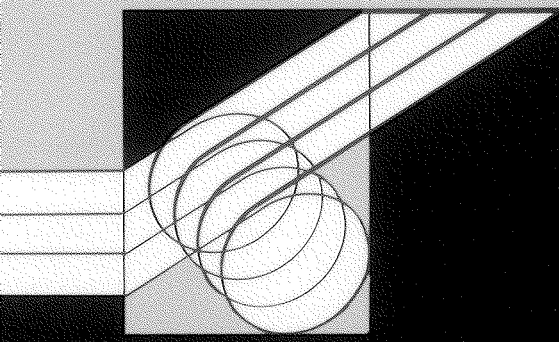
HEALTH EFFECTS INSTITUTE

H E I

RESEARCH REPORT No. 9



Biochemical and Metabolic Response to Nitrogen Dioxide-Induced Endothelial Injury

Jawaharlah M. Patel and Edward R. Block

Division of Pulmonary Medicine
College of Medicine, University of Florida
Gainesville, Florida 32610

Includes the Report of the Institute's Health Review Committee

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ABBREVIATIONS

DPH —	1,6-diphenyl-1,3,5-hexatriene
G6PDH —	glucose-6-phosphate dehydrogenase
GSH —	glutathione
GSH-per —	glutathione peroxidase
GSH-red —	glutathione reductase
HBSS —	Hanks' Balanced Salt Solution
5-HT —	5-hydroxytryptamine
LDH —	lactate dehydrogenase
TMA-DPH —	trimethylamino-DPH
τ —	excited state lifetime

PREFACE

THE HEALTH EFFECTS INSTITUTE AND ITS RESEARCH PROCESS

The Health Effects Institute (HEI) is an independent nonprofit corporation which, according to its charter, is "organized and operated . . . specifically to conduct or support the conduct of, and to evaluate, research and testing relating to, the health effects of emissions from motor vehicles."

It is organized in the following ways to pursue this purpose:

INDEPENDENCE IN GOVERNANCE

HEI is governed by a four-member board of directors whose members are William O. Baker, Chairman Emeritus of Bell Laboratories and Chairman of the Board of Rockefeller University; Archibald Cox, Carl M. Loeb University Professor (Emeritus) at Harvard University; Donald Kennedy, President of Stanford University; and Charles Powers, President, Clean Sites, Incorporated. Professor Cox chairs the Board. These individuals, who select their own successors, were chosen initially after consultations with industry and other individuals, by then Environmental Protection Agency Administrator, Douglas M. Costle.

TWO-SECTOR FINANCIAL SUPPORT

The Institute receives half of its funds from the United States government through the Environmental Protection Agency and half from the automotive industry. Twenty-six leading manufacturers of vehicles or engines that are certified for use on U.S. highways contribute to the Institute's budget, in shares proportionate to the number of vehicles or engines that they sell.

RESEARCH PLANNING AND PROJECT EVALUATION

HEI is structured to define, select, support, and review research that is aimed at investigating the possible health effects of mobile source emissions. Its research program is devised by the Health Research Committee, a multi-disciplinary group of scientists knowledgeable about the complex problems involved in determining the health effects of mobile source emissions. The Committee seeks advice from HEI's sponsors and from other sources prior to independently determining the research priorities of the Institute.

After the Health Research Committee has defined an area of inquiry, the Institute announces to the scientific community that research proposals are being solicited on a specific topic. Applications are reviewed first for scientific quality by an appropriate expert panel. Then they are reviewed by the Health Research Committee both for quality and for relevance to the mission-oriented research program. Studies recommended by the Committee undergo final evaluation by the Board of Directors, which also reviews the procedures, independence, and quality of the selection process.

When a study is completed, a draft final report is reviewed by a separate HEI Committee, the Health Review Committee. Members are expert scientists representing a broad range of experience in environmental health sciences. The Review Committee has no role in the review of applications or the selection of projects and investigators for funding. This Committee assesses the scientific quality of each study and evaluates its contribution to unresolved scientific questions.

Each funded proposal is assigned in advance of completion to a member of the Review Committee, who acts as "primary reviewer." When the draft report is received, the primary reviewer directs a peer review that involves: (1) referral of the report to appropriate technical experts and, when appropriate, (2) involvement of the Review Committee biostatistician to determine the appropriateness of the statistical methods used to evaluate the data. After the investigator has had a chance to comment on the technical evaluations, the primary reviewer drafts a review. This document is sent to the investigator for comment

It is subsequently is examined by the full Review Committee and revised as necessary. The investigator's final report, as well as the Review Committee's report, are then made available to the sponsors and to the public after evaluation by the HEI Board of Directors.

All HEI investigators are urged to publish the results of their work in the peer-reviewed literature. The timing and nature of HEI report releases are tailored to ensure that the Review Committee's report does not interfere with the journal publication process. The report of the Review Committee will be as thorough as necessary to evaluate any individual report.

INTRODUCTION

In the summer of 1982, HEI issued a Request for Applications (RFA 82-2) soliciting proposals to address the need for "the development, calibration, and application of in vitro and in vivo biological assays which measure the possible health effects of automotive emissions," particularly in reference to non-neoplastic chronic lung disease. In the fall of 1982, Dr. Jawaharlal M. Patel of the University of Florida proposed a project entitled "Biochemical and Metabolic Response to NO_2-Induced Endothelial Injury." HEI approved the three-year project and authorized expenditure of $308,000. The project began in April, 1983, and the final report was accepted

by the Health Review Committee in January, 1987. The Health Review Committee's report, which follows the investigators' report, is intended to place the investigators' final report in perspective as an aid to the sponsors of HEI and to the public.

THE CLEAN AIR ACT

The Environmental Protection Agency (EPA) sets standards for motor vehicle emissions of oxides of nitrogen (and other pollutants) under Section 202 of the Clean Air Act, as amended in 1977. Section 202 (a) (1) directs the Administrator of EPA to "prescribe (and from time to time revise) . . . standards applicable to the emission of any air pollutant from any class or classes of new motor vehicles or new motor vehicle engines, which in his judgment cause, or contribute to, air pollution which may reasonably be anticipated to endanger public health or welfare." Section 202 (a) (3) and 202 (b) (1) impose specific requirements for reductions in motor vehicle emissions of oxides of nitrogen (and other pollutants), and provide EPA with limited discretion to modify those requirements.

The determination of the appropriate standards for emissions of oxides of nitrogen depends in part on an assessment of the risks to health they present. Research on the biophysical and biochemical parameters of the effects of nitrogen dioxide in cell cultures may contribute to such risk assessment and, therefore, to informed regulatory decisionmaking.

In addition, Section 109 of the Clean Air Act provides for the establishment of national ambient air quality standards to protect the public health. The current standards include nitrogen dioxide. Research on the effects of the type described above may contribute to the assessment of the appropriateness of the standards.

BACKGROUND

In order to assess the potential of a single toxicant to produce disease, it is desirable to develop laboratory assays that will detect early effects at the cellular level. Cell cultures are used in carefully controlled artificial systems to help us understand how different cell functions relate to one another.

The cells that line the blood vessels (vascular endothelium) are typical of most cells in being delimited by a 10 nanometer-thick surface membrane. The transport of molecules at the cellular level is highly dependent upon the composition of the surface membrane and its biophysical status, including fluidity. The present model of the cell membrane is that of a fluid mosaic, where two phospholipid bilayers join to present their hydrophobic (water-immiscible) faces to the interior of the membrane and their hydrophilic (water-miscible) surfaces to the external milieu. Embedded in and traversing the bilayers are various proteins, some of which are receptors for external molecules or signals, and others which are enzymes that play a role in carrying out the mechanics of cell function.

The structure, composition, and function of the membrane are linked in a dynamic state that is influenced by the environment, and which directs a variety of control mechanisms in cell metabolism. Modifications in the electrical charge of the membrane, the proportion of component phospholipids, and the presence and activation of surface-bound enzymes can profoundly influence the behavior of the cell. A change in one function, for instance that caused by an environmental agent such as nitrogen dioxide, can affect other functions at the cellular level. These in turn may influence the performance of the cells, tissues, and organs in disease processes.

INVESTIGATORS' REPORT

by Jawaharlal M. Patel and Edward R. Block

Biochemical and Metabolic Response to Nitrogen Dioxide-Induced Endothelial Injury

ABSTRACT

Nitrogen dioxide (NO_2), a major oxidant constituent of vehicle emissions, is toxic to lung cells including endothelial cells. Since NO_2 is a reactive free radical, one of the postulated mechanisms of NO_2-induced pulmonary injury involves the peroxidation of membrane lipids. Therefore, this study evaluated the dose- and time-dependent effects of nitrogen dioxide exposure by measuring the biochemical and biophysical parameters, as well as the metabolic function, in porcine pulmonary artery and aortic endothelial cells in monolayer cultures. To evaluate the biochemical changes, the antioxidant enzyme GSH-reductase (GSH-red), GSH- peroxidase (GSH-per), and glucose-6-phosphate dehydrogenase (G6PDH) activities, as well as the lipid peroxide formation, glutathione (GSH) content, and lactate dehydrogenase (LDH) release were measured. Biophysical changes were measured by monitoring lipid fluidity in both the hydrophobic and hydrophilic regions of the plasma membrane. The uptake of 5-hydroxytryptamine (5-HT) was measured as a metabolic function of endothelial cells.

Confluent porcine pulmonary artery and aortic endothelial cells were exposed to 3 or 5 ppm NO_2 or air (control) for 3-24 hours. After 3-, 6-, or 12-hour exposures to 3 or 5 ppm NO_2, the GSH-red and G6PDH activities, as well as the lipid peroxide formation and LDH release, were not different from those of controls in both pulmonary artery and aortic endothelial cells. Exposure of the cells to 3 or 5 ppm NO_2 for 24 hours resulted in significant increases in GSH-red ($p < 0.05$) and G6PDH ($p < 0.001$) activities in both cell types. Exposure to 5 ppm NO_2 for 24 hours significantly ($p < 0.05$) increased lipid peroxide formation and increased ($p < 0.01$) LDH release in both the pulmonary artery and aortic endothelial cells. GSH-per activity and GSH content in NO_2-exposed pulmonary artery and aortic endothelial cells were not different from those of controls, irrespective of NO_2 concentration and exposure time.

Fluorescence spectroscopy was used to measure the membrane lipid fluidity. Membrane fluidity in the hydrophobic region was measured by 1,6-diphenyl-1, 3, 5-hexatriene (DPH), an aromatic hydrocarbon that partitions into the hydrophobic interior of the lipid bilayer. Fluidity changes in the hydrophilic region of the plasma membrane was measured by 1-(4-trimethly-aminophenyl)-6-phenyl-1, 3, 5-hexatriene (TMA-DPH), a cationic probe that is anchored at the lipid-water interface, as well as by fluorescamine (FM), a fluorescent molecular probe which covalently binds with amino groups of phospholipids and proteins at the external surface of the plasma membrane. After exposure, the plasma membrane fluidity in intact cells, as well as in lipid vesicles prepared from total lipid extracts of these cells, was measured. NO_2 caused a significant decrease in fluidity in both the hydrophobic and hydrophilic regions of the plasma membrane of endothelial cells as early as 3 hours; the decrease in fluidity progressed with further exposure to NO_2. Exposure to NO_2 for 24 hours, but not 3 or 12 hours, significantly ($p < 0.05$) reduced the 5-HT uptake in both the pulmonary artery and aortic endothelial cells.

These results indicate that 1) antioxidant enzyme activities are increased in the pulmonary artery and aortic endothelial cells exposed to NO_2. This response is comparable, in part, to that in the lungs from animals exposed to NO_2; and 2) oxidant injury due to NO_2 changes the physical state of membrane lipids, impairs membrane function, and contributes to biochemical and metabolic abnormalities in the cells.

INTRODUCTION

Nitrogen dioxide (NO_2) is a common indoor and outdoor environmental pollutant arising from gas- and wood-burning stoves, emissions from automotive and other combustion engines, and cigarette smoke, as well as from coal-burning plants (Kaldor et al., 1984; Horvath et al., 1970; Shy, 1973). There is considerable evidence that NO_2 exposure can cause biochemical, physiologic, and morphologic alterations in mammalian lungs (Mustafa and Tierney, 1978; Guidotti, 1978). Although epithelial cells are a major target in NO_2-induced injury, morphologic studies also identify damage to pulmonary endothelial cells in animals exposed to NO_2 (Goldstein et al., 1977; Ohata and Wakisawa, 1979; Ohata et al., 1980). The fact that inhaled NO_2 diffuses into the blood through the endothelium has been supported by recent studies that clarify the fate of inhaled NO_2 in mammals. For example, NO_2 or its intermediates was found in extrapulmonary sites in rhesus monkeys (Goldstein et al., 1977) and rats (Ohata and Wakisawa, 1979; Ohata et al., 1980) exposed to NO_2. Similarly, alterations in various components of red cell membranes and membrane enzyme activity have been demonstrated in rats exposed to NO_2 (Kunimoto et al., 1984). Some investigators believe that mammals' earliest cytotoxic changes following acute exposure to NO_2 are selective endothelial cell toxicity affecting the plasma membrane and organelles of these cells (Guidotti, 1978; Sherwin and Carlson, 1978). The development of pulmonary edema in guinea pigs, mice, rats, dogs, and rabbits following their exposure to NO_2 appears to be due to increased lung microvasculature permeability and is believed to occur as a result of endothelial injury (Guidotti, 1978).

The precise mechanism of NO_2-induced injury is unknown. However, one of the major theories involves free radical-mediated peroxidative cleavage of membrane lipids (Mustafa and Tierney, 1978; Guidotti, 1978). Free radicals generally are highly reactive and participate in physicochemical reactions such as hydrogen abstraction, radical addition, bond scission, and annihilation reactions that can oxidize unsaturated fatty acids in cell membranes, damage DNA, and oxidize protein amino side chains (Proctor and Reynolds, 1984). Since NO_2 itself is a free radical and has been reported to cause peroxidative cleavage of membrane lipids in mammalian lung cells (Sagai et al., 1984; Sevanian et al., 1979; Thomas et al., 1968), these investigators evaluated the effect of NO_2 exposure on 1) membrane fluidity and function, and 2) antioxidant defense response to oxidant injury in cultured mammalian endothelial cells.

The fluidity of a membrane generally is considered to reflect the relative motion of the constituents of the membrane (Singer, 1974; Shinitzky, 1984). Fluidity primarily depends on the physical state of the fatty acyl chains constituting the membrane bilayer structure, and optimal membrane function requires the lipids to be in a fluid state (Shinitzky, 1984). The cellular defense mechanisms involved in the detoxification of lipid hydroperoxides include the antioxidant tripeptide glutathione (GSH) and a series of GSH-dependent antioxidant enzymes, including GSH, GSH-reductase, GSH-peroxidase, and glucose-6-phospate dehydrogenase.

To study the effect of NO_2-induced injury, a tissue culture model was used because it allows the examination of the direct effects of NO_2 apart from influences from other lung cells or inflammatory cells. The endothelial cell was chosen as a model for three reasons: first, NO_2 exposure is known to cause morphologic alterations in the endothelial cells of mammalian lungs; some investigators believe that endothelial injury is the earliest manifestation of NO_2-induced lung injury (Guidotti, 1978, 1980; Brown et al., 1983). Second, vascular endothelial cells in culture have been used successfully by a number of investigators as a model for studying oxidant injury to mammalian cells (Autor et al., 1984; Block et al., 1985; Ody and Junod, 1985). Third, endothelial cells from the pulmonary artery and other major vessels (e.g., the aorta) can be grown readily in tissue culture, and they possess a number of well defined membrane-dependent properties that can be used as indices of membrane injury (Block et al., 1985; Ody and Junod, 1985; Block and Stalcup, 1981; Ryan and Ryan, 1977). Elucidation of fundamental molecular interactions can be applicable to any other cells that are injured by NO_2.

SPECIFIC AIMS

Since NO_2 is a free radical and has been reported to cause peroxidative cleavage of membrane lipids in mammalian lung cells, we hypothesized that the earliest manifestations of NO_2 injury initiate at the plasma membrane, affect the physical state of cell membrane lipids, and lead to altered biochemical and metabolic functions of the cells. To test this hypothesis, specific aims of this study were: 1) to characterize the dose-dependent early effects of NO_2 on endothelial cell membrane structure and lipid fluidity; 2) to evaluate the effect of NO_2 on the biochemical and metabolic functions of the endothelial cells; 3) to establish a relationship between NO_2-induced biophysical, biochemical, and metabolic alterations and changes in the ultrastructural morphology of endothelial cells; and 4) to assess the recovery pattern of cell injury following NO_2 exposure.

MATERIALS AND METHODS

Tissue Culture. Using methods described by Block et al. (1985), we obtained endothelial cells from the main pulmonary artery or thoracic aorta of 6- to 7- month-old pigs. 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), which contained 100 U/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 U/mg; Worthington Biochemicals, Freehold, NJ) in HBSS that contained 1x-antimicrobial agents, and was incubated at 37°C for 20 minutes (aortas) or 25 minutes (pulmonary arteries). These incubation times were chosen because they produced the best cell yields from aortas and pulmonary arteries, respectively.

At the end of the incubation period, the detached cell-enzyme mixture was transferred to a centrifuge tube that contained RPMI 1640 medium (KC Biological, Lenexa, KA) supplemented with 15% fetal bovine serum (Flow Laboratories, McLean, VA) and 1x-antimicrobial agents. The cell-enzyme mixture was then centrifuged at 160 xg for 5 minutes at 4°C. The pellet was resuspended in fresh medium, and the suspension was seeded into sterile plastic culture dishes at densities of 1-2 x 10^4 cells/cm^2 and incubated at 37°C with humidified 5% CO_2 in air.

After 60 minutes, the nonadherent cell suspension and medium were removed, and fresh medium was added to the dishes. The medium was changed every 48 hours until primary confluence was reached (4 to 7 days), after which the concentration of fetal bovine serum in the culture medium was decreased to 5%. Endothelial cell monolayers were subcultured (i.e., passaged) 4 to 5 days after confluence by incubation for 1 minute with 0.1% trypsin (VMF Trypsin, Worthington Diagnostic Systems, Inc., Freehold, NJ) in calcium-magnesium-free HBSS (KC Biological, Lenexa, KA). Preconfluent subcultures were incubated in RPMI 1640 culture medium containing 15% fetal bovine serum and 1x-antimicrobial agents. Second to fifth passage cells in post-

confluent monolayers were used for all experiments. Each dish of control cells was matched with a dish of NO_2 cells for exposure time and for variables due to tissue culture techniques, e.g., seeding density of endothelial cells, time to monolayer confluence, cell density at confluence, number of subcultures, and number of days after confluence. In both pulmonary artery and aortic endothelial cells, seeding densities were 1-2 x 10^4 cells/cm^2, time to monolayer confluence was 2 or 3 days, and cell densities at confluence were 1.25 $\pm$ 0.03 (SE) x 10^6 cells per dish. Cells were studied one or two days after confluence.

Identification of Endothelial Cells. All cell monolayers in culture initially were 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.

Exposure to NO_2. Culture dishes containing endothelial cell monolayers were exposed to a continuous flow of 3 or 5 ppm NO_2 in air containing 5% CO_2 in an airtight 12 x 12 inch stainless steel chamber (Brinkmann Instruments, Westbury, NY) housed inside a CO_2 incubator (Hotpack, Philadelphia, PA) and maintained at 37°C. The stainless steel chamber contained a water-filled tray on its bottom and had one inlet and one outlet port. Pre-mixed gas cylinders were obtained from Air Products, Jacksonville, FL. Exposures were carried out for 3-, 6-, 12-, or 24-hour periods. Equal numbers of matched dishes (i.e., matched for confounding tissue culture variables) were exposed to air containing 5% CO_2 for comparable times and served as controls. NO_2 concentrations in the chamber were monitored by the Saltzman method (1954).

The concentration of NO_2 used in this study is higher than the ambient level but is much lower than levels used in many in vivo studies (Mustafa and Tierney, 1978; Guidotti, 1978; Sagai et al., 1984; Thomas et al., 1968). We chose 3 and 5 ppm NO_2 because it allowed us to define the mechanisms of NO_2-induced injury at the molecular level by using a tissue culture model. Nitrogen dioxide is of limited solubility in an aqueous medium; therefore, the amount of NO_2 directly interacting with the cells is much less than the 3 or 5 ppm flowing into the chamber. The exact amount of NO_2 reaching the cells is technically difficult to ascertain 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. Therefore, in some experiments, the effect of medium and medium components on membrane fluidity was evaluated after exposure of cells to NO_2. In addition, serum concentration of the medium was reduced from 10% to 3% to evaluate potential interaction of NO_2 with medium components. For the recovery study, which took place immediately after the NO_2 exposure, the medium was changed and cells were kept in a control chamber with fresh medium and monitored up to 48 hours.

Measurement of Membrane Fluidity. We used fluorescence spectroscopy to evaluate the effects of NO_2 exposure on membrane fluidity in cultured endothelial cells. The principle of this method is that the 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 (Lakowicz, 1983). As the fluidity of the surrounding medium (i.e., the cell membrane) decreases, the mobility of the fluorescent probe is hindered, which results in a change in its fluorescence polarization.

Immediately after exposure, cells were washed twice with HBSS, scraped and suspended in 0.1 M phosphate buffer (pH 7.4) that contained 0.15 M KCl (KCl buffer). Suspension of cells in 1 ml of the KCl buffer were mixed with 1 ml of a 20- μM dispersion of 1,6-diphenyl-1,3,5,-hexatriene (DPH), a fluorescent aromatic hydrocarbon with an all *trans* polyene system that partitions into the hydrophobic interior of the cell membrane (Pessin et al., 1978), or with 1 ml of a 10- μM trimethylamino-DPH (TMA-DPH) to assess the fluidity of the lipid-water interface of the plasma membrane (Prendergast et al., 1981), or with 0.25 ml of a 100- μM dispersion of fluorescamine, which covalently binds with amino groups of proteins and phospholipids (ethanolamine and serine) to assess the external surface of the plasma membrane (Underfriend et al., 1972). After 30 minutes of incubation with DPH, 20 minutes with TMA-DPH, and 5 minutes with fluorescamine at 25°C, the cells were washed twice with KCl buffer, homogenized, resuspended in 2 ml of KCl buffer, and used for spectroscopic measurements.

Ultraviolet-visible spectra were determined with a Cary 210 spectrophotometer equipped with a cell holder maintained at 37°C. Steady-state fluorescence excitation and emission spectra and polarization were measured with an SLM 4800 subnanosecond spectrofluorometer (SLM Instruments, Inc., Urbana, IL). Fluorescence spectra were corrected for wavelength-dependent variation in light-source output, phototube response, and monochromator efficiency (Angelides and Nutter, 1983). 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 (Lentz et al., 1979).

Rotational relaxation time is a sensitive index of membrane fluidity that accurately reflects the rotational motion of fluorescent probes (e.g., DPH) incorporated into the lipid bilayer of membranes (Lakowicz, 1983; Pessin et al., 1978). In biophysical terms, it is the time it takes for a given probe molecule within the membrane to rotate through an angle θ so that $\cos \theta = e^{-1}$ (Lakowicz, 1983; Pessin et al., 1978). This time varies inversely with the fluidity of the membrane surrounding the probe. The rotational relaxation time has distinct advantages over other fluidity parameters, such as microviscosity, because it quantifies both rate and range of the rotational motion of the membrane probe, and does not depend on calibration curves derived from reference solvents (Pessin et al., 1978).

Rotational relaxation times for DPH were calculated as a function of temperature, at 5° increments, from 5°C to 40°C,

using the Perrin equation as modified by Van Blitterswijk et al. (1981):

$$r_s = (r_0 - r_\infty)/(1 + 3\tau/\rho) = r_\infty$$

where r_s is the measured steady-state anisotropy at a given temperature, r_0 is the limiting anisotropy (i.e., the anisotropy in the absence of rotational motion), and r_∞ is the infinitely slow decaying component of DPH. Since the values of r_s ranged from 0.13 to 0.28, the values for r_∞ were determined by the empirical relation between r_∞ and r_s by using equation, $r_\infty = 4/3\, r_s - 0.1$ (Van Blitterswijk et al., 1981). τ is the excited-state lifetime of the fluorescent probe, and ρ is the rotational relaxation time in nanoseconds. r_0 is 0.362 for DPH (Shinitzky and Barenholz, 1984). According to this equation, ρ depends on three parameters: r_s, the steady state anisotropy; r_∞, the slow-decaying or static component of r_s (Van Blitterswijk et al., 1981); and τ, the excited-state lifetime.

Excited-state lifetimes were determined by phase-demodulation methods and calculated as before (Angelides and Nutter, 1983). The emission polarizer was set at 55° from the vertical position with vertically polarized light, to eliminate the effects due to Brownian rotation on the observed lifetime.

Fluorescence anisotropies were measured for DPH, TMA-DPH, and fluorescamine by 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 polarized $(V/H)_v$ or horizontally polarized $(V/H)_h$. The steady-state anisotropy 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. For measurements of r_s, the emission wave length was 430 nm for DPH (362 nm excitation), 430 nm for TMA-DPH (360 nm excitation), and 470 nm for fluorescamine (385 nm excitation).

Measurement of Fluidity in Lipid Vesicles. To demonstrate further that the NO_2-induced changes are in the lipid bilayer, we measured fluidity in lipid vesicles prepared from lipid extracts of control and NO_2-exposed cells. Since fluorescamine reacts with amino groups of proteins and phospholipids, this also allows us to confirm that the NO_2-induced changes are in lipids rather than in proteins.

Preparation of Vesicles. Immediately after exposure, cells were washed twice and lipids were extracted by the method of Folch et al. (1957). Multilamellar dispersions were prepared from the chloroform solutions of total lipid extracts from control and NO_2-exposed endothelial cells. The lipid extracts were evaporated to dryness with a stream of N_2. The dried samples were reconstituted with 1.0 ml of 0.1 M phosphate buffer (pH 7.4). The lipids were allowed to swell in an N_2 atmosphere at room temperature. The suspensions then were sonicated in a Branson water-bath sonicator for 10 minutes under N_2 to disperse the lipids. Characterization of vesicle stability was done by measuring release of entrapped carboxyfluorescein at room temperature (25°C) and at 37°C (Luke et al., 1980). The vesicle suspensions also were subjected to gel filtration on a Sepharose 4B column, which is known to exclude single lamellar vesicles (Gilmore et al., 1979).

The lipid vesicles prepared from the total lipid extracts from control or NO_2-exposed endothelial cells were used to measure fluidity, as described above.

Biochemical Assays. 5-Hydroxytryptamine (5-HT) is a biologically active amine that is removed from the circulation by pulmonary endothelial cells. Removal of 5-HT involves a sodium- and energy-dependent, carrier-mediated plasma membrane transport process (Block et al., 1985; Block and Stalcup, 1981). 5-HT uptake was measured immediately after exposure in control and NO_2-exposed cells in a monolayer culture. Uptake by the cells was measured after a 10-minute incubation in the presence of 1×10^{-6} M 5-[^{14}C]-HT (5-[2-^{14}C] hydroxytryptamine binoxalate (New England Nuclear, Boston, MA) at 37°C in 20% O_2-75% N_2-5% CO_2. Uptake of 5-HT was calculated from the disappearance of 5-[^{14}C]-HT from the culture medium during the 10-minute incubation (Block et al., 1985). Total ^{14}C-radioactivity and 5-[^{14}C]-HT and its radiolabeled metabolites in the culture medium were analyzed using ion exchange columns, thin layer chromatography, and lipid scintillation spectrometry. Uptakes were corrected for nonspecific loss and nonenzymatic degradation. Uptake of 5-HT is expressed as picomoles of 5-HT taken up per hour per 10^6 cells. Release of LDH into the medium, expressed as LDH in medium/(LDH in medium + cellular LDH) x 100, was used as an index of cytotoxicity and plasma membrane leakiness (Autor et al., 1984; Block et al., 1985). The LDH activity was measured by spectrophotometric analysis of NADH oxidation (Bergmeyer et al., 1966). Corrections were made for the LDH activity of fetal bovine serum. Lipid peroxide formation was estimated in cell homogenates immediately after NO_2 exposure by measuring malonaldehyde formation by the thiobarbituric acid reaction (Bernheim et al., 1948.)

For antioxidant enzyme activities, immediately after exposure to NO_2 or air, the endothelial cell monolayers were washed twice with 4 ml of sterile phosphate buffered saline (pH 7.2) and then scraped into 1.5 ml of 50 mM phosphate buffer (pH 7.4). The cells were homogenized using a motor-driven Teflon pestle to glass homogenizer (Eberbach Corp, Ann Arbor, MI). Total GSH content of the cell was measured using 0.5 ml of the cell homogenate, and the remaining homogenates were centrifuged at 1000 rpm for 10 minutes at 4°C. Enzyme activities were measured in the homogenate supernatant. There was no measurable enzyme activity in the pellet.

The GSH-red activity was measured by following NADPH oxidation at 340 nm, as described by Bergmeyer et al. (1966). One unit of activity is equivalent to 1 nmol NADPH oxidized per minute per milligram of protein. The GSH-per activity

was measured by coupling the reduction of GSH-red as described by Little et al. (1970). One unit of activity is equivalent to 1 nmol NADPH oxidized per minute per milligram of protein. The G6PDH activity was measured by following the reduction of $NADP^+$ during the oxidation of glucose-6-phosphate at 340 nm, as described by Lohr and Waller (1974). One unit of activity is the equivalent of 1 nmol NADP+ reduced per minute per milligram of protein. All enzyme assays were conducted at room temperature (25°C) using a spectrophotometer (Model 2400, Gilford Instruments, Oberlin, OH). Total nonprotein sulfhydryl groups in cell homogenate was estimated as described by Sedlak and Lindsay (1968). Protein concentration was determined by the method of Lowry et al. (1951).

Data Analysis. For each cell type, rotational relaxation time, fluorescence anisotropies, 5-HT uptake, lipid peroxidation, LDH release, GSH content, and antioxidant enzyme activities were measured in a minimum of 6 to 8 control and NO_2-exposed dishes for each exposure time and NO_2 concentrations. Within each experiment, control and NO_2-exposed dishes were matched for seeding density, time to monolayer confluence, cell density at confluence, number of subcultures, and number of days after confluence. This experimental paradigm conforms to a split-plot design (Winer, 1971), where pairs of matched control and NO_2-exposed dishes form whole plots; individual dishes are subplots. Rotational relaxation time or fluorescence anisotropy is linearly related to temperature. Therefore, y-intercepts and slopes for the plots of τ or r_s against temperature were estimated (Winer, 1971) for each dish, and were used in multivariate split-plot analysis of variance to test for the effects of cell type, NO_2 exposure, and their possible interactions on fluidity. If significant effects were identified, univariate analysis of variance or *post hoc* pairwise comparisons of means were performed using the Bonferroni correction for equality (Winer, 1971). Analysis of variance was used to test the effects of NO_2 exposure and endothelial cell type on antioxidant enzyme activities, lipid peroxide formation, LDH release, 5-HT uptake, and GSH content. If significance was found, Student's paired *t*-test was used (Winer, 1971). All computations were performed using the Statistical Analysis System (SAS) package of programs, with the aid of the Biostatistics Unit or with the aid of a Hewlett-Packard programmable calculator (HP-41CV).

RESULTS

General Characteristics of Cells. After exposure to 3 or 5 ppm NO_2 for 3 to 24 hours, the structure of both pulmonary artery and aortic endothelial cells was not different from that of the control cells, as assessed by phase contrast and electron microscopy. Cell protein per culture dish was comparable in control and NO_2-exposed pulmonary artery and aortic endothelial cells (Table 1). Exposure to 3 or 5 ppm NO_2 for up to 24 hours had no significant effect on cell number in either cell type (Table 2).

Physical Properties of Fluorescent Molecular Probes Incorporated Into Endothelial Cells. When control and NO_2-exposed (5 ppm, 24 hours) pulmonary artery endothelial cells were incubated with an aqueous dispersion of DPH (Figure 1) or TMA-DPH (Figure 2), the time-dependent increases in fluorescence intensities were parallel and achieved equilibrium after 20 to 40 minutes or 15 to 20 minutes, respectively. Thus, the rates of membrane incorpora-

Table 1. Effect of NO_2 Exposure on Protein Content in Pulmonary Artery (PA) and Aortic (AO) Endothelial Cells

		Protein (μg/dish)*		
			NO_2	
Exposure time (hour)	Cell type	Control	3 ppm	5 ppm
3	PA	198 ± 7	190 ± 4	197 ± 7
	AO	207 ± 10	198 ± 9	205 ± 10
6	PA	208 ± 8	209 ± 6	199 ± 8
	AO	198 ± 6	215 ± 12	204 ± 6
12	PA	216 ± 11	218 ± 9	229 ± 6
	AO	196 ± 9	210 ± 10	221 ± 10
24	PA	224 ± 10	216 ± 10	208 ± 9
	AO	210 ± 7	211 ± 7	211 ± 11

*Data represent mean ± SE (n = 6). Differences between control and NO_2 groups at each exposure time were not significant.

Table 2. Effect of NO_2 Exposure on Cell Counts of Pulmonary Artery (PA) and Aortic (AO) Endothelial Cells

		Cell Counts* (10^6 cells/dish)		
			NO_2	
Exposure time (hour)	Cell type	Control	3 ppm	5 ppm
3	PA	1.20 ± 0.02	1.22 ± 0.02	1.17 ± 0.03
	AO	1.30 ± 0.02	1.25 ± 0.03	1.33 ± 0.02
6	PA	1.23 ± 0.02	1.24 ± 0.02	1.27 ± 0.03
	AO	1.26 ± 0.03	1.25 ± 0.02	1.26 ± 0.01
12	PA	1.24 ± 0.04	1.26 ± 0.03	1.27 ± 0.03
	AO	1.26 ± 0.01	1.24 ± 0.01	1.37 ± 0.04
24	PA	1.34 ± 0.03	1.28 ± 0.04	1.31 ± 0.02
	AO	1.24 ± 0.03	1.23 ± 0.02	1.21 ± 0.02

*Data represent mean ± SE (n = 6). Differences between control and NO_2 groups at each time were not significant.

tion of DPH and TMA-DPH, and the equilibrium fluorescence intensity of DPH or TMA-DPH incorporated into control and NO_2-exposed pulmonary artery endothelial cells, were identical. Since fluorescamine instantly reacts with amino groups, and excess probe is hydrolyzed to nonfluorescent product in seconds, it is not possible to monitor time-dependent fluorescence intensities for fluorescamine. The fluorescence emission spectra for DPH (Figure 3), and TMA-DPH (Figure 4), with emission wavelength maxima of 430 for both, also were identical in control and NO_2-exposed pulmonary artery endothelial cells. Emisson spectra for fluorescamine (Figure 5) were different for control and NO_2-exposed cells, even though wavelength maxima of 470 were the same for both. Similar results were obtained with aortic endothelial cells (not shown). There were no NO_2 concentration and exposure time-dependent effects on the physical properties of these probes.

To evaluate the effect of NO_2 on the spectroscopic properties of DPH, we measured the excited-state lifetime (τ) of the probe in control and NO_2-exposed cells. Table 3 shows the effect of NO_2 on the τ values for DPH in both pulmonary artery and aortic endothelial cells. Although the τ in the NO_2-exposed endothelial cells was slightly higher than in the control cells, the differences were not significant and did not affect the τ values. Therefore, differences in DPH between control and NO_2-exposed endothelial cells can be attributed to changes in the intrinsic characteristics of the membrane components that affect the rotational motion of the probe, rather than to changes in the amount or rate of DPH incorporated or to changes in τ.

NO_2-Induced Membrane Fluidity Changes in Endothelial Cells. Effects of NO_2-induced alterations in lipid fluidity in hydrophobic (measured by DPH) and hydrophilic (measured by TMA-DPH and fluorescamine) membrane constituents are described below.

Exposure to 5 ppm NO_2 for 3 hours caused significant increases ($p < 0.05$) in the rotational relaxation time of DPH in both pulmonary artery and aortic endothelial cells (Figure 6A). Exposure to NO_2 for 12 hours resulted in differences similar to those observed after 3 hours (data not shown). However, prolonged exposure to NO_2 for 24 hours caused further increases in the rotational relaxation time of DPH ($p < 0.001$ versus 3-hour and 12-hour exposures) in both pulmonary artery and aortic endothelial cells (Figure 6B). Exposure to 3 ppm NO_2 resulted in similar changes (data not shown).

Exposure to 5 ppm NO_2 for 12 hours caused a significant ($p < 0.05$) increase in r_s for TMA-DPH in both pulmonary artery and aortic endothelial cells (Figure 7). Prolonged exposure to NO_2 for 24 hours caused further increases in r_s ($p < 0.001$) for TMA-DPH in both pulmonary artery and aortic endothelial cells (Figure 7). Similarly, exposure to 5 ppm NO_2 for 12 and 24 hours caused significant ($p < 0.05$ and $p < 0.001$) increases in r_s for fluorescamine in pulmonary artery and aortic endothelial cells, respectively (Figure 8).

Effect of NO_2 on Membrane Phospholipid Content of Endothelial Cells. Pulmonary artery endothelial cells were exposed to 5 ppm NO_2 for 24 hours, and total lipids were extracted and proteins were separately collected. Lipid and protein fractions were separately reacted with fluorescamine, which reacts with amino groups of proteins and phospholipids (ethanolamine and serine). As shown in Table 4, NO_2 exposure significantly ($p < 0.01$) increased cell phospholipid content. Total protein content of NO_2-exposed cells was not different from the controls. These results, together with alterations in fluidity, suggest that NO_2 exposure results in an increase of membrane phospholipid content, which increases the rigidity and decreases the fluidity of endothelial cell membrane in the hydrophobic and hydrophilic regions.

Effect of NO_2 Exposure Measured as Fluidity in Lipid Vesicles. Figures 9, 10 and 11 show r_s values for DPH, TMA-DPH, and fluorescamine, respectively, in lipid vesicles prepared from total lipid extracts of control and NO_2-exposed pulmonary artery endothelial cells. NO_2 exposure (5 ppm for 24 hr) significantly ($p < 0.001$) reduced lipid fluidity in hydrophobic and hydrophilic regions of the vesicles compared to the controls. These results are similar to those observed in intact endothelial cells, suggesting that the NO_2-induced alterations are in the membrane lipid bilayer and are related to structural changes in specific areas of the bilayer.

Table 3. Effect of 24-Hour Exposure to 5 ppm NO_2 on Excited-State Lifetimes (τ) for DPH Measured at Three Temperatures in Pulmonary Artery (PA) and Aortic (AO) Endothelial Cells*

	PA		AO	
Temp.	Control	NO_2	Control	NO_2
5°C	9.99 ± 0.13	10.33 ± 0.15	9.69 ± 0.13	10.48 ± 0.09
25°C	9.07 ± 0.09	9.61 ± 0.14	8.73 ± 0.10	9.15 ± 0.11
40°C	9.23 ± 0.12	8.94 ± 0.09	8.27 ± 0.14	8.94 ± 0.08

*Values for τ are in nanoseconds and represent the mean ± SE ($n = 5$). Differences between control and NO_2 groups at each temperature were not significant.

Table 4. Effect of NO_2 Exposure on Phospholipid and Protein Content of Pulmonary Artery Endothelial Cells*

	Phospholipids (Ethanolamine & Serine)		Proteins (Total)
	Total lipid extract	Separated phospholipids	μg/dish
	μg/μg protein	μg/μg protein	
Control	0.071 ± 0.009	0.062 ± 0.006	363.2 ± 47
NO_2	0.089 ± 0.013**	0.081 ± 0.005**	360.0 ± 36

*Cells were exposed to 5 ppm NO_2 for 24 hours. Amino groups of phospholipids and proteins were estimated by fluorescamine.
**$p < 0.01$ vs control.

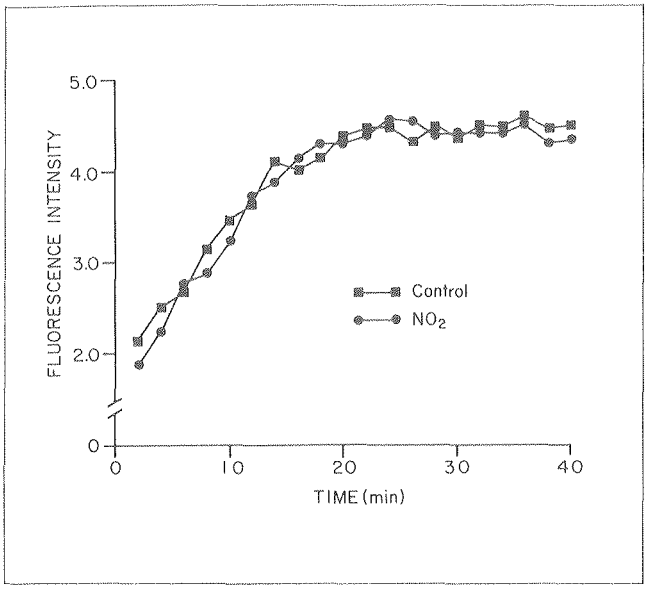

Figure 1. Effect of NO_2 exposure (5 ppm for 24 hours) on kinetics of DPH incorporation into pulmonary artery endothelial cells. Equilibrium fluorescence intensities, achieved between 20 and 40 minutes, are identical and indicate that the amount of probe incorporated into control and NO_2-exposed cells was identical. Similar results were obtained with aortic endothelial cells.

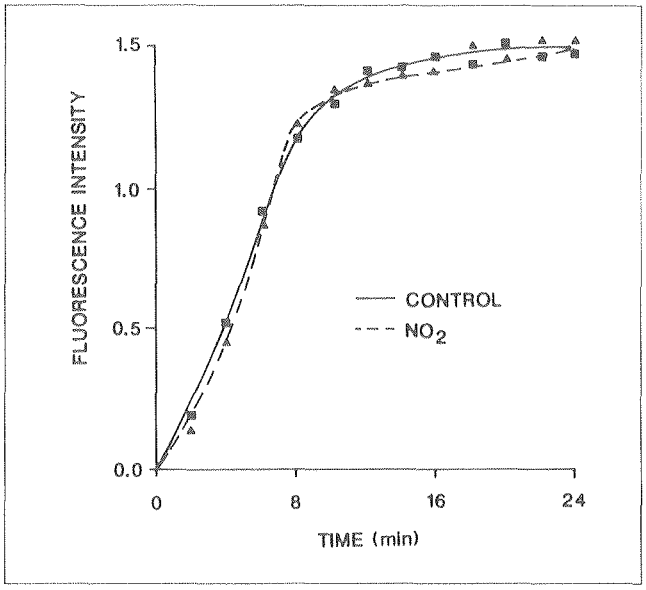

Figure 2. Effect of NO_2 exposure (5 ppm for 24 hours) on kinetics of TMA-DPH incorporation into pulmonary endothelial cells. Equilibrium fluorescence intensities, achieved between 15 and 20 minutes, are identical and indicate that the amount of probe incorporated into control and NO_2-exposed cells was identical.

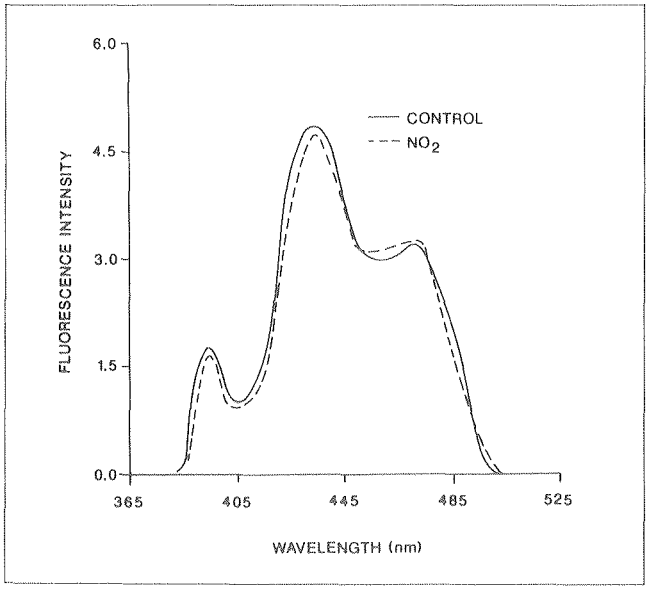

Figure 3. Effect of NO_2 exposure (5 ppm for 24 hours) on fluorescence emission spectra for DPH in pulmonary artery endothelial cells.

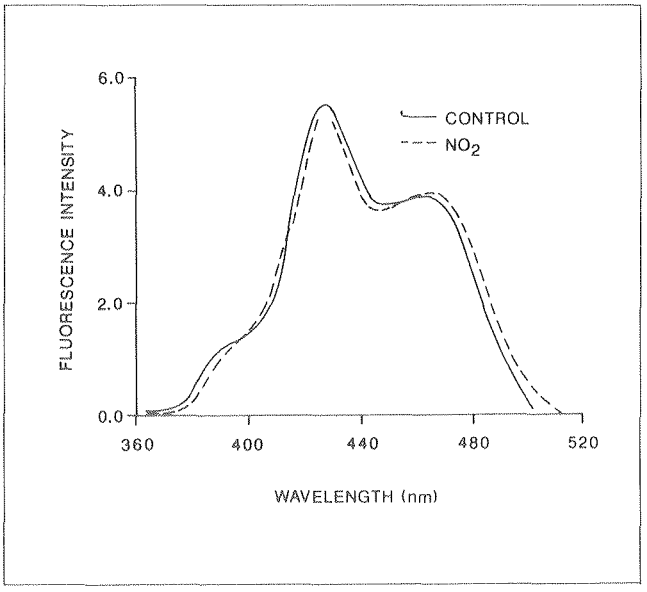

Figure 4. Effect of NO_2 exposure (5 ppm for 24 hours) on fluorescence emission spectra for TMA-DPH in pulmonary artery endothelial cells.

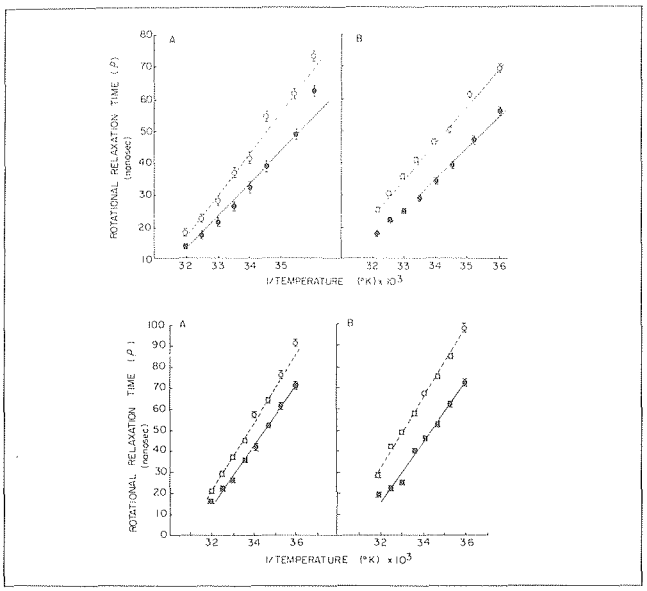

Figure 5. Effect of NO_2 exposure (5 ppm for 24 hours) on fluorescence emission spectra for fluorescamine in pulmonary artery endothelial cells.

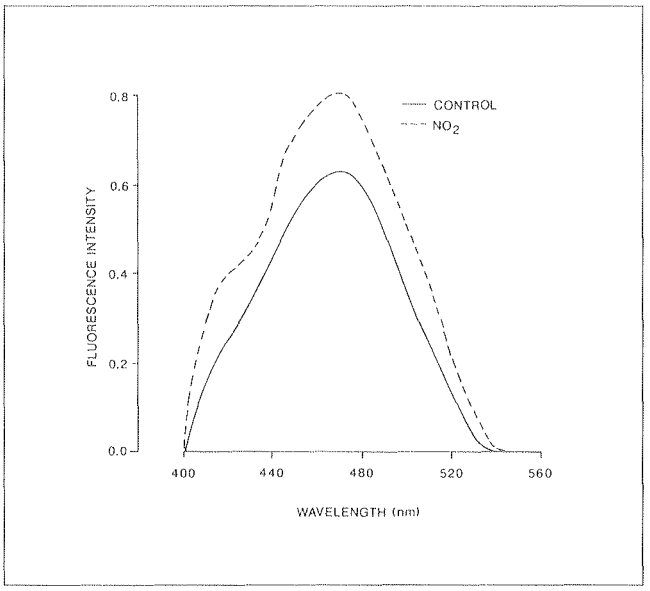

Figure 6. Effect of 5 ppm NO_2 exposure (O----O) on the rotational relaxation times of DPH. Upper panel shows effect on pulmonary artery and lower panel aortic endothelial cells. *A*, 3 hour-; and *B*, 24-hour exposure in both panels. Control cells are represented by (●——●) in each panel. The results shown in each panel are an average of 4 to 8 experiments. Data represent mean ± SE.

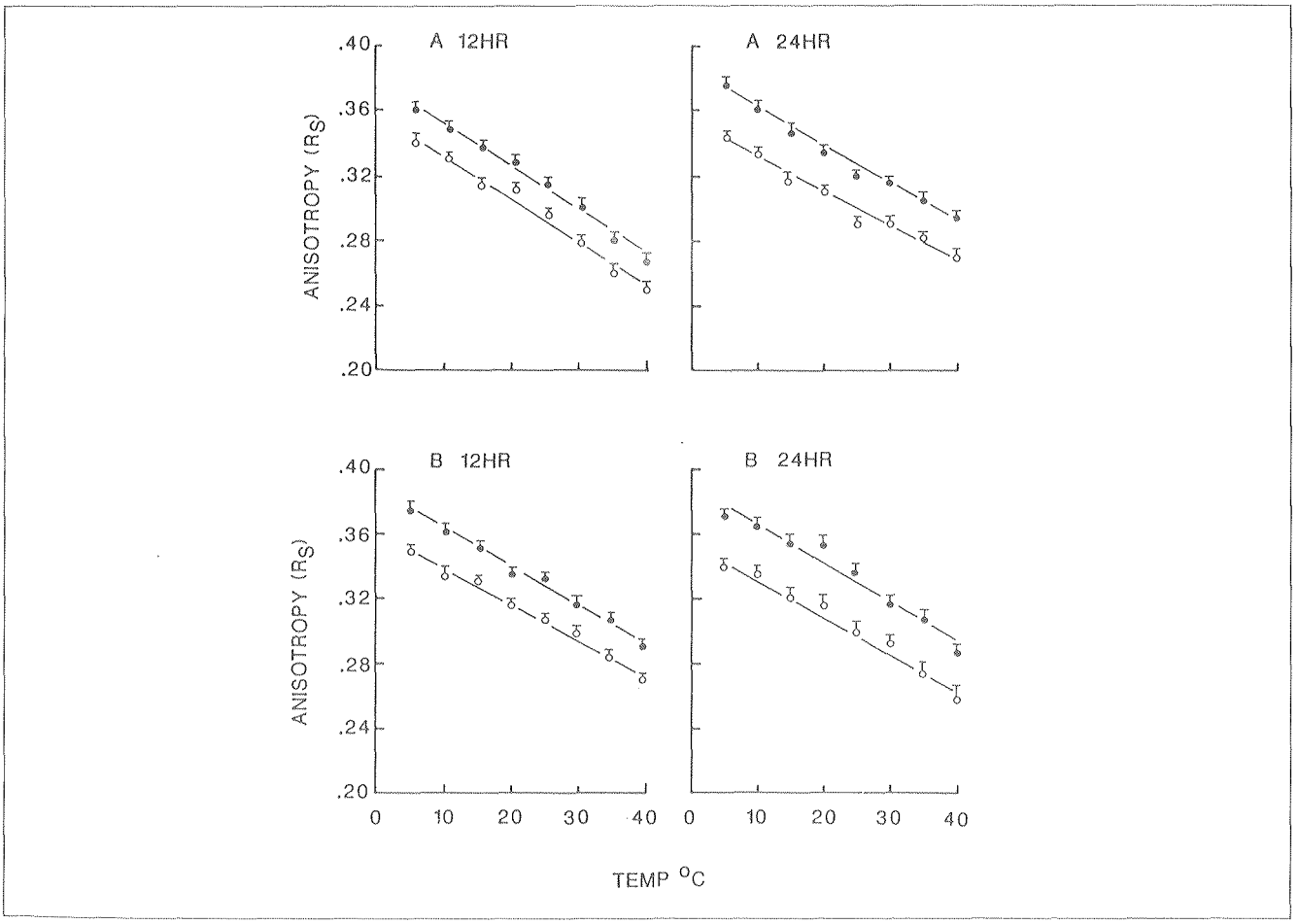

Figure 7. Effect of 12- and 24-hour exposure of NO_2 on steady-state fluorescence anisotropies (r_s) for TMA-DPH in pulmonary artery (panel A) and aortic (panel B) endothelial cells. Cells were exposed to either air (——) or 5 ppm NO_2 (* ---- *).

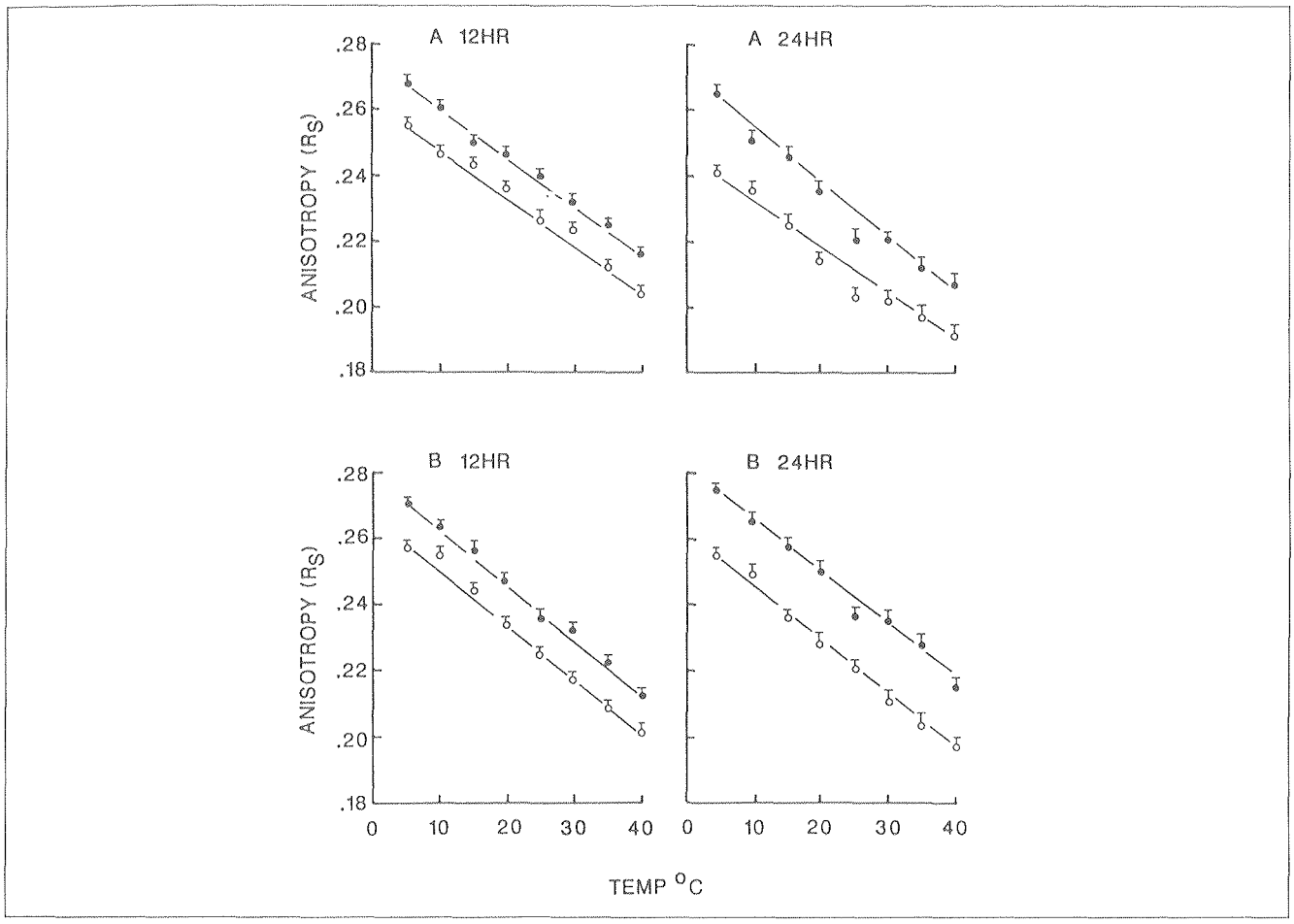

Figure 8. Effect of 12- and 24-hour exposure of NO_2 on steady-state fluorescence anisotropies (r_s) for fluorescamine in pulmonary artery (panel A) and aortic (panel B) endothelial cells. Cells were exposed to either air (□——□) or 5 ppm NO_2 (■----■).

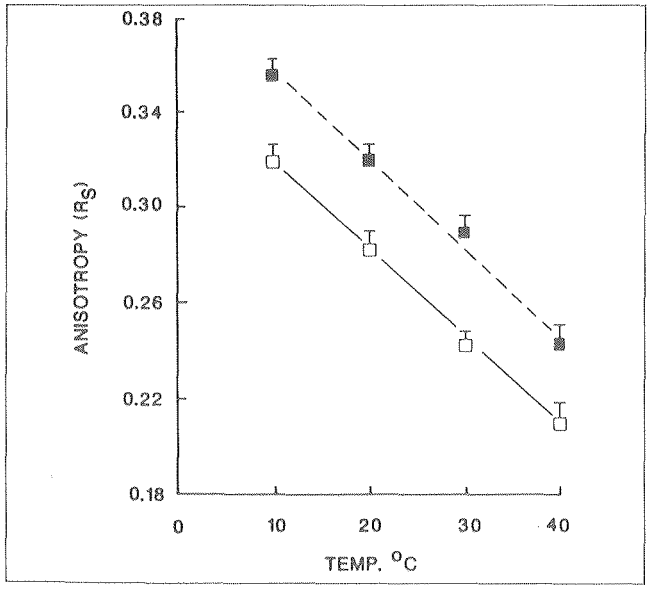

Figure 9. Steady-state fluorescence anisotropy (r_s) for DPH in lipid vesicles prepared from total lipid extracts of control (□——□) and NO_2 (■----■)-exposed pulmonary artery endothelial cells. Cells were exposed to 5 ppm NO_2 for 24 hours.

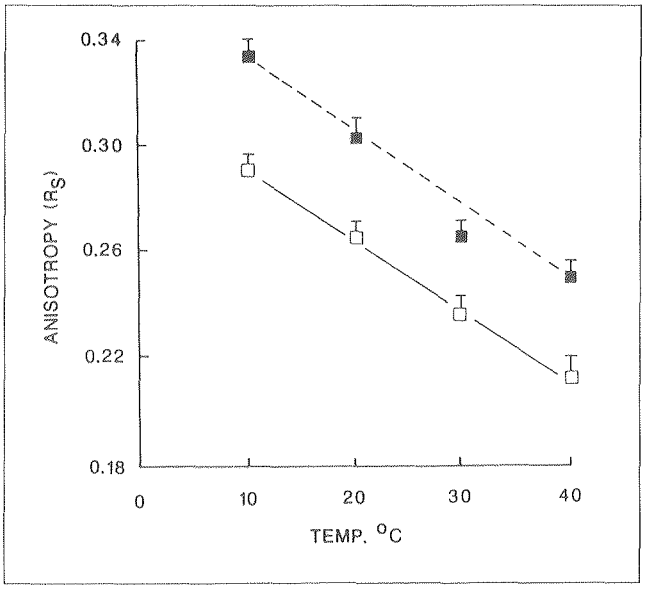

Figure 10. Steady-state fluorescence anisotropy (r_s) for TMA-DPH in lipid vesicles prepared from total lipid extracts of control (□——□) and NO_2 (■----■)-exposed pulmonary artery endothelial cells. Cells were exposed to 5 ppm NO_2 for 24 hours.

Effect of NO_2 on Culture Medium Components. Presently, there is no widely accepted method for culturing endothelial cells in serum-free culture media. Therefore, NO_2 might react with fetal calf serum or other components of the culture medium and generate reactive products in the medium that might, in turn, react with endothelial cells and contribute to cytoxicity.

To evaluate this possibility, we used two approaches. First, we exposed (A) cells + medium and (B) medium only to NO_2 for 12 hours. At the end of the exposure, membrane fluidity was measured in the cells from (A). The media from (A) and (B) were added to two new sets of cells, (C) and (D), that were incubated under controlled conditions for 12 hours. After the 12 hours of controlled incubation, membrane fluidity was measured in the cells from (C) and (D). As shown in Figure 12, it is clear that the media from (A) and (B) that were exposed to NO_2 did not alter the membrane fluidity in the cells in sets (C) and (D).

Second, endothelial cells were directly exposed, in the absence of a culture medium, either to control conditions or to 5 ppm NO_2 for 3 hours. At the end of the exposure, membrane fluidity was measured in both control and NO_2-exposed cells. As shown in Figure 13, direct exposure to NO_2 significantly decreased the fluidity of the plasma membrane ($p < 0.001$). The decrease in fluidity after 3 hours of direct exposure to NO_2 was greater than the decreases that occurred after 24 hours of exposure to 5 ppm in the presence of medium (Figure 6B).

Effects of NO_2 Exposure on 5-HT Uptake. Exposure to 5 ppm for 3 hours (Figure 14) or 12 hours (data not shown) did not significantly alter the uptake of 5-HT by pulmonary artery or aortic endothelial cells. However, exposure to NO_2 for 24 hours significantly ($p < 0.05$) reduced 5-HT uptake in both pulmonary artery and aortic endothelial cells (Figure 14). 5-HT uptake in cells exposed to 3 ppm NO_2 was not different from the controls.

Effect of NO_2 Exposure on LDH Release and Lipid Peroxide Formation. LDH release (Table 5) and lipid peroxide formation (Table 6) were comparable in control and NO_2-exposed pulmonary artery and aortic endothelial cells at 3, 6 and 12 hours. However, after 24 hours of NO_2 exposure, significant increases in LDH release ($p < 0.01$) and lipid peroxide formation ($p < 0.05$) were observed in both pulmonary artery and aortic endothelial cells.

Effect of NO_2 Exposure on Antioxidant Enzyme Activities of Endothelial Cells. The enzyme activities of GSH-red, G6PDH, and GSH-per in control pulmonary artery and aortic endothelial cells are shown in Figures 15 and 16, respectively. The GSH-red and GSH-per activities in control pulmonary artery endothelial cells were significantly higher ($p < 0.02$) than those in controlled aortic endothelial cells at all exposure times.

Exposure to 3 or 5 ppm NO_2 for 3 to 24 hours had no significant effect on GSH-per activity in either pulmonary artery or aortic endothelial cells (data not shown). Similarly, exposure to 3 or 5 ppm NO_2 for 3, 6, or 12 hours had no significant effect on GSH-red and G6PDH activities in either cell type (Figures 17 and 18). In contrast, exposure to 3 or 5 ppm NO_2 for 24 hours caused significant increases in GSH-red activity ($p < 0.05$) and in G6PDH activity ($p < 0.001$). The NO_2-induced increases were significantly ($p < 0.02$) greater in 5 ppm NO_2 than in 3 ppm NO_2 in both pulmonary artery and aortic endothelial cells. NO_2-induced increases in antioxidant enzyme activities appear to be time dependent in both pulmonary artery and aortic endothelial cells.

Table 5. Effect of NO_2 Exposure on LDH Release by Pulmonary Artery (PA) and Aortic (AO) Endothelial Cells*

		LDH Release (% Total)		
			NO_2	
Exposure time (hour)	Cell type	Control	3ppm	5 ppm
3	PA	8.1 ± 0.8	8.2 ± 0.5	8.6 ± 1.0
	AO	7.4 ± 0.9	8.0 ± 0.7	7.8 ± 0.6
6	PA	8.6 ± 0.8	7.6 ± 0.4	8.0 ± 0.7
	AO	7.5 ± 0.4	7.8 ± 0.6	7.4 ± 0.6
12	PA	7.9 ± 0.8	8.3 ± 0.7	8.3 ± 1.2
	AO	7.3 ± 0.7	8.1 ± 0.5	9.0 ± 1.5
24	PA	7.0 ± 0.8	9.6 ± 1.2	16.7 ± 1.0**
	AO	7.7 ± 0.5	9.0 ± 1.3	15.4 ± 0.8**

*Data represent mean ± SE (n = 6)

**$p < 0.01$ vs control

Table 6. Effect of NO_2 Exposure on Lipid Peroxide Formation by Pulmonary Artery (PA) and Aortic (AO) Endothelial Cells*

		Lipid Peroxide Formation nmol malonaldehyde /hr/mg protein		
			NO_2	
Exposure time (hour)	Cell type	Control	3ppm	5ppm
3	PA	0.35 ± 0.05	0.39 ± 0.07	0.29 ± 0.03
	AO	0.30 ± 0.05	0.33 ± 0.05	0.34 ± 0.09
6	PA	0.39 ± 0.07	0.32 ± 0.03	0.39 ± 0.07
	AO	0.36 ± 0.08	0.36 ± 0.07	0.35 ± 0.04
12	PA	0.29 ± 0.06	0.32 ± 0.06	0.32 ± 0.04
	AO	0.33 ± 0.07	0.31 ± 0.08	0.28 ± 0.03
24	PA	0.34 ± 0.04	0.41 ± 0.09	0.59 ± 0.03**
	AO	0.30 ± 0.05	0.38 ± 0.07	0.53 ± 0.07**

*Data represent mean ± SE (n = 6)

**$p < 0.05$ vs control

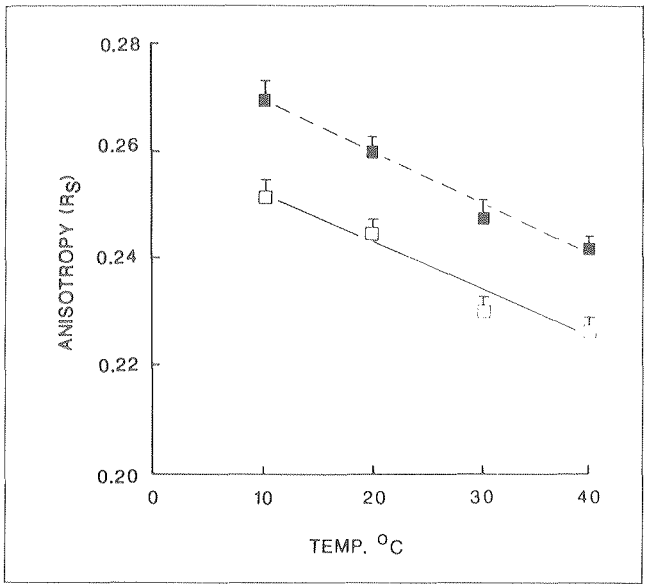

Figure 11. Steady-state fluorescence anisotropy (r_s) for fluorescamine in lipid vesicles prepared from total lipid extracts of control (□——□) and NO_2 (■---■)-exposed pulmonary artery endothelial cells. Cells were exposed to 5 ppm NO_2 for 24 hours.

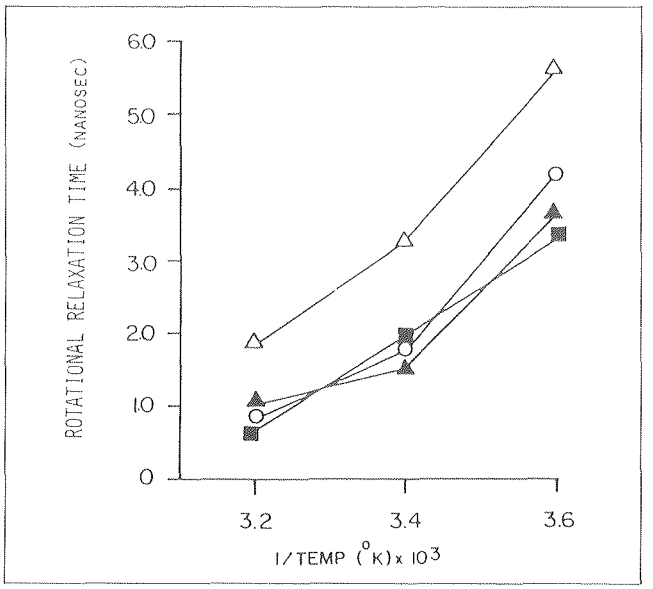

Figure 12. Effect of NO_2-exposed medium components on rotational relaxation times of DPH in pulmonary artery endothelial cells. *Control* cells were exposed to air containing 5% CO_2 (■). *A:* cells and medium were exposed to 5 ppm NO_2 for 12 hours (Δ). *B:* medium alone was exposed to 5 ppm NO_2 for 12 hours. *C:* medium from *A* was poured onto a fresh set of cells (Δ). *D:* medium from *B* was poured onto another fresh set of cells (○). Cells from *C* and *D* were exposed to control conditions for 12 hours. Rotational relaxation times were measured at 5°, 25°, and 40°C.

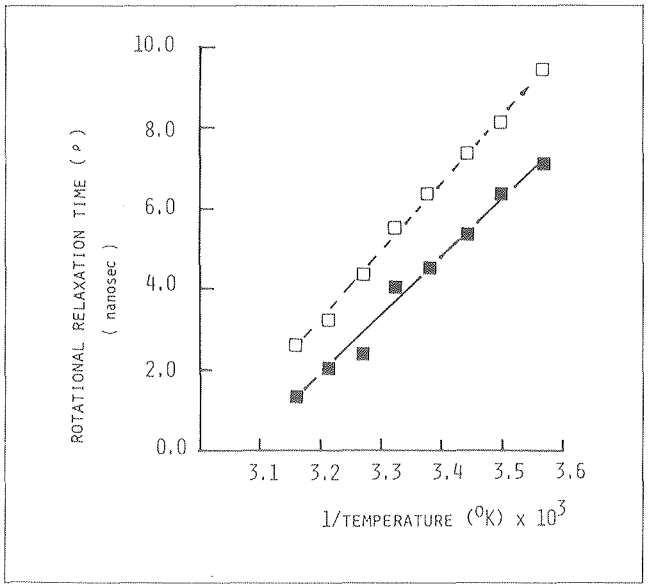

Figure 13. Effect of direct exposure (in the absence of culture medium) to 5 ppm NO_2 (□) for 3 hours on rotational relaxation times of DPH in pulmonary artery endothelial cells. Controls (■) were exposed to air containing 5% CO_2 under identical conditions.

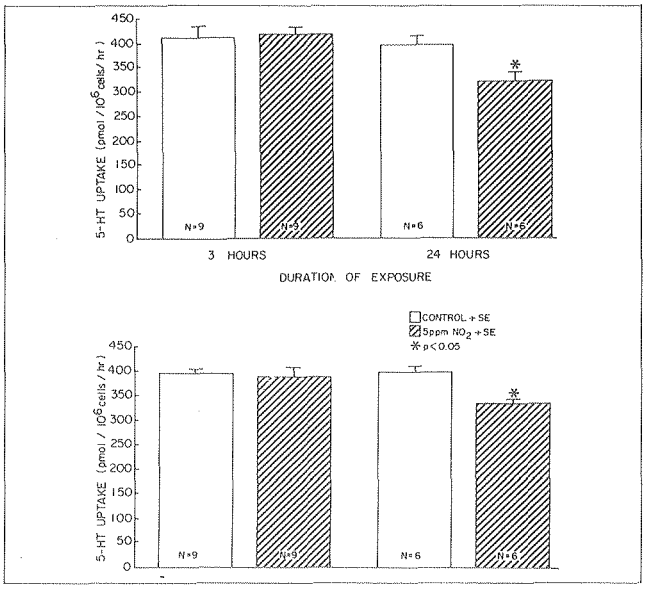

Figure 14. Effect of NO_2 exposure (5 ppm for 24 hours) on 5-HT uptake in pulmonary artery (upper panel) and aortic (lower panel) endothelial cells, measured as the disappearance of 5-[^{14}C]-HT from the culture medium.

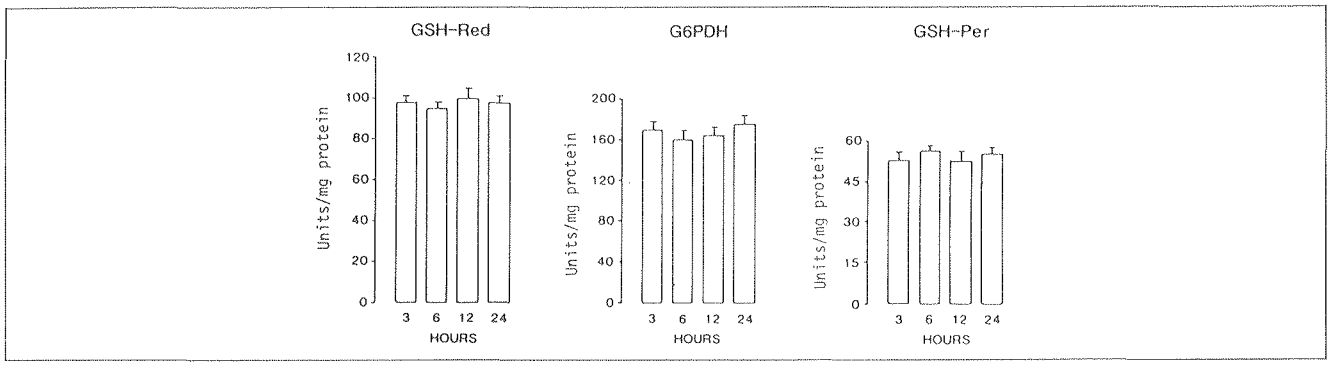

Figure 15. Enzyme activities of GSH-red, G6PHD, and GSH-per in control pulmonary artery endothelial cells after 3, 6, 12 and 24 hours of exposure to 20% 0_2-5% CO_2-75% N_2. Data represent mean ± SE (n = 8). There were no time-dependent differences in activities.

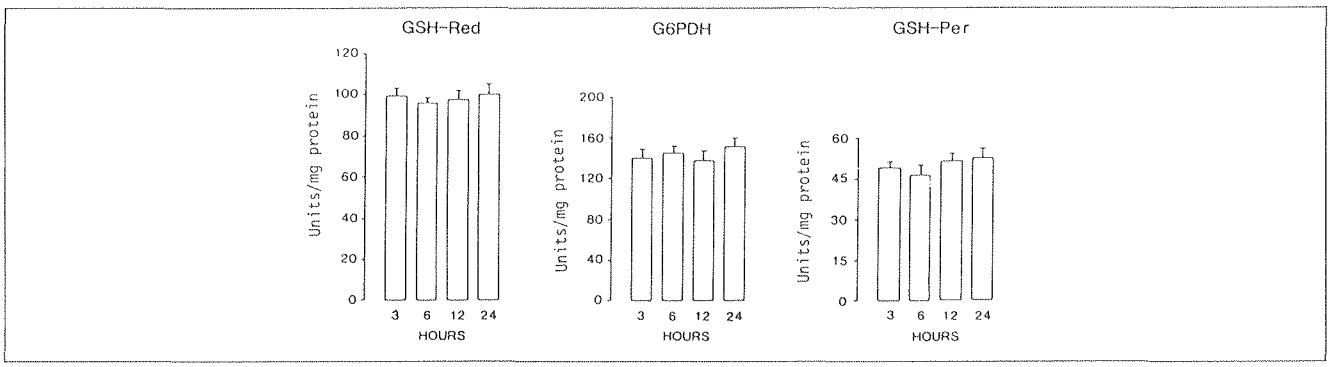

Figure 16. Enzyme activities of GSH-red, G6PDH, and GSH-per in control aortic endothelial cells after 3, 6, 12, and 24 hours of exposure to 20% 0_2-5% CO_2-75% N_2. Data represent mean ± SE (n = 8). There were no time-dependent differences in activities.

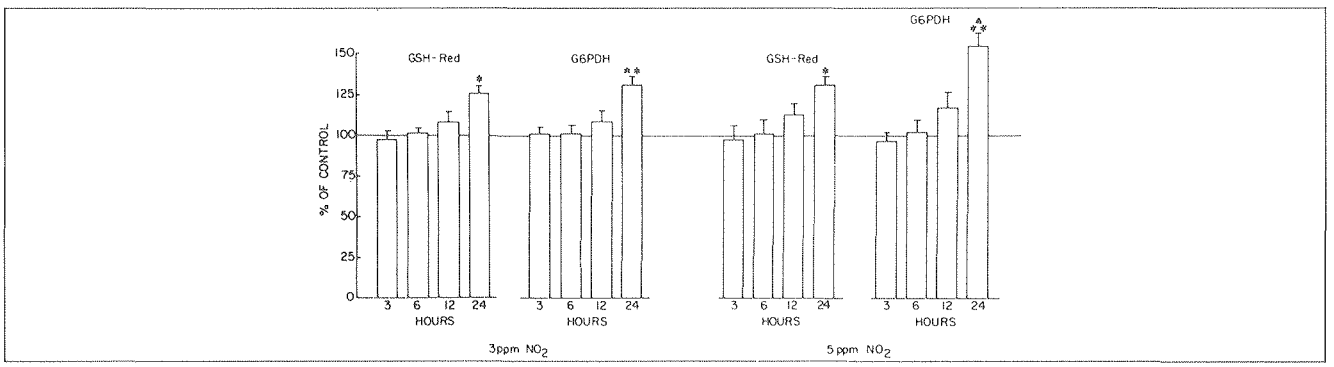

Figure 17. Time-dependent effect of 3 and 5 ppm NO_2 exposure on GSH-red and G6PDH activities as a percentage of control in pulmonary artery endothelial cells. Data represent mean ± SE (n = 8). *p < 0.05, **p < 0.001 vs. control, Δ p < 0.02 vs. 3 ppm NO_2.

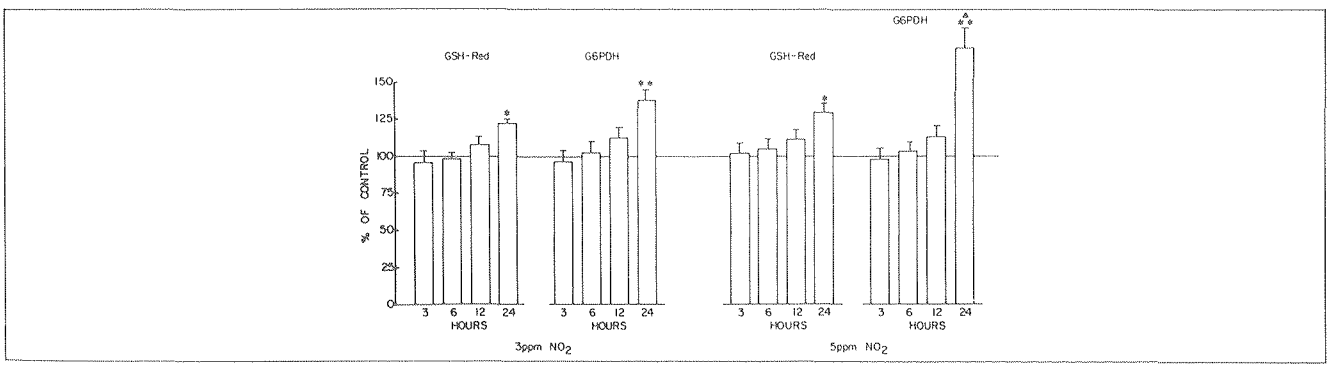

Figure 18. Time-dependent effect of 3 and 5 ppm NO_2 exposure on GSH-red and G6PDH activities as a percentage of control in aortic endothelial cells. Data represent mean ± SE. (n = 8). *p < 0.05. **p < p.001 vs. control. Δp < 0.02 vs. 3 ppm NO_2.

Effect of NO_2 Exposure on GSH Content. The effect of NO_2 exposure on the GSH content of endothelial cells is given in Table 7. The values of GSH, a naturally occurring, biologically active antioxidant, were not significantly different in control and NO_2-exposed pulmonary artery and aortic endothelial cells.

Table 7. Effect of NO_2 Exposure on GSH Content in Pulmonary Artery (PA) and Aortic (AO) Endothelial Cells

		GSH μg/mg protein		
			NO_2	
Exposure time (hour)	Cell type	Control	3ppm	5ppm
3	PA	3.3 ± 0.3	3.2 ± 0.3	3.6 ± 0.4
	AO	3.4 ± 0.5	3.0 ± 0.4	4.1 ± 0.6
6	PA	3.0 ± 0.2	3.9 ± 0.2	3.7 ± 0.5
	AO	3.6 ± 0.3	4.0 ± 0.6	3.2 ± 0.4
12	PA	4.0 ± 0.6	3.3 ± 0.8	3.0 ± 0.3
	AO	3.8 ± 0.4	3.0 ± 0.3	3.7 ± 0.7
24	PA	3.3 ± 0.1	3.6 ± 0.7	4.1 ± 0.5
	AO	3.9 ± 0.7	3.1 ± 0.5	4.0 ± 0.6

Data represent mean ± SE (n = 6). Differences between control NO_2 groups at each exposure time were not significant.

Recovery of NO_2-induced Injury in Endothelial Cells. Recovery from lipid fluidity changes, 5-HT uptake, and LDH release was monitored up to 48 hours after exposure to 5 ppm for 24 hours in both pulmonary artery and aortic endothelial cells. As shown in Table 8, lipid fluidity in the hyrophobic region of the lipid bilayer was significantly ($p < 0.001$) decreased after a 24-hour exposure to 5 ppm NO_2 in both cell types. The decrease in fluidity remained significantly reduced ($p < 0.001$) up to 12 hours of recovery. Fluidity changes recovered slightly between 12 and 36 hours of recovery, but still remained significantly ($p < 0.05$) decreased compared to controls. Fluidity changes were almost completely recovered in 48 hours after the NO_2 exposure period in both pulmonary artery and aortic endothelial cells.

Table 9 shows the recovery of 5-HT uptake after NO_2 exposure in both pulmonary artery and aortic endothelial cells. 5-HT uptake was significantly ($p < 0.05$) reduced after 24 hours of exposure to 5 ppm NO_2, and remained reduced at that level up to 24 hours in the recovery period. However, 5-HT uptake was significantly recovered within 36 to 48 hours in both pulmonary artery and aortic endothelial cells.

Recovery of LDH release after NO_2 exposure to pulmonary artery and aortic endothelial cells is shown in Table 10. LDH release was significantly ($p < 0.01$) higher after a 24-hour exposure to 5 ppm NO_2 in both cell types, and remained elevated for up to 12 hours of recovery. However, almost total recovery was achieved in a 24-hour recovery period in both pulmonary artery and aortic endothelial cells.

Table 8. Recovery of Membrane Lipid Fluidity in NO_2-Exposed Pulmonary Artery (PA) and Aortic (AO) Endothelial Cells

		Anisotropy (r_s) for DPH at 25°C	
Recovery time (hour)	Cell type	Control	NO_2
0*	PA	0.270 ± 0.003	0.305 ± 0.008**
	AO	0.263 ± 0.002	0.308 ± 0.004**
12	PA	0.281 ± 0.005	0.298 ± 0.001**
	AO	0.265 ± 0.002	0.300 ± 0.005**
24	PA	0.277 ± 0.008	0.289 ± 0.006***
	AO	0.270 ± 0.006	0.291 ± 0.003***
36	PA	0.270 ± 0.004	0.283 ± 0.004***
	AO	0.273 ± 0.007	0.285 ± 0.005***
48	PA	0.275 ± 0.008	0.280 ± 0.003
	AO	0.268 ± 0.004	0.270 ± 0.007

*Time represents fluidity change immediately after NO_2 exposure period (5 ppm, 24 hours).
**$p < 0.001$ vs control
*** $p < 0.05$ vs control

Table 9. Recovery of 5-HT Uptake in NO_2-Exposed Pulmonary Artery (PA) and Aortic (AO) Endothelial Cells

		5-HT Uptake (pmol 5-HT/hr/10^6 Cells	
Recovery time (hour)	Cell type	Control	NO_2
0*	PA	439 ± 18	328 ± 28**
	AO	415 ± 19	320 ± 20**
12	PA	435 ± 20	335 ± 20**
	AO	422 ± 14	326 ± 17**
24	PA	444 ± 15	370 ± 26**
	AO	412 ± 21	353 ± 20**
36	PA	440 ± 23	400 ± 28
	AO	425 ± 20	390 ± 18
48	PA	450 ± 26	428 ± 16
	AO	422 ± 23	405 ± 18

*Time represents 5-HT uptake immediately after NO_2 exposure period (5 ppm, 24 hours).
**$p < .05$ vs control

Table 10. Recovery of LDH Release in NO_2-Exposed Pulmonary Artery (PA) and Aortic (AO) Endothelial Cells

Recovery time (hour)	Cell type	LDH Release (% Total) Control	NO_2
0*	PA	7.5 ± 0.8	15.9 ± 1.0**
	AO	7.8 ± 0.4	16.4 ± 0.8**
12	PA	7.4 ± 0.3	11.3 ± 0.8**
	AO	8.0 ± 0.1	12.0 ± 0.5**
24	PA	7.7 ± 0.3	8.2 ± 0.6
	AO	7.8 ± 0.2	8.0 ± 0.3
36	PA	8.4 ± 0.4	7.4 ± 0.4
	AO	8.0 ± 0.2	8.9 ± 0.8
48	PA	7.9 ± 0.4	8.3 ± 0.3
	AO	7.3 ± 0.5	7.5 ± 0.2

*Time represents LHD Release immediately after NO_2 exposure period (5 ppm, 24 hours).
**$p < 0.01$ vs control

DISCUSSION

The basic structure of biological membranes can be represented by a phospholipid bilayer with inlays and overlays of proteins and phospholipids (Singer, 1974). According to the fluid mosaic model of membrane structure, membrane components are capable of rapid rotational and translational diffusion within the fluid lipid matrix of the membrane (Singer, 1974; Shinitzky, 1984). Alterations in membrane fluidity have been shown to affect a variety of fundamental cellular functions, including transmembrane transport, enzyme action, growth characteristics, and receptor-ligand interaction (Shinitzky, 1984).

The present study demonstrates that NO_2 exposure results in a progressive decrease in plasma membrane fluidity in both pulmonary artery and aortic endothelial cells in culture, as demonstrated by the decreased rotational motion of DPH, a fluorescent probe that partitions into the hydrophobic interior of the membrane lipid bilayer. In addition, this study also demonstrates that NO_2 exposure decreased anisotropies for TMA-DPH, a fluorescent molecular probe that anchors at the lipid-water interface, and for fluorescamine, which reacts with surface membrane amino groups of phospholipids and proteins, indicating changes in the hydrophilic region of the plasma membrane of endothelial cells. Although many molecular probes including DPH are used extensively for labeling the plasma membrane of mammalian cells, it has been reported that DPH can partition into the membranes of intracellular organelles of intact cells (Grunberger et al., 1982). Such partitioning is time-dependent. We used relatively short incubation times in this study; we previously observed (Block et al., 1986) that alterations in DPH in intact endothelial cells are identical to those occurring in isolated plasma membrane vesicles derived from the same cells. These data indicate that the fluidity changes in this study reflect changes primarily, if not exclusively, in the plasma membrane. Similarly, TMA-DPH, due to its cationic charge, resists partitioning in other than the lipid-water surface on the membrane (Sheridan and Block, 1985). Fluorescamine has been shown to covalently bind to the surface membrane amino groups of proteins and phospholipids. It does not label internal components of the cells, as evidenced by separation of intracellular proteins by polyacrylamide gel electrophoresis (Hawkes et al., 1976). Finally, fluorescence microscopy has disclosed the specific localization of DPH and TMA-DPH in the plasma membranes of the cells. These probes remain in the plasma membrane for at least 60 minutes, and even longer in the case of TMA-DPH (Sheridan and Block, 1985)

The use of lipid vesicles in this study confirms that NO_2-induced changes in the endothelial cell membrane are associated with membrane lipids. These results indicate that significant decreases in the fluidity of the hydrophobic and hydrophilic regions of the plasma membrane were accompanied by an increase in the phosphatidylethanolamine and phosphatidylserine content of the cells. The increase in lipid content increases the bilayer rigidity of the cell membranes. The increase in rigidity is known to cause abnormal cell functions (Shinitzky, 1984; Stubbs and Smith, 1984).

The results of the present study also indicate that the fluidity changes are due to direct exposure to NO_2, rather than to indirect action mediated through medium components or their generated reactive products. If one assumes that NO_2 reacts with components of the medium and generates a reactive species that in turn interacts with endothelial cells, the production of reactive species, as well as the degree of membrane injury, should be proportional to the volume of the medium. Although we have not identified any reactive products, our data clearly demonstrate that NO_2 exposure altered endothelial cell plasma membrane fluidity in the absence of culture medium. The alteration in membrane fluidity, which was greater in the absence of the medium, can be explained by the limited solubility of NO_2 in aqueous media, which reduces the concentration of NO_2 at the plasma membrane.

In addition to decreasing plasma membrane fluidity, NO_2 exposure caused significant decreases in 5-HT uptake and significant increases in LDH release. 5-HT uptake and LDH release depend on the integrity of the plasma membrane and have been used as indices of plasma membrane injury in endothelial cells (Autor et al., 1984; Ody and Junod, 1985; Ryan and Ryan, 1977). Alterations in these membrane-dependent properties support the fluidity data and indicate that the plasma membrane is an important early target in NO_2-induced cytotoxicity. It is not surprising that the detection of alterations in plasma membrane fluidity precedes the detection of alterations in 5-HT uptake and LHD release, since

tions in 5-HT uptake and LDH release, since the sensitivity of the fluorescence spectroscopic method is much greater than the sensitivities of the 5-HT uptake and LDH methods. In addition, it is not unexpected that alterations in the physical state of the plasma membrane lipids, as reflected in the changes in fluidity, precede derangements in plasma membrane function. However, the sequence of precise events in the mechanism of NO_2 cell injury needs to be firmly established.

The preceding results support our hypothesis that NO_2 affects the physical state of membrane lipids leading to alterations in membrane function. The increase in thiobarbituric acid-reactive material in the cultured cells exposed to NO_2 implies a role for peroxidative cleavage of lipids in the pathogenesis of NO_2-induced membrane injury. NO_2 and NO_2-derived free radicals can readily react with the unsaturated bonds of plasma membrane cholesterol and fatty acids, can initiate lipid peroxidation, and can alter the molecular organization of the lipid bilayer, which lead to derangements in the membrane function.

According to this hypothesis, the peroxidation of membrane lipids should precede the alterations in membrane fluidity and function. However, we did not detect evidence of lipid peroxide formation until exposure to NO_2 was extended to 24 hours, whereas decreases in fluidity were evident after a 3-hour exposure to NO_2. There are several possible explanations for this apparent inconsistency. First, the sensitivity of the fluorescence spectroscopic methods far exceeds the sensitivity of the colorimetric methods used to measure thiobarbituric acid-reactive material. Second, the accumulation of malonaldehyde in cells is a function of the rate at which it is generated and the rate at which it disappears. In addition to the loss into the culture medium that could not be detected, malonaldehyde may be metabolized by endothelial cells, as has been reported for other mammalian cells in culture (Bird, 1982). Either of these possibilities would account for the delayed detection of thiobarbituric acid-reactive material. Finally, NO_2-induced lipid peroxidation may generate byproducts that, unlike malonaldehyde, do not react with thiobarbituric acid (Dahle et al., 1962).

The exact relation between the detection of altered membrane fluidity and the demonstration of increased peroxidation of membrane lipids requires clarification. However, the increase in lipid peroxide formation in the present study is consistent with earlier reports that NO_2 exposure promotes lipid peroxidation in mammalian lungs in vivo (Sagai et al., 1984; Sevanian et al., 1979; Thomas et al., 1968) and in vitro (Mustafa et al., 1978; Pryor et al., 1981). Moreover, several reports indicate that NO_2 exposure alters lung phospholipid and total lipid composition (William et al., 1971; Thomas et al., 1970; Blank et al., 1978). For example, lipid epoxide formation was increased in triglyceride, cholesterol, and phospholipid fractions derived from the lungs of rats exposed to NO_2 (Sevanian et al., 1979). In addition, NO_2 exposure resulted in an increase in the ratio of saturated species of phospholipids to unsaturated species of phospholipids in rat and hamster lungs (William et al., 1971; Blank et al., 1978). This is particularly relevant to the present study, because an increase in membrane saturated lipids would be expected to result in a decrease in membrane fluidity (Shinitzky, 1984).

The cellular defense mechanisms involved in the detoxification of lipid hydroperoxide include the antioxidant tripeptide GSH and a series of GSH-dependent antioxidant enzymes. The present study indicates that GSH content and GSH-red and GSH-per activities are comparable in pulmonary artery and aortic endothelial cells in culture. In contrast, G6PDH activity was greater in pulmonary artery cells than it was in aortic endothelial cells. These results are consistent with earlier observations under similar conditions of tissue culture technique and exposure (Block et al., 1985). The reason for greater G6PDH activity in pulmonary artery endothelial cells is not clear. The present study also demonstrates the time-dependent effects of NO_2-exposure on the antioxidant enzyme response in endothelial cells. The NO_2 exposure for 24 hours increased GSH-red and G6PDH activities in both pulmonary artery and aortic endothelial cells but it had no significant effect on GSH-per activity in either cell type. Similarly, in this report we demonstrated a significant increase in lipid peroxide formation in cells exposed to 5 ppm NO_2 for 24 hours in both pulmonary artery and aortic endothelial cells.

The increase in antioxidant enzyme activities appears to be an appropriate adaptive response to increased oxidant stress associated with lipid peroxidation and lipid hydroperoxide formation. GSH-red activity is essential for maintaining the level of GSH, and G6PDH is necessary to generate NADPH as a reducing equivalent. These results are consistent with those concerning the lungs of animals exposed to NO_2. For example, NO_2 exposure promotes lipid peroxide formation in mammalian lungs (Sagai et al., 1984; Sevanian et al., 1979; Thomas et al., 1968) as well as increases in lung GSH content and activities of GSH-red, GSH-per, and G6PDH (Mustafa and Tierney, 1978; Sagai et al., 1984; Tyson et al., 1982). Since NO_2 is a free radical, it reacts directly with membrane lipids and generates lipid hydroperoxide. Therefore, other antioxidant enzymes of the lung, such as catalase and superoxide dismutase, are ineffective in providing protection against injury from lipid hydroperoxides (Flohe, 1982).

Despite an adequate supply of all nutrients, including cysteine and selenium, in the growth medium, we did not observe any change in GSH-per activity or GSH content in pulmonary artery and aortic endothelial cells exposed to NO_2. This is inconsistent with in vivo observations of intact lungs of rats exposed to NO_2. There are at least three possible explanations for the discrepancy between our observations regarding GSH-per activity and GSH content in vitro and those of others in vivo. First, the lung is composed of more than 40 different cell types. Thus, it is possible that observed increases in GSH-per activity and GSH content in lung tissue fractions from animals exposed to NO_2 are due to one of the other cell types. Second, differences in NO_2 concentration and

exposure time between the in vitro and in vivo studies may account for the differences. Third, the NO_2-induced differences in some antioxident enzyme activities in vivo and in vitro may be due to differences in species or age of the animals.

Although it is known that NO_2 causes injury to lung cells, very little is known about the specific cellular and molecular events that initiate and propagate the injury. Unlike previous studies that used whole lung homogenates (DeNicola et al., 1981) or 9000 x g and microsomal preparations (Mustafa and Tierney, 1978; Thomas et al., 1968) to evaluate the fundamental molecular mechanisms of NO_2-induced injury, the present study used a tissue culture model of pure endothelial cells. Our results demonstrate that alterations in the lipid bilayer of the plasma membrane are early and functionally significant manifestations of NO_2-induced cellular injury. To our knowledge, this is the first report to implicate a role for plasma membrane injury in the pathogenesis and manifestations of NO_2 cytotoxicity.

The precise nature of the NO_2-induced dynamic changes in the plasma membrane and their relation to altered cell and organ function are not fully defined, but represent fertile areas for future investigations that may increase our understanding of the mechanisms responsible for NO_2 toxicity. Besides being involved in vital transmembrane transport processes, the plasma membrane provides a semipermeable barrier for the cell and participates in mitogenesis, cell cycling, differentiation, proliferation, and transmembrane signal transduction (Shinitzky, 1984). Alterations in these fundamental cellular processes could have serious consequences for cell and organ function and viability. The development of pulmonary edema in many mammalian species after NO_2 exposure may be caused by increased permeability secondary to NO_2-induced injury to the surface membrane of airway epithelial or capillary endothelial cells (Guidotti, 1978, 1980). Similarly, altered membrane fluidity could account for the alterations in various properties of the red cell membrane, including decreases in enzyme activity, that have been demonstated in rats exposed to NO_2 (Kunimoto et al., 1984). Finally, elevations in the lysosomal enzymes acid phosphatase and β-glucuronidase in hamster lungs after NO_2 exposure can be explained by their release through perturbed portions of the plasma membrane into the extracellular fluid during active phagocytosis by macrophages and neutrophils (DeNicola et al., 1981).

To evaluate the effect of NO_2 exposure on lung endothelial cells, it would be most appropriate to study endothelial cells derived from the pulmonary capillary bed. However, development of techniques for selectively culturing pure populations of endothelial cells from the pulmonary capillary bed have met with limited success. Several investigators have shown that the biochemical and metabolic characteristics of pulmonary capillary endothelial cells in vivo appear similar to pulmonary artery and aortic endothelial cells in culture (Block and Stalcup, 1981; Ryan and Ryan, 1977). Thus, in analyzing the results of the present study, we assumed that endothelial cells from the pulmonary artery and thoracic aorta are a good model for endothelial cells from the pulmonary capillary bed.

In summary, this study demonstrates that a decrease in membrane fluidity is an early manifestation of NO_2-induced endothelial cell injury. With more prolonged exposure to NO_2, the decrease in membrane fluidity was greater and was associated with alterations in biochemical and metabolic functions of these cells. These results support the hypothesis that oxidant injury due to NO_2 alters the physical state of membrane lipids and leads to impairment of membrane function, and suggest that membrane injury may contribute to biochemical, physiologic, and metabolic abnormalities after exposure to NO_2. The precise events that are initiated at the plasma membrane and lead to the intracellular components in the mechanism of NO_2 cell injury need to be firmly established.

ACKNOWLEDGMENTS

We would like to thank Dr. Randy Carter of the Biostatistics Unit of the University of Florida for analysis of the data, and Dr. Kimon Angelides of the Biochemistry and Neuroscience Departments at the University of Florida, for their continued advice and support for the fluorescence molecular probe study. We also wish to thank Mr. Bert Herrera for technical assistance in tissue culture, and Mrs. Chris Cannon for secretarial assistance.

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HEALTH REVIEW COMMITTEE'S REPORT

BACKGROUND

Nitrogen dioxide is an important contributor to the air pollution effects of fossil-fueled internal combustion engines, and is associated with biological activity and human health effects. The proposed study explores the effects of nitrogen dioxide at a level of 3 to 5 ppm introduced into tissue culture flasks that contain vascular endothelial cells, and measures various biophysical and biochemical parameters of nitrogen dioxide (NO_2) effects. Changes at the cellular level may be detectable far earlier than the major symptoms of disease, and could elucidate the primary nature of the injury produced by the toxicant.

OBJECTIVES OF THE PROPOSED STUDY

Endothelial cells from the pig aorta and pulmonary artery were isolated and cultured as monolayers and exposed to 3 or 5 ppm of nitrogen dioxide or air as control. Membrane fluidity, 5-hydroxytryptamine transport, and several glycolytic and glutathione enzymes that are involved in antioxidant defense were measured at 3, 12, and 24 hours of exposure. Specifically, the objectives of the research were:

1. to measure in the cultured endothelial cells the enzyme activities of glucose-6-phosphate dehydrogenase, glutathione reductase, and glutathione peroxidase;
2. to measure the rate of lipid peroxide formation in endothelial cells exposed to NO_2 or air;
3. to assess biofluorescence labeling techniques, utilizing hydrophobic and amine-binding fluorescent probes, to measure membrane fluidity in endothelial cells exposed to NO_2;
4. to measure 5-hydroxytryptamine uptake by monolayer cultures in the NO_2 protocol studies, to determine the effects of NO_2 on the kinetics of 5- hydroxytryptamine uptake;
5. to measure the effects of NO_2 exposure on sodium-potassium ATPase activity as a membrane enzyme potentially susceptible to NO_2, thereby reflecting other changes in membrane function;
6. to assess, by morphologic and morphometric studies, the effects of the NO_2 dose levels on the endothelial cells in vitro;
7. to attempt to protect the cells against the effects of NO_2 as measured by these studies by the addition of vitamin E or glutathione to the culture medium;
8. to correlate in vitro NO_2-induced endothelial injury with in vivo studies of rats, evaluating the ability of isolated perfused lungs obtained from rats exposed to NO_2 to clear 5-hydroxytryptamine and assessing the morphology of the lungs.

All objectives except number 8 (the studies using the in vivo-exposed, perfused isolated lung), were attempted, and the preliminary results were included in progress reports by the principal investigator. The in vivo studies were abandoned during the second year upon the recommendation of the site visit committee, which favored strengthening the in vitro studies. Objectives number 5, the ATPase measurements; number 6, morphologic and morphometric assessment of NO_2 dose levels on the endothelial cells in vitro; and number 7, protection by vitamin E or glutathione, were not studied to a sufficient degree to be included in the final report.

TECHNICAL EVALUATION

OBJECTIVES

Except as stated above, the objectives of the original proposal were met in the final report. The emphasis of the study shifted from biochemical considerations of antioxidant defense mechanisms to the more sensitive changes in membrane fluidity identified during the course of the study. It was found that the biophysical changes in membrane fluidity occurred earliest, at three hours of exposure, while 5-hydroxytryptamine uptake and biochemical changes in antioxidant defense enzymes occurred later. A very interesting feature not included in the original objectives was the finding of recovery in 24 to 48 hours from virtually all abnormal biophysical parameters induced by NO_2 exposure. By finding a highly sensitive indicator of exposure in membrane fluidity, the investigators may have discovered an appropriate marker of early exposure and response to NO_2 (and possibly other oxidants).

In general, the methodology, with respect to the preparation and characterization of cell cultures, assay of biochemical and enzymatic parameters, and determination of membrane lipid fluidity, appears to be appropriate. The investigators might have done better in the study design if they had included another lung cell type rather than aortic endothelial cells.

A second concern about the study design is the relevance of these in vitro studies to in vivo events. The initial review body was concerned that it would be difficult to interpret the results from NO_2 exposures of in vitro cell culture systems. This problem was to be addressed by comparing in vivo exposure in isolated lung perfusion studies with the in vitro cell exposures studies. Because in vivo studies were not done, the question of the relevance of these results to the in vivo state remains acute.

Third, since membrane lipid peroxidation is perceived to be responsible for changes in the membrane fluidity, more extensive studies of membrane lipid peroxidation other than the malonaldehyde-thiobarbiturate color reaction should have been performed, because that method is not a sensitive marker for lipid peroxidation. Perhaps for this reason, in the membrane fluidity studies, abnormalities preceded the apparent appearance of lipid peroxidation products.

A fourth methodologic concern is the potential desiccation effects of continuous administration of NO_2 to the exposed cell cultures. The controls, gassed by standard methods, might not show desiccation artifacts.

Two other factors were not considered by the investigators. One was the possible formation of NO_2 adducts to membrane lipids, rather than only split products of lipid peroxidation. The other consideration was the problem of the amount of NO_2 absorbed by the cell culture; it was partially addressed in the experiments in which the NO_2-exposed medium was transferred to fresh cells without affecting the membrane fluidity of those cells. In isolated perfused lung preparations, inhaled NO_2 is reduced to NO_2^- in lung tissues, and its oxidation to NO_3apparently occurs in the presence of erythrocytes. Because the cell culture system used here is devoid of erythrocytes, it might not totally reflect in vivo systems.

In maintaining a focused approach to the research questions, the investigators did not pursue some of the initial aims to completion. In particular, an electron microscopic survey of the exposed and control cells should have documented the condition and distribution of organelles that may have been affected by injury to the surface membrane. Phase contrast microscopy is insufficient to prove that the cells were viable, and it should be established whether or not intracellular edema occurs. Another set of experiments not pursued, because preliminary results indicated no differences in control and NO_2-exposed cells, were the ATPase assays. In retrospect, the study design may have prohibited cogent data analysis of the effect of NO_2 on ATPase levels. Since DNA strand breaks caused by oxidants can block ATP synthesis[1], it would not have been clear whether changes in ATPase levels reflected a direct effect on enzyme function or a simple decrease in substrate level.

RESULTS

The results of the study are presented clearly, and are generally in accordance with the study design and methods. The raw data have been analyzed and presented in a thorough and illuminating manner. Most of the biochemical changes did not occur for 24 hours; the biophysical changes of membrane fluidity could be detected in three hours. The increase in lipid and phospholipid content remain unexplained. That glutathione content and glutathione peroxidase activity were not altered should raise the question of whether lipid peroxides were formed or antioxidant defense was involved, or both. Both the LDH release data and the 5-hydroxytryptamine uptake depression reflect functional alterations after membrane damage.

The most sensitive parameter measured in this study is the change in membrane lipid fluidity. The alterations observed in fluidity are convincing evidence that membrane lipids may be an early target of NO_2 attack. One might question the exactitude with which the diphenylhexatriene fluorescence labels reflect membrane lipid fluidity changes. However, this does not affect the overall interpretations of the study, because alterations in membrane protein fluidity, which this label could also report, could ultimately be expressed as cellular metabolic or functional alterations. However, the mechanistic relationship leading from NO_2 exposure through membrane fluidity changes to biochemical and functional derangements of the cell membrane are not worked out in this study. Rather, the principal value of the study is that it identifies the sensitivity of membrane fluidity studies in detecting NO_2 effects.

INTERPRETATIONS AND CONCLUSIONS

The research by Patel and Block on the effects of NO_2 fluidity and other membrane properties makes a serious attempt to relate the physical measurement of membrane fluidity with biological reality in the form of various physiological functions of membranes. Thus, one comprehends that fluidity is not just an abstract, functionless construct, but a central player in biological activity. The data are convincing, the methodology sound, and the conclusions clearly reasoned from the results. The degree of speculation made could be diminished by additional studies, as indicated above and below.

Although changes in plasma membrane fluidity were noted and the effects were ascribed to NO_2 interaction with lipids in the hydrophilic region of the membrane, the nature of this interaction, including the actual changes in the lipid or NO_2 molecules, remained unclear. Identification of the reaction products would have eliminated much speculation made otherwise. The body of completed research would suggest interesting future comparisons between the response of vascular endothelium and alveolar or tracheobronchial epithelium in vivo.

Additional studies should attempt specifically to clarify the relation between the membrane fluidity changes and the subsequent abnormalities in membrane integrity and membrane biochemical function. It has not been demonstrated whether the biochemical changes, which occur many hours after the onset of the membrane fluidity changes, are directly related to the changes in the membrane fluidity or are secondary to more extensive cell injury. The ability of the cells to recover both biochemically and biophysically is of extreme interest, and the mechanisms of recovery, as well as the time courses and dose responses, would be exceedingly interesting studies to pursue. The most important observation is that the biophysical changes in the membrane preceded by many hours the metabolical uptake and biochemical and enzymatic changes in the cells.

1. Marx, J.L. 1987. Science 235:529-531.

Additional, more sensitive studies to detect whether or not lipid peroxidation products precede membrane fluidity changes, as speculated by the authors, would be crucial to prove the hypothesis that the sequence of events that NO_2 exposure initiates begins with lipid peroxidation and proceeds to membrane fluidity changes that are followed by functional, enzymatic, biochemical, and metabolic changes in the cells. This sequence is not proven by the current observations and thus, the underlying hypothesis of the investigators still requires supporting evidence.

BROAD OVERVIEW

Overall, this study met HEI and investigator goals in identifying a measure of biophysical response to NO_2 exposure in vitro that preceded any measurable changes in lipid peroxidation, membrane function, or measured biochemical and metabolic changes in the cells. The experimental approach employed sophisticated cell biology and biophysical measurements, and demonstrated an overall elegant approach to the study of biochemical toxicology. The investigators have not, however, provided unequivocal evidence of a causative connection between lipid peroxidation, membrane biophysical changes, membrane dysfunction, and biochemical and metabolic changes.

This study extends our knowledge of the possible importance, but uncertain pre-eminence, of membrane changes in nitrogen dioxide toxicity. The significance of these findings, with respect to environmental exposures to NO_2, remains in question. The similar effects in vitro on endothelial cells from aortic or pulmonary artery sources may be expected only under in vitro conditions, and not necessarily in vivo in pulmonary capillaries. Further research is required to establish whether or not endothelial cells in culture provide an appropriate model for NO_2 effects in vivo, where other defense mechanisms are present as well as alternative mechanisms for enhancing toxicity. The specificity and sensitivity of the observed response in other lung cell types must be investigated.

These methods can be exploited in future studies as a model system for the design of agents to protect individuals before or after specific accidental NO_2 exposure, and for nutritional or other studies aimed at defining how humans can best equip themselves to survive accidental toxic oxidant exposure. The project's results will ultimately lend insight into the connection between the physical state of membrane components and the functional capability of membranes, such as the uptake and metabolism of vasoactive amines by membrane transport systems. Extension of the investigation to in vivo NO_2 exposures and an examination of pulmonary endothelial metabolic function in isolated perfused lung models will provide key information about the comparability of these in vitro data with in vivo responses to oxidant exposure. Thus, this project is an interesting beginning of a line of inquiry that requires both additional basic research as outlined above, extension to in vivo findings, and potential application of techniques to identify significant early responses to NO_2 and other oxidant exposures.

RECOMMENDATIONS

The identification of biophysical markers sensitive to early changes in cell responses to NO_2, as well as possibly to other oxidant agents, offers a promising line of additional investigation. In addition to clarifying the mechanisms of membrane injury and their possible relation to lipid peroxidation products, techniques might be developed that would allow such biophysical monitoring of in vivo response in human populations.

That these membrane changes are reversible in vitro is an important consideration in assessing their biological significance. However, the data are too premature to have a bearing on risk assessment or public policy at this time. Further HEI considerations should be given to the solicitation and support of additional work to explore the mechanisms and significance of the NO_2-induced biophysical changes in cells exposed to NO_2 and other oxidant pollutant substances.

JOURNAL PUBLICATIONS AND ABSTRACTS BASED ON THIS RESEARCH

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ABOUT THE INVESTIGATORS

Jawaharlal M. Patel received a Ph.D. in biochemistry from Marathwada University in Aurangabad, India in 1973, and was a visiting fellow at the National Institute of Environmental Health Sciences in Research Triangle Park, North Carolina. He is currently an assistant research scientist in the Pulmonary Medicine Division of the Department of Medicine at the University of Florida. Dr. Patel's primary research interests include drug metabolism by microsomal enzymes, biochemical toxicity of inhaled compounds, and cell biology of the lung.

Edward R. Block was awarded an M.D. by Johns Hopkins School of Medicine in 1968. He served as a clinical associate at the National Institute of Allergy and Infectious Diseases and was a senior staff fellow at the National Institute of Health. Dr. Block is presently Associate Chief of Staff for Research at the Veterans Administration Medical Center in Gainesville and is professor of medicine at the University of Florida College of Medicine. His research emphasizes the metabolic affects of oxygen and hyperoxia in pulmonary function.

HEALTH EFFECTS INSTITUTE
215 First Street • Cambridge, MA 02142
(617) 491-2926

RESEARCH REPORT No. 9: June, 1987