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# MODULATION OF PARTICULATE NITRIC OXIDE SYNTHASE ACTIVITY AND PEROXYNITRITE SYNTHESIS IN CHOLESTEROL ENRICHED ENDOTHELIAL CELL MEMBRANES

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**Abstract**—Endothelium-derived relaxing factor/nitric oxide (EDRF/NO) is produced by the vascular wall and is a key modulator of vascular tone and blood pressure. Since reduced EDRF/NO release from the endothelium is a major key event in the development of atherosclerosis, we investigated the effect of cholesterol on endothelial cell particulate (membrane-bound) NO synthase activity. Low concentrations (up to 0.2 mM) of liposomal cholesterol progressively activated plasma membrane-bound NO synthase. Increasing cholesterol concentration above that which maximally stimulated enzyme activity produced a progressive inhibition with respect to the control value. In time course experiments using endothelial cell plasma membranes enriched with cholesterol, changes in NO production were followed by analogous changes in soluble guanylate cyclase activity (sGC). *N*-Monomethyl-L-arginine (L-NMMA) (1 mM) inhibited particulate NO synthase activity at all cholesterol concentrations used with subsequent decreases in cGMP production. Egg lecithin liposomes (free of cholesterol) had no effect on NO synthase activity. A three-fold increase in superoxide ($O_2^-$) and a 2.5-fold increase in NO formation followed by an eight-fold increase in peroxynitrite ($ONOO^-$) production by cholesterol-treated microsomes isolated from endothelial cells was observed, one which rose further up to eight-fold in the presence of superoxide dismutase (SOD) (10 U/mL). Cholesterol had no effect on Lubrol-PX solubilized membrane-bound NO synthase or on cytosolic (soluble) NO synthase activities of endothelial cells. Cholesterol modulated lipid fluidity of plasma membranes labelled with 1,6-diphenyl-1,3,5-hexatriene (DPH) as indicated by the steady state fluorescence anisotropy $[(r_o/r) - 1]^{-1}$. Arrhenius plots of $[(r_o/r) - 1]^{-1}$ indicated that the lipid phase separation of the membranes at 26.2 ± 1.5° was elevated to 34.4 ± 1.9° in cholesterol-enriched membranes, consistent with a general decrease in membrane fluidity. Cholesterol-enriched plasma membranes treated with egg lecithin liposomes showed a lipid phase separation at 27.5 ± 1.6°, indicating the reversible effect of cholesterol on membrane lipid fluidity. Arrhenius plots of NO synthase activity exhibited break point at 26.9 ± 1.8° which rose to 35.6 ± 2.1° in 0.5 mM cholesterol-treated plasma membranes and decreased to 21.5 ± 1.4° in plasma membranes treated with 0.2 mM cholesterol. The allosteric properties of plasma membrane-bound NO synthase inhibited by $Mn^{2+}$ (as reflected by changes in the Hill coefficient) were changed by cholesterol, consistent with modulations of the fluidity of the lipid microenvironment of the enzyme. Our findings suggest that incorporation of high concentrations of cholesterol into endothelial cell membranes causes down-regulation of NO synthase by producing an increased packing of bulk lipids. In contrast, cholesterol incorporation at low concentrations up-regulates NO synthase by increasing the fluidity of the lipid microenvironment of the enzyme. The present studies concerning the behaviour of particulate NO synthase and rate of NO release with respect to the structure and function of the biomembranes provide important new clues as to the role of this fascinating molecule in atherosclerosis.

*Key words:* particulate NO synthase; cholesterol; nitric oxide (NO); peroxynitrite ($ONOO^-$); superoxide ($O_2^-$); membrane fluidity; endothelial cells; fluorescence polarization

Studies in many experimental models and in humans have shown that atherosclerosis induces an early selective impairment of endothelium-derived relaxation; the proposed mechanism of this defect is thought to be a decrease in EDRF/NO† release from the endothelium [1]. Studies *in vitro* show that native LDL and oxLDL inhibit EDRF responses, while antioxidants such as probucol reduce the formation of free radicals and the oxidative modification of LDL that lead to the impairment of EDRF responses and may prevent this dysfunction in hypercholesterolaemia and atherosclerosis [2, 3].

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† Abbreviations: L-NMMA, *N*-monomethyl-L-arginine; DPH, 1,6-diphenyl-1,3,5-hexatriene; sGC, soluble guanylate cyclase; EDRF, endothelium-derived relaxing factor; NOS, NO synthase; NO, nitric oxide; $ONOO^-$ peroxynitrite; ONOOH, peroxynitrous acid; HBSS, Hanks balanced salt solution; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethane sulphonic acid; oxLDL, oxidized low-density lipoproteins; L-NMMA·AcOH, *N*-monomethyl-L-arginine monoacetate; SOD, superoxide dismutase; TLC, thin-layer chromatography.

It has been reported that endothelium-derived relaxation can be preserved in hypercholesterolaemic rabbits by an L-arginine (2.25%) supplement diet [4]. Recent studies, however, suggest an abnormal endothelium-derived NO system in hypercholesterolaemia and indicate that decreased availability of NO substrate is not responsible for the impaired endothelial function [5, 6]. NO is considered an important protective agent produced by the endothelium to preserve the integrity of the endothelium and may protect it against atherogenesis [7].

NO is enzymatically formed from a terminal guanidino nitrogen of L-arginine by a gene family of NO synthase (EC 1.14.23). Although several NO synthase isoforms have been isolated, all are homologous and may be divided into two categories with different regulation and activities. The constitutive isoforms in neuronal or endothelial cells are always present. These NO synthase isoforms are inactive until intracellular calcium levels increase; then, the calcium-binding protein calmodulin binds to calcium, and the calcium–calmodulin complex binds to and activates NO synthase. In contrast, the inducible NO synthase isoform is normally absent from macrophages and hepatocytes, but when these cells are activated by specific cytokines, an inducible NO synthase enzyme is produced [8, 9].

It has been demonstrated that calmodulin-dependent EDRF/NO synthase activity is also present in the particulate fractions of bovine aortic endothelial cells [10, 11]. Detailed subcellular fractionation of isolated pig endothelial cells revealed that the distribution of constitutive NO synthase activity closely resembled that of the plasma membrane marker 5′-nucleotidase, suggesting that most, if not all, of the constitutive NO synthase activity in these cells is associated with the plasma membrane [12]. This localization might render the enzyme more susceptible to activation by physical stimuli, such as a shear stress-induced change in membrane fluidity [13].

Peroxynitrite ($ONOO^-$), a product of NO reaction with superoxide ($O_2^-$), has recently been defined as a potent oxidant and potential mediator of vascular tissue injury [14]. It has been suggested that both the independent reactions of $O_2^-$ and NO and their reaction product $ONOO^-$ are critical in the initiation and maintenance of the atherosclerotic state and contribute to the defect in vasorelaxation [14, 15].

Cholesterol is a major component of biological membranes and is responsible for some of their physico-chemical features. One of the best-known effects of cholesterol on the properties of the phospholipid bilayer is the dramatic change in the enthalpy and cooperativity of the gel to liquid crystalline phase transition in the phospholipid bilayer. Cholesterol causes the elimination of the sharp, highly cooperative phase transition in dipalmitoylphosphatidylcholine bilayers while the size of the cooperative unit for the sharp transition also decreases as cholesterol content of the membrane increases. On the other hand depletion of cholesterol from biomembranes produces a progressive disordering of lipid packing, i.e. an increase in membrane fluidity with subsequent changes in membrane-bound enzyme activity [16, 17].

The present studies were undertaken to explore the mechanisms responsible for the cholesterol-modulated alterations of the NO production by the particulate (membrane-bound) NO synthase of endothelial cells. The experiments below demonstrate that cholesterol was able to manifest its effects on the enzyme in a short time (≈2 hr) and did not require prolonged preincubation. Cholesterol at high concentrations decreased the activity of the particulate NO synthase by decreasing the membrane fluidity, thereby altering the partitioning of the NO synthase molecules between the membrane and aqueous phase and/or altering the partitioning between the fluid and solid domain of the endothelial cell membranes. At low concentrations, however, it increased the activity of the enzyme by increasing the fluidity of its lipid microenvironment. Furthermore, it was shown that endothelial cell microsomal membranes treated with cholesterol were subjected to higher oxidative stress by $ONOO^-$, resulting in a consequential decrease in membrane fluidity as compared to normal microsomal membranes. Such changes may be an early key event in endothelial dysfunction leading to atherosclerosis.

## MATERIALS AND METHODS

### *Materials*

[$^3$H]L-Arginine (54 Ci/mmol; 1 Ci = 37 GBq) was obtained from the Radiochemical Centre (Amersham, Bucks, U.K.). NO (99.99% pure) was obtained from Messer Griesheim (Germany). L-NMMA·AcOH and SOD were obtained from Calbiochem (Switzerland). Cholesterol (Merck AG) was routinely recrystallized twice from 95% ethanol and its purity tested by TLC. Egg lecithin was obtained from Serva Feinbiochemica GmbH and Co., Germany, and GTP-agarose and DPH from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

*Cell culture.* Bovine aortic endothelial cells were harvested and grown on Cytodex-3 microcarrier beads (Pharmacia/LKB) in Dulbecco's modified Eagle's medium supplemented with 4 mM L-glutamine and 10% (v/v) foetal calf serum as previously described [18]. Approximately $1 \times 10^9$ cells were suspended in 50 mM Tris–HCl (pH 7.4) containing 10 mM EDTA, 5 mM glucose, 1.15% (w/v) KCl, 0.1 mM DL-dithiothreitol, leupeptin (2 mg/L), pepstatin A (2 mg/L), trypsin inhibitor (10 mg/L) and phenylmethylsulphonyl-fluoride (44 mg/L) (buffer 1). The cell suspension was bubbled with helium for 15 min and sonicated and the homogenate centrifuged in three subsequent steps at 1000 *g*, 10,000 *g* and 105,000 *g* for 10, 20 and 60 min, respectively. The 105,000 *g* pellet (microsomal fraction) was resuspended in 50 mM Tris–HCl (pH 7.4) containing 0.1 mM EDTA, 0.1 mM dithiothreitol, leupeptin (2 mg/L), pepstatin A (2 mg/L), trypsin inhibitor (10 mg/L), phenylmethylsulphonyl fluoride (44 mg/L) and 10% (v/v) glycerol (buffer 2). For the separation of the plasma membranes from the microsomes aliquots (2 mL) of the microsomal fraction (2.0–2.5 mg of protein) were layered over a 20 mL cushion of 27% (w/v) sucrose in 10 mM Tris–HCl buffer containing 1 mM EDTA

and centrifuged for 3 hr at 65,000 *g* in a SW-27 rotor of a Beckman L5–75 ultracentrifuge. The band (2 mL) at the sucrose interface containing plasma membranes was diluted with 8 mL of buffer 2 (without glycerol) collected by centrifugation at 100,000 *g* for 60 min, resuspended in homogenization buffer 2 and stored at −70°.

The designation of the subcellular fractions as microsomes and plasma membranes was based on the determination of corresponding marker enzyme activities: i.e. NADH-dependent cytochrome C reductase (microsomes) and 5′-nucleotidase (plasma membranes) with 25–50 μg aliquots of each fraction as previously described [19]. Solubilization of membranes was performed using 0.1% (w/v) Lubrol-PX at 4° for 12 hr under magnetic stirring, in a medium consisting of HBSS pH 7.4, 200 mM mannitol, 0.1 mM dithiothreitol, phenylmethyl-sulphonyl-fluoride (44 mg/L) and 0.35 mg of membrane protein. Protein concentration was measured by the Bradford method [20] using reagents from Bio-Rad (Richmond, CA, U.S.A.) and BSA as a standard.

*Assay of particulate* NO *synthase activity*

*Determination of* NO. NO synthase activity of endothelial cell membranes was measured by monitoring either NO and/or [$^3$H]-L-citrulline production as previously described [18, 21]. Reactions (1 mL sample volumes) were carried out for 10 min at 37°. Standard reaction mixtures contained: HBSS-EDTA (1 mM); NO synthase (50 μg of membrane protein); L-arginine (100 μM); NADPH (100 μM); FAD (5 μM); FMN (5 μM); tetrahydrobiopterin (5 μM) and calmodulin (1 μM). Reactions were terminated by adding 10 U of L-lactic dehydrogenase and 100 μL of Na pyruvate (10 mM). NO determination was carried out by mixing the incubates with 100 μL of a reagent consisting of: 20% sulphanilamide in 20% $H_3PO_4$ and 25 μM scopoletin. NO was monitored at room temperature (22°) with an excitation wavelength at 350 nm and an emission at 460 nm in an Aminco SPF-500 fluorescence spectrophotometer. Fluorescence was monitored continuously in time until the slope of the line could be measured (approx. 8 min). Slope measurements were then converted to pmol of NO using a standard curve constructed with various concentrations of pure NO. For the assay of the allosteric inhibition by $Mn^{2+}$ of the plasma membrane NO synthase the reaction mixture contained increasing amounts of $MnCl_2$ as indicated in Fig. 8 (see below).

Solutions of acidified saline (9 mg/mL NaCl, 0.1 mM HCl, pH 4.0) were deoxygenated by bubbling with 100% nitrogen for 2 hr. NO gas was bubbled into 10 mL of deoxygenated acidified saline at a rate of one bubble per sec for 20 min, while the gas above was flushed away with $N_2$. The solubility of NO in water is 7.34 cm$^3$/100 mL and assuming saturation, the maximum concentration of NO in the solution is 3.3 mM [22]. Stock solutions of nitrite ($NO_2^-$) were prepared by dissolving appropriate amounts of sodium nitrite in acid saline to produce a 3.3 mM stock solution (i.e. identical to the estimated stock concentration of the NO saturated solutions). The stock solutions of $NO_2^-$ were prepared in open, ambient air-exposure tubes. The NO and $NO_2^-$ solutions were stored on ice and used within 3–4 hr. The present fluorophotometric method is highly sensitive with a resolution of less than 5 pmol of NO. The method is approx. 100 times more sensitive for NO than for $NO_2^-$.

*Determination of* L-*citrulline.* L-Citrulline was measured in 50 mM HEPES (pH 7.4) containing the same cofactors as described for NO measurements in the presence of NO synthase (50 μg of membrane protein). [$^3$H]L-Arginine (100,000 cpm) was mixed with cold L-arginine (100 μM) in a final volume of 200 μL. After incubation (10 min at 37°), assays were terminated with 2 mL of ice-cold 100 mM HEPES buffer (pH 5.5) containing 10 mM EGTA and 500 mg of Dowex AG 50W-X8 ($Na^+$ form), which were eluted with 2 mL of water. [$^3$H]L-Citrulline was measured in a liquid scintillation counter. Blank values were determined in the absence of added enzyme.

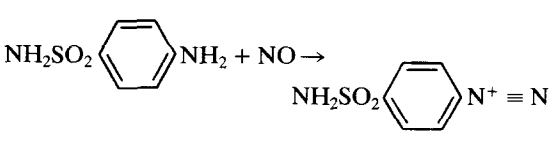
*Determination of superoxide anion* ($O_2^-$)

$O_2^-$ production by microsomes (50 μg protein) was measured by monitoring cytochrome C (10 μM) reduction spectrophotometrically at 550 nm over 10 min at 37°. An extinction coefficient of 21 mM$^{-1}$ cm$^{-1}$ was used to calculate the rate of $O_2^-$ production, and corrected for cytochrome *C* reduction non-specifically inhibited by SOD (10 U/mL).

*Determination of* $ONOO^-$

$ONOO^-$ was synthesized, titrated and stored as previously described [22, 23]. The reaction mixture (total volume 1 mL) consisted of: HBSS-EDTA (1 mM); microsomes (50 μg of protein); L-arginine (100 μM); NADPH (100 μM); FAD (5 μM); FMN (5 μM); tetrahydrobiopterin (5 μM); calmodulin (1 μM) and 25 μM scopoletin. Incubations were carried out at 37° for 10 min. Reactions were terminated by the addition of 10 U of L-lactic dehydrogenase and 100 μL of Na pyruvate (10 mM). $ONOO^-$ was estimated at room temperature (22°) with an excitation wavelength of 350 nm and an emission of 460 nm in an Aminco SPF-500 Fluorescence Spectrophotometer. Fluorescent intensity was then converted to nmol of $ONOO^-$ using a standard curve constructed with various concentrations of pure $ONOO^-$.

Scopoletin, employed in the present study for the assay of both NO and $ONOO^-$, is a fluorescence substance which has been used for the determination of hydrogen peroxide ($H_2O_2$) in the presence of horseradish peroxidase. For the NO assay sulphanilamide reacts with NO at low pH (≈2) and forms a diazonium ion according to the reaction:




This electron acceptor is capable of oxidizing the hydroxyl group of scopoletin with subsequent fluorescence decay at 460 nm (excitation 350 nm).

The rate of this decrease in fluorescence increased with increasing NO concentrations.

In our present experiments scopoletin was also found to be an efficient probe for the estimation of $ONOO^-$ at physiological pH in the absence of sulphanilamide. $ONOO^-$ induced the oxidation of scopoletin in a linear fashion over the range 0–10 $\mu$M. The effect of pH on $ONOO^-$ mediated oxidation of scopoletin was evaluated between 2 and 10. Oxidation of scopoletin was faster at pH between 2 and 7 (approx. 15 sec) than at pH between 7 and 10 (approximately 60 sec) and the final fluorescence intensity was stable over time. $ONOO^-$ becomes protonated at acidic pH to peroxynitrous acid (ONOOH), which is an unstable species. ONOOH can undergo homolysis to a molecule with hydroxyl radical ($OH^\cdot$) like reactivity [24].

At acidic pH, $ONOO^-$ oxidizes scopoletin through the $OH^\cdot$ radical formation while at alkaline pH it oxidizes scopoletin by directly reacting with its hydroxyl group. NO, $H_2O_2$ and xanthine/xanthine oxidase do not directly oxidize scopoletin without metal catalysis. $H_2O_2$-dependent oxidation of scopoletin occurs via metal-catalysed secondary oxidant formation in the presence of horseradish peroxidase, cytochrome *C* or $Fe^{3+}$ EDTA. In the present experiments it was shown that L-cysteine (1 mM), as a source of free sulphydryl groups, resulted in a 97% inhibition of $ONOO^-$ mediated oxidation of scopoletin. L-Cystine (1 mM), which lacks free sulphydryl groups, reduced scopoletin oxidation by only 20%. At pH 7.4 the use of free radical scavengers and specific enzyme inhibitors makes it possible to distinguish between oxidation induced by $ONOO^-$ versus other oxidant species, making scopoletin a sensitive and efficient trap for cellular $ONOO^-$ production.

*Purification of sGC*

sGC from endothelial cells was purified by GTP-agarose chomatography. Samples of cytosol (10 mg protein) in 12 mL of HBSS containing 10 mM $MnCl_2$ were added to a GTP-agarose column (1.8 × 9 cm) pre-equilibrated with 25 mM Tris–HCl buffer pH 7.6, containing 250 mM sucrose and 10 mM $MnCl_2$. After application of the sample, the column was washed with five column volumes of equilibration buffer. sGC was then eluted from the column with 5 mL equilibration buffer plus 10 mM GTP. The eluted enzyme was immediately concentrated using Centricon-30 microconcentrators (Amicon) and resuspended in 25 mM Tris–HCl, pH 7.6 containing 250 mM sucrose [22].

*cGMP determination*

Concentrations of cGMP were determined by radioimmunoassay after acetylation of the samples with acetic anhydride. The reaction mixture contained triethanolamine/HCl (50 mM), creatine phosphate (5 mM), $MgCl_2$ (3 mM), isobutylmethylxanthine (1 mM), creatine kinase (0.6 U), GTP (1 mM) and 0.085–1.0 $\mu$g purified sGC; total volume was 150 $\mu$L. The reactions were initiated by the addition of GTP and samples incubated for 10 min at 37°. The incubation medium was aspirated and cGMP extracted by the addition of ice-cold HCl (0.1 M). After 10 min, the samples were transferred to a new plate, dried, and reconstituted in 5 mM sodium acetate (pH 4.75) for cGMP determination. cGMP formation was determined using a cGMP assay kit (Amersham).

*Preparation of liposomes*

Liposomes were prepared essentially as previously described [25]. Briefly, egg lecithin and cholesterol in a molar ratio 1:2 and dissolved in chloroform were evaporated under vacuum onto the walls of a glass flask. Liposomes were formed by adding 2.0 mL physiological saline to the dried film, and the mixture was then dispersed by shaking and subsequent sonication. Aliquots of liposomes were extracted with $CHCl_3/CH_3OH$ (2:1, v/v) and the extracts chromatographed on silica gel G with appropriate standards using $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (20:12:3:1, v/v) as developing solvent. The content of lipid phosphorus was measured in the spots after digestion with 70% $HClO_4$ at 180° [26] and the cholesterol content using the reaction of Liebermann. For the purpose of obtaining liposomes of uniform size, gel filtration and/or Millipore filtration were used. Thus, liposomes were passed through a Sepharose 4B column and eluted in a homogeneous profile at approx. 50% of the column volume. Membrane filtration of the liposomes was performed on a 0.45 $\mu$m Millipore filter simply by forcing the preparation through the membranes.

*Steady-state fluorescence polarization studies*

Lipid fluidity was assessed by the steady-state fluorescence polarization of DPH according to the method described by Shinitzky and Barenholz [27]. Measurements were made in an AMINCO SPF-500 spectrofluorometer with a polarization accessory for fluorescence-polarization measurements at an excitation wavelength of 360 nm and an emission wavelength of 430 nm. The polarization of fluorescence was expressed as the fluorescence anisotropy, $r$, and the anisotropy parameter $[(r_o/r) - 1]^{-1}$ was calculated using the value of $r_o = 0.365$ for DPH as previously described [28]. The anisotropy parameter varies directly with the apparent rotational relaxation time of the probe and thus inversely with fluidity. The fluorescence anisotropy was calculated according to the equation:

$$[r = I_{VV} - I_{VH}/I_{VV} + 2I_{VH}]$$

where $I_{VV}$ and $I_{VH}$ are the intensities of the emitted light orientated parallel and perpendicularly to the plane of the exciting beam, respectively. Light-scattering corrections were always <5% of the total signal and did not differ significantly in the various preparations. Measurements were made at least in triplicate. The movements of DPH within the medium depend on both the lifetime ($\tau$) and viscosity of the medium ($n$). It is generally assumed that $\tau$ is constant at one temperature and fluorescence polarization is therefore directly related to $n$. However, the presence of a third compound can modify $\tau$ if this compound acts as a quencher. A direct measurement of $\tau$ in the presence or absence of quencher checks this possibility. An estimate can be obtained by measuring the intensity of total

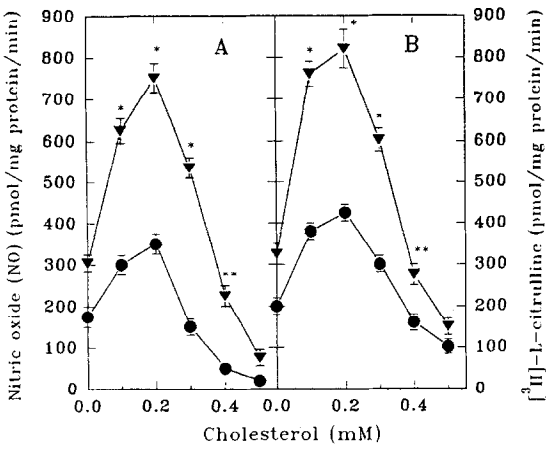



Fig. 1. Effect of cholesterol on NO (A) and [$^3$H]L-citrulline (B) produced by particulate (membrane-bound) NO synthase of endothelial cell plasma membranes (▼). The effect of cholesterol on NO synthase in the presence of L-NMMA (1 mM) is also shown (●). Experimental details are given in the text. Points and bars represent means ± SD from three different experiments. A statistically significant difference from the plasma membranes in the presence of L-NMMA was determined (*$P < 0.001$, **$P < 0.001$).

fluorescence $F = I_{VV} + 2I_{VH}$. Identical fluorescence intensities measured in the presence and absence of liposomal cholesterol allow us to conclude that they have no effect on $\tau$ and that consequently, the observed variation in $[(r_o/r) - 1]^{-1}$ values represents modulation of the lipid membrane bilayer. The temperature dependence of $[(r_o/r) - 1]^{-1}$ was determined over the 4–40° range. Endothelial cell plasma membranes were warmed initially to 40° and fluorescence polarization was estimated every 1–2° as the suspension cooled slowly to 4°. Plots of log $[(r_o/r) - 1]^{-1}$ versus $1/T$ were constructed to detect thermotropic transition temperatures.

*Treatment of membranes*

Endothelial cell plasma membranes or microsomes (100 μg membrane protein) were incubated in polystyrene tubes at 25° for 2 hr in the aforementioned homogenization buffer 2 under gentle shaking with different amounts of liposomal cholesterol as specified in Fig. 1. The incubation mixture (2 mL) was removed and after dilution with the same buffer was centrifuged for 30 min at 105,000 *g*. The precipitate was resuspended in 20% (w/v) sucrose–phosphate buffer 0.1 M, pH 7.4 and layered at the interface between 27% sucrose-phosphate buffer 0.1 M, pH 7.4 and 1.15% KCl-phosphate buffer 0.1 M, pH 7.4. The gradient was centrifuged for 1 hr at 105,000 *g* and the liposome-free membranes recovered as a pellet at the bottom of the gradient. The fluorescence hydrocarbon DPH was used as a fluorescence probe to monitor the degree of fluidity in the plasma membrane lipid core. Labelling of plasma membranes was performed with $2 \times 10^{-6}$ M DPH dispersion in PBS obtained by injection of 0.1 mL of $2 \times 10^{-3}$ M DPH in tetrahydrofuran into 100 mL PBS that had been stirred vigorously. This DPH dispersion is practically clear and void of fluorescence. A volume of 1 mL plasma membrane suspension in homogenization buffer 2 at a concentration of 100 μg/mL was incubated with 2 mL DPH dispersion for 30 min at 25°. The labelled membranes were then washed twice with the same buffer, resuspended in PBS at a concentration of 50 μg/mL and immediately used for the fluorescence studies.

*Statistical analysis*

All experiments were performed in triplicate. Computer analysis for curve fitting was performed. The correlation coefficients ($r$) for the straight lines were above 0.98. Mean value and standard deviation were calculated. Comparisons between the two groups were performed by the use of a paired standard Student's $t$-test. A value of $P < 0.05$ was considered significant. When two different curves were made with the same membrane preparation under the same experimental conditions, the individual points and the slopes obtained showed a maximum variability of 5%. At different membrane preparations, maximum variability was less than 10%.

## RESULTS

*NO and L-citrulline determination after cholesterol treatment of endothelial cell plasma membranes*

The curves representing the changes in plasma membrane NO synthase activity of endothelial cells at different concentrations of liposomal cholesterol are shown in Fig. 1. NO and [$^3$H]L-citrulline production showed a 2.5-fold increase at 0.2 mM of liposomal cholesterol. Higher concentrations of cholesterol (up to 0.5 mM), however, led to a progressive inhibition of enzyme activity (approx. 80% for the NO and 70% for the [$^3$H]L-citrulline production) with respect to the control (untreated with cholesterol) plasma membranes. In the presence of L-NMMA (1 mM) both NO and [$^3$H]L-citrulline production were inhibited at all cholesterol concentrations used. The production of NO by endothelial cell plasma membranes in the presence of a saturating concentration of L-arginine (100 μM) was dependent on NADPH. D-Arginine (100 μM) failed to stimulate enzyme activity, indicating the specificity of the activation by L-arginine. Concentrations of NADPH up to 1 mM did not increase the production of NO beyond that seen with 100 μM NADPH. Enzyme activity is also $Ca^{2+}$/calmodulin and tetrahydrobiopterin-dependent. In a $Ca^{2+}$-free solution containing 1 mM EDTA the activity of NO synthase was very low (25 ± 4 pmol NO/mg protein/min).

*NO and cGMP determination using an incubation chamber*

Figure 2 shows the amounts of NO and cGMP when (a) plasma membranes (50 μg) with the appropriate substrates and cofactors, and (b) HBSS were placed in two adjacent compartments of a chamber which were separated by a thin Teflon membrane (poly-tetrafluoroethylene, 0.0015 in (1 in = 25.4 mm) in thickness, Dupont, Wilmington,

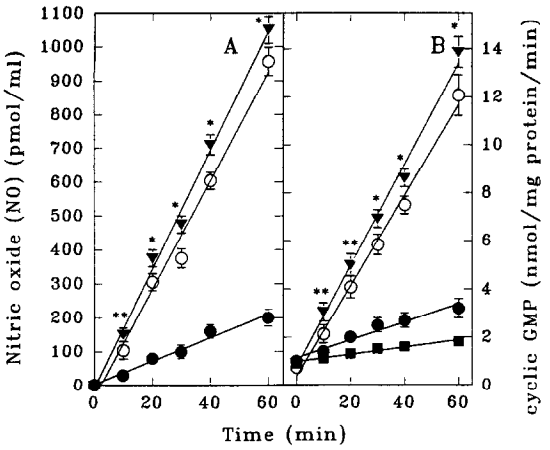



Fig. 2. Endothelial cell plasma membranes and HBSS were placed in the two compartments of a chamber separated by a thin Teflon membrane (poly-tetrafluoroethylene, 0.0015 in (1 in = 25.4 mm) in thickness), one which permits only NO diffusion. (A) NO was determined in HBSS when plasma membranes (50 μg) with the appropriate cofactors were placed in the opposite compartment (▼). Cholesterol (0.5 mM) treatment of plasma membranes resulted in an 80% decrease in the amount of NO that was diffused into the HBSS compartment (●). Egg lecithin (10 mg/mL) treatment of plasma membranes had no effect on the amount of NO diffused into the HBSS compartment (○). (B) Replacement of HBSS with sGC resulted in a seven-fold increase of cGMP in the compartment containing sGC (▼) as compared to the basal sGC activity (■). When plasma membranes were treated with 0.5 mM cholesterol an approximately 75% decrease in cGMP production was observed (●). Egg lecithin treated plasma membranes showed no statistically significant differences ($P > 0.05$) in cGMP production as compared to untreated (control) membranes (○). Experimental details are given in the text. Each point represents the mean ± SD of three independent experiments. A statistically significant difference from cholesterol-treated plasma membranes was determined (*$P < 0.001$, **$P < 0.05$).

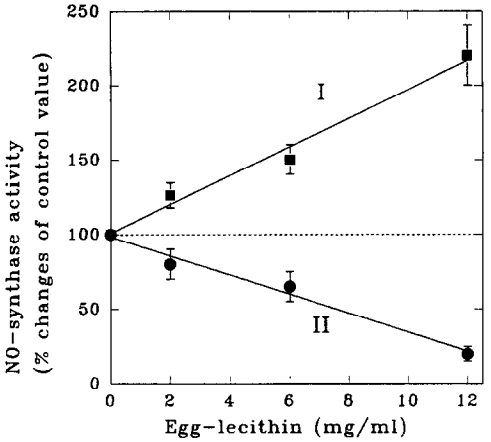



Fig. 3. Restoration of plasma membrane-bound NO synthase activity of endothelial cells by removing the excess cholesterol from the membranes enriched with cholesterol. Curve I (■) represents experiments in which plasma membranes were pretreated with cholesterol at a concentration of 0.5 mM capable of decreasing enzyme activity. Curve II (●) represents experiments in which plasma membranes were pretreated with cholesterol at a concentration of 0.2 mM capable of increasing the specific activity of the enzyme (see Fig. 1). Controls were membranes enriched with cholesterol incubated under the same conditions but in the absence of egg lecithin liposomes. The specific activity of the NO synthase was estimated by determining the NO production per mg protein per min. Values are the mean ± SD of three different experiments.

DE, U.S.A.) permitting NO diffusion through the membrane. The amounts of NO produced by particulate NO synthase with and without treatment of the plasma membranes with cholesterol were estimated periodically.

A time-dependent increase in NO in the compartment containing HBSS was observed to reach an equilibrium with NO present in the adjacent compartment containing plasma membranes within 60 min. When plasma membranes were treated with 0.5 mM of liposomal cholesterol the accumulation of NO in the HBSS compartment decreased approximately 80% as was seen in plasma membranes untreated with cholesterol (control). Finally, HBSS in the first compartment was replaced by purified sGC isolated from endothelial cells for periodic bioassay of NO over a 60 min period. In control (untreated with cholesterol) plasma membranes we noted that the diffusion of NO across the Teflon membrane into the sGC compartment resulted in the production of a significant amount of cGMP (13.95 ± 1.85 nmol/mg protein/min). When the procedure was repeated with plasma membranes treated with 0.5 mM cholesterol an approximately 75% decrease in cGMP production was observed as compared to membranes untreated with cholesterol. Control experiments using plasma membranes treated with egg lecithin liposomes (10 mg/mL) had no effect on either NO or cGMP production, indicating that liposomal cholesterol incorporated into the membrane lipid matrix changed the membrane bound-NO synthase activity.

*Reversible effect of cholesterol on* NO *synthase activity*

To examine whether the cholesterol effect on particulate NO synthase activity could be reversed by removing the added cholesterol from the membranes, plasma membranes enriched with cholesterol were incubated with egg lecithin liposomes. Figure 3 shows a linear increase in NO production by membranes treated with 0.5 mM cholesterol and a linear decrease in NO production by membranes treated with 0.2 mM of cholesterol at different amounts of egg lecithin. These findings clearly indicate that the cholesterol effect on NO synthase activity can be reversed by removing the additional cholesterol from the membranes and that cholesterol does not exert its action directly on the protein molecule, but rather through its binding to the lipid matrix of the membrane bilayer causing a modulation of enzyme activity. Furthermore cholesterol's lack of effect (0.2 mM) on Lubrol-PX

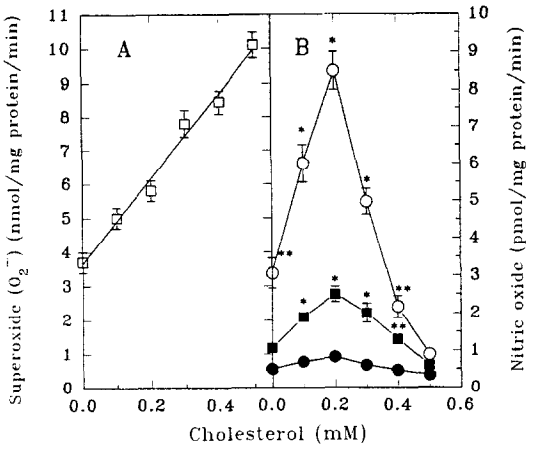



Fig. 4. Effect of cholesterol on (A) superoxide ($O_2^-$) (□) and (B) nitric oxide (NO) (■) production by particulate NO synthase of endothelial cell microsomal membranes. The effect of cholesterol on NO production in the presence of L-NMMA (1 mM) (●) and SOD (10 U/mL) (○) by microsomal membranes is shown. Each point represents the mean ± SD of three independent experiment. A statistically significant difference from membranes in the presence of SOD (*$P < 0.001$, **$P < 0.01$) and L-NMMA was determined (*$P < 0.001$, **$P < 0.01$).

solubilized particulate NO synthase as well as on cytosolic (soluble) NO synthase activities (Table 1) reveals that cholesterol does not affect the enzyme directly, but that the evoked functional changes in the membrane-bound enzyme are mediated through changes in the physical state of the membrane lipid bilayer [29].

### NO *and* $O_2^-$ *production by endothelial cell microsomal membranes*

The effect of liposomal cholesterol on NO and $O_2^-$ production by microsomal membranes is shown in Fig. 4. NO production followed a biphasic curve while $O_2^-$ was linearly increased at various concentrations of liposomal cholesterol. Low concentrations of cholesterol (up to 0.2 mM) increased NO production approximately 2.5-fold; however, high concentrations of cholesterol (up to 0.5 mM) decreased NO production by approximately 90% with respect to untreated (control) membranes. In experiments when SOD (10 U/mL) was included in the incubation mixture, NO production by microsomes at various concentrations of liposomal cholesterol followed a biphasic curve with a dramatic increase up to four-fold and up to eight-fold as compared to cholesterol-enriched microsomes in the absence of SOD and untreated (control) membranes, respectively. These findings clearly indicate that cholesterol incorporation into the endothelial cell microsomal membranes at high concentrations inhibited NO synthase activity by changing their physico-chemical characteristics.

### $ONOO^-$ *production by endothelial cell microsomal membranes*

Figure 5(A) shows the effect of various concentrations of liposomal cholesterol on $ONOO^-$ production by microsomal membranes. Low concentrations of cholesterol (up to 0.2–0.3 mM) increased the production of $ONOO^-$ approximately eight-fold. Higher concentrations, however (up to 0.5 mM) caused a decrease of $ONOO^-$ production with respect to the maximum stimulation. In the presence of L-NMMA (1 mM) $ONOO^-$ production was decreased by 90% at various concentrations of liposomal cholesterol. Figure 5(B) shows a time-dependent increase in $ONOO^-$ production by microsomes treated with liposomal cholesterol (0.2 mM) and untreated (control) microsomes. An approximately four-fold increase of $ONOO^-$ production within 60 min in cholesterol-treated microsomes was observed as compared either to untreated (control) microsomes or to microsomes treated with egg lecithin liposomes (10 mg/mL). Desferrioxamine (100 μM), a well known scavenger of $ONOO^-$ [24], decreased $ONOO^-$ production by

Table 1. Activities of NOS in the subfractions of endothelial cells

| Subfractions | NO synthase activity (pmol NO/mg protein/min) |
|---|---|
| Plasma membrane-bound NOS (PMB) | 305 ± 35 |
| PMB treated with cholesterol (0.2 mM) | 785 ± 72* |
| PMB solubilized with Lubrol-PX 0.1% (w/v) | 495 ± 52* |
| Lubrol-PX solubilized PMB treated with cholesterol (0.2 mM) | 545 ± 57* |
| Microsome-bound NOS (MB) | 1150 ± 95 |
| MB treated with cholesterol (0.2 mM) | 2675 ± 215** |
| MB solubilized with Lubrol-PX 0.1% (w/v) | 1770 ± 155** |
| Lubrol-PX solubilized MB treated with cholesterol (0.2 mM) | 1725 ± 165** |
| Cytosolic NO synthase (sNOS) (105,000 *g* supernatant) | 1905 ± 175 |
| sNOS treated with cholesterol (0.2 mM) | 2118 ± 195*** |

Results were obtained from six separate experiments.
* Statistically significant difference ($P < 0.05$) compared to PMB.
** Statistically significant difference ($P < 0.05$) compared to MB.
*** No statistically significant difference ($P > 0.05$) compared to sNOS.

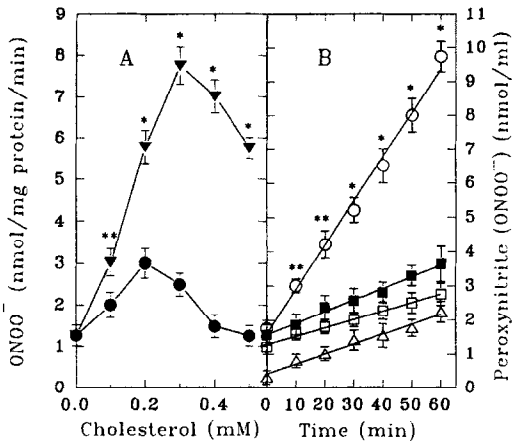



Fig. 5. (A) Effect of cholesterol on peroxynitrite ($ONOO^-$) production by particulate NO synthase of endothelial cell microsomal membranes (▼). The effect of cholesterol on $ONOO^-$ production in the presence of L-NMMA (1 mM) is also shown (●). (B) Time-dependence of $ONOO^-$ production by endothelial cell microsomal membranes treated (○), untreated (△) with liposomal cholesterol (0.2 mM) and treated with egg lecithin liposomes (10 mg/mL) (□). The scavenging effect of desferrioxamine (100 μM) on $ONOO^-$ production by microsomes is also shown (■). Experiments were carried out in the presence of 50 μg microsomal protein with the appropriate substrates and cofactors in HBSS pH 7.4 and scopoletin 25 μM. The oxidation of scopoletin by $ONOO^-$ was followed at various time intervals and the concentrations of $ONOO^-$ were estimated as described in the Experimental section. Each point represents the mean ± SD of three independent experiments. A statistically significant difference from membranes treated with egg lecithin liposomes in the presence of L-NMMA and desferrioxamine was determined (*$P < 0.001$, **$P < 0.01$).

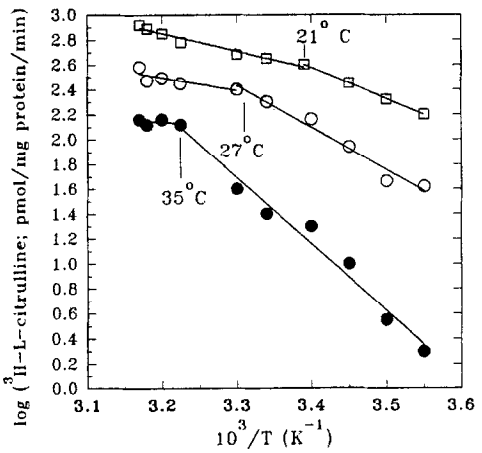



Fig. 6. Arrhenius plots of particulate NO synthase activity from 0.5 mM cholesterol treated (●), 0.2 mM cholesterol treated (□) and untreated (controls) (○) plasma membranes isolated from endothelial cells. A transition temperature at ≈27° in control membranes was elevated to ≈35° in 0.5 mM cholesterol-treated membranes and depressed to ≈21° in 0.2 mM cholesterol-treated membranes. Each point represents the average value of duplicate determinations from a typical experiment that was repeated three times. The straight lines were fitted by the method of least squares.

approximately 70% as compared to cholesterol (0.3 mM)-treated membranes.

*Temperature dependence of endothelial cell plasma membrane-bound* NO *synthase*

The temperature dependence of NO synthase activity in plasma membranes treated with either 0.5 mM or 0.2 mM liposomal cholesterol is shown in Fig. 6. Arrhenius plots of plasma membrane NO synthase activity (pmol of [$^3$H]L-citrulline/mg membrane protein/min) showed a transition temperature at 26.9 ± 1.8°. Plasma membranes treated with 0.5 mM liposomal cholesterol showed an elevation of the break point to 35.6 ± 2.1°, approx. 8–9° above that of untreated (control) membranes, consistent with a decrease in the fluidity of the lipid microenvironment of the enzyme. In contrast, plasma membranes treated with 0.2 mM liposomal cholesterol showed a depression of the break point to 21.5 ± 1.4°, approximately 5–6° below that of untreated (control membranes), consistent with an increase in the fluidity of the lipid microenvironment of the enzyme.

*Temperature dependence of fluorescence anisotropy of endothelial cell plasma membranes*

The effects of temperature on the fluorescence anisotropy parameter, $[(r_o/r - 1]^{-1}$, of DPH in plasma membranes are illustrated by representative Arrhenius plots in Fig. 7. An increase in temperature produces a concomitant diminution in $[(r_o/r) - 1]^{-1}$ values, which means an increase in membrane

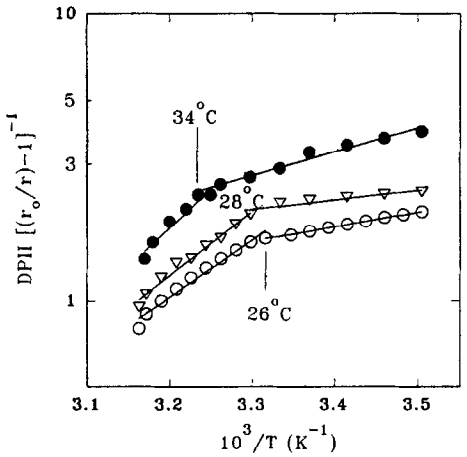



Fig. 7. Temperature dependence of the fluorescence anisotropy of DPH in the control (○), in cholesterol-treated plasma membranes (●) and in cholesterol-enriched endothelial plasma membranes treated with egg lecithin liposomes (▽). The ordinate is the fluorescence anisotropy and the abscissa is the reciprocal of the absolute temperature. A separation phase temperature at ≈26° in control membranes was elevated to ≈34° in cholesterol-treated membranes and restored to ≈28° in egg lecithin treated membranes. Experimental details are given in the text. This experiment is representative of three that were performed. The straight lines were fitted by the method of least squares.

fluidity. However, the evolution of the fluidity was not linear; a thermotropic transition temperature (separation phase) was indeed observed at 26.2 ± 1.5° in untreated membranes. Treatment of membranes with 0.5 mM liposomal cholesterol caused a statistically significant ($P < 0.01$) increase in $[(r_0/r) - 1]^{-1}$ compared with untreated (control) membranes. It must be realized that the corresponding decrease in membrane fluidity is constant over the full range of temperatures studied. Furthermore, the thermotropic transition temperature was elevated to 34.4 ± 1.9°. This upward shift, which corresponds to the increase of 8–9° in the break point temperature of DPH fluorescence anisotropy, is consistent with a general decrease in plasma membrane fluidity. Treatment of cholesterol-enriched plasma membranes with egg lecithin liposomes (10 mg/mL), which are capable of withdrawing cholesterol from a given membrane, caused a decrease of the lipid phase separation temperature from 34.4 ± 1.9° to 27.5 ± 1.6°, indicating the reversible effect of cholesterol on membrane lipid fluidity.

*Cooperative inhibition of the endothelial cell plasma membrane* NO *synthase by* $Mn^{2+}$ *ions*

Evidence that low concentrations of cholesterol (0.2 mM) resulted in an increase in the fluidity of the lipid microenviroment of particulate NO synthase while high concentrations of cholesterol (0.5 mM) resulted in a decrease in the fluidity of its lipid microenvironment was obtained from the allosteric inhibition of enzyme activity by $Mn^{2+}$. Figure 8 shows the curves obtained when the relative rates of enzymatic activity were plotted against different concentrations of $Mn^{2+}$ in control plasma membranes and in membranes preincubated with 0.2 mM and 0.5 mM cholesterol for 2 hr at 25°. The Hill coefficient ($h$) (slopes) in the control (untreated plasma membranes) was $h = 1.76 \pm 0.17$ indicating the presence of cooperativity; this was decreased to $h = 1.06 \pm 0.12$ in plasma membranes treated with 0.2 mM cholesterol, suggesting a loss in the cooperativity of the enzyme, which is consistent with an increase in the fluidity of the annular lipids of the enzyme. In contrast, plasma membranes treated with 0.5 mM cholesterol showed a Hill coefficient $h = 2.31 \pm 0.26$ suggesting a higher cooperativity of the enzyme consistent with a decrease in the fluidity of the annular lipids of the enzyme [28, 30].

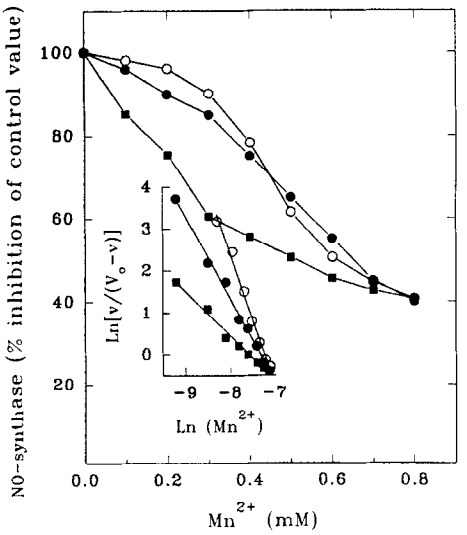



Fig. 8. Effect of $Mn^{2+}$ on the reaction rate of NO synthase (pmol NO/mg protein/min) for cholesterol (0.2 mM) treated (■), cholesterol (0.5 mM) treated (○) and untreated (control) (●) endothelial cell plasma membranes. The insert shows Hill plots of the same data. Corresponding Hill coefficients ($h$) are: $h = 1.76 \pm 0.17$ (●), $h = 1.06 \pm 0.12$ (■), and $h = 2.31 \pm 0.26$ (○). The correlation coefficients ($r^2$) for the straight lines in the inserts are >0.98; $v$ is the reaction velocity and $V_0$ is the rate of the reaction in the absence of $Mn^{2+}$. Points of the curves drawn are mean values of duplicate determinations from a typical experiment repeated three times.

## DISCUSSION

Impaired production of NO either as a result of endothelial injury or dysfunction has been implicated in the pathology of a variety of cardiovascular diseases, such as hypertension, hypercholesterolaemia, atherosclerosis and diabetes [31]. Therefore, the prevention and/or reversal of the functional and morphological changes in the endothelium associated with these diseases as well as the increase in our understanding of the regulation of the L-arginine/NO pathway may lead to the development of new therapeutic agents. Evidence suggests accelerated degradation of endothelium-derived NO by $O_2^-$ in hypercholesterolaemic vessels [6].

The reaction product of $O_2^-$ and NO is $ONOO^-$ which is a relatively long-lived strong oxidant. Thus, NO may substantially increase the toxicity of $O_2^-$ by converting a transmembrane signal mediator into a potent and relatively long lived oxidant [32]. The rate of $ONOO^-$ formation is determined by the product of concentrations of NO and $O_2^-$ which are low under normal circumstances. Because many pathological processes can simultaneously stimulate the production of NO and $O_2^-$ the rate of $ONOO^-$ formation can become substantial under pathological conditions [33].

The ability of exogenous cholesterol to be incorporated *in vitro* into endothelial cell membranes and cause significant changes in membrane-bound NO synthase activity by altering membrane fluidity was investigated in the present study. Cell membranes exist as a dynamic matrix which responds to various physiological or pathological conditions by modifying its physicochemical properties, allowing movement or conformational changes in membrane-bound proteins in a specific fashion.

The concept that alterations in membrane fluidity result in changes in the detectability of membrane-associated receptors and enzyme proteins has now been established and this thesis is supported by data correlating the activity of membrane-bound enzymes with membrane fluidity [34]. The curves representing the changes in plasma membrane NO synthase

activity at different concentrations of exogenous cholesterol have biphasic character (Fig. 1). Low concentrations of cholesterol progressively activated particulate NO synthase, whereas increasing cholesterol concentration above that maximally stimulating enzyme activity caused a progressive inhibition with respect to control value.

A time dependence of NO production by plasma membranes was verified by placing plasma membranes and HBSS in a special incubation chamber with two compartments separated by a thin Teflon membrane through which NO gas could pass. NO accumulation in the HBSS was decreased approximately 80% in cholesterol-treated plasma membranes as compared to non-treated (controls). When HBSS was replaced by purified sGC, cGMP production decreased approximately 75% when plasma membranes were treated with cholesterol (0.5 mM) (Fig. 2). These experiments clearly indicate that NO release from endothelial cell plasma membranes is identical to gaseous NO and not to any nitrosothiol compound.

That cholesterol has no direct action on the membrane-bound NO synthase of endothelial cells but rather exerts its effects through its penetration in the lipid matrix of the bilayer was shown by removing cholesterol from the membranes after incubation with egg lecithin liposomes of cholesterol-loaded plasma membranes. All of these functional effects were fully reversible (Fig. 3). Therefore cholesterol does not irreversibly change membrane-bound NO synthase activity and its presence in the membrane causes a modulation in enzyme activity.

Cholesterol caused an increase in both NO and $O_2^-$ production by endothelial microsomes which react to form $ONOO^-$ (Figs 4 and 5). $O_2^-$ is produced by microsomes in redox reactions of mixed-function oxidases through cytochrome $P_{450}$. In our previous study we showed that dietary cholesterol increases hepatic drug oxidase and cytochrome $P_{450}$ activities in guinea pigs [19]. In Fig. 4(B) the decline of the curve showing NO production by microsomal membranes at high cholesterol concentrations, in the presence of SOD, supports the view that cholesterol decreases NO synthase activity by changing the membrane structure. However, the decline of the curve, in the absence of SOD, is apparently due to both decreased NO synthase activity and neutralization of NO by $O_2^-$ to form $ONOO^-$.

In many pathological conditions, including atherosclerosis, simultaneous cellular production of $O_2^-$ and NO occur, potentially leading to the formation of $ONOO^-$ [14]. Peroxynitrite anion is a strong oxidant in its own right ($E^\circ$ ($ONOO^-/NO_2$, $H_2O$) = 1.4 V at pH 7.0), oxidizing sulphydryl groups at a rate that is substantially faster than would be possible via its first-order decay. It also reacts with transition metals, including the copper of SOD and metals of other proteins, to form a strong nitrating species resembling nitronium ion ($NO_2^+$) [33]. In the present study enrichment of microsomal membranes with various amounts of cholesterol resulted in an eight-fold increase in $ONOO^-$ production (Fig. 5A). In time-dependent experiments (within 60 min), a four-fold increase in $ONOO^-$ production by cholesterol (0.3 mM) treated microsomes as compared to untreated (control) microsomes was observed (Fig. 5B). The decline of the curve showing $ONOO^-$ production by microsomes at cholesterol concentrations higher than 0.3 mM, is apparently due to a decrease in NO synthase activity with a subsequent decrease in NO production and hence $ONOO^-$ formation. In our previous studies we demonstrated that $ONOO^-$ increased cGMP synthesis in cultured human keratinocytes [23]. Furthermore, a recently-presented publication [34] suggests that $ONOO^-$ produces coronary vasorelaxation in dogs and that the mechanism of $O_2^-$ inactivation of NO involves converting it to a shorter-lived and less potent vasorelaxant species. High concentrations of $ONOO^-$ prolonged exposure to a lower steady-state concentration of $ONOO^-$, however, may be directly damaging to tissues and possibly result in abnormal vasoregulation *in vivo*. Previous reports indicate that $ONOO^-$ initiates lipid peroxidation and causes oxidation of plasma lipoproteins [35]. $ONOO^-$ or its by-products released within the endothelial cells may interact with the different components of these cells. Therefore, we reasoned that $ONOO^-$ may damage endothelial cell membranes by modulating their lipid and protein components.

The characteristic temperature ("break points") of Arrhenius plots of the fluorescence anisotropy of DPH and/or particulate NO synthase activity were employed as measures of changes in membrane fluidity (Figs 6 and 7). Arrhenius plots of plasma membrane NO synthase activity showed a characteristic temperature at ≈26.9°. Cholesterol (0.5 mM) increased this temperature to ≈35.6°; this is matched by a similar effect on thermotropic transition temperatures estimated by the DPH fluorescence anisotropy (≈26.2° and ≈34.4°, respectively). Low concentrations of cholesterol (0.2 mM) decreased the transition temperature of NO synthase from ≈26.9° to ≈21.5°. The thermotropic separation phase temperature of cholesterol-enriched plasma membranes (≈34°) was restored to that of untreated (control) plasma membranes (≈28°) when these membranes were treated with egg lecithin liposomes in order to withdraw the cholesterol added (Fig. 7).

These findings clearly indicate that plasma membrane NO synthase activity is governed by both bulk lipid fluidity and the fluidity of its annular lipids. High concentrations of cholesterol caused an increased packing (decrease in fluidity) of the bulk lipids, whereas low concentrations of cholesterol produce an opposite effect on the lipid annulus (increase in fluidity) of the enzyme. The increase in the activity of the enzyme in plasma membranes treated by low concentrations of cholesterol (up to 0.2 mM) may be due to the increase in the lipid fluidity of the immediate environment of the enzyme, which would relieve a constraint on the protein molecule imposed by the annular lipids and increase its conformational flexibility and hence its activity. The decrease in enzyme activity by higher concentrations of cholesterol (<0.2 mM) may be due to the extensive rigidification of the membranes [36].

In the present study further evidence that

cholesterol at low concentrations (>0.2 mM) affects the fluidity of the lipid microenvironment of plasma membrane NO synthase was obtained from the alterations in the cooperativity behaviour of this enzyme [28, 29]. As shown in Fig. 8 the value of the Hill coefficient for the allosteric inhibition of NO synthase by $Mn^{2+}$ was decreased in plasma membrane treated with cholesterol 0.2 mM, indicating a loss in the cooperativity of the enzyme consistent with an increase in the fluidity of its annular lipids; high concentrations of cholesterol (0.5 mM) increased the Hill coefficient indicating higher cooperativity of the enzyme consistent with a decrease in the fluidity of the annular lipids of the enzyme [30].

NO synthase is a haem-protein showing a 29–39% overall identity to NADPH-cytochrome $P_{450}$ reductase. NO synthase reduces cytochrome $P_{450}$ as indicated by the fact that *in vitro* NO synthase supports the hydroxylation of *N*-ethylmorphine by cytochrome $P_{450}$. This suggests that NO synthase may participate in similar electron transfer processes *in vivo*. However, since NADPH-cytochrome $P_{450}$ reductase is a membrane-bound enzyme, this characteristic may be only of physiological significance for the membrane-bound type of NO synthase [37]. The close similarity between NO synthase and the NADPH-cytochrome $P_{450}$ reductase/cytochrome $P_{450}$ system suggests that $Mn^{2+}$ ions inhibited NO synthase activity either by a nonspecific oxidation of sulphydryls or by replacement of the haem-iron.

Alterations in cell membrane fluidity with concomitant upregulation or downregulation of the membrane syndromic and/or antidromic proteins modulate the transduction mechanism translating the external signal into an internal signal carried by second messengers [38]. A variety of agents could affect the fluidity of various lipid domains within endothelial cell membranes and these alterations in fluidity may be responsible for altering the NO/cGMP signalling system designed for both intra- and inter-cellular regulation [39].

An attractive hypothesis was recently presented to explain the mechanism of endothelial dysfunction in atherosclerosis and hypercholesterolaemia [40]. Physiological NO formation is a receptor-linked phenomenon involving G-protein-associated mobilization of calcium in endothelial cells. According to the hypothesis the G protein would be inactivated in early atherosclerosis and hypercholesterolaemia, yielding decreased NO formation in response to a certain level of stimulation of endothelial surface (flow) receptors. Later, when atherosclerosis is manifest and/or hypercholesterolaemia has become more advanced, NO breakdown would be enhanced possibly by the increasing formation of oxygen radicals. This hypothesis may reflect receptor-mediated changes in membrane bound-NO synthase activity: the transmembrane signal transduction mediated through G proteins. In various systems, transmembrane $Ca^{2+}$ flux is initiated by the binding of a receptor agonist to its membrane receptor. Thus, receptor-mediated changes in NO synthase activity and increased $Ca^{2+}$ influx can be linked to increased NO and $ONOO^-$ formation in cells. Damage caused by $ONOO^-$ on endothelial cell membranes (e.g. increased permeability, decreased activity of membrane-bound $Ca^{2+}$-ATPase) would contribute to the derangement of calcium homeostasis engendering further $ONOO^-$ and $O_2^-$ production. Thus scavenging free radicals or preventing an increase in intracellular $Ca^{2+}$ would provide protection against injury by interrupting this deleterious cycle.

The studies reported here indicate that cholesterol, as a building stone of biomembranes, modulates particulate NO synthase activity of endothelial cells. Alterations in cholesterol content on endothelial cell membranes could be mediated both intrinsically by changes in cholesterol metabolism or extrinsically by changes in cholesterol blood concentrations (for example, in hypercholesterolaemia). The presence of intimal lesions has been confirmed long before any recognizable endothelial denudation. Thus, hypercholesterolaemia appears to cause a more subtle form of injury without morphologic alterations. Hypercholesterolaemia can increase the cholesterol to phospholipid ratio of endothelial cell membranes and subsequently membrane fluidity and this change may be important in enhancing monocyte adhesion [41].

In conclusion the present study suggests that an increase in cholesterol content of endothelial cell membranes could be involved as an initiator in changes in membrane-bound NO synthase activity resulting in deviations from normal endothelium function. Furthermore, cholesterol causes an imbalance in endothelium-derived NO and $O_2^-$. $ONOO^-$ as a product of the reaction between NO and $O_2^-$ can then participate in the pathogenesis of atherosclerotic lesions by causing lipoprotein oxidation which then contributes to the formation of fatty streaks and subsequent plaque formation characteristic of atherosclerotic lesions.

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