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EFFECT OF NITROGEN DIOXIDE ON SURFACE MEMBRANE FLUIDITY AND INSULIN RECEPTOR BINDING OF PULMONARY ENDOTHELIAL CELLS

JAWAHARLAL M. PATEL,*† DEBORAH A. EDWARDS,† EDWARD R. BLOCK†‡ and MOHAN K. RAIZADA§

Departments of †Medicine and §Physiology, University of Florida College of Medicine, and ‡Veterans Administration Medical Center, Gainesville, FL 32610, U.S.A.

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Abstract—Nitrogen dioxide (NO_2), an environmental oxidant pollutant, is known to peroxidize membrane lipids of lung cells. We evaluated the ability of NO_2 to alter the surface membrane fluidity, lipid composition, and insulin receptor binding of porcine pulmonary artery endothelial cells in culture. After 3- to 24-hr exposure to 5 ppm NO_2, cells were labeled with either 1-(4-trimethylaminophenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH), a cationic fluorescent aromatic hydrocarbon that anchors at the lipid–water interface, or fluorescamine, a fluorescent molecular probe that covalently binds with amino groups of surface phospholipids and proteins. Membrane fluidity was measured by monitoring changes in the steady-state fluorescence anisotropies (r_s) for TMA-DPH and fluorescamine. Insulin specific receptor binding was monitored by measuring time-dependent binding of ^{125}I-insulin. Following NO_2 exposure, r_s values for TMA-DPH and fluorescamine were increased significantly in a time-dependent fashion, with maximum increases at 24 hr ($P < 0.001$). Similar increases in r_s values were observed in isolated plasma membranes as well as in lipid vesicles prepared from total lipid extracts of endothelial cells or their plasma membranes. Phosphatidylethanolamine plus phosphatidylserine content in lipid extracts from 24-hr but not 3- to 12-hr NO_2-exposed cells was increased significantly ($P < 0.01$) compared to control cells. Specific binding of ^{125}I-insulin to cells exposed to NO_2 for 12 and 24 hr (but not 3 and 6 hr) was reduced significantly ($P < 0.05$) compared to binding in control cells. Scatchard analysis of the binding data indicated that NO_2 exposure caused a 5-fold reduction in insulin receptor binding sites in endothelial cells. Recovery was achieved 24 hr after NO_2 exposure with, but not without, changing culture medium. These results indicate that NO_2 exposure causes reversible changes in the physical state of lipids in the superficial lipid domains of the pulmonary endothelial cell plasma membrane, and these alterations may interfere with plasma membrane-dependent functions such as receptor–ligand interaction.

Nitrogen dioxide (NO_2) is an oxidant constituent of indoor and outdoor environmental pollution arising from cigarette smoke, wood-burning stoves, combustion of fossil fuels, and emissions from automotive and other combustion engines [1–4]. There is considerable evidence that NO_2-induced oxidant injury causes damage to lung cells and results in alterations of various biochemical, physiologic, and metabolic functions of mammalian lungs [2, 3, 5–7]. The precise mechanism of NO_2-induced lung toxicity is unknown. However, one of the major theories involves free radical mediated peroxidative cleavage of membrane lipids [8–11]. NO_2 is known to cause lipid peroxidation in the mammalian lung [8–11], and peroxidative cleavage of membrane lipids can alter the physical state of the lipids, resulting in alterations in membrane structure and function.

* Address for correspondence: J. M. Patel, Ph.D., Division of Pulmonary Medicine, Health Center Box J-225, University of Florida, Gainesville, FL 32610.

‖ Abbreviations: LDH, lactate dehydrogenase; r_s, steady-state fluorescence anisotropy; TMA-DPH, 1-(4-trimethylaminophenyl)-6-phenyl-1,3,5-hexatriene; 5-HT, 5-hydroxytryptamine; HEPES, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; and PBS, phosphate-buffered saline.

The fluidity of membranes is generally considered to reflect the molecular order and relative motion of the membrane constituents [12, 13]. Fluidity depends primarily on the physical state of the fatty acyl chains comprising the membrane lipid bilayer structure. Optimal membrane function requires the lipids to be in a fluid state [13].

NO_2 exposure has been reported to injure both pulmonary epithelial and endothelial cells [2, 14, 15]. Although the degree of injury may be different in these cell types, the molecular mechanism responsible for toxicity appears to be similar [2, 3, 16, 17]. We recently reported that NO_2 exposure decreases fluidity in the hydrophobic interior of the plasma membranes of pulmonary artery and aortic endothelial cells, with subsequent increases in lactate dehydrogenase (LDH‖) release and lipid peroxide formation and decreases in plasma membrane-dependent 5-hydroxytryptamine (5-HT) transport [18]. These results are the first to suggest a cause-and-effect relationship between NO_2-induced alterations in endothelial cell plasma membrane fluidity and derangements in plasma membrane function. Since NO_2 is a free radical and reacts directly with membrane lipids [11, 19], it is likely to have an even greater effect on the physical state of the lipids in

the more superficial lipid or surface domains of the plasma membrane bilayer. Similarly, if alterations in membrane fluidity are responsible for derangements in membrane function, then NO_2-induced decreases in surface membrane fluidity would be expected to result in alterations in surface membrane-dependent functions such as receptor–ligand interaction. Recent work from several laboratories indicates the presence of insulin receptors on the surface of pulmonary endothelial cells and the ability of these receptors to mediate the rapid transport of insulin across these cells [20–22]. In the present study, we evaluated the effect of NO_2 exposure on the fluidity of the surface lipid domains of the plasma membrane and on insulin receptor binding in cultured mammalian pulmonary endothelial cells.

MATERIALS AND METHODS

Tissue culture. Using methods described by Block *et al.* [23], we obtained endothelial cells from the main pulmonary artery of 6- to 7-month-old pigs. In brief, fresh blood vessels were obtained from the slaughterhouse and transported on ice in phosphate-buffered saline (pH 7.2). Each vessel was washed twice with sterile Hanks' balanced salt solution (HBSS) containing 100 μg/ml penicillin, 100 μg/ml streptomycin, 10 μg/ml gentamicin, and 2.5 μg/ml Fungizone (1x-antimicrobial agents). The vessels were meticulously trimmed of fat and serosa, and branch vessels were ligated. The lumen of each vessel was then filled with 0.3% (w/v) collagenase (type I CLS, specific activity 145 units/mg; Worthington Biochemical, Freehold, NJ) in HBSS containing 1x-antimicrobial agents and was incubated at 37° for 25 min. This incubation time was chosen because it produced the best cell yields from the pulmonary arteries. At the end of the incubation period, the detached cell–enzyme mixture was transferred to a centrifuge tube containing RPMI 1640 medium (Hazleton Research Products, Inc., Lenexa, KS) supplemented with 10% fetal bovine serum (Flow Laboratories, McLean, VA) and 1x-antimicrobial agents. The vessels were then cut open, and the luminal surface was gently scraped with a No. 10 sterile scalpel. The scraped cells were suspended in fresh culture medium. These scraped cells as well as the collagenase-treated cells were then centrifuged at 160 *g* for 5 min at 4°, the pellet was resuspended in fresh medium, and the suspension was seeded into sterile plastic culture dishes at densities of 1–2×10^4 cells/cm^2 and incubated at 37° with humidified 5% CO_2 in air. After 60 min, the nonadherent cell suspension and medium were removed, and fresh medium was added to the dishes. Medium was changed every 48 hr until primary confluence was reached (4–7 days), after which the concentration of fetal bovine serum in the culture medium was decreased to 3%. Endothelial cell monolayers were subcultured (i.e. passaged) 4–5 days after confluence by incubation for 1 min with 0.1% trypsin (Hazleton Research Products, Inc.) in $Ca^{2+}Mg^{2+}$-free HBSS (Hazleton Research Products, Inc.). Preconfluent subcultures were incubated in RPMI 1640 culture medium containing 10% fetal bovine serum and 1x-antimicrobial agents, and postconfluent subcultures were maintained in RPMI 1640 medium supplemented with 3% fetal bovine serum and 1x-antimicrobial agents. Second-to-fifth passage cells in postconfluent monolayer were used for all experiments.

Identification of endothelial cells. All cell monolayers in culture were initially identified as endothelial cells by phase contrast microscopy. Representative culture dishes from each experiment were characterized further by electron microscopy or by indirect immunofluorescent staining for factor VIII antigen, or both. On the basis of these criteria, monolayer cultures used in these experiments were estimated to be pure cultures of endothelial cells.

Exposure to NO_2. Culture dishes containing endothelial cell monolayers were exposed to a continuous flow of 5 ppm NO_2 in air containing 5% CO_2 in an airtight 12 × 12 inch stainless steel chamber (Brinkmann Instruments, Westbury, NY) housed inside a CO_2 incubator (Hotpack, Philadelphia, PA) and maintained at 37°. The stainless steel chamber contained a water-filled tray on its bottom and had one inlet and one outlet port. Premixed gas cylinders were obtained from Air Products, Jacksonville, FL. Exposures were carried out for 3–24 hr. NO_2 concentrations in the chamber were monitored by the method of Saltzman [24]. Each dish of NO_2-exposed cells was matched with a dish of control cells (i.e. matched for cell line, time to monolayer confluence, cell density, number of subcultures, and number of days postconfluence) that was exposed to air containing 5% CO_2 (control condition) for comparable times. For recovery studies, cells were exposed to NO_2 or control conditions for 24 hr, after which all cells were transferred to control conditions with and without changing the culture medium. Recovery was monitored for 24 hr after the transfer to control conditions.

The concentration of NO_2 used in this study was slightly higher than ambient levels but much lower than levels used in many *in vivo* studies [2, 3, 5, 7–10]. We chose 5 ppm NO_2 because it allowed us to define the mechanisms of NO_2-induced injury at the molecular level without causing gross cellular injury or death. Because NO_2 has a limited solubility in an aqueous medium, the amount of NO_2 directly interacting with the cells is much less than the 5 ppm flowing into the chamber. The exact quantitation of NO_2 reaching the cells is technically difficult to achieve unless cells are exposed in the absence of medium. In the case of vascular endothelial cells, direct exposure to NO_2 in the absence of culture medium deviates from the *in vivo* situation.

Isolation of cell plasma membranes. Plasma membranes from control and NO_2-exposed endothelial cells were isolated using the dextran-polyethylene glycol aqueous two-phase system of Brunette and Till [25] as described by Block *et al.* [23]. Isolated plasma membrane purity was characterized by monitoring marker enzyme activities of (Na^+–K^+)ATPase, cytochrome *c* oxidase and cytochrome *c* reductase as described by Block *et al.* [23].

Lipid extraction. Immediately after exposure, cells were washed twice, resuspended in 0.1 M sodium phosphate buffer (pH 7.4) containing 0.15 M KCl buffer, and centrifuged at 2000 rpm for 10 min. The

resulting cell pellet as well as isolated plasma membranes were then extracted by the method of Folch *et al.* [26] to separate the total lipid and protein fractions. The protein fractions were dissolved in 1.0 ml of 0.5 N NaOH and stored at 5° prior to use. Lipid fractions were stored under N_2 at −20° until use. Phospholipids were separated from the total lipid fraction by thin-layer chromatography on silica gel G according to Skipski and Barclay [27] and used to prepare vesicles for fluidity and amino group determinations.

In some experiments, cells were labeled with fluorescamine prior to the isolation of the lipid and protein fractions. Since fluorescamine binds covalently with amino groups of both lipids and proteins [28, 29], the estimation of amino groups could be done directly on the labeled total lipid and protein fractions.

Preparation of vesicles. Aliquots of the total lipid extracts and the separated phospholipids were evaporated to dryness with a stream of N_2. The dried samples were then reconstituted with 2.0 ml of KCl buffer and allowed to swell in an N_2 atmosphere at room temperature. The suspensions were sonicated in a Branson Water-Bath Sonicator (Branson Cleaning Equipment Corp., Shelton, CT) for 5 min under N_2 to disperse the lipids. Homogeneous vesicles were obtained by centrifugation and gel filtration as described by Gilmore *et al.* [30]. These vesicles were immediately used to measure fluidity or phospholipid amino group content, or both.

Estimation of amino groups of phospholipids and proteins. Amino groups of phospholipids (phosphatidylethanolamine and phosphatidylserine) and proteins were estimated using a fluorescamine assay [28]. The vesicles prepared from the total lipid extracts or from the separated phospholipids (2.0 ml volume) and the protein pellets (diluted to 2.0 ml with KCl buffer) were mixed with 0.25 ml of a 1 mM solution of fluorescamine in absolute ethanol. After a 10-min incubation at room temperature, fluorescence intensity was measured using an SLM 4048s spectrofluorometer (SLM Instruments, Inc., Urbana, IL) with excitation and emission wavelengths of 385 and 470 nm respectively. Standardizations were done using 99% pure phosphatidylethanolamine (Supelco, Inc., Bellefonte, PA) or bovine serum albumin (Sigma Chemical Co., St. Louis, MO) for phospholipid and protein respectively.

Measurement of membrane fluidity. We used fluorescence spectroscopy to evaluate the effects of NO_2 exposure on membrane fluidity in cultured endothelial cells, isolated plasma membranes, and in vesicles prepared from the total lipid extracts of these cells as well as plasma membranes. The principle of this method is that polarization of the light emitted by a fluorescent probe depends on its rotational motion, which is a function of the fluidity of the surrounding medium [31]. As the fluidity of the surrounding medium (i.e. the cell membrane) decreases, the mobility of the fluorescent probe is hindered, resulting in a change in its fluorescence polarization. The probes we chose to use localize at the surface of the plasma membrane but are sensitive to different physical properties of the lipid bilayer. For example, 1-(4-trimethylaminophenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) is a cationic fluorescent aromatic hydrocarbon that anchors at the lipid–water interface and reflects changes in the polar head group region at the surface of the plasma membrane [32, 33], whereas fluorescamine binds covalently with the amino groups of proteins and phospholipids (phosphatidylethanolamine and phosphatidylserine) at the hydrophilic surface of the endothelial cells [28, 29].

Immediately after exposure, cells were washed twice with HBSS, scraped, and suspended in KCl buffer. Suspensions of 3×10^6 cells, of isolated plasma membranes, or of vesicles prepared from total lipid extracts in 2.5 ml of KCl buffer were mixed with 0.5 ml of a probe solution in KCl buffer containing either 50 μl of 0.5 mM stock solution of TMA-DPH in dimethyl sulfoxide or 50 μl of 50 mM stock solution of fluorescamine in acetone. The stock solution of fluorescamine was prepared fresh each time and used immediately. The final concentrations of TMA-DPH and fluorescamine were 10 and 1 μM respectively. The rate of probe incorporation at 25° was monitored by measuring fluorescence intensities every 2 min for 24 min for TMA-DPH (excitation/emission = 360 nm/430 nm) and every 5 sec for 120 sec for fluorescamine (excitation/emission = 385 nm/470 nm). Labeling of cells (or plasma membranes and lipid vesicles) with TMA-DPH or fluorescamine was complete after 20 and 1 min respectively (see Fig. 1), and these incubation times were used in all experiments. After incubation was complete, cells, plasma membranes, or vesicles were washed twice with KCl buffer, resuspended in 2.5 ml of KCl buffer, and used for fluorescence measurements. To verify that TMA-DPH was localized to the plasma membrane of the intact endothelial cells and did not translocate to intracellular organelles, fluorescence intensities were measured in washed intact cell suspensions and in isolated mitochondrial and microsomal fractions derived from these cells. Mitochondria and microsomes were isolated by the method of Hook *et al.* [34]. Previous investigators have shown that fluorescamine binds covalently to surface membrane amino groups of phospholipids and proteins and does not label internal components of the cells, as demonstrated by polyacrylamide gel electrophoresis [28, 29].

Steady-state fluorescence excitation and emission spectra and polarization were measured with an SLM 4048s subnanosecond spectrofluorometer (SLM Instruments, Inc.). Fluorescence spectra were corrected for wavelength-dependent variation in light source output, phototube response, and monochromator efficiency [35]. Light scattering by membranes was reduced to very low levels by the use of cutoff filters, and fluorescence measurements were corrected for residual light scattering according to Lentz *et al.* [36].

Steady-state fluorescence anisotropy (r_s) is widely used as an index of membrane fluidity [13, 31]. Although there has been recent controversy regarding the interpretation of steady-state fluorescence measurements of anisotropy, it is now recognized that the value of r_s is primarily a function of the molecular packing or order of the membrane lipids

rather than the rotational rate of the fluorescent probe [13, 37, 38]. Increases in r_s reflect increases in order, which are associated with decreases in membrane fluidity [13, 37, 38]. Thus, r_s varies inversely with the fluidity of the membrane surrounding the probe and allows us to make comparisons between the physical state of the lipids in plasma membranes from control and NO_2-exposed cells.

Fluorescence anisotropies were measured using the T format of the spectrofluorometer, which simultaneously measures the ratio of the vertical and horizontal components of the emitted light with the exciting light either vertically $(V/H)_v$ or horizontally polarized $(V/H)_h$. The steady-state anisotropy (r_s) is defined by the following equation: $r_s = (V/H)_v - (V/H)_h/(V/H)_v + 2(V/H)_h$, where $(V/H)_h$ corrects for unequal transmission of horizontally and vertically polarized light. Simultaneous measurement of both emitted components is rapid and serves to eliminate the contributions of cell settling to the anisotropy values.

Measurement of ^{125}I-insulin binding. Specific binding of ^{125}I-labeled insulin to membrane receptors was measured in control and NO_2-exposed endothelial cells by the previously described method of Boyd and Raizada [39]. Briefly, immediately after exposure, cell dishes were washed twice with 4.0 ml of phosphate-buffered saline (PBS), pH 7.4. Triplicate dishes were incubated at 24° with 1.0 ml of HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid) buffer, pH 7.4 (110 mM HEPES, 30 mM NaCl, 10 mM glucose), containing 0.28 nM (150,000 cpm) B26-labeled ^{125}I-labeled porcine insulin (Eli Lilly Laboratories, Indianapolis, IN) and 1.6% bovine serum albumin to determine total binding. In addition, duplicate dishes were also incubated with 16.6 μM unlabeled porcine insulin (Elano Products Co., Indianapolis, IN) in the reaction mixture to determine nonspecific binding. Following incubation, the cell dishes were washed rapidly four times with a total volume of 20 ml of ice-cold PBS. The attached cells were dissolved in 1.0 ml of 0.2 N NaOH and transferred to tubes. The dishes were rinsed with 1 ml of distilled water, which was combined with the original samples. Radioactivity was determined in a Beckman 5500 γ-counter with a counting efficiency of 73% for ^{125}I. Specific binding was calculated by subtracting nonspecific binding obtained in the presence of an excess of unlabeled insulin from total binding. The protein content of each sample was determined in duplicate by the method of Lowry *et al.* [40].

For Scatchard analysis [41], binding of ^{125}I-insulin to control and NO_2-exposed endothelial cells was carried out in the presence of increasing concentrations of unlabeled insulin. The bound/free ratio of labeled insulin was calculated and plotted as a function of insulin bound to cells. The binding parameters were calculated by a computerized analysis of the fitted curve by the method of Munson and Rodbard [42].

Data analysis. Fluorescence anisotropies were measured in a minimum of six control and six NO_2-exposed dishes. Within each experiment, control and NO_2 dishes were matched for appropriate tissue culture variables. Fluorescence anisotropy data were analyzed using multivariate split-plot analysis of variance [43] to test for the effects of exposure time and NO_2 and their possible interactions on anisotropies. If any significant effects were identified, univariate analyses of variance or *post-hoc* pairwise comparisons of means were performed using the Bonferroni correction [44]. Student's paired *t*-test was used to compare the effects of control and NO_2 exposure on ^{125}I-insulin binding and phospholipid composition [44].

RESULTS

General characteristics of cells. After exposure to 5 ppm NO_2 for up to 24 hr, the structure of pulmonary artery endothelial cells was not different from that of control cells as assessed by phase-contrast microscopy. Similarly, exposure to 5 ppm NO_2 for up to 24 hr had no significant effect on cell number or cell protein per culture dish in pulmonary artery endothelial cells (Table 1).

Effect of NO_2 exposure on phospholipid content of cells. Free amino group-containing phospholipids, i.e. phosphatidylethanolamine and phosphatidylserine, were measured in the total lipid extracts as well as in the separated phospholipids from control and NO_2-exposed cells. Exposure to 5 ppm for 3–12 hr had no effect on phospholipid amino group

Table 1. Effect of NO_2 (5 ppm) exposure on cell count and phospholipid and protein contents of endothelial cells

Exposure time (hr)	Group	Cell count (10^6 cells/dish)	Protein (μg/dish)	Phospholipids (ethanolamine + serine) (μg/μg protein)	
				Total lipid extract	Phospholipid fraction
3	Control	1.28 ± 0.05	352 ± 17	0.071 ± 0.005	0.063 ± 0.002
	NO_2	1.27 ± 0.03	360 ± 26	0.074 ± 0.007	0.068 ± 0.005
24	Control	1.33 ± 0.02	363 ± 47	0.071 ± 0.006	0.062 ± 0.006
	NO_2	1.29 ± 0.03	360 ± 36	0.089 ± 0.003*	0.081 ± 0.005*

Phospholipid and protein amino groups were estimated by using the fluorescamine assay (see Materials and Methods). Data represent mean ± SE (N = 6).

* $P < 0.01$ compared to control.

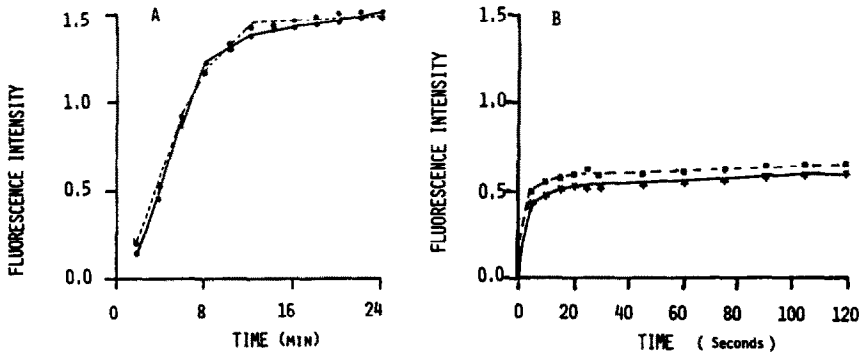

Fig. 1. Kinetics of TMA-DPH (A) and fluorescamine (B) incorporation in control (——) and NO_2-exposed (5 ppm × 24 hr) (– – – – –) pulmonary artery endothelial cells.

content. In contrast, exposure to 5 ppm NO_2 for 24 hr significantly increased ($P < 0.01$) phosphatidylethanolamine plus phosphatidylserine contents in both total lipid extracts and separated phospholipids compared to control cells (Table 1). Phosphatidylethanolamine plus phosphatidylserine contents in the total lipid extracts of plasma membranes isolated from control and NO_2-exposed (5 ppm, 24 hr) cells were 0.856 and 1.56 $\mu g/\mu g$ protein respectively.

Spectroscopic and kinetic properties of TMA-DPH and fluorescamine. When control and NO_2-exposed pulmonary artery endothelial cells were incubated with aqueous dispersions of TMA-DPH, increases in the fluorescence intensity of TMA-DPH were nearly identical and appeared to level off after 15 min (Fig. 1A). Increases in fluorescence intensity for fluorescamine in control and NO_2-exposed pulmonary artery endothelial cells were much more rapid, leveling off after 30 sec (Fig. 1B). The slight increase in fluorescence intensities in NO_2-exposed cells was due to increased amino group-containing phospholipids in these cells. Fluorescamine intensities in cells exposed to 5 ppm NO_2 for 12 hr, which was prior to the increase in amino group-containing phospholipids, were identical to controls (data not shown).

Representative fluorescence emission spectra for TMA-DPH and fluorescamine incorporated into control and NO_2-exposed pulmonary artery endothelial cells are shown in Fig. 2, A and B respectively. The emission wavelength maxima for TMA-DPH and fluorescamine were 430 and 470 nm, respectively, in both control and NO_2-exposed endothelial cells. The fluorescence emission spectra for TMA-DPH were identical in control and NO_2-exposed cells. The increase in fluorescence intensities in NO_2-exposed cells (Fig. 2B) was due to the increased content of amino group-containing phospholipids in these cells. Fluorescamine intensities in cells exposed to 5 ppm NO_2 for 12 hr were identical to control intensities as were the phospholipid contents of these cells (data not shown).

These results demonstrate that there are no differences in the amount of probe incorporated per number of lipid molecules, in the rate of the incorporation, or in the spectroscopic properties of incorporated probe between control and NO_2-exposed cells. Therefore, differences in r_s between control and NO_2-exposed endothelial cells can be attributed to changes in the physical state of the lipids in the plasma membrane bilayer.

Location of TMA-DPH in cells. Endothelial cells were incubated with TMA-DPH for 30 min. After

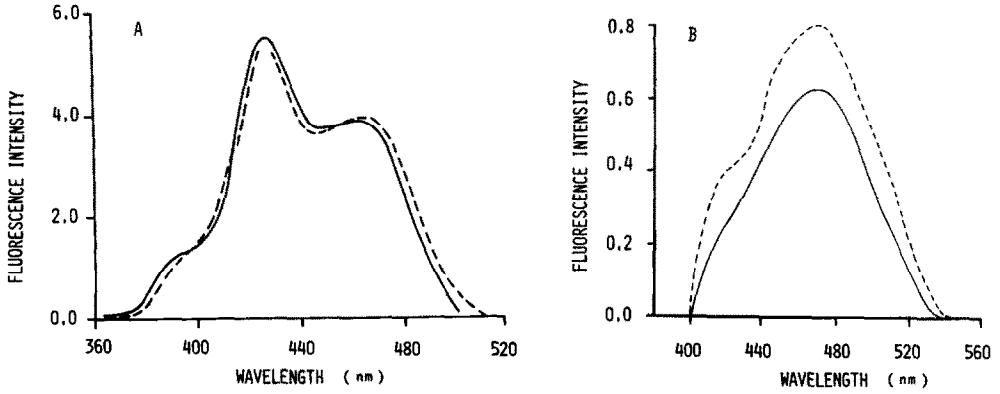

Fig. 2. Fluorescence emission spectra for TMA-DPH (A) and fluorescamine (B) in control (——) and NO_2-exposed (5 ppm × 24 hr) (– – – – –) pulmonary artery endothelial cells.

Table 2. Distribution of TMA-DPH in endothelial cells

Time* (min)	Fluorescence intensity		
	Cell suspension	Mitochondria	Microsomes
30	198	025	026
60	195	026	025
120	193	026	025

Cells (6×10^6) were incubated with 10 μM TMA-DPH. Fluorescence intensities for blanks were: buffer = 025 and buffer + TMA-DPH = 025 (TMA-DPH is nonfluorescent until it partitions in the lipid bilayer). Each sample used to measure fluorescence intensity contained 25 ± 4 μg protein. Results represent an average of duplicate experiments.

* This is the time after the cells were washed to remove excess unincorporated probe.

incubation, cells were washed twice to remove excess unincorporated probe, and translocation of probe from the plasma membrane to intracellular organelles, e.g. mitochondria and microsomes, was monitored over the next 120 min. As shown in Table 2, fluorescence intensities in the intact cell suspensions after removal of excess probe were maximal at 30 min and did not change over the next 90 min. Fluorescence intensities for TMA-DPH in isolated mitochondrial and microsomal fractions were comparable to blanks at all times tested. These results indicate that TMA-DPH incorporated into the plasma membrane at the end of the 30-min incubation period did not translocate into intracellular components during the subsequent 120 min. These results are supported by previous studies using fluorescence microscopy, which demonstrate that TMA-DPH remains localized in the plasma membranes of a variety of mammalian cells [33], including porcine pulmonary artery endothelial cells (E. R. Block and N. P. Sheridan, unpublished observations).

Effect of NO_2 exposure on membrane fluidity changes in endothelial cells and lipid vesicles. Exposure to 5 ppm NO_2 for 3 hr caused a significant increase ($P < 0.05$) in r_s for TMA-DPH in pulmonary artery endothelial cells (Fig. 3A). More prolonged exposure to NO_2 (i.e. 24 hr) resulted in even greater increases in r_s for TMA-DPH ($P < 0.001$, Fig. 3A). Membrane fluidity changes in lipid vesicles prepared from the total lipid extracts of control and NO_2-

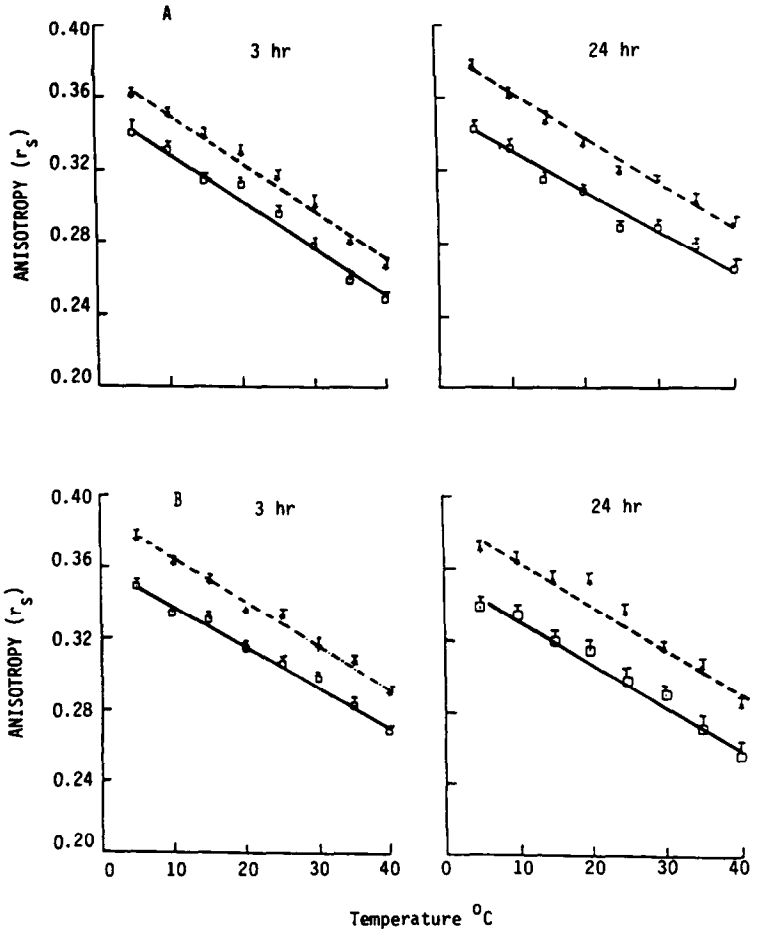

Fig. 3. Steady-state fluorescence anisotropies (r_s) for TMA-DPH in intact cells (A) and lipid vesicles (B) from control (——) and NO_2-exposed (– – – –) pulmonary artery endothelial cells. The results shown in each panel are the mean of four experiments ± SE.

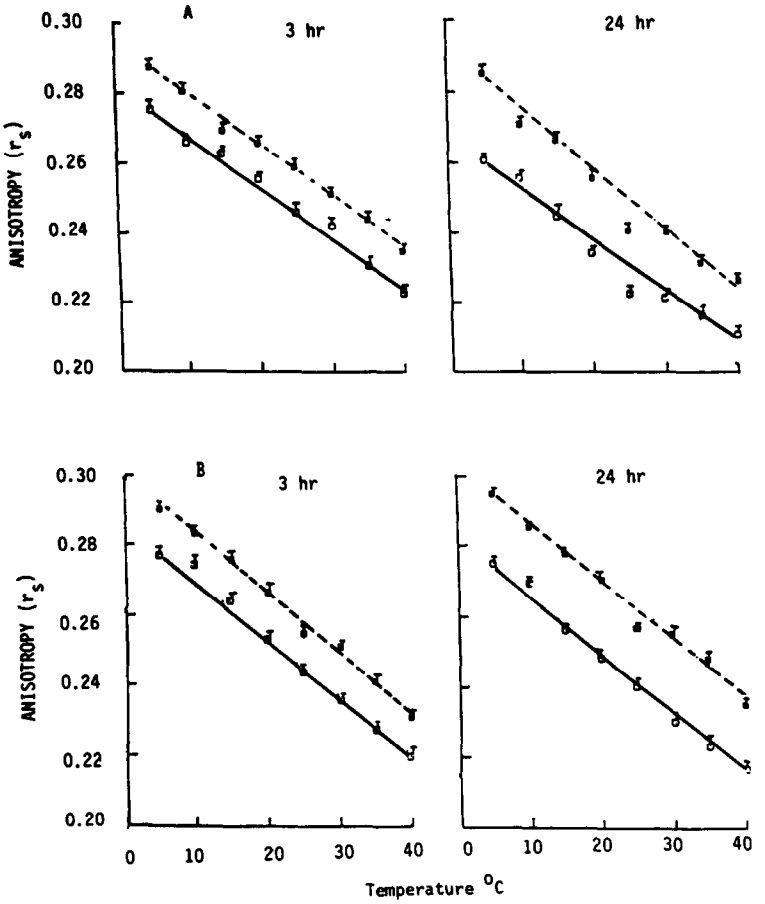

Fig. 4. Steady-state fluorescence anisotropies (r_s) for fluorescamine in intact cells (A) and lipid vesicles (B) from control (——) and NO_2-exposed (– – – –) pulmonary artery endothelial cells. The results shown in each panel are the mean of four experiments ± SE.

exposed cells were comparable to r_s values observed in intact cells (Fig. 3B). Exposure to 5 ppm NO_2 for 6 or 12 hr caused increases in r_s for TMA-DPH similar to those observed after a 3-hr NO_2 exposure (data not shown). Identical results were obtained when NO_2-induced surface membrane fluidity changes were examined using fluorescamine in intact cells (Fig. 4A) and in lipid vesicles prepared from total lipid extracts of these intact endothelial cells (Fig. 4B).

Fluidity changes in plasma membranes isolated from endothelial cells. To confirm that NO_2 exposure caused fluidity changes in the lipid bilayer of the plasma membrane of endothelial cells, we measured r_s for both TMA-DPH and fluorescamine in isolated plasma membranes, as well as in vesicles prepared from total lipid extracts of these membranes from control and NO_2-exposed (5 ppm, 24 hr) cells. NO_2 exposure caused increases in r_s for TMA-DPH ($P < 0.001$) in isolated plasma membranes (Fig. 5A) and in lipid vesicles prepared from total lipid extracts of these plasma membranes (Fig. 5B). Similar increases in r_s for fluorescamine were observed in isolated plasma membranes from NO_2-exposed cells and in their lipid vesicles (Fig. 5, C and D respectively). The magnitudes of the increases were comparable to those observed in intact NO_2-exposed endothelial cells (Figs. 3 and 4).

Effect of NO_2 exposure on ^{125}I-insulin binding in endothelial cells. The specific binding of ^{125}I-insulin to membrane receptors of control and NO_2-exposed endothelial cells increased with incubation time (Fig. 6). After a 2-hr incubation with ^{125}I-insulin, receptor binding to cells exposed to 5 ppm NO_2 for 12 or 24 hr was significantly less ($P < 0.05$) than binding to control cells (Fig. 7). Receptor binding of ^{125}I-insulin to cells exposed to NO_2 for 3 or 6 hr was comparable to controls (Fig. 7).

Scatchard analysis of the binding data provided a curvilinear plot (Fig. 8) that is in agreement with data on insulin receptors from other mammalian cells, including endothelial cells [20–22, 39]. The resolution of these curves by computer characterization [42] gave high-affinity binding sites (K_a $9.12 \times 10^8\ M^{-1}$ and K_a $1.37 \times 10^{10}\ M^{-1}$ for control and NO_2-exposed endothelial cells respectively). The number of high-affinity binding sites in NO_2-exposed cells was reduced 5-fold compared to control cells. The low-affinity binding sites in control (K_a $9.12 \times 10^6\ M^{-1}$) and in NO_2-exposed cells (K_a $8.2 \times 10^6\ M^{-1}$) were comparable.

Recovery of NO_2-induced changes in endothelial

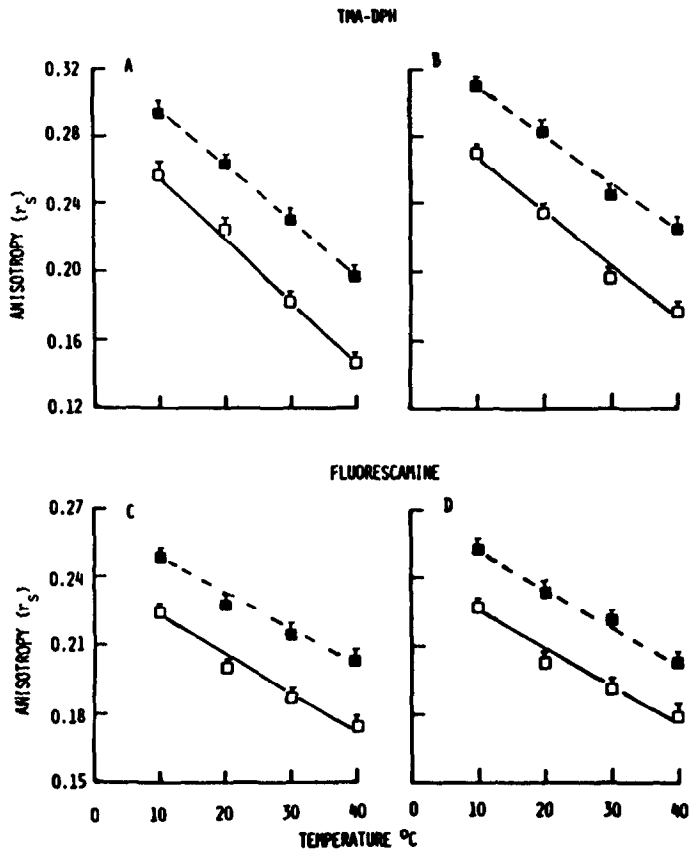

Fig. 5. Steady-state fluorescence anisotropies (r_s) for TMA-DPH and fluorescamine in isolated plasma membranes (panels A and C) and in plasma membrane lipid vesicles (panels B and D) respectively. Control and NO_2-exposed cells are represented by (——) and (– – – –), respectively, in all panels. The results shown in each panel are the mean of triplicate determinations ± SE.

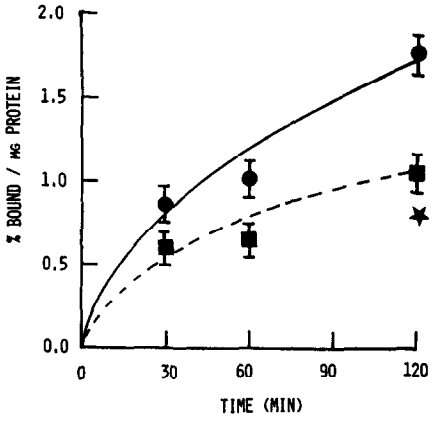

Fig. 6. Time-dependent effect of ^{125}I-insulin binding to control (——) and NO_2-exposed (5 ppm, 24 hr) (– – – –) pulmonary artery endothelial cells. The results shown are the mean of four experiments ± SE. Key: (*) $P < 0.05$ versus control.

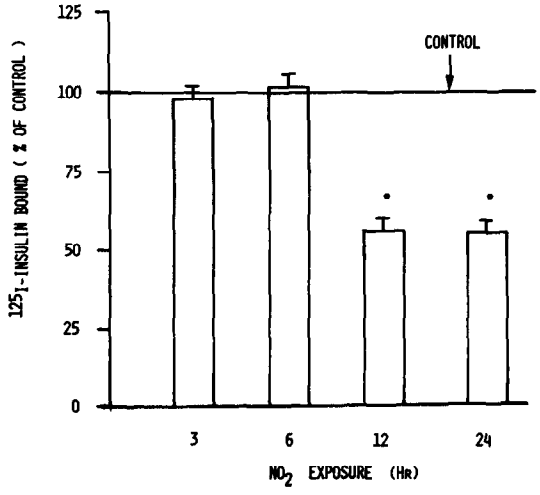

Fig. 7. Effect of NO_2 exposure time on ^{125}I-insulin binding to pulmonary artery endothelial cells. Cells were incubated with ^{125}I-insulin for 2 hr prior to measuring binding. Data are expressed as percent of control (mean ± SE for N = 4). Specific binding in controls was 1.65 ± 0.13% bound/mg protein. Key: (*) $P < 0.05$ versus control.

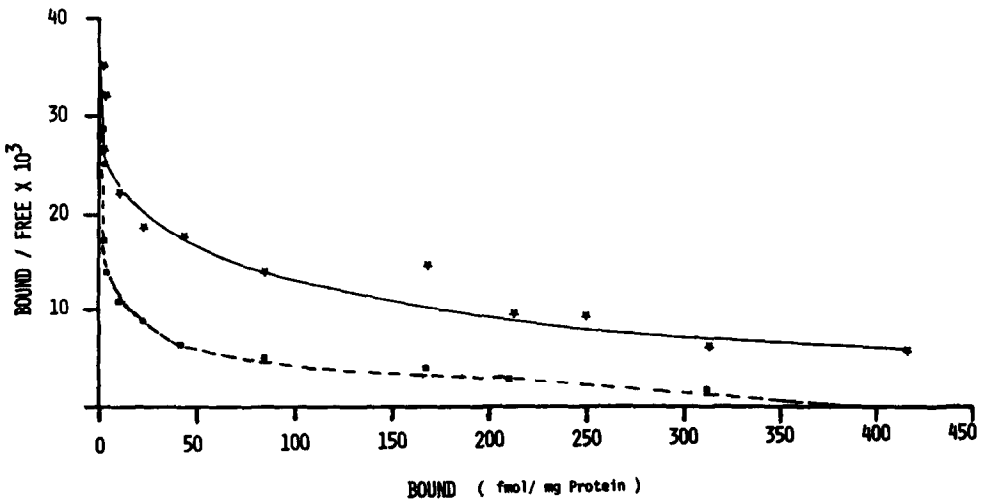

Fig. 8. Scatchard analysis of binding of ^{125}I-insulin to control (——) and NO_2-exposed (– – – –) endothelial cells. Data are means of triplicate determinations.

cells. Following exposure to 5 ppm NO_2 for 24 hr, cells were monitored for recovery. In some experiments, cells were provided with fresh culture medium during the 24-hr recovery period. As shown in Table 3, phospholipid (ethanolamine and serine) content, r_s for TMA-DPH and for fluorescamine at 25°, and ^{125}I-insulin binding returned to control values within 24 hr in cells given fresh medium immediately after completion of the NO_2 exposure. In contrast, in NO_2-exposed cells whose medium was not changed after NO_2 exposure, phospholipid content, r_s for TMA-DPH and for fluorescamine, and ^{125}I-insulin binding remained significantly different from controls.

DISCUSSION

The basic structure of mammalian cell membranes consists of a lipid bilayer composed primarily of phospholipids and cholesterol [12]. Proteins that are involved in fundamental cellular functions, such as receptor–ligand interaction, carrier-mediated transport, and enzyme activity, are embedded in the lipid bilayer [12, 13]. Optimum membrane function requires the membrane lipids to be in a fluid state [13, 37]. Alterations in the dynamic state of membrane lipids have been shown to interfere with a number of fundamental cellular functions [36, 45, 46]. Changes in the physical state of distinct lipid domains within the plasma membrane can be identified using fluorescent probes that partition into the plasma membrane lipid bilayer [13, 31, 37, 45]. We used TMA-DPH and fluorescamine, two well-defined fluorescent probes that localize at the lipid–water interface of the surface membrane of mammalian cells and have no membrane perturbing effects [27, 28, 32, 33]. Localization of TMA-DPH to the plasma membrane has been demonstrated in

Table 3. Recovery of NO_2-induced changes in endothelial cells

	Exposure condition	Change of medium after NO_2 exposure −	+
Phospholipids (μg/μg protein) (ethanolamine + serine)	Control	0.074 ± 0.006	0.079 ± 0.004
	NO_2	0.092 ± 0.003*	0.078 ± 0.006
r_s for TMA-DPH at 25°	Control	0.290 ± 0.004	0.296 ± 0.003
	NO_2	0.330 ± 0.008†	0.291 ± 0.006
r_s for fluorescamine at 25°	Control	0.244 ± 0.005	0.247 ± 0.007
	NO_2	0.293 ± 0.008†	0.252 ± 0.004
[^{125}I]Insulin binding (% bound/mg protein)	Control	1.50 ± 0.07	1.45 ± 0.04
	NO_2	1.15 ± 0.06‡	1.48 ± 0.06

Cells were exposed to 5 ppm NO_2 or control conditions for 24 hr. After exposure was completed, cells were monitored for recovery for 24 hr. In some experiments, cells were provided with fresh culture medium (+) during the 24-hr recovery period. Data represent mean ± SE (N = 4).

* $P < 0.01$ versus control.

† $P < 0.001$ versus control.

‡ $P < 0.05$ versus control.

model membranes as well as in mammalian cells, including porcine endothelial cells ([32, 33] and E. R. Block and N. P. Sheridan, unpublished observations), using fluorescence spectroscopy and microscopy. Our results confirm that TMA-DPH remains localized in the plasma membrane of porcine pulmonary artery endothelial cells for at least 120 min after completion of incubation and does not translocate into the mitochondrial or microsomal membranes under the experimental conditions used in the present studies. In the case of fluorescamine, it has been clearly demonstrated that the physicochemical properties of this probe prevent its translocation beyond the surface membrane of mammalian cells [28, 29].

The results of the present study using two fluorescent probes that partition in two distinct surface lipid domains within the plasma membrane demonstrate that exposure to NO_2 decreases surface membrane fluidity in pulmonary artery endothelial cells in monolayer culture. In addition to a decrease in surface membrane fluidity, NO_2 exposure caused a significant decrease in ^{125}I-insulin receptor binding and a significant increase in the amino group-containing phospholipids, phosphatidylethanolamine and phosphatidylserine, in these cells. The nonlinear Scatchard plots obtained from insulin binding to control and NO_2-exposed endothelial cells could account for the presence of sites with two different affinities for insulin binding [39]. In the present study, the number of high-affinity binding sites in NO_2-exposed cells was reduced 5-fold compared to control cells. These results are comparable to those recently reported by Simon *et al.* [47], in which alterations in lipid composition of Friend erythroleukemia cells resulted in reductions in both plasma membrane fluidity and high- but not low-affinity binding sites of insulin receptors.

NO_2 exposure also caused alterations of endothelial cell plasma membrane lipids by at least two different mechanisms: (1) by increasing the phosphatidylethanolamine plus phosphatidylserine content of the plasma membrane lipids (this paper), and (2) by perturbing the molecular order of the membrane lipids through peroxidation of unsaturated fatty acids [18]. The alterations in cell lipid composition and in surface membrane-dependent receptor properties support the fluidity data and indicate that the surface membrane is an important site-specific target in NO_2-induced cytotoxicity. Although the time courses of the surface membrane fluidity changes and of the alterations in receptor function and phospholipid content are not identical, this is not unexpected for at least two reasons. First, membrane lipids are very sensitive to oxidant injury [3, 8, 9]. The formation of lipid oxidation products would be expected to alter the physical state of the membrane lipids initially and the composition of membrane lipids subsequently [45, 46]. These changes in physical state would also be expected to antedate alterations in membrane function, such as receptor–ligand interaction. Second, the sensitivity of the fluorescence spectroscopic method is much greater than the sensitivity of the assays for insulin receptor binding and lipid composition.

The preceding results support our hypothesis that NO_2 exposure results in injury to the surface of the plasma membrane of endothelial cells and also results in alterations in surface membrane structure and function. The increase in membrane phospholipid content noted in the present study can be compared, in part, with earlier reports that NO_2 exposure increases the phospholipid content of rat and hamster lungs [48, 49]. NO_2-induced lipid changes in the intact lung can result from multiple factors. However, it is relevant to the present study because alterations in lung lipid composition would be expected to change the physical state, i.e. fluidity, of membrane lipids and to alter surface function, including receptor–ligand interaction [13, 45, 46]. For example, Luly and Shinitzky [50] have reported insulin receptor binding with increased unsaturation of membrane lipids in fatty acid-supplemented erythroleukemia cells. In addition, the polar head groups of membrane lipids are known to influence the binding properties of the insulin receptors [51].

The alterations in surface membrane fluidity, insulin receptor binding, and phospholipid content disappeared after a 24-hr recovery in cells supplemented with fresh culture medium. The concordant recovery patterns provide additional evidence in support of a causal relationship between NO_2-induced alterations in surface membrane fluidity and function. The failure to reverse the alterations after the 24-hr recovery in cells whose medium was not replaced after NO_2 exposure is surprising. We have reported previously that culture medium serves to protect against NO_2-induced endothelial cell injury [18]. Therefore, it is possible that the relative deficiency of some essential protective nutrient in the medium delays the recovery process. Alternatively, it is possible that NO_2 exposure results in the generation of a substance that inhibits or delays recovery. Additional studies are required to resolve the factors responsible for recovery from NO_2-induced endothelial cell injury. However, it is now apparent that it is important to define quite precisely the culture conditions used in recovery experiments involving NO_2.

In summary, the present study demonstrates that NO_2 exposure perturbed surface lipid domains of the plasma membrane of pulmonary artery endothelial cells, resulting in alterations in the physical state and composition of plasma membrane lipids. These alterations were associated with alterations in plasma membrane receptor function. Since the structural integrity of membrane components is essential for normal cellular function, the exact nature of the NO_2-induced changes in the plasma membrane lipid bilayer and their precise relationship to altered cell and organ function need to be firmly established.

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