Exposure to perflubron is associated with decreased Syk phosphorylation in human neutrophils

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Fernandez, Rosemarie, Vidya Sarma, Ellen Younkin, Ronald B. Hirschl, Peter A. Ward, and John G. Younger. Exposure to perflubron is associated with decreased Syk phosphorylation in human neutrophils. J Appl Physiol 91: 1941–1947, 2001.—Liquid ventilation with perflubron is associated with reduced neutrophil recruitment into the lung during acute injury. Perflubron also reduces chemotactic responses, the respiratory burst, and cytokine production in neutrophils and in alveolar macrophages in vitro. In the current studies, the effect of perflubron on neutrophil chemotaxis to formyl-Met-Leu-Phe (fMLP) and phagocytosis of opsonized sheep erythrocytes (EA) correlated with decreased phosphorylation of Syk, an important intracellular second messenger in pathways regulating neutrophil functional responses. Brief (5 min) exposure of neutrophils to perflubron resulted in a dose-dependent reduction in chemotaxis to fMLP and reduced phagocytosis of EA but no apparent morphological changes as seen by electron microscopy. Concurrently, there was a reduction in both total cytosolic tyrosine phosphorylation and Syk phosphorylation. Binding studies indicated that this effect was neither a result of impaired ligand-receptor affinity nor a change in the number of fMLP receptors available on the neutrophil surface. These results suggest that perflubron nonspecifically affects cellular activation as measured by tyrosine phosphorylation perhaps by interfering with transmembrane signal transduction.

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perflubron in a dose-dependent manner reduces neutrophil chemotaxis to an fMLP gradient. We observed a similar effect on chemotaxis when neutrophil suspensions were exposed to varying surface-area (rather than volume-volume) doses. Phagocytosis of foreign erythrocytes was also reduced. Tyrosine phosphorylation was nonspecifically reduced after exposure to perflubron, and the Syk protein was among those proteins affected. Lastly, measurement of fMLP binding on perflubron-exposed neutrophils demonstrated no detectable differences in either the dissociation constant \((K_d)\) or the number of available fMLP binding sites.

**MATERIALS AND METHODS**

Alliance Pharmaceutical (San Diego, CA) provided sterile medical grade perflubron (LiquiVent) for all experiments. Anti-Syk polyclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and antiphosphotyrosine monoclonal antibody (MAB) (4G10) was obtained from Upstate Biotechnology (Lake Placid, NY). Horseradish peroxidase (HRP)-conjugated sheep anti-mouse and anti-rabbit antibodies were obtained from Amersham (Arlington Heights, IL), and rabbit control IgG was purchased from Organon-Teknika-Cappel (Malvern, PA). All other reagents were obtained from Sigma Chemical (St. Louis, MO) unless otherwise stated.

**Isolation of human neutrophils.** Fresh whole blood was obtained by venipuncture from healthy volunteers, immediately added to acid citrate dextrose, and subjected to dextran sedimentation. Neutrophils were purified by hypotonic lysis to remove remaining erythrocytes followed by centrifugation through Ficoll-Hypaque (Pharmacia Biotech, Piscataway, NJ) to remove contaminating mononuclear cells. This process yielded ≥98% neutrophils with >95% viability as determined by trypan blue exclusion. In experiments requiring cell lysis and tyrosine phosphorylation analysis, neutrophils were also treated with 5 mM diisopropyl fluorophosphate for 5 min on ice and washed three times with PBS.

**Neutrophil migration after perflubron exposure.** Dose-response relationships to perflubron were determined by exposing cell suspensions to various volumetric ratios of perflubron and in other experiments by exposing a 50% volumetric ratio of suspended cells and perflubron to various interface areas. Interface area was varied by incubating cells and perflubron in polypropylene tubes of various internal diameters. In all cases, neutrophils \((2 \times 10^6/ml)\) suspended in Eagle’s minimum essential medium (EMEM) were incubated with perflubron at room temperature for 5 min, during which time suspended cells were intermittently gently mixed with the perflubron phase with the use of a large-bore transfer pipette. More vigorous agitation of cell suspensions was carefully avoided.

After incubation, chemotaxis assays were carried out in micro-Bouyden chambers (Neuroprobe, Cabin John, MD) by using 3-μm, polystyrene/porous polycarbonate filters (Nucleopore, Pleasanton, CA). Perflubron-exposed and control cells were centrifuged, rinsed with EMEM, and resuspended at 2 × 10^6/ml in EMEM + 0.1% BSA. The chemottractant fMLP (40 nM) was added to the lower wells of each chamber, and a polycarbonate filter was interposed between the upper from lower wells. Neutrophils \((1 \times 10^5)\) were added to the upper wells, and the chambers were placed in a humidified incubator at 37°C with 5% CO₂ for 30 min. Filters were then fixed with propanol, stained with hematoxylin, mounted on slides, and evaluated for cell migration. Three replicate wells were prepared for each condition tested, and five fields were scored for each well. Filters were scored by counting the number of cells that migrated through the filter per high power field \((×400)\). Data were expressed as a migration index, the ratio of the number of migrated cells in a test chamber to the number of migrated cells in a control chamber containing no fMLP. Values are means ± SE for four experiments.

**Neutrophil phagocytosis of opsonized erythrocytes.** Isolated human neutrophils \((2 \times 10^6)\) were incubated with either 50% perflubron or PBS alone for 5 min at room temperature. Cells were then rinsed, resuspended at 2 × 10^6/ml in PBS + 1 mM CaCl₂, 1 mM MgCl₂, and activated with fMLP (40 nM) for 10 min at 37°C. Opsonized erythrocytes (EA), which had been prepared by incubating preserved sheep erythrocytes with rabbit anti-sheep erythrocyte IgG, were then added to the neutrophil suspensions. Phagocytosis was quantitated microscopically from serial aliquots of the neutrophil-erythrocyte suspension as the percentage of neutrophils containing phagocytosed red blood cells. Because of variability in phagocytic activity between blood donors, results at each time point were reported as the percentage of total phagocytosis achieved in perflubron unexposed control neutrophils at 30 min. All assays were performed in triplicate, and reported values represent means ± SE for three experiments.

**Total cytosolic tyrosine phosphorylation.** Phagocytosis assays were executed as described above. At designated time points, 2-ml samples were taken for analysis. Residual unopsonized erythrocytes were removed by hypotonic lysis, and the neutrophils pelleted in a centrifuge. Neutrophils were then lysed by resuspension in buffer containing 1% Triton X-100, 50 mM Tris, pH 8.0, 100 mM NaCl, 1 mM Na₃VO₄, 1 mg/ml leupeptin, 1 mg/ml aprotinin, 10 mg/ml soybean trypsin inhibitor, and 1 mM phenylmethylsulfonyl fluoride. Zero-minute time points represented cells that had been activated with fMLP but had not been exposed to EA. Lysates were clarified by centrifugation at 22,000 g for 10 min and combined with 4× SDS-PAGE sample buffer containing Na₃VO₄, boiled for 5 min, and separated by 7.5% SDS-PAGE. Proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Schleicher and Schuell, Keene, NH) and blocked with 2% BSA in PBS containing 1 mM EDTA, 0.05% Tween 20, and 1 mM Na₃VO₄. The membrane was probed with antiphosphotyrosine MAb 4G10 in blocking buffer (1:1,500), washed three times with 0.2% Tween 20 in 50 mM Tris and 100 mM NaCl, and then incubated with HRP-conjugated sheep anti-mouse antibody (Ab) (1:10,000) in wash buffer containing 5% nonfat dry milk. Phosphorylated bands were visualized with the use of the enhanced chemiluminescence system and Hyperfilm (Amersham). Experiments were performed in triplicate, and photographs of representative results are reported.

**Cytosolic Syk phosphorylation.** Neutrophil lysates were prepared as described above. Lysates (∼100 μg protein) were precleared with protein-A Sepharose beads for 30 min and then incubated overnight at 4°C with either 2 μg anti-Syk Ab or 2 μg rabbit IgG. Next, protein-A Sepharose was added to each sample and incubated for 2 h with rotation at 4°C. The beads were washed thoroughly with lysis buffer, and adsorbed proteins were solubilized in sample buffer and separated on 7.5% SDS-PAGE. Transfer to PVDF and subsequent immunoblotting with 4G10 antiphosphotyrosine MAb were then conducted as outlined above. The membranes were photographed and then were stripped with 100 mM β-mercaptoethanol, 2% SDS, and 62.5 mM Tris (pH 6.7) at 50°C. Finally, the membranes were reprobed with anti-Syk Ab to
demonstrate equivalent loading of immunoprecipitated protein.

Perflubron interference with electrophoresis and the detection of phosphorylated cytosolic proteins. Perflubron is a liquid immiscible in aqueous media. However, because of its density, the material is difficult to separate from cell pellets during washing by centrifugation. Trace quantities of perflubron, therefore, likely contaminated the cell lysates used to determine cytosolic protein phosphorylation in our experiments. Additional experiments were conducted to ensure that any changes in protein phosphorylation noted were not a result of perflubron interference with the immunoblotting process (including protein migration during PAGE, protein transfer to nylon membrane, and Ab detection of membrane-immobilized protein). Cell lysates from the human squamous cell carcinoma cell line A341 (Upstate Biotechnology) that had been incubated with epidermal growth factor (known to induce cytosolic tyrosine phosphorylation) were exposed to perflubron in a one-to-three ratio for 5 min. Next, 10 μl of the aqueous phase were subjected to PAGE, membrane immobilization, and probing with 4G10 antiphosphotyrosine as described above. Simultaneous controls of cell lysates exposed to perflubron were also studied.

Neutrophil fMLP receptor kinetics. Isolated human neutrophils were incubated with 50% perflubron or PBS alone and suspended in cold EMEM + 1% BSA before initiation of binding experiments. Cells then were rinsed and resuspended in EMEM + 0.1% BSA. Suspended cells (1 × 10^7) were placed into polypropylene tubes containing various concentrations of [3H]-labeled fMLP and either unlabeled fMLP or buffer alone. Tubes were incubated on ice for 30 min with periodic shaking. Binding was halted by centrifuging cell suspensions through a 25% sucrose gradient and then flash freezing the cell pellet. Cell pellets were counted in a gamma counter. Nonspecific binding was defined as ligand binding not inhibited in the presence of 1,000-fold excess unlabeled fMLP. Specific binding was defined as the mathematical difference between nonspecific and total radioactivity bound. The number of receptor sites per neutrophil and the K_d were determined using Scatchard analysis of triplicate measurements. The reported results reflect ≈4 separate experiments.

Neutrophil morphology after perflubron exposure. A previous report has noted the presence of numerous cellular inclusions in phagocytes exposed to perflubron, presumably representing perfluorocarbon-filled vacuoles (17). To determine if phagocytosis of perflubron was occurring in our system, some neutrophil suspensions were reserved for electron microscopy. Cells were isolated and incubated with perflubron as described above and then fixed in a 4% glutaraldehyde in cacodylate buffer. Pelleted cells were then mounted, stained, and photographed using transmission electron microscopy at a magnification of ×10,000.

Statistical analysis. All experiments were performed at least in triplicate on neutrophils from at least three different donors. The local institutional review board approved the experimental protocol. The inhibition of chemotaxis seen with both volumetric and interface surface area dosing was tested with linear regression. Differences in phagocytosis following exposure to perflubron were examined using repeated-measures ANOVA followed by Tukey’s post hoc testing at individual time points. The effects of perflubron fMLP binding parameters were tested with two-tailed nonpaired t-tests. All results are presented as means ± SE, and a P value < 0.05 was considered statistically significant.

RESULTS

Perflubron inhibits fMLP-stimulated chemotaxis of human neutrophils. Brief incubation with perflubron produced dose-dependent inhibition of neutrophil migration in response to 40 nM fMLP (Fig. 1). Because neutrophils were washed with fresh buffer after perflubron exposure and before being placed in the chemotactic chamber, the reduced activity would appear to be because of an effect other than physical coating of the cells. Maximal inhibition was achieved with a 1:1 dilution of perflubron and could not be overcome by increased concentrations of fMLP. A dose–response effect was also observed in experiments varying the physical area of perflubron, i.e., aqueous interface, rather than the volume dose of perflubron (Fig. 1). Trypan blue exclusion assays demonstrated that cell viability was not affected between perflubron-treated and untreated cells (data not shown).

Perflubron inhibits neutrophil phagocytosis and tyrosine phosphorylation. Phagocytosis was detectable within 30 s of addition of EA (Fig. 2A). Neutrophils that had been incubated with perflubron demonstrated reduced phagocytosis at each measured time point, a difference that was statistically significant by 30 min.
Corresponding to the blunted phagocytic response was a global decrease in tyrosine phosphorylation in neutrophil lysates obtained at varying time points (Fig. 2B).

In vitro incubation with perflubron reduces Syk phosphorylation in stimulated neutrophils. Evidence for reduced tyrosine phosphorylation of Syk after neutrophil exposure to perflubron is shown in Fig. 3. Western blot analysis of immunoprecipitated Syk from neutrophil lysates showed increased tyrosine phosphorylation within 30 s of neutrophil exposure to erythrocytes. This response was almost completely abolished in neutrophils pretreated with perflubron, except for faint evidence of tyrosine phosphorylation at the 10- and 30-min intervals.

Exposure to perflubron does not interfere with immunoblotting. Cell lysates from EGF-stimulated A341 cells were subjected to PAGE, membrane immobilization, and Ab probing for phosphotyrosine. Exposure to perflubron did not alter either the apparent molecular weight or the intensity of the final probed bands (Fig. 4).

Fig. 2. Decreased responsiveness to sensitized sheep erythrocytes. A: phagocytosis was measured by the number of neutrophils containing erythrocytes at various time points. Plotted values are expressed as the percentage of maximal response, i.e., the number of phagocytosis-positive control neutrophils at the 30-min time point. Repeated-measures ANOVA demonstrated differences between the curves (P < 0.05), and Tukey's post hoc testing was positive at 30 min; n = 4 in each group. B: Western blot analysis (WB) of total cytosolic phosphotyrosine (PY) residues in neutrophil lysates. A nonspecific reduction in PY-containing proteins is seen in the perflubron-treated group. The image shown is representative of 3 separate experiments. *Significant difference (P < 0.05). MM, molecular mass.

Fig. 3. Altered Syk phosphorylation after treatment with perflubron. Lysates from control and perflubron-treated neutrophils were immunoprecipitated (IP) with anti-Syk antibodies and then examined with WB for PY (top) or for total Syk content (bottom) to confirm uniform yield during immunoprecipitation and blotting. In control cells, phosphorylated Syk was evident within 30 s of exposure to sensitized erythrocytes, whereas perflubron-treated cells had a delayed and diminished response. Image shown is representative of 3 separate experiments.

Fig. 4. Effect of perflubron exposure on immunoblotting of PY-containing cytosolic proteins. To confirm that changes in cytosolic tyrosine phosphorylation in our studies were not a result of nonspecific interference by perflubron, cell lysates from epidermal growth factor-stimulated A341 cells were incubated with perflubron and then subjected to PAGE, membrane immobilization, and probing with 4G10 anti-phosphotyrosine antibody. No differences in apparent molecular mass or band intensity were noted between perflubron-exposed (left) and -unexposed (right) lysates. Image shown is representative of 3 separate experiments.
Exposure to perflubron does not alter fMLP binding kinetics on neutrophils. The apparent nonspecific reduction in tyrosine phosphorylation among perflubron-treated neutrophils raised the possibility of impaired fMLP binding to its surface receptor after perflubron exposure. To address this question, fMLP binding studies were performed. As shown in Table 1, exposure to perflubron produced no detectable change in either the number of fMLP binding sites per cell or the receptor-ligand $K_d$.

Functional changes in neutrophils are not associated with morphological changes. Examination of perflubron-treated cells by transmission electron microscopy demonstrated no clear morphological changes compared with neutrophils that were not exposed to perflubron. Specifically, there was no evidence of perflubron-containing vacuoles or obvious abnormalities of the plasma membrane (Fig. 5).

**DISCUSSION**

Numerous studies have demonstrated decreased neutrophil accumulation in the lung and in models of neutrophil-mediated injury of lung during liquid ventilation with perflubron (2, 11, 22). Experiments in vitro using either isolated neutrophils or alveolar macrophages have similarly shown changes in function in the presence of perflubron (16, 19, 20). Our data demonstrate that the decreased chemotaxis and phagocytosis in part may be because of reduced intracellular tyrosine phosphorylation and, in particular, decreased phosphorylation of an intracellular signaling protein necessary for normal chemotaxis and phagocytosis, namely Syk. It has been reported that macrophages derived from Syk-deficient mice are defective in phagocytosis and FcγR-mediated signaling events (3). Furthermore, downregulation of Syk expression in monocytes by antisense oligonucleotides leads to abrogation of FcγRIIA-mediated phagocytosis (12). These findings further support our studies that Syk activation is required for phagocytosis.

The way in which perflubron interferes with cytosolic phosphorylation events is unclear. Our results indicate that perflubron did not interfere with ligand binding during fMLP priming of neutrophils. Rather, available data point to membrane-related effects of perflubron exposure. It has recently been shown that exposure to numerous perfluorocarbon liquids reduces osmotic fragility in erythrocytes and also decreases the collagen-stimulated aggregation of platelets, both of which are membrane-associated phenomena (14). Interestingly, the magnitude of both effects is directly proportional to the lipid solubility of the perfluorocarbon tested, suggesting the liquids may exert their effect by partitioning into and altering the physical behavior of biological membranes. Similar alterations at the cell membrane during transmembrane signaling leading to Syk phosphorylation would explain our findings. Recent studies indicate that membrane-associated integrins can also play a role in phagocytosis (4) and that Syk activation is coupled to integrins (13). The downregulation of Syk activation seen in our studies may in part be due to perturbation of integrin signaling by perflubron. Further study is needed to more completely define the effect of perflubron during receptor-ligand interactions as well as transmembrane signaling, which could potentially mitigate in vivo inflammatory response in a lung-injured patient.

**Table 1. fMLP receptor parameters on perflubron-treated neutrophils**

<table>
<thead>
<tr>
<th>Control Neutrophils ($n = 5$)</th>
<th>Perflubron-Treated Neutrophils ($n = 4$)</th>
<th>95% Confidence Interval for Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Binding site/cell 22,000 ± 2,700</td>
<td>24,300 ± 3,200</td>
<td>2,400, −7,000</td>
</tr>
<tr>
<td>$K_d$, nM 31 ± 4</td>
<td>29 ± 2</td>
<td>7, −3</td>
</tr>
</tbody>
</table>

Values are means ± SE. $K_d$, dissociation constant; fMLP, formyl-Met-Leu-Phe.
There are several limitations to the present studies. It is possible that, in addition to altering transmembrane signaling, perflubron may also affect other membrane-related or intracellular processes necessary for normal phagocytosis. That exposure of neutrophils to perflubron reduces functional cell responses to FMLP (without altering the binding parameters of FMLP) and phagocytic responses after exposure to EA suggests that perflubron may be exerting its effects at a point beyond the ligand-receptor interaction. Whether this could be inhibition of G protein signaling, generation of IP3, and so forth, remains to be determined. Extensive membrane remodeling is known to occur during phagocytosis (18), and perflubron may somehow interfere with this process. Our experiments examined only events related to the signaling pathway that initiates phagocytosis. Effects of perflubron on the cytoskeleton or other effector portions of the phagocytic process are also possible. This question might be addressed in future studies by examining cellular responses that require a membrane-independent stimulus and a membrane-dependent response. Furthermore, although our work and previous studies have concentrated on inflammatory cells, perflubron’s effects may not be limited to hematopoietic cells; effects on other lung cellular constituents deserve further study.

Lastly, the in vitro model used in our studies does not completely reflect the in vivo conditions of partial liquid ventilation. Tidal breaths of gas during mechanical ventilation constantly agitate intrapulmonary perflubron during partial liquid ventilation. As a result, resident inflammatory cells may physically encounter more turbulent conditions than those experienced by the neutrophils in our studies. Cellular activation through continuous physical deformation might explain why others have described intracytoplasmic inclusions of perflubron (presumably a result of phagocytosis), whereas electron microscopy of cells in our experiments revealed no such inclusions. Furthermore, early in acute injury, neutrophils are extravascular, not intraalveolar, such that direct contact between cells and the perflubron residing in the airspaces may not occur. Work with cultured endothelial cells has shown that perflubron is capable of partitioning into cell membranes when the cells and the liquid are brought into close proximity without direct contact (21).

In conclusion, incubation of neutrophils with perflubron reduced chemotaxis and phagocytosis in an in vitro system. The reduction in function corresponded to decreased Syk phosphorylation in the setting of diffusely and apparently nonspecifically reduced intracellular tyrosine phosphorylation. These results represent the first direct evidence that perflubron affects intracellular signaling in inflammatory cells, which could potentially lead to a decrease in the inflammatory response. Further efforts to localize this agent’s mechanism of action should lead to a better understanding of its effects during partial liquid ventilation in injured lungs.

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