NO₂ interfacial transfer is reduced by phospholipid monolayers

LYDIA M. CONNOR,¹ AKHIL BIDANI,¹ JON GOERKE,²,³ JOHN A. CLEMENTS,²,⁴ AND EDWARD M. POSTLETHWAIT¹
¹Pulmonary and Critical Care Medicine, Department of Internal Medicine, University of Texas Medical Branch, Galveston, Texas 77555; and ²Cardiovascular Research Institute, Departments of ³Physiology and ⁴Pediatrics, University of California at San Francisco, San Francisco, California 94143

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Connor, Lydia M., Akhil Bidani, Jon Goerke, John A. Clements, and Edward M. Postlethwait. NO₂ interfacial transfer is reduced by phospholipid monolayers. J Appl Physiol 91: 2024–2034, 2001.—Nitrogen dioxide (NO₂) is a ubiquitous, pollutant gas that produces a broad range of pathological and physiological effects on the lung. Absorption of inhaled NO₂ is coupled to near-interfacial reactions between the solute gas and constituents of the airway and alveolar epithelial lining fluid. Although alveolar surfactant imparts limited resistance to respiratory gas exchange compared with that contributed by either the pulmonary membrane or uptake in red blood cells, resistance to NO₂ flux could have a significant effect on NO₂ absorption kinetics. To investigate the effect of interfacial surfactant on NO₂ absorption, we designed an apparatus permitting exposure of variously compressed monolayers. Our results suggest that compressed monolayers enriched in 1,2-dipalmitoyl-sn-3-glycero-phosphocholine present significant resistance to NO₂ absorption even at surface tensions greater than those achieved in vivo. However, monolayers composed of pure unsaturated phospholipids failed to alter NO₂ absorption significantly when compressed, in spite of similar reductions in surface tension. The results demonstrate that phospholipid monolayers appreciably limit NO₂ absorption and further that monolayer-induced resistance to NO₂ flux is related to physicochemical properties of the film itself rather than alterations within the aqueous and gas phases. On the basis of these findings, we propose that pulmonary surfactant may influence the intrapulmonary gas phase distribution of inhaled NO₂.

nitrogen dioxide; reactive absorption; pulmonary surfactant; interfacial resistance; lung epithelial lining fluid; dipalmitoyl phosphatidylcholine

NITROGEN DIOXIDE (NO₂) is a free radical atmospheric toxicant that initiates a broad range of pulmonary pathophysiological responses. Inhalation of NO₂ induces dose-dependent effects on both conducting airway and alveolar epithelia. Acute low-level exposures result in oxidative stress, the induction of characteristic epithelial lesions spanning the bronchoalveolar region, and inflammation (13, 44). Chronic NO₂ exposure produces alterations in lung architecture and obstructive lesions that result in a reduction of both available alveolar surface area and lung compliance (13, 44). Although the pathological consequences of both acute and chronic NO₂ intoxication have been extensively documented, the mechanisms that govern NO₂ intrapulmonary dispersion and the induction of tissue injury have not been fully characterized.

The distribution of acute epithelial injury is related, in part, to the intrapulmonary airspace concentration of NO₂ and its local rate of absorption. The pulmonary epithelium is overlain by a continuous but inhomogeneous aqueous layer, epithelial lining fluid (ELF), which covers all airspace surfaces and protrusions (2). Absorption of inhaled NO₂ is governed by near-interfacial reactions with ELF constituents that serve to maintain the net driving force for the flux of NO₂ from the gas phase into the ELF, a process termed “reactive absorption” (33). Because absorption is directly coupled to reaction, diffusion of NO₂ through the ELF is presumed to be limited (3, 35, 52). Products formed as a consequence of reactive absorption likely initiate the cascade of events leading to cell injury.

The small-molecular-weight antioxidants glutathione, ascorbic acid, and uric acid have been identified as primary substrates for NO₂ reactive absorption (35, 52). Interestingly, although unsaturated fatty acids as components of phospholipids appear in abundance in the lung aqueous surface compartment, under environmentally relevant conditions, NO₂ reacts less with unsaturated fatty acids than with other available substrates (14, 35, 37, 38). NO₂ reactive absorption proceeds most rapidly during electron transfer reactions with antioxidant anions, which can exist in significant concentration at lung ELF pH (33, 35, 52). Under quasi-steady-state, well-mixed exposure conditions (NO₂ inflow constant), NO₂ absorption rapidly attains an aqueous substrate concentration-dependent rate that does not increase with time although the

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uptake efficiency is <100% (33). This phenomenon can be observed under transient exposure conditions (NO₂ as the limiting reagent) in which the first-order rate constant for the disappearance of NO₂ from the gas phase plateaus despite further increases in aqueous-phase substrate concentration or mixing (34). These in vitro studies are in agreement with theoretical analyses that suggest that appreciable mass transfer limitations are present at the gas-liquid interface (3, 10).

Pulmonary surfactant functions to prevent alveolar collapse at end-expiratory lung volumes by lowering surface tension and, thereby, maintaining high lung compliance (9, 47). The most abundant surfactant active component is 1,2-dipalmitoyl-sn-3-glycero-phosphocholine (DPPC), which may be enriched within the monomolecular film present at the air-liquid interface (30, 51). In general, the flux of respiratory gases across the serial resistances encountered in transit from the air space to red blood cells (e.g., surface fluid compartment, tissue, plasma, and red blood cell) is thought to proceed with limited restriction (53). However, previous experimental evidence suggests that the physicochemical characteristics of the gas-liquid interface may influence the flux of molecules between the gas and aqueous phases. For example, interfacial films of oils and aliphatic alcohols have been demonstrated to limit significantly the in vitro rate of water evaporation (1, 23), CO₂ absorption (4), and O₂ transport (21) across gas-liquid interfaces and to restrict the rate of gas-liquid reactions (10). On the basis of these previous observations, we investigated whether monolayers of surface active phospholipids alter the interfacial flux (absorption) of NO₂. We utilized an in vitro exposure apparatus, which facilitated 1) control of phospholipid monolayer and aqueous phase composition, 2) continuous measurement of surface tension, and 3) determination of steady-state NO₂ absorption rates.

METHODS

Reagents. DPPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1,2-dipalmitoyl-sn-glycero-3-phosphorac-glycerol (DPPG), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-rac-glycerol (POPG), and 1,2-dilinoleoyl-sn-glycero-3-phosphocholine (DLPC), all in powder form, were purchased from Avanti Polar Lipids (Alabaster, AL) and used as received. Glutathione (GSH), ascorbic acid (AH₂), desferrioxamine, and SeKem agarose were purchased from Sigma Chemical (St. Louis, MO). Tween 80, HPLC-grade chloroform, methanol, and isopropanol and all other reagents, of analytical grade, were purchased from Fisher Scientific (Houston, TX). NO₂ and nitric oxide cylinders were purchased from Liquid Carbonic (Pasadena, TX) as certified standards in N₂.

Surfactant isolation. Surfactant was isolated from viral antigen-free, 250–275 g, male Sprague-Dawley rats (Harlan Sprague Dawley, Houston, TX). The protocol for harvesting bronchoalveolar lavage fluid (BALF) was approved by the Animal Care Use Committee of the University of Texas Medical Branch, Galveston. Animals were allowed free access to water and food before induction of anesthesia. Animals were anesthetized with 70 mg/kg intraperitoneal pentobarbital sodium and, after tracheal cannulation and midline thoracotomy, lungs were lavaged in situ five times with 9.0 ml of 0.15 M NaCl. The BALF was pooled, centrifuged at 150 × g for 10 min to remove cells and debris and subsequently at 60,000 × g for 2.5 h to isolate the surface-active components (modified from Stevens et al., Ref. 50). The pellet was extracted by a modified Bligh-Dyer method (5), with subsequent double reextraction of the upper aqueous phase. The pooled crude extract was dried under N₂ and redissolved in chloroform for gravimetric estimation of total extractable material. These weights probably overestimate the true phospholipid weights because of the presence of other lipids and hydrophobic proteins. For surface deposition, solutions (1 mg/ml) were prepared in chloroform, stored under N₂ at −20°C, and used within 72 h.

Compressed monolayer studies. An exposure probe-surface balance with a pantographic barrier was utilized to examine NO₂ absorption across phospholipid monolayers at several surface tensions (24 ± 1°C) (Fig. 1A). Surface tension was measured as described below. The glass probe was modeled after a previously described design (48). Briefly, pollutant gas entered the probe chamber via a sidearm port and exited around a disk such that flow proceeded centrally over a defined surface area (28.3 cm²) (Fig. 1B). The sampling line of the nitrogen oxides (NOx) analyzer (see below) was connected to the exit port to actively withdraw gas through the central tube. Gas flow into the exposure system exceeded the sam-

![Fig. 1. Schematic representation of the exposure probe-surface balance apparatus. Individual components are identified by letters. A: Pantograph barrier. Glass exposure probe (a); hinged, deformable stainless steel frame with Teflon ribbon lining (maximum surface area = 387 cm²), minimum surface area = 157 cm²) (b); platinum plate (c); and manual leadscrew (d). Flexiglas dish (in which the compression frame was mounted), adjustable platform, force transducer assembly and enclosure are not shown. B: Vertical cross-section of the probe and aqueous interface: gas inlet port (e); has exit port to nitrogen oxides (NOx) analyzer (f), central restriction disk (g), height = 5 mm (h), and clearance = 1 mm (i).](https://jap.physiology.org/doi/10.1152/jappl.00015.2002)
plunging rate by 10–15%. The compression apparatus consisted of a stainless steel, Teflon ribbon-lined pantograph frame (17 × 25 cm) mounted in a Plexiglas dish (20 × 45 cm), which contained the aqueous phase. Phospholipids were deposited within the pantograph frame from chloroform solutions (1 mg/ml) by use of a 25-µl syringe to achieve an initial area per molecule of 121 Å²/molecule (−0.1 µg/cm²). The compression system was mounted on a moveable platform to permit adjustment of the distance between the aqueous surface and the bottom edge of the probe. The entire apparatus was isolated in an enclosure to prevent room air currents from disturbing the flow pattern under the probe.

\[ \text{NO}_2 \text{ exposure protocols.} \] For exposure of compressed films, 500 ml of buffer (10 mM potassium phosphate, pH 7.0) with or without GSH (1.0 mM) was placed in the Plexiglas dish, and the aqueous surface was cleaned by aspiration. The system was raised to achieve a 1-mm separation between the bottom surface of the probe and the aqueous surface. The probe gas inlet and exit lines were connected, and the system enclosure was closed. After a 10-min control exposure period, a phospholipid monolayer was deposited from solvent through a port and allowed to equilibrate for 5 min. Subsequently, three full compression-expansion cycles were performed before compression to the final set area(s) to achieve a uniform phosphatidylcholine (PC) film devoid of bare areas. Alternatively, exposures were conducted in a different apparatus utilizing 50-ml flat-bottom Erlenmeyer flasks (33) when it became necessary either to limit the aqueous phase volume or to prevent contamination of the compression system. For these exposures, 10 ml of buffer (10 mM or 50 mM potassium phosphate, pH 7.0) with or without GSH (1.0–50.0 mM) or AH₂ (1.0 mM) was introduced into the flask. A phospholipid film was deposited at a nominal surface density of 0.5 µg/cm² onto a surface area of 15.9 cm² and allowed to equilibrate for 10 min before NO₂ delivery. Flasks were equipped with a Teflon-lined stopper with inlet and exit ports that extended within 1.0 and 5.0 cm above the interface, respectively, with the outlet port being sampled by the NOx analyzer. Films were not compressed after spreading in this system. Exposures generally lasted 30–45 min. Inlet NO₂ concentrations \([\text{NO}_2]_i\) were determined before and after each exposure with the probe or flask out of line.

\[ \text{NO}_2 \text{ exposures.} \] Exposures conducted in both the probe and flask systems were performed under unstirred, quasi-steady-state conditions (NO₂ inflow constant) at 25°C unless stated otherwise (34). For most studies, \([\text{NO}_2]_i\) was <10 ppm. In this range, NO₂ absorption displays first-order kinetics with respect to \([\text{NO}_2]_i\) (34). GSH (1.0 mM) was used most often as the aqueous substrate because it effectively drives NO₂ absorption (35, 52) and can be maintained in stable concentration with limited autoxidation. Exposure atmospheres were generated by countercurrent injection of high-concentration NO₂ (in N₂) into temperature-equilibrated, humidified air by using mass flow controllers (Scott Specialty Gas, Houston, TX) adjusted to achieve the appropriate initial surface density from a single exposure, after the three initial compression-expansion cycles. Surface tension was measured directly on all monolayers studied in the probe-surface balance apparatus. Because of geometric limitations, surface tension measurements for the flask studies were determined separately on films contained in open glass vessels of equivalent interfacial geometry.

\[ \text{Reaction product analyses.} \] Both probe and flask aqueous pools were sampled for reaction products. Nitrite (NO₃⁻) concentrations were determined via the Greiss reaction (36) using standards containing 1.0 mM GSH. Glutathione analyses were performed via HPLC (18). Briefly, immediately after the termination of an exposure, 1.5 ml of aqueous phase was withdrawn, diluted 1:2 in HPLC mobile phase, and stored at 4°C in the dark. Samples (15 µl) were injected onto a 15 × 150-mm Waters C18 Novapak column and eluted isocratically at 1.0 ml/min by using 3% methanol in 10 mM phosphate buffer (pH 3.0) containing 50 µM tetrabutyl-ammonium hydrogen sulfate. Peaks were detected at 220 nm and integrated to compute peak area. GSH was estimated on the basis of standard curve peak areas. Each sample was analyzed in triplicate, and a mean peak area was used to compute concentration.

\[ \text{Data analysis.} \] All values are expressed as means ± SD. Mean differences between groups were tested by ANOVA and Dunnett’s test post hoc (49). Significance was defined as \( P < 0.05. \)

\[ \text{RESULTS} \]

Effect of DPPC monolayers on NO₂ absorption. DPPC monolayers were deposited onto 1.0 mM GSH solutions and exposed to NO₂ for up to 1 h. During the course of a single exposure, after the three initial compression-expansion cycles, monolayers were compressed to four set areas from an initial molecular area of 121 to 49 Å²/molecule. The presence of the probe prevented further reductions in interfacial surface area or surface tension. Between 10 and 15 min were allotted at each stage of compression to permit determination of NO₂ absorption as well as measurement of stable surface tensions. As demonstrated in Fig. 2, when the DPPC monolayers were cycled compressively and reexpanded, NO₂ absorption rates were coupled to surface tension. NO₂ uptake decreased linearly with surface tension (Fig. 3; \( r = 0.99 \)), attaining a maximal reduction of 47% from the monolayer-free surface value (\( P < 0.05 \)). On compression, NO₂ uptake rates were stable over >45-min periods.
Similar results were obtained when exposures were conducted at elevated temperatures (∼30°C, [NO₂] = 7.6 ± 0.2 ppm, 44.3 ± 1.7, and 34.5 ± 2.3 ng·min⁻¹·cm⁻² without and with compressed DPPC, respectively, data not shown). In contrast to previous observations (33), under the experimental conditions employed we observed only a modest rise in NO₂ uptake when the temperature of uncompressed DPPC systems was increased. Background NO₂ loss was approximately twofold greater at the elevated temperature, likely because of condensation of water on system surfaces, increased NO₂ reaction with water, and increased diffusivity of solute NO₂ and nitrite within the aqueous phase. The limited temperature effect likely resulted from an inability to maintain the elevated temperatures (initial temperature ≈ 37°C) so that analyses were conducted under somewhat lower actual temperatures (gas phase ≈ 31°C, bulk liquid temperature ≈ 29°C). However, compression consistently induced significant decreases in the NO₂ absorption rates.

Surface tension dependence. Because NO₂ uptake fell with surface tension, we investigated whether this was related to surface tension or the nature of the interfacial film. To do this, we used a soluble detergent, Tween 80, to reduce surface tension, and we conducted NO₂ exposures using GSH + Tween 80 mixtures. To avoid possible contamination of the compression system by the Tween 80, we used the exposure flasks. A 0.1% solution of Tween 80 satisfied the criteria of significantly reducing basal surface tensions (70.3 ± 3.6 to 39.5 ± 2.2 mN/m, P < 0.05) while also permitting the deposition of stable phospholipid films. Despite reductions in surface tension, Tween 80 solutions failed to reduce the rate of GSH-mediated NO₂ absorption (Fig. 4). By comparison, when DPPC monolayers (Fig. 3) were compressed to the same average surface tension achieved by 0.1% Tween 80 alone (γ = 39.5 ± 2.2 mN/m), DPPC monolayers reduced NO₂ absorption ∼32%. Similarly, when DPPC films were deposited (0.5 µg/cm²) onto Tween 80 solutions, NO₂ uptake significantly declined by 52% (Fig. 4), suggesting that the presence of saturated interfacial phospholipids, rather than surface tension per se, inhibited NO₂ flux.

Fig. 2. Effect of cyclic surface compression on simultaneously recorded surface tension (A) and NO₂ concentration ([NO₂]) at exit port ([NO₂]e) (B). 1,2-Dipalmitoyl-sn-3-glycero-phosphocholine (DPPC) monolayers were deposited onto 1.0 mM glutathione (GSH) and exposed to 5.2 ppm NO₂. Monolayers were compressed in the surface balance reducing surface area by 60% at maximum compression (from an initial phospholipid surface density of 121 Å²/molecule) through 3 compression and 2 expansion cycles. Plots represent typical compression/expansion cycles that preceded monolayer exposures at selected set area(s). Decreasing the area in the absence of a surface film did not change either surface tension or [NO₂] in the exit gas. The plot of [NO₂] slightly lags the plot of monolayer surface tension because of delayed NO₂ analyzer response.

Fig. 3. Relationship between NO₂ absorption and surface tension with graded compression of DPPC monolayers in the surface balance. DPPC was deposited at an initial surface density of 121 Å²/molecule (−0.1 µg/cm²) onto 1 mM GSH solutions and exposed to 5.2 ppm NO₂ for ∼45 min at 25°C. Monolayers were compressed in stepwise fashion while [NO₂]e was monitored for ∼10 min at each compression point. Surface tensions of 71.4 ± 1.5, 60.6 ± 1.3, 53.8 ± 2.0, 47.2 ± 4.5, and 26.5 ± 4.4 were recorded at areas of 387, 230, 203, 186, and 157 cm², respectively. NO₂ absorption decreased linearly (r = 0.99) with surface tension. At the end of each experiment, [NO₂]e returned to baseline levels on surface reexpansion. Values are means ± SD for n ≥ 6.
Gas-phase NO₂ and aqueous-phase substrate concentration dependence. The gas-phase concentration dependence of monolayer-induced effects was investigated utilizing the probe/surface balance apparatus with [NO₂]ᵢ between 4 and 125 ppm. The Saltzman procedure for NO₂ analysis was employed for [NO₂]ᵢ.

DPPC monolayers were deposited onto GSH solutions and exposed to 4, 9, 19, or 125 ppm NO₂ for 30 min. DPPC-induced reductions in NO₂ uptake were significant (*P*, 0.05) at all NO₂ concentrations studied (Fig. 5). Exposures were also conducted to determine whether substrate composition and concentration influenced the observed monolayer-induced effect on NO₂ absorption. Flask exposures were conducted with DPPC films deposited onto solutions containing 1.0 mM AH₂ and exposed to 5 ppm NO₂ for 30 min. Desferrioxamine (50 μM) was added to AH₂ solutions to limit autoxidation. As in the experiments with GSH, AH₂-mediated NO₂ absorption was significantly reduced from 48.0 ± 6.0 to 16.6 ± 1.2 ng·min⁻¹·cm⁻², suggesting that the monolayer-induced effects were independent of aqueous substrate composition. In a separate series of experiments, the effect of interfacial films was assessed over a broad range of substrate concentrations. Again DPPC films were deposited onto solutions containing 1, 10, or 50 mM GSH. Relative to the GSH controls, the addition of DPPC films significantly decreased NO₂ absorption regardless of substrate concentration (Fig. 6).

**Reaction product analysis.** To investigate whether monolayer-induced decrements in NO₂ absorption were related to altered reaction pathways, we measured GSH utilization and the reaction products of NO₂. Under physiological conditions, NO₂ undergoes quantitative univalent reduction to NO. Both probe and flask exposures were conducted to determine whether surface films modulated the stoichiometry between NO₂ uptake and NO formation (Table 1). For the probe exposures, a round Teflon dish (66 × 4 mm

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Fig. 4. Effect of detergent on NO₂ absorption. Buffer or 0.1% Tween 80 solutions with or without 1 mM GSH were exposed to 4.6 ppm NO₂ for 15 min in the presence (hatched bar) or absence (open bars) of a DPPC film in the flask system. DPPC was deposited at an initial surface density of 0.5 μg/cm² and allowed to equilibrate for 15 min before exposure initiation. Despite achieving low surface tensions (listed above bars), 0.1% Tween 80 had no effect on NO₂ absorption. NO₂ absorption was significantly reduced, however, when DPPC monolayers were deposited onto the Tween 80 solutions. Although surface tension was further reduced by the DPPC (24.3 mN/m), it did not differ from surface tensions achieved with buffer + DPPC in the absence of Tween 80 (25 mN/m). *NO₂ uptake was significantly lower when DPPC was added to the Tween-GSH-containing system (ANOVA; *P* < 0.05). Values are means ± SD for *n* ≥ 5.

Fig. 5. Relationship between NO₂ gas phase concentration at inlet port ([NO₂]ᵢ) and monolayer-induced effects on NO₂ absorption (A) and fractional uptake (B). DPPC monolayers, deposited at an initial surface density of 121 Å²/molecule onto 1 mM GSH in the surface balance, were exposed to 4, 10, 20, 90, or 125 ppm NO₂ (DPPC uncompressed = open bars, DPPC compressed = hatched bars). Exposures of uncompressed monolayers proceeded for 10 min followed by maximal compression (achieving a 60% reduction in surface area) and continued exposure for an additional 20 min. At [NO₂] > 20 ppm, monolayers were allowed to equilibrate in the absence of NO₂ gas flow, and gas phase analysis was performed by using Saltzman reagent (17). Values are means ± SD for *n* ≥ 3. *Uptakes are significantly lower for compressed DPPC systems compared with matched uncompressed systems (ANOVA; *P* < 0.05).
Effect of monolayer composition on NO₂ absorption. Although it is generally accepted that pulmonary surfactant present at the interface is enriched in DPPC (8, 19), whether unsaturated fatty acyl moieties are retained throughout compression-expansion cycles remains equivocal (24, 39). We investigated the effects of interfacial unsaturated fatty acids on NO₂ uptake utilizing monolayers composed of either DLPC or POPC. Although not a component of natural surfactant, DLPC was of particular interest both as a potential direct reaction substrate for NO₂ (because of the abundant unsaturated bonds) and because of molecular packing properties induced by the two unsaturated fatty acyl moieties. On compression, despite surface tension reductions, monolayers of DLPC and POPC did not significantly decrease NO₂ absorption rates (Fig. 7).

Alveolar surfactant is a complex mixture containing both saturated and unsaturated lipids. Consequently, studies utilizing BALF extracts and phospholipid mixtures were also conducted to examine monolayer systems more representative of natural surfactant. Rat BALF lipid extract, although a crude preparation, was used as a surrogate of natural surfactant, and DPPC-POPG (9:1; wt/wt) and DPPC-POPC-DPPG (70:27:3; wt/wt/wt) mixtures were prepared to simulate published compositions of human surfactant phospholipids and fatty acids (41). Phospholipid stock solutions (1 mg/ml in chloroform) were prepared by using an averaged molecular weight based on the percent composition of each phospholipid in the solution. All three composed monolayers significantly reduced NO₂ ab-

Table 1. Effect of interfacial DPPC on NO₂-derived NO₂ production

<table>
<thead>
<tr>
<th>Exposure Conditions</th>
<th>NO₂ Produced, nmole</th>
<th>NO₂ Uptake, nmole</th>
<th>Ratio Nitrite/NO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flask – PC</td>
<td>1.23 ± 0.03</td>
<td>0.98 ± 0.04</td>
<td>1.25 ± 0.03</td>
</tr>
<tr>
<td>Flask + PC</td>
<td>0.61 ± 0.05</td>
<td>0.53 ± 0.08</td>
<td>1.16 ± 0.07</td>
</tr>
<tr>
<td>Probe + PC</td>
<td>0.63 ± 0.21</td>
<td>0.65 ± 0.27</td>
<td>0.99 ± 0.13</td>
</tr>
</tbody>
</table>

Values are means ± SD (n ≥ 3). After exposures, aqueous-phase aliquots were withdrawn for immediate nitrite (NO₂) analysis as described in METHODS.
Fig. 8. Effect of interfacial phospholipid composition on NO₂ absorption in the surface balance. Mixed monolayer systems in which the relative concentration of DPPC varied were exposed to NO₂ (4.6 ± 0.2 ppm) as described in Fig. 7. Stock solutions of the phospholipid mixtures (1 mg/ml in chloroform) were prepared on the basis of either a weighted average or estimated molecular weights for pure chemical and bronchoalveolar lavage fluid (BALF) systems, respectively. DPPC, 1,2-dipalmitoyl-sn-glycero-3-phospho-rac-glycerol; POPG, 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-rac-glycerol. Double-hatched bars indicate uptakes in the presence of films. *NO₂ absorption was decreased significantly in all cases (ANOVA; P < 0.05). Surface tensions achieved on compression (listed above bars) were similar among the pure chemical systems. Although surface tension remained high for BALF extract, NO₂ uptake was significantly decreased. Values are means ± SD for n ≥ 3.

DISCUSSION

Investigations into the influences of surface films on gas-vapor transport processes began in the mid-1920s. Initial studies were directed at evaluating the effect of monomolecular oil films on the rate of water evaporation (23, 27, 40). In the early 1940s, Langmuir and Schaefer (23) designed and utilized an apparatus for evaluating monolayer effects on the rate of water evaporation. These studies, along with those that followed, suggested that interfacial monolayers of some fatty acids and aliphatic alcohols significantly reduced the rate of water evaporation (1, 42). However, several years passed before the concept of lung surfactant film permeability was first introduced: it was initially discussed in the context of a mathematical model (7) evaluating the stability of bubbles expressed from lung by Pattle (29). Although studies assessing interfacial lung surfactant permeability to water vapor have been conducted (25), monolayer effects on the rate of gas flux from the gas to the aqueous phase have received limited attention (e.g., 4, 21). This may be due in large part to the fact that O₂ and CO₂ partial pressures normally equilibrate rapidly between alveolar gas and pulmonary capillary blood (43).

Characterizing the influences of interfacial phospholipids on NO₂ absorption required an exposure model that would enable measurement of changes in NO₂ gas phase concentration over a defined surface during controlled compression of the surface film. We were limited to an in vitro approach because interfacial conditions within the lung cannot be tightly controlled. Although studies were conducted under quasi-steady-state exposure conditions, intrapulmonary NO₂ concentrations vary throughout ventilation. However, previous studies have demonstrated that processes governing NO₂ absorption were equivalent under quasi-steady-state and transient (NO₂-limiting) exposure conditions (32, 34). Despite the design limitations of the compression-exposure apparatus that prevented compression of surface films to very low tensions (Fig. 1), the system permitted reproducible control and study of the effects of interfacial, gas-phase, and aqueous-phase conditions on NO₂ absorption.

Using the probe exposure apparatus, we observed that compression of DPPC monolayers to surface tensions still substantially above those reached in vivo (e.g., 25 vs. <1 mN/m) significantly reduced the rate of NO₂ absorption (Figs. 3, 6, 7). Increases in monolayer packing, indicated by lower surface tensions, were rapidly followed by reduced NO₂ absorption (Fig. 2). We therefore felt it important to evaluate whether the decreased uptake was due to additional resistance imposed directly at the interface or to changes in the gas- and/or aqueous-phase conditions. NO₂ diffuses through the gas-phase boundary layer, dissolves in the aqueous-phase boundary layer, and reacts within a near-interfacial zone. Because of the limited aqueous solubility of NO₂, saturation of the aqueous boundary layer should occur rapidly in the absence of reactive substrates (3). The driving force for the continued net flux of NO₂ is maintained by diffusion of both NO₂ and reactive substrates (e.g., GSH, AH₂) into the reaction zone. Chemical reaction of NO₂ serves to limit both its diffusion into the bulk aqueous phase and the development of backpressures resulting from accumulation of dissolved NO₂. Previous kinetic studies, demonstrating that NO₂ gas-phase disappearance rates were independent of aqueous bulk-phase volume and equivalent for both wetted filter material and aqueous bulk-phase systems, support these concepts (34). Studies employing 1% agarose to gel the aqueous phase demonstrated that impeding aqueous-phase convection did not alter the steady-state rates of NO₂ uptake compared with unstirred controls (data not shown). In the probe exposure studies, stable uptake rates were observed over prolonged periods with or without either compressed monolayers or reaction substrates. It is unlikely that monolayer compression caused a backpressure-induced decrease in NO₂ flux due to aqueous substrate limitations because this would imply that solute diffusion would be constrained to a new, lower steady-state level.
Although previous NO₂ studies have shown limitations to mass transfer in the gas phase (3), we concluded that the addition of a monolayer would not affect gas-phase NO₂ transport. Nevertheless, appreciable mass transfer resistance is associated with the gas phase as is shown by the saturation of NO₂ absorption rates in the presence of vigorous interfacial stirring and aqueous substrates in great excess (e.g., [NO₂] ≈ 10 ppm, GSH concentration ≈ 10 mM) (33, 34). NO₂ absorption in the presence of an uncompressed DPPC film did reach maximal values (Fig. 6) similar to those reported previously under steady-state, well-stirred conditions (33). We attributed the reduction in NO₂ absorption observed with DPPC to resistance introduced by the interfacial films themselves rather than effects of the films on the adjacent bulk phases. We based this conclusion on the following considerations: 1) the constant background loss of NO₂ in buffer systems despite the absence or presence of DPPC, 2) the theoretical lack of monolayer-induced diffusion effects within the aqueous reaction zone, 3) the lack of direct surface tension effects on absorption (Fig. 4), 4) a film-induced reduction in NO₂ absorption at various aqueous substrate concentrations and compositions, and NO₂ gas-phase concentrations (Fig. 5), and 5) the consistent stoichiometry of NO₂ formation from NO₂ (Table 1). It is noteworthy that DPPC films reduced NO₂ uptake significantly even at 90 and 125 ppm (~60%, Fig. 5). These data clearly demonstrate that interfacial monolayers limit NO₂ transfer.

Despite their lowering of surface tension to ~25 mN/m, unsaturated PC moieties did not restrict NO₂ transfer (Fig. 7). The area per molecule of unsaturated PC in a fully compressed monolayer remains quite high (e.g., POPC > 70 Å²/molecule) (11) compared with disaturated PC (<49 Å²/molecule). Moreover, had exposure-induced oxidation of the unsaturated fatty acid occurred, the products would have further disordered molecular packing. NO₂ reaction with unsaturated fatty acids proceeds via either allylic hydrogen abstraction or double-bond addition (15, 16). For polyunsaturated fatty acids, the molecule isomerizes and adds O₂ to produce a cis-trans conjugated diene fatty acid hydroperoxide. For both mono- and polyunsaturates, NO₂ addition products may potentially result (22, 38). The conformational changes induced by these molecular modifications could have introduced further disorder into the films (20).

Others have reported monolayer-induced resistance to transport processes ranging from <1 to 10³ s/cm (4, 23, 48). Estimates of film resistance, which we calculated from the data of Sebba and Briscoe (48) (for C₁₆–C₂₀ fatty acid and aliphatic alcohols), ranged from <0.1 to 25 s/cm, with the exception of n-docosanol, which achieved a resistance of 180 s/cm when maximally compressed. Blank and Roughton (4) reported resistance to CO₂ transport across (C₁₆–C₁₈ fatty acid and aliphatic alcohol) films of 80–392 s/cm. Clements (7) estimated the film resistance of Patte’s bubbles (surfactant bubbles extruded from whole lung) to be ~10³ s/cm (180 s/cm when the solubility coefficient of air in water is taken into account). A plot of film resistance vs. surface pressure (Fig. 9) clearly demonstrates that film resistance is related to the degree of DPPC monolayer compression. Under the exposure conditions employed in these studies, monolayer resistance to NO₂ absorption increased from 0.3 to 12.3 s/cm when the film was compressed from 121 to 49 Å²/molecule. Because the surface tensions achieved in these studies were still higher than those that occur in vivo, and film molecular packing was lower, it is reasonable to speculate that physiological resistance to NO₂ transfer within the pulmonary gas exchange regions could exceed those observed in this study. Differences in estimates of film resistance between previous studies and those reported herein likely reflect variations in experimental materials and methods and in the definition of film resistance.

We cannot be sure that NO₂ absorption would have continued to decrease during surface compression to lower, physiological levels of surface tension; however, the relation is reasonably linear in the range of surface tension measured (Fig. 3). Nevertheless, it is likely that the low resistance of mixed monolayers would be raised by further compression, which produces a more DPPC-enriched film. Furthermore, alveolar surface tension varies throughout the ventilation cycle even during tidal breathing: large increases in lung volume increase alveolar surface tension above equilibrium (25 mN/m) (46), at which point renewal of the surfactant monolayer begins. Interfacial lipids are probably also responsible for the low surface tension of conducting airway lining fluid (30 mN/m) (45). As demonstrated by the Tween 80 (Fig. 4) and unsaturated fatty acid (Fig. 7) studies, mere reduction in surface tension is not sufficient to restrict NO₂ transfer. Consequently, because airway interfacial properties have not been fully characterized, it is difficult to predict whether significant film resistance to NO₂ transfer might occur in these anatomic sites.
The implications of these results are intriguing. If NO₂ absorption in proximal air spaces is limited by interfacial surfactant, then NO₂ movement into more distal regions would occur. In contrast, the absence of exposure-induced injury has been accepted as prima facie evidence for the lack of NO₂ distribution into distal alveoli. The intrapulmonary distribution of inhaled reactive gases is related to the balance between removal at the airway walls and longitudinal transport (3, 6). Dosimetry models predict that the gas-phase concentration of inhaled NO₂ rapidly declines beyond the proximal alveolar spaces (26, 28). Most models, which attempt to characterize the tissue dose of inhaled oxidants, predict a rapid equilibrium between gas and aqueous (ELF) phases on the basis of Henry’s law solubility coefficients (3). Data from our studies lead us to speculate that the resistance of surfactant films to NO₂ diffusion could result in increased NO₂ transport to more distal airspaces. If uptake were sufficiently restricted, cell injury would not directly correlate with the local gas-phase [NO₂] concentration. Furthermore, because in vitro exposure models (e.g., cell cultures) generally do not include an interfacial film of surfactant, dose-effect relationships may differ appreciably from those occurring in vivo. Consequently, differing relationships between gas-phase concentration and cell injury may confound direct extrapolations between in vitro exposure models and from models to animals.

APPENDIX

Validation of probe exposure apparatus. Because of the design characteristics of the probe apparatus, some NO₂ loss to the atmosphere was inevitable (Fig. 1). To evaluate NO₂ flow characteristics under the probe and to determine optimal probe positioning, round filters wetted with Saltzman reagent (17) were placed on a solid support and positioned directly beneath the probe, and the pattern of color development was examined. When 1) the central restriction disc was recessed 4 mm from the probe lip and 2) the probe lip was positioned 1 mm above the wetted filter, a circular pattern of homogeneous staining (34.1 ± 1 cm²) formed on the wetted filters. The surface area of the colored circle was similar to that of the probe itself (28.3 cm²) and was not altered when exposures were conducted with the pantograph barrier set to the smallest allowable surface area.

The background (buffer without GSH) rate of NO₂ gas phase disappearance ([(NO₂)₀ − [NO₂]ᵣ] · exit flow · surface area⁻¹) represents the combined contributions of aqueous solubility and reaction with water (which is relatively slow; Ref. 12). The background rates among buffer only, buffer plus DPPC (121 Å²/molecule), and buffer plus compressed DPPC (49 Å²/molecule) systems were comparable (data not shown), indicating that monolayer-induced effects on transfer were small under these conditions. In addition, the rates of NO₂ gas-phase disappearance were comparable for GSH solutions with or without uncompressed DPPC or POPC (121 Å²/molecule) (23.0 ± 0.4, 23.1 ± 0.3, and 23.1 ± 0.4 ng·min⁻¹·cm⁻² for GSH, DPPC, and POPC, respectively). Collectively, the data suggest that the gas phase distribution under the probe was not appreciably affected by the presence of a monolayer.

When GSH solutions were exposed to NO₂ under the probe, the exit concentration remained constant for >45 min, indicating steady-state uptake over the time intervals used in our experiments and suggesting that the thickness of the NO₂ reaction zone remained constant, allowing a calculation of the NO₂ gradient across the monolayer.

Derivation of film resistance. We derived a mathematical expression for film resistance under the probe on the basis of the following assumptions: 1) a steady state existed during the measurements of uptake, 2) the profile of changing stagnant layer thickness in the gas phase was not affected by the surface films, 3) an edge-to-center NO₂ gradient existed, 4) vertical gradients in gas-phase [NO₂] were negligible, 5) the effective depth of the reaction zone was constant, and 6) specific film resistance was equal to the difference between total resistance (probe-flask system with PC deposited onto GSH solution, R total) and probe resistance (probe-flask system with GSH solution alone, R apparatus).

We used a washer-shaped control volume, of thickness h with inner radius r and outer radius r + Δr, to perform a steady-state mole balance on NO₂ (Eq. 1).

\[ [C]_{r+\Delta r} - [C]_r = 2\pi r R \frac{dN}{dt} \]

Taking the limit as \( \Delta r \to 0 \), this equation becomes

\[ \frac{dC}{dr} = 2\pi r \frac{dN}{dt} \]

We then defined the rate equation for interfacial flux as

\[ N = \frac{1}{R} (C - \lambda C_{\text{liquid}}) \]

where R is the interfacial resistance (s/cm), C liquid is \([NO₂]₀\) at the aqueous surface, and \( \lambda \) is the solubility of NO₂ in the aqueous phase. Assuming that the backpressure from the liquid is negligible (\( N \approx C/R \)) and

\[ \frac{dC}{dr} = \frac{2\pi}{QR} r \frac{N}{C} \]

integrating Eq. 5 yields

\[ \ln \left( \frac{C_i}{C_f} \right) = \frac{\pi}{QR} (r_f^2 - r_i^2) \]

where subscripts i and f refer to initial and final parameters, (i.e., radii or concentration) so that

\[ R = 60 A \left[ \frac{Q}{\ln \left( \frac{C_i}{C_f} \right)} \right] \]

We then defined specific film resistance as the difference between \( R_{\text{total}} \), the system resistance (GSH with PC deposited) and \( R_{\text{apparatus}} \), probe resistance (GSH alone). \( R \), therefore, represents a composite coefficient that includes the effects of surface adsorption, aqueous solubility, diffusion, and chemical reaction of NO₂.

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