubation time, 24 h; final lipopolysaccharide concentration, 1 ng/ml to 10 μg/ml, not shown). In addition, both TNFα and IL-1β displayed similar kinetics of secretion after maximum lipopolysaccharide stimulation of MBCs, as shown in figures 3A and 3B, respectively. Highest levels of cytokines were observed at 6–12 h of incubation for TNFα (fig. 3A) and at 12–24 h for IL-1β, respectively (fig. 3B). When MBCs were stimulated with lipopolysaccharide in the presence of perfluorohexane (n = 3, 30 % [vol/vol]), after 6 and 24 h TNFα and IL-1β secretion into supernatants was

reduced strikingly as compared with samples without perfluorohexane.

The mean percentage (normalized to the corresponding controls without perfluorohexane = 100%) of TNFα and IL-1β levels in supernatants of lipopolysaccharide-stimulated MBCs exposed to different amounts of perfluorohexane is presented in figures 3C and 3D, respectively. We observed a significant (n = 17; $P < 0.001$) decrease of TNFα levels to 81% of the controls after 6 h of incubation, whereas a less pronounced inhibition was observed after 24 h. However, we did not observe any significant dependency of this effect from the perfluorohexane doses applied (Kruskal-Wallis test, $P > 0.2$; fig. 3C). IL-1β release of perfluorohexane-exposed cells was inhibited by perfluorohexane in a similar manner (fig. 3D). Perfluorohexane reduced IL-1β release of MBCs significantly (n = 17; $P < 0.001$) to 77–89%. In perfluorohexane-exposed MBCs, we could exclude any accu-
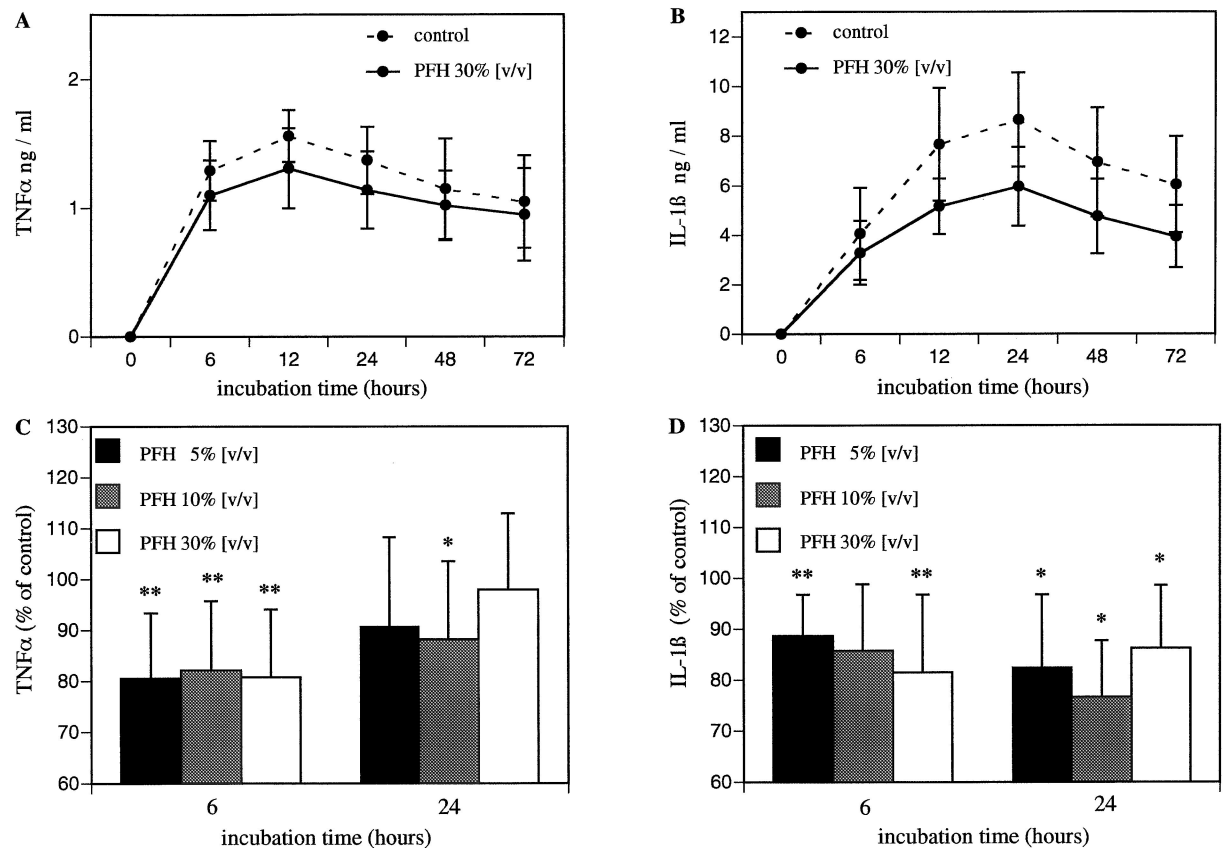
Fig. 3. Inhibition of the release of proinflammatory cytokines in supernatants of lipopolysaccharide-stimulated mononuclear blood cells by perfluorohexane (PFH). Kinetics of tumor necrosis factor (TNF)α (*A*) and interleukin (IL)-1ß (*B*) concentrations in supernatants of lipopolysaccharide-stimulated mononuclear blood cells in the presence of 30% perfluorohexane [vol/vol] compared with control samples without perfluorohexane (mean ± SD of three independent experiments). Inhibition of TNFα (*C*) and IL-1ß (*D*) release in supernatants of mononuclear blood cells after 6 and 24 h of lipopolysaccharide stimulation (10 μg/ml), respectively, in the presence of increasing amounts of perfluorohexane. Results are presented as mean percentage of cytokine content of control supernatants without perfluorohexane (± SD) from 17 independent experiments (paired *t* test, *P < 0.05, **P < 0.001).

mulation of both TNFα and IL-1β in cell lysates (data not shown). In addition, we did not find any significant difference between values of samples, which were gently agitated, and samples incubated without shaking.

Interaction of Perfluorohexane with Lipopolysaccharide or Cytokines

The influence of a potential interaction of lipopolysaccharide with perfluorohexane in our model was studied by preincubation of lipopolysaccharide-containing RPMI medium (10 μg/ml) with different amounts of perfluorohexane followed by stimulation of MBCs with this conditioned medium. In comparison to MBCs incubated with lipopolysaccharide-containing medium without perfluorohexane exposure, we did not find any significant difference in lipopolysaccharide-stimulated release of TNFα as well as TF expression (table 1). Therefore, we could exclude that the observed inhibitory effect of perfluorohexane, at least after maximum lipopolysaccharide stimulation, is caused by nonspecific binding or sequestering of significant amounts of lipopolysaccharide.

By preincubation of TNFα-containing supernatants

with different amounts of perfluorohexane before TNFα detection by ELISA, we could exclude a direct effect of perfluorohexane on TNFα αmeasurements (not shown).

Effect of Perfluorohexane on Alveolar Macrophages from Bronchoalveolar Lavage

Samples from diagnostic bronchoalveolar lavages contained an average of $5.1 \pm 1.1 \times 10^6$ cells, and $54 \pm 11\%$ of these were macrophages. Studied under identical con-

Table 1. Effect of Preincubation of LPS-containing RPMI with PFH on MBC Stimulation (in Comparison with Controls without PFH = 100%)*

	% PFH [v/v]		
	5	10	30
TF	104 ± 14.2	98 ± 11.6	97 ± 5.6
TNF	103 ± 8.0	100 ± 7.1	102 ± 8.5

* Results are presented as mean of control (%) ± SD of five independent experiments.

LPS = Lipopolysaccharide; PFH = perfluorohexane; MBC = mononuclear blood cells; TF = tissue factor; TNF = tumor necrosis factor.
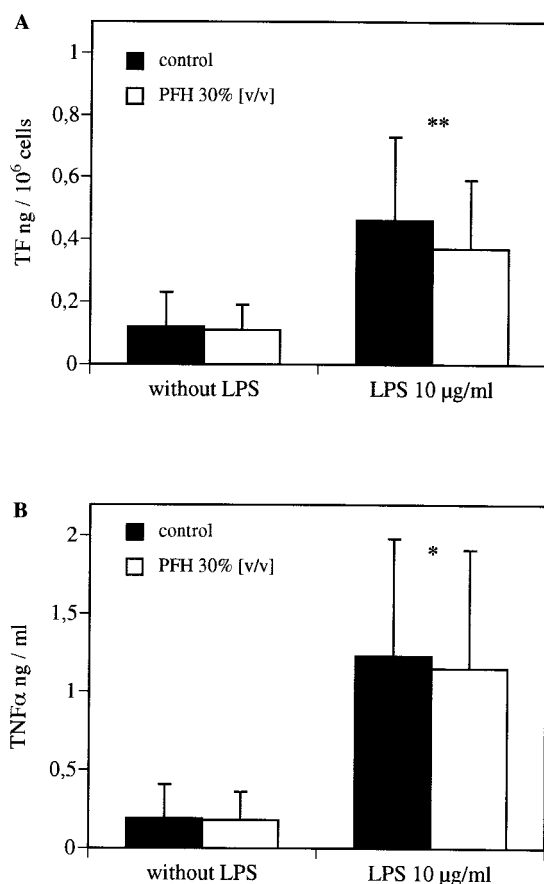
Fig. 4. Effect of perfluorohexane (PFH) on tissue factor (TF) content in cell lysates and on tumor necrosis factor (TNF)α concentrations in supernatants after *ex vivo* lipopolysaccharide (LPS) stimulation (10 μg/ml) of human alveolar macrophages prepared from diagnostic bronchoalveolar lavage fluid for 6 h. (*A*) Note the increased TF antigen content in lipopolysaccharide-stimulated alveolar macrophages compared with nonstimulated controls, which was significantly inhibited by perfluorohexane ([vol/vol] 30%). Results are presented as mean cellular TF concentration ($\pm$ SD) from 10 independent experiments; **$P < 0.01$. (*B*) Note the increased TNFα concentration in supernatants of lipopolysaccharide-stimulated alveolar macrophages compared with nonstimulated controls, which was significantly inhibited by perfluorohexane ([vol/vol] 30%). Results are presented as mean cellular TNFα concentration ($\pm$ SD) from 10 independent experiments; *$P < 0.05$.

ditions, lipopolysaccharide-stimulated AMs showed similar levels of interference by perfluorohexane (30 % [vol/vol]) as obtained for MBCs. There was a significant inhibition for TF ($P < 0.01$) antigen expression in cell lysates and for TNFα release in supernatants ($P < 0.05$; figs. 4A and 4B). We did not find any effect of perfluorohexane on AMs without *ex vivo* lipopolysaccharide stimulation.

Discussion

Perfluorocarbons are currently under investigation both as pure substances used for liquid ventilation and as blood substitutes in form of emulsified perfluorooctyl-

bromide.[4–6,26] Apart from their oxygen-carrying properties, an anti-inflammatory potential of these substances has been hypothesized.[2,8,27] Several studies have shown decreased chemotaxis, adherence, and superoxide anion generation of neutrophils after exposure to perfluorocarbon emulsions *in vitro*.[10–13] However, it was not entirely clear from these reports whether in the tested emulsions, perfluorocarbon itself or its additives might be responsible for the observed effects. Only a few studies could show that certain pure perfluorocarbons such as perfluorooctylbromide influenced directly peripheral-blood neutrophil or AM function.[12,28]

It was recently described that vaporized perfluorohexane improved pulmonary function in an ovine model of acute lung injury.[7] Although the mechanism of its action is still poorly understood, it was suggested that vaporized perfluorohexane, as other perfluorocarbons, develops beneficial effects beside improving gas exchange. To elucidate an assumed interaction of perfluorohexane with inflammatory cells in the alveolar space, we examined the *in vitro* effect of perfluorohexane on isolated MBCs and AMs. In the present report we were able to demonstrate that perfluorohexane treatment affected the release of proinflammatory cytokines (TNFα, IL-1β) as well as the expression of the procoagulant protein TF in *ex vivo* stimulated phagocytic cells. A potential interference of emulsifying agents could be excluded because we used perfluorohexane without additives. The perfluorohexane doses applied in our *in vitro* study were comparable to the volume-to-volume ratio found in the lavage fluid of animals treated with vaporized perfluorohexane *in vivo* (fluorocrit, 10–30%).[7] Therefore, the in *ex vivo* stimulated cells during the first 2–6 h observed inhibition of expression of early response genes such as TF and TNFα, which are known to be mediators rapidly activated in ARDS,[14–17,29] underline the biologic relevance of the analyzed perfluorohexane–cell interaction. Using trypan blue exclusion and flow cytometry, we could exclude that the effects of perfluorohexane treatment were caused by putative cytotoxic properties of perfluorohexane. In addition, we did not find any hints of direct perfluorohexane cytotoxicity in morphologic analysis of perfluorohexane-exposed MBCs and AMs by electron as well as video-enhanced vital microscopy. However, we observed that both activated monocytes in the MBC cultures and AMs actively phagocytosed perfluorohexane in the early contact period (1–4 h). These findings are in line with observations from other investigators, that ingested perfluorocarbon particles were detectable in phagocytic cells after perfluorocarbon exposure *in vivo*, whereas in the surrounding tissues edema, inflammation, necrosis, or fibrosis did not occur as signs of cellular damage.[30–33] Long-term application of perfluorooctylbromide in experimental studies of ARDS provided additional evidence of good biocompatibility of perfluorocarbons.[31]

To analyze the perfluorohexane effect on stimulated monocytes and AMs, we incubated the cells with increasing amounts of lipopolysaccharide. The observed perfluorohexane-mediated inhibition of lipopolysaccharide-induced cytokine release and TF expression was striking but less pronounced as compared with other cellular effects that were found in studies using both pure perfluorocarbons and perfluorocarbon emulsions.[10-13] This might be explained, in part, by the observation of Lane *et al.*,[34] that certain perfluorocarbon effects such as TNFα release by monocytic cells could be overcome by high doses of lipopolysaccharide through binding of considerable amounts of lipopolysaccharide by perfluorocarbon. In contrast, we could exclude that such an influence may cause the significant perfluorohexane-mediated inhibition of cytokine release and TF expression, at least in case of maximally stimulated cells using a large dose of lipopolysaccharide (10 μg/ml).

Although we could show that perfluorohexane droplets were phagocytosed by lipopolysaccharide-stimulated monocytes–macrophages, it was not possible to determine the cellular mechanism of the observed inhibitory perfluorohexane effect. However, our results indicate that neither a conceivable block of mediator release with consecutive intracellular accumulation nor altered kinetics of the release or the expression of proinflammatory markers are responsible for the inhibition. Further evidence of a real decrease of protein formation was found by the down-regulation of TF on the mRNA level. Taken together, from the current results it could be concluded that perfluorohexane interferes with stimulant–receptor interaction or with intracellular signal transduction pathways. Because a direct interaction of the inert perfluorohexane particles with intracellular regulator proteins or metabolic products seems unlikely, we suppose that the observed adherence and phagocytosis of perfluorohexane partially prevents endotoxin stimulation by an alteration of cell membrane structures. Thus, the known surface tension reducing effect and lipid solubility of perfluorocarbons may play a role.[35] Confirming this, Mathy-Hartert *et al.*[36] reported a time-dependent increase of endothelial cell membrane surface after incubation with perfluorooctylbromide. Such nonpharmacologically specific changes would also likely explain that the effects of perfluorohexane on cytokine release in our study did not reveal any clear dose dependency. Further support of this mechanism is given by the fact that perfluorocarbon was found to influence the expression and release of very different proinflammatory mediators and surface molecules in various cell populations stimulated with lipopolysaccharide, formyl-Met-Leu-Phe, or cytokines.[12,34,37] On the other hand, several studies that found an impaired neutrophil function caused by perfluorocarbon exposure during stimulation with phorbol myristate acetate, contradict, at least in part, the hypothesis of perfluorocarbon-induced alter-

ations on cell surface.[11-13,34] Because phorbol myristate acetate develops its stimulating effect intracellularly *via* activation of protein kinase C pathway, the contention of a modified interaction between the stimulant and the extracellular membrane receptor alone may not explain the inhibitory effects of perfluorocarbons in phorbol myristate acetate-stimulated cells. Interestingly, other surface-active compounds such as surfactants have been shown to affect transcription factor nuclear factor-κB activation.[28,38] After lipopolysaccharide stimulation, IL-1β, TNFα, and TF gene expression are known to be controlled by nuclear factor-κB.[39,40] Therefore, a suppression of gene transcription may contribute to the observed perfluorohexane effects. Further studies are required to elucidate perfluorocarbon interactions with intracellular signal transduction.

In view of therapeutic consequences, the immune modulating effects of perfluorocarbons, which have been demonstrated, might be one possible protective mechanism in tissue injury caused by local and systemic activation of inflammatory and procoagulant processes. Because a fulminant inflammatory reaction triggers the pathophysiologic course of ARDS and sepsis, the observed down-regulation of proinflammatory cytokines and the procoagulant protein TF may attenuate the severity of organ dysfunction. On the other hand, there is no evidence that the risk of nosocomial pneumonia is increased after liquid ventilation.[41] The instillation of perfluorocarbons has been shown even to reduce bacterial infiltration and to improve survival in experimental pneumonia.[42]

In summary, we found an inhibition of expression and release of procoagulant and proinflammatory mediators by *in vitro*–stimulated monocytes and AMs after exposure to biologic relevant doses of perfluorohexane. With respect to therapeutic strategies, these direct anti-inflammatory properties provide further evidence for a promising and safe therapeutic approach of perfluorohexane in acute lung injury and ARDS.

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