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European Journal of Pharmacology 307 (1996) 233–241

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# Evidence for modulation of hydrogen peroxide-induced endothelial barrier dysfunction by nitric oxide in vitro

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Received 7 February 1996; revised 18 March 1996; accepted 22 March 1996

**Abstract**

Acute effects of the nitric oxide (NO) donors sodium nitroprusside and glyceryl trinitrate on hydrogen peroxide ($H_2O_2$)-induced increases in endothelial monolayer permeability to trypan blue-labelled bovine serum albumin have been investigated in vitro. Exposure of bovine pulmonary artery endothelial cell monolayers to 0.2 mM $H_2O_2$ for 20 min caused a significant increase in percentage trypan blue-labelled albumin transfer from the lumenal to the ablumenal compartment (basal 6.0 ± 0.6 to 25.4 ± 0.9%, $n = 4$, $P < 0.0005$). In separate experiments 100 μM sodium nitroprusside significantly enhanced the effect of 0.2 mM $H_2O_2$ (from 7.4 ± 1.4 to 11.9 ± 1.5%, $n = 9$, $P < 0.0001$) but did not alter albumin transfer in the absence of $H_2O_2$. This additive effect appeared to be due to NO release from sodium nitroprusside, since nitrite concentration in the medium overlying cells treated with 100 μM sodium nitroprusside was 19.9 ± 1.8 μM ($n = 12$). Significantly less nitrite (3.5 ± 0.5 μM, $n = 12$, $P < 0.0001$) was found in the medium overlying cells treated with 100 μM glyceryl trinitrate, which in contrast to sodium nitroprusside, inhibited the permeability increase caused by $H_2O_2$ (from 15.6 ± 3.3 to 13.8 ± 3.1%, $n = 6$, $P < 0.001$). Furthermore 10 μM sodium nitroprusside, which released comparable amount of nitrite (4.5 ± 0.4 μM, $n = 6$) to 100 μM glyceryl trinitrate, also inhibited the permeability increase caused by $H_2O_2$ (from 20.7 ± 0.4 to 19.4 ± 0.3%, $n = 9$, $P < 0.01$). We conclude that relatively large amounts of NO released from 100 μM sodium nitroprusside exacerbate the barrier dysfunction caused by $H_2O_2$, while lower amounts of NO give a small amount of cytoprotection.

*Keywords:* Endothelium; Albumin transfer; Hydrogen peroxide; Sodium nitroprusside; Glyceryl trinitrate

## 1. Introduction

Reactive oxygen species have been implicated in acute vascular injury associated with a variety of conditions such as inflammation and ischaemia-reperfusion injury (Thies and Autor, 1991). In acute inflammatory lung disease the vascular endothelium constitutes an important target of such reactive oxygen species which disturb the pro- and anti-oxidant balance normally maintained by the endothelial monolayer. The resulting acute oxidative stress is associated with alterations in a number of endothelial properties, collectively known as endothelial dysfunction (Rubanyi, 1993). Longer term exposure to reactive oxygen species produces more extensive injury to both endothelium and underlying smooth muscle, which is implicated in the pathogenesis and progression of atherosclerosis (Kozar et al., 1994). It has been proposed that the principal reactive oxygen species responsible for oxidant injury to the endothelium is the hydroxyl radical, derived from superoxide anion and/or hydrogen peroxide ($H_2O_2$) in the presence of iron (Thies and Autor, 1991), though iron-independent pathways for hydroxyl radical generation from superoxide also exist (Beckman et al., 1990). Additionally there is evidence that $H_2O_2$ can directly injure vascular endothelium (Li and Lau, 1993).

Endothelium-derived nitric oxide (NO) released under physiological conditions is thought to have free radical scavenging properties (Rubanyi, 1993) and might therefore be expected to constitute part of the endothelial cell's protective armamentarium against reactive oxygen species generated during injury. On the other hand unphysiologically high quantities of NO such as those generated by the inducible NO synthase, can be cytotoxic and may be directly responsible for the local endothelial damage associated with endotoxin shock (Palmer et al., 1992). Alternatively NO released in this way may generate more reactive

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0014-2999/96/$15.00 Published by Elsevier Science B.V.
*PII* S0014-2999(96)00271-3

radicals, by combination with superoxide to yield large amounts of peroxynitrite (Beckman et al., 1990) or with $H_2O_2$ to produce singlet oxygen (Noronha-Dutra et al., 1993).

The barrier function of the vascular endothelium is central to its role in the regulation of cardiovascular homeostasis. Injurious stimuli such as reactive oxygen species damage the integrity of the monolayer and thereby expose the underlying tissue to further insult. Endothelial monolayers grown on porous membrane supports have been used as in vitro models to investigate modulation of endothelial barrier function and in particular $H_2O_2$ has been shown to increase monolayer permeability to macromolecules (Berman and Martin, 1993). The role of NO in modulation of endothelial barrier function is a matter of debate, but from information available to date, it would appear that in large vessel endothelium at least, NO can offset the permeabilising action of mediators such as thrombin via generation of cGMP (Draijer et al., 1995).

The purpose of the present study was to examine the effects of NO generation on endothelial barrier dysfunction evoked by acute (short term) exposure to the oxidant $H_2O_2$. The bovine pulmonary artery endothelial cells used were chosen to reflect the fact that this endothelial cell type constitutes a primary target for oxidants released during acute inflammatory lung disease. The sources of NO used were the NO donors sodium nitroprusside and glyceryl trinitrate; barrier dysfunction was assessed as the effect of $H_2O_2$ on monolayer permeability to trypan blue-labelled bovine serum albumin. Generation of NO from donors was confirmed indirectly as the formation of nitrite in the medium overlying treated cells.

## 2. Materials and methods

### 2.1. Cell culture

Bovine pulmonary artery endothelial cells were cultured according to the method of Burke-Gaffney and Keenan, 1993 with modifications. For this study 12 pulmonary arteries (one per cow) were obtained from a local abattoir. Tissues were transported to the laboratory (on ice) in Medium 199 supplemented with 200 U/ml penicillin, 200 μg/ml streptomycin, 0.8 μg/ml gentamicin, 10 μg/ml fungizone and 0.02 M Hepes (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulphonic acid), pH 7.2. Vessels were cleared of fat and connective tissue, cut open along one side and laid flat. Endothelial cells were isolated by exposing the lumenal side of the arteries to 0.5 mg/ml collagenase (type II) in serum-free minimum essential medium (MEM) for 20 min at 37°C. Remaining loosely attached cells were removed by washing with phosphate-buffered saline (PBS). The combined collagenase digest/PBS wash was harvested by centrifugation (1000 × *g*, 10 min, 20°C) and resuspended in complete MEM (containing non-essential amino acids, 15% heat-inactivated foetal calf serum, 75 U/ml penicillin, 75 μg/ml streptomycin, 4 mM L-glutamine, 18 μg/ml endothelial cell growth supplement and 1 U/ml heparin) which additionally contained 10 μg/ml fungizone and 50–100 μg/ml gentamicin. The following day this medium was removed, adherent cells washed with PBS and fresh complete MEM added. When a significant population of endothelial cells had become established, contaminating smooth muscle cells were removed by treatment with pancreatin (2.5% in 0.85% NaCl) for 5–20 min. The remaining endothelial cells were allowed to grow to confluence and subcultured using trypsin/EDTA (0.5%/0.2%). Confluent cells displayed typical cobblestone morphology and the presence of factor VIII-related antigen was established by indirect immunofluorescence. Cells of passage 8–18 (derived from six of the 12 arteries processed) were used in experiments.

### 2.2. Measurement of trypan blue-labelled albumin transfer across endothelial monolayers

#### 2.2.1. Monolayer preparation and treatment

Confluent cells (4–7 days in culture) were trypsinized and seeded at a density of $2.3 \times 10^5$ cells/cm$^2$ onto transwell polycarbonate membrane assemblies (6.5 mm diameter, 0.4 μm pore size; Costar), suspended in 24 well culture plates. Before seeding, membranes were coated with 25 μg/ml bovine plasma fibronectin (3 h at 37°C) followed by an equilibration period of 1 h where membranes were incubated with complete MEM to ensure even wetting of the membrane. 600 μl of complete MEM was then added to the lower compartment, followed by 100 μl of cell suspension to the upper compartment. 4 h after seeding medium was replaced in both upper and lower chambers with fresh medium. Monolayers were fed daily and 72 h after seeding were incubated (lumenally and ablumenally) with test reagent(s) in Locke's buffer (mM: NaCl 154, KCl 5.6, $CaCl_2 \cdot 2H_2O$ 2.0, $MgCl_2 \cdot 6H_2O$ 1.0, $NaHCO_3$ 3.6, glucose 5.6, Hepes 10.0, pH 7.4) containing 2 mM L-glutamine, at the indicated concentrations for the indicated times under sterile conditions.

#### 2.2.2. Trypan blue-labelled albumin transfer

This was adapted from the methods of Rotrosen and Gallin (1986) and Gudgeon and Martin (1989). Following removal of test reagent(s), monolayers were washed and equilibrated with Hanks' balanced salt solution (HBSS) containing 0.02 M Hepes (HBSS/Hepes) for 30 min at 37°C. One hundred μl of 4% trypan blue-labelled albumin (prepared by the method of Rotrosen and Gallin (1986), trypan blue > 99% protein-bound as judged by acid precipitation) was added to the upper compartment of the membrane assembly, 600 μl of HBSS/Hepes to the lower compartment and the system incubated at 37°C for 60 min in a shaking water bath. Trypan blue-labelled albumin transfer across endothelial cell monolayers was quantified

by measuring the absorbance at 590 nm (using a Pharmacia LKB. Ultraspec III spectrophotometer) of aliquots sampled from the lower compartment and was expressed as either the percentage transfer of trypan blue-labelled albumin (the absorbance of the solution in the lower chamber as a percentage of the solution initially added to the upper chamber) or as the fold increase in transfer (ratio of percentage transfer across the treated monolayer to that across an untreated monolayer). Basal transfer rate across untreated monolayers was also measured as μg trypan blue-labelled bovine serum albumin transferred over the time course of the experiment (μg/h; a value of 4 mg/h represents 100% transfer, since the upper chamber was initially exposed to 100 μl of a 4% albumin solution).

### 2.3. Nitrite measurement

Nitrite production as an indicator of NO production from NO donors, was measured by the Griess reaction. Cells grown in culture plates were incubated with sodium nitroprusside (1, 10, 100 μM) or glyceryl trinitrate (100 μM) in Locke's buffer containing 2 mM L-glutamine, 10 μM indomethacin and 100 U/ml superoxide dismutase for 20 min at 37°C. (Concentrations of nitroprusside up to 100 μM are widely used in the literature (e.g. to activate bovine coronary artery guanylyl cyclase (Ignarro et al., 1981); to cause tolerance in cultured vascular cells (Zhang et al., 1993) and 100 μM glyceryl trinitrate has been shown to stimulate cGMP production in vascular cells (Feelisch and Kelm, 1991)). The supernatant overlying cells was then assayed for nitrite content (after reduction of any nitrate present in the sample) by a modification of the method of Yu et al. (1994). Briefly 200 μl of supernatant was incubated for 1 h at 25°C with 100 μl nitrate reductase from *Aspergillus niger* (196 mU/ml, Boehringer Mannheim), 100 μl NADPH (0.56 mM, Sigma), 100 μl FAD (70 μM, Sigma) and 200 μl Locke's buffer containing 2 mM L-glutamine. The reaction was stopped by boiling for 3 min. An equal volume (700 μl) of Griess reagent (1 : 1 mixture of 2% sulphanilamide in 5% phosphoric acid and 0.2% *N*-(1-naphthyl)ethylenediamine dihydrochloride in distilled water) was added to the reduced samples and incubated at 60°C for 10 min. The absorbance of the chromophore formed by reaction of nitrite with the Griess reagent was measured at 546 nm. Standard curves for 0–10 nmol/tube nitrate and nitrite were included in each assay.

### 2.4. Cell viability assay

The methylthiazol tetrazolium assay used, which uses mitochondrial respiration as an indicator of cell viability, was adapted from the method of Mossman (1983). Following incubation ± $H_2O_2$ for 20 min or 1 h, cells in 6 well plates were washed with 2 ml PBS, then 2 ml MEM followed by 200 μl methylthiazol tetrazolium (5 mg/ml) were added to each well. After a 3 h incubation at 37°C the medium overlying cells was aspirated and cells were solubilised with 1.5 ml dimethylsulphoxide (DMSO). A 500 μl aliquot of the DMSO solution was added to 2 ml distilled water in a borosilicate tube and the absorbance of the resulting solution was measured at 600 nm. Results were expressed as percentage of control (absorption of solutions derived from cells treated with Locke's/glutamine buffer taken to represent 100% viability).

### 2.5. Electron microscopy

Electron microscopy of endothelial cells was carried out on samples fixed in phosphate buffer (pH 7.4) containing 2.5% glutaraldehyde and post-fixed in osmium tetroxide. Transmission electron micrographs were produced with a Phillips 201 electron microscope.

### 2.6. Data analysis

For statistical analysis permeability data were expressed as percentage transfer of trypan blue-labelled albumin, nitrite data were expressed in concentration units (μM) and viability data as percentage of control. All data were expressed as mean values ± S.E.M. of $n$ measurements per treatment. These were made with cells of > 1 passage number derived from the same artery, unless otherwise stated in the figure and table legends. Permeability and nitrite data were compared by one way analysis of variance to determine if any significant differences existed between treatment groups. This was followed by a Bonferroni or $F$ test as appropriate, to determine where differences lay. A paired Student's $t$ test was used to compare 48 h post treatment permeability data to control. For viability data confidence intervals were constructed (95%, 99%, 99.9%) and an unpaired Student's $t$ test was used to assess statistical significance. In all cases a $P$ value $< 0.05$ was taken to be significant. For clarity of presentation, permeability data were graphed as fold increases in trypan blue-labelled albumin transfer over basal or percentage increases in transfer.

### 2.7. Materials

Fibronectin (from bovine plasma), endothelial cell growth supplement, collagenase (type II), goat anti-rabbit immunoglobulin G, bovine serum albumin, trypan blue, catalase, superoxide dismutase, $H_2O_2$, indomethacin, sulphanilamide, naphthylethylenediamine dihydrochloride, sodium nitroprusside, sodium nitrite, sodium nitrate, methylthiazol tetrazolium, DMSO and potassium ferricyanide were obtained from Sigma (UK). Minimum essential medium, nonessential amino acids, foetal calf serum, penicillin/streptomycin, fungizone, gentamicin, pancreatin, L-glutamine, trypsin, EDTA, Hepes (1 M, cell culture grade) and HBSS were obtained from Gibco (UK). Heparin

(mucous) injection BP was obtained from Leo Laboratories (Ireland). Rabbit anti-human factor VIII antigen was from Nordic Immunology. Glyceryl trinitrate was from David Bull Laboratories (UK). All other chemicals were of the highest grade commercially available.

## 3. Results

### 3.1. Effect of $H_2O_2$ on trypan blue-labelled albumin transfer across endothelial monolayers

We first investigated the effect of $H_2O_2$ on trypan blue-labelled albumin transfer across endothelial cell monolayers. A 20 min exposure to 0.2 mM $H_2O_2$ significantly increased transfer over basal levels (from $6.0 \pm 0.6$ to $25.4 \pm 0.9\%$, $P < 0.0005$, $n = 4$). This effect was not further increased by exposure times of up to 60 min (Fig. 1). The effects seen with this concentration of $H_2O_2$ at 20 min were not explicable in terms of a loss in cell viability as judged by uptake of methylthiazol tetrazolium into mitochondria which was not significantly reduced (Table 1), or by transmission electron microscopy: though cells had become rounded and retraction was evident, suggesting alterations to intercellular adherent junctions, mitochondrial cristae appeared intact (data not shown). The increase in albumin transfer was partly reversible, since removal of $H_2O_2$ and re-introduction of complete medium led to a significant reduction in transfer across those monolayers 48 h post treatment (Fig. 2a). There was also a significant reduction in transfer across untreated monolayers over the 48 h period. Monolayers exposed to 0.2 mM $H_2O_2$ for 1 h did not recover barrier function by 48 h later

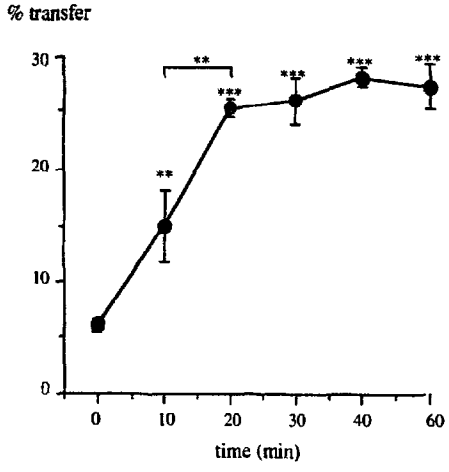


Fig. 1. The effect of 0.2 mM hydrogen peroxide ($H_2O_2$) on percentage transfer of trypan blue-labelled albumin across bovine pulmonary artery endothelial cell monolayers with time. The ordinate measures mean (±S.E.M.) percentage transfer recorded from two experiments each in duplicate ($n = 4$). Cells used were of different passage numbers derived from two different arteries. Basal albumin transfer rate was $240 \pm 22$ μg/h. **, $P < 0.005$; ***, $P < 0.0005$ denotes significant difference from control (0 time), unless otherwise indicated.

Table 1
Lack of effect of 0.2 mM hydrogen peroxide ($H_2O_2$) on viability of bovine pulmonary artery endothelial cells

| $[H_2O_2]$(mM) | Treatment time(min) | Viability(% control) |
|---|---|---|
| 0 | 20 | 100 |
| | 60 | 100 |
| 0.2 | 20 | $91.3 \pm 2.6$ [a] |
| | 60 | $97.0 \pm 5.6$ [a] |

Cellular viability was assessed using the methylthiazol tetrazolium assay. Results are expressed as % of control (absorption of solutions derived from cells treated with vehicle alone taken to represent 100% viability). Data are presented as mean ± S.E.M. from three experiments each in triplicate ($n = 9$). Cells used were of > 1 passage number derived from > 1 artery. [a] Not significantly different from respective controls, as assessed using confidence intervals and an unpaired Student's $t$ test.

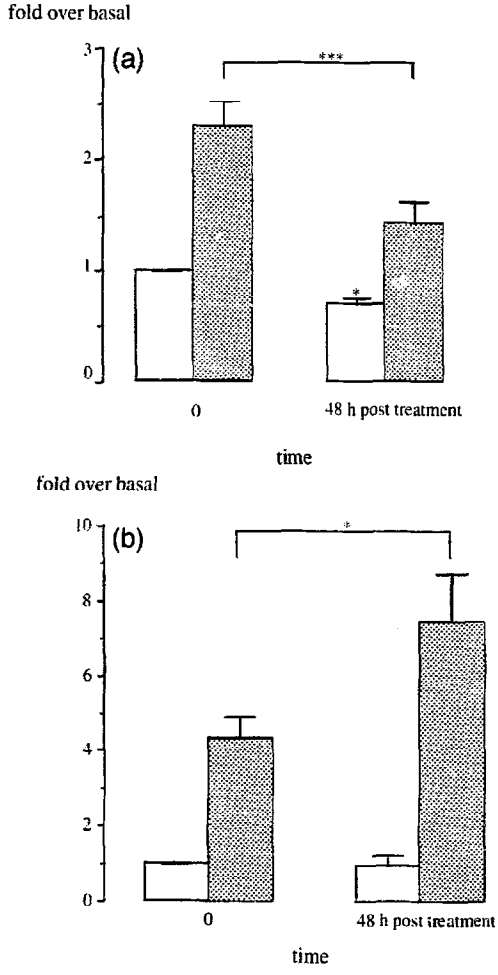


Fig. 2. Extent of recovery of endothelial barrier function following treatment of monolayers with 0.2 mM $H_2O_2$ for (a) 20 min, (b) 1 h. Trypan blue-labelled albumin transfer was assessed immediately after treatment (0 time) and again 48 h post treatment. The ordinate measures mean (±S.E.M.) fold increases in transfer over basal values at time 0. a: five experiments each in triplicate ($n = 15$); basal albumin transfer rate was $323 \pm 73$ μg/h. b: two experiments, one in duplicate, one in triplicate ($n = 4$–5); basal albumin transfer rate was $173 \pm 38$ μg/h. Untreated and treated monolayers are represented by open and stippled columns respectively. *, $P < 0.05$; ***, $P < 0.0005$, significantly different from basal (at time 0), unless otherwise indicated.

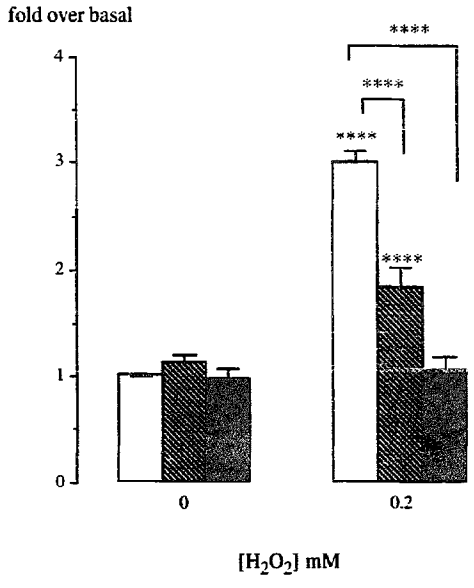


Fig. 3. The effect of a 20 min treatment with 0.2 mM $H_2O_2$ on trypan blue-labelled albumin transfer across endothelial monolayers in the absence (open columns) or presence of catalase, 3 U/ml (hatched columns), 30 U/ml (closed columns). The ordinate measures mean (±S.E.M.) fold increases in transfer over basal values in the absence of catalase. Data are from two to four experiments each carried out in triplicate ($n = 6–12$). Basal albumin transfer rate was 256±24 μg/h. ****, $P < 0.0001$, significantly different from basal, unless otherwise indicated.

(Fig. 2b) and though there was significant evidence of more dramatic morphological changes than above, with the appearance of large intercellular gaps, there was still no evidence of mitochondrial damage or loss of viability (Table 1). Evidence that the increase in albumin transfer was mediated by $H_2O_2$ rather than by a breakdown product was provided by the observation that catalase abolished the $H_2O_2$ effect (Fig. 3). We also established that the $H_2O_2$-mediated increases in transfer seen were not due to dissociation of trypan blue from bovine serum albumin and migration of trypan blue through the filters; incubation of trypan blue-labelled albumin solutions with 0.2 mM $H_2O_2$ did not reduce the percentage of protein-bound trypan blue as judged by trichloroacetic acid precipitation.

### *3.2. Effect of 100 μM sodium nitroprusside on $H_2O_2$-induced increases in trypan blue-labelled albumin transfer*

We first established that sodium nitroprusside could release NO when incubated with bovine pulmonary artery endothelial cells, by measuring nitrite production in the supernatants overlying cells treated with 100 μM sodium nitroprusside. In the absence of sodium nitroprusside no nitrite was detectable, however when cells were incubated with sodium nitroprusside for 20 min, a nitrite concentration of 19.9 ± 1.8 μM was recorded (Table 2). We next investigated the effects of sodium nitroprusside on trypan blue-labelled albumin transfer. At (1, 10, 100 μM) sodium nitroprusside did not itself alter albumin transfer across

Table 2
Nitrite concentrations in the medium overlying bovine pulmonary artery endothelial cells treated with 1, 10, or 100 μM sodium nitroprusside (SNP) or with 100 μM glyceryl trinitrate (GTN) for 20 min

| Treatment | [Drug](μM) | Treatment time(min) | Nitrite produced(μM) |
|---|---|---|---|
| Cells alone | 0 | 20 | n.d. |
| SNP | 1 | 20 | n.d. |
| SNP | 10 | 20 | 4.5±0.4 [a] |
| SNP | 100 | 20 | 19.9±1.8 |
| GTN | 100 | 20 | 3.5±0.5 [a,b] |

Nitrite production was assessed using the Griess assay. Results are expressed as μM nitrite produced. Data are presented as mean±S.E.M. from 2–4 experiments each in triplicate ($n = 6–12$). Cells used were of > 1 passage number derived from > 1 artery. n.d. not detectable. [a] Significantly different release compared to 100 μM sodium nitroprusside, $P < 0.0001$. [b] Not significantly different compared to 10 μM sodium nitroprusside.

endothelial monolayers (data not shown); however 100 μM sodium nitroprusside significantly increased the $H_2O_2$ effect (Fig. 4). As had been the case with $H_2O_2$ alone, the 'combination' effect was partly reversible by 48 h (Fig. 5). To exclude the possibility that 100 μM sodium nitroprusside (500 μM with respect to cyanide) might be mediating its action via release of cyanide, we measured basal and $H_2O_2$-stimulated albumin transfer in the presence of a concentration of potassium ferricyanide which was also 500 μM with respect to cyanide (83 μM). As can be seen in Fig. 6, neither basal nor stimulated transfer was affected by this treatment.

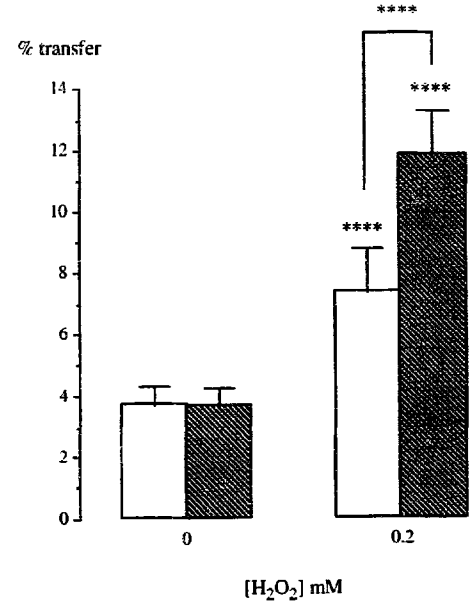


Fig. 4. The effect of a 20 min treatment with 0.2 mM $H_2O_2$ on trypan blue-labelled albumin transfer across endothelial monolayers in the absence (open columns) or presence of 100 μM sodium nitroprusside (hatched columns). The ordinate measures mean (±S.E.M.) percentage transfer recorded from three experiments each in triplicate ($n = 9$). Cells used were of > 1 passage number derived from > 1 artery. Basal albumin transfer rate was 147±26 μg/h. ****, $P < 0.0001$, significantly different from basal, unless otherwise indicated.

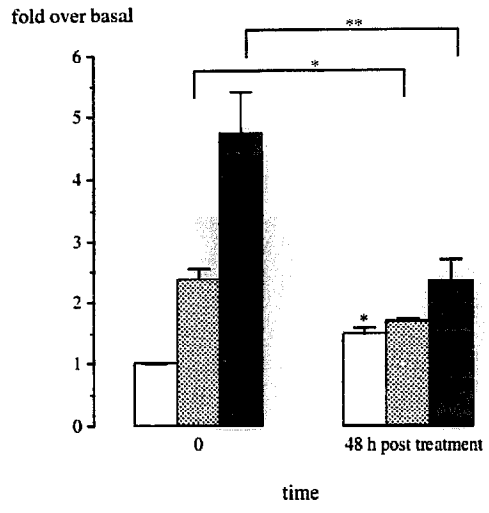


Fig. 5. Partial recovery of endothelial barrier function following treatment of monolayers with 0.2 mM $H_2O_2$ for 20 min in the absence (stippled columns) or presence (closed columns) of 100 $\mu$M sodium nitroprusside. Untreated monolayers are represented by open columns. Trypan blue-labelled albumin transfer was assessed immediately after treatment (0 time) and again 48 h post treatment. The ordinate measures mean ($\pm$S.E.M.) fold increases in transfer over basal values at time 0 recorded from two experiments each in triplicate ($n = 6$). Basal albumin transfer rate was $117 \pm 33$ $\mu$g/h. *, $P < 0.05$; **, $P < 0.01$, significantly different from basal, unless otherwise indicated.

### 3.3. Effect of 100 $\mu$M glyceryl trinitrate on $H_2O_2$-induced increases in trypan blue-labelled albumin transfer

Having demonstrated that the NO donor sodium nitroprusside (100 $\mu$M) enhanced the barrier dysfunction caused by $H_2O_2$, we next investigated the action of glyceryl trinitrate, which unlike sodium nitroprusside does not spontaneously release NO (Lopez-Belmonte et al., 1993). Monolayers treated with 100 $\mu$M glyceryl trinitrate released significantly lower amounts of nitrite into the overlying medium ($3.5 \pm 0.5$ $\mu$M, $n = 12$, $P < 0.0001$) than

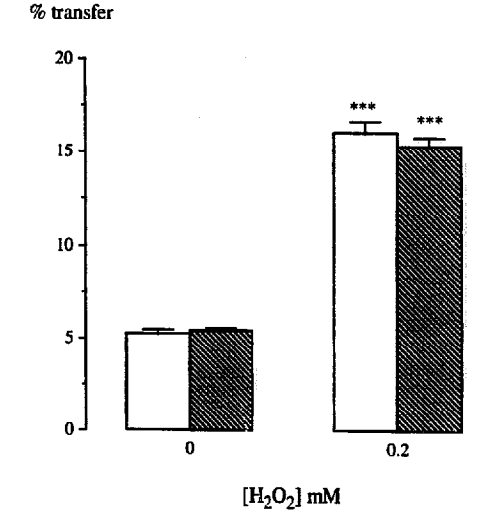


Fig. 6. The effect of a 20 min treatment with 0.2 mM $H_2O_2$ on trypan blue-labelled albumin transfer across endothelial monolayers in the absence (open columns) or presence of 83 $\mu$M potassium ferricyanide (hatched columns). The ordinate measures mean ($\pm$S.E.M.) percentage transfer recorded from two experiments each in triplicate ($n = 6$). Basal albumin transfer rate was $210 \pm 9$ $\mu$g/h. ***, $P < 0.001$, significantly different from basal.

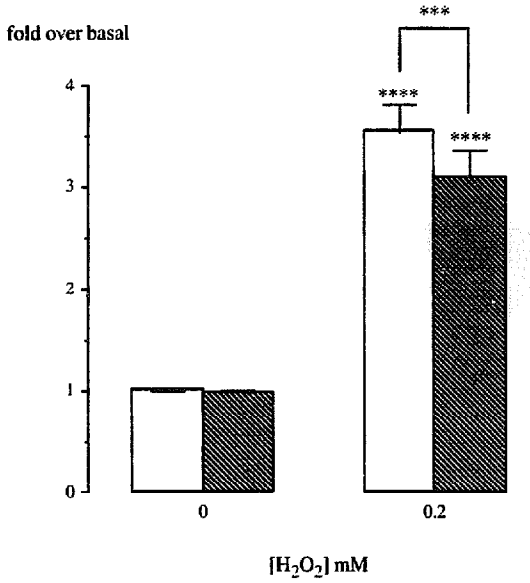


Fig. 7. The effect of a 20 min treatment with 0.2 mM $H_2O_2$ on trypan blue-labelled albumin transfer across endothelial monolayers in the absence (open columns) or presence of 100 $\mu$M glyceryl trinitrate (hatched columns). The ordinate measures mean ($\pm$S.E.M.) fold increases in transfer over basal values recorded from two experiments each in triplicate ($n = 6$). Basal albumin transfer rate was $168 \pm 26$ $\mu$g/h. ***, $P < 0.001$; ****, $P < 0.0001$, significantly different from basal unless otherwise indicated.

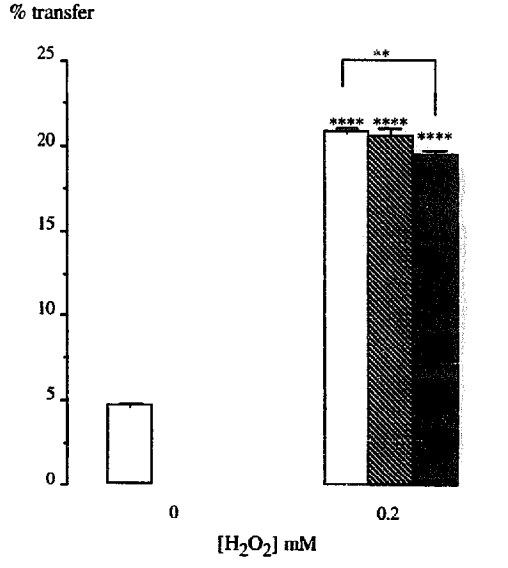


Fig. 8. The effect of a 20 min treatment with 0.2 mM $H_2O_2$ on trypan blue-labelled albumin transfer across endothelial monolayers in the absence (open columns) or presence of 1 or 10 $\mu$M sodium nitroprusside (hatched, closed columns respectively). The ordinate measures mean ($\pm$S.E.M.) percentage transfer recorded from three experiments each in triplicate ($n = 9$). Basal albumin transfer rate was $187 \pm 11$ $\mu$g/h. **, $P < 0.01$; ****, $P < 0.0001$, significantly different from basal unless otherwise indicated.

had been the case with sodium nitroprusside (Table 2). In addition neither 1, 10, or 100 μM glyceryl trinitrate altered basal albumin transfer (data not shown). However when monolayers were treated with 100 μM glyceryl trinitrate + 0.2 mM $H_2O_2$, transfer was significantly reduced, compared to that stimulated by $H_2O_2$ alone (see Fig. 7 and Section 4 below).

### 3.4. Effect of 1 or 10 μM sodium nitroprusside on $H_2O_2$-induced increases in trypan blue-labelled albumin transfer

We next investigated whether sodium nitroprusside at concentrations less than 100 μM would modulate $H_2O_2$-mediated increases in albumin transfer in a manner similar to 100 μM glyceryl trinitrate. Endothelial monolayers treated with 10 μM nitroprusside released similar amounts of nitrite into the overlying medium (4.5 ± 0.4 μM) to those released by 100 μM glyceryl trinitrate (Table 2), while nitrite release from 1 μM nitroprusside was undetectable. As had been the case with glyceryl trinitrate, 10 μM sodium nitroprusside significantly reduced trypan blue-labelled albumin transfer stimulated by 0.2 mM $H_2O_2$; however at 1 μM, nitroprusside did not alter the $H_2O_2$ effect. (See Fig. 8 and for comparison, the enhancement of the $H_2O_2$ effect by 100 μM sodium nitroprusside shown in Fig. 4).

## 4. Discussion

This study has provided evidence for the involvement of NO in modulation of endothelial barrier dysfunction caused by short term oxidant exposure. The nature of this modulation appears to depend on the amount of NO produced.

We first defined the parameters by means of which we wished to examine acute endothelial dysfunction in vitro. The model system we used, that of measuring alterations of endothelial monolayer permeability to trypan blue-labelled albumin is well established (Rotrosen and Gallin, 1986; Gudgeon and Martin, 1989). The concentration of $H_2O_2$ we chose was comparable to that previously shown to increase trypan blue-labelled albumin transfer across bovine aortic endothelial cell monolayers (Berman and Martin, 1993) and to cause oxidant damage to bovine pulmonary artery endothelial cells in culture (Li and Lau, 1993). We carried out albumin transfer experiments following a 20 min incubation of monolayers with $H_2O_2$ as we wished to investigate the effects of 'acute' oxidant exposure. The loss of barrier function we observed was not accompanied by indications of cytotoxicity, in agreement with the data presented by Li and Lau (1993) for bovine pulmonary artery endothelial cells treated with $H_2O_2$ for 1 h. In that study, only slight losses in viability (by the methylthiazol tetrazolium assay) were recorded by 1 h, with maximal effects requiring 4 h exposure; significant increases in lactate dehydrogenase release and lipid peroxidation were evident only by 2 h. In our study we did however observe morphological changes consistent with loss of barrier function (see Section 3.1) in cells treated with $H_2O_2$ for 20 min or 1 h.

Though the mechanism by which acute exposure to $H_2O_2$ increased permeability of endothelial cells to trypan blue-labelled albumin has not been addressed here, it is possible that a calcium ($Ca^{2+}$)-dependent protein kinase C may be involved, for the following reasons: (i) $H_2O_2$ has been shown to stimulate rapid (within minutes) influx and release of $Ca^{2+}$ from intracellular stores in vascular endothelium (Doan et al., 1994); (ii) protein kinase C activation has been shown to be an important determinant of increases in pulmonary microvascular endothelial permeability following exposure to $H_2O_2$ for 1 h (Siflinger-Birnboim et al., 1992); (iii) protein kinase C activation has been shown to result in phosphorylation of cytoskeletal proteins and enhanced albumin permeability of endothelial monolayers (Stasek et al., 1992). While it is attractive to speculate that $H_2O_2$ may acutely compromise barrier function in the above manner, a multifactorial role is more likely, since additional effects of short term exposure have been described in the literature. For example, it has been shown that $H_2O_2$ increases sodium-potassium pump activity (Meharg et al., 1993) and inhibits organic anion transport (Hinshaw et al., 1992) in endothelial cells. Both of these effects are associated with depletion of cellular ATP which may also significantly contribute to dysfunction and cytotoxicity.

The apparent reversibility of $H_2O_2$-induced permeability increases 48 h after the 20 min exposure may have been due to the secretion of extracellular matrix by the cells during the extra 48 h in culture. Such a matrix, which may play an important role in regulating the permeability of the vessel wall to macromolecules is reportedly laid down only after a number of days in culture (Malik et al., 1989). If such a matrix is responsible for the 'recovery' observed, then the significant enhancement of barrier function seen with untreated monolayers over the same 48 h period would be expected. The lack of recovery of monolayers treated with $H_2O_2$ for 1 h may be due to a more fundamental cytotoxic effect of the longer exposure time. The fact that cells were initially grown on filters coated with fibronectin, would not necessarily prevent the subsequent laying down of a matrix. An alternative explanation for the reversal of $H_2O_2$-induced increases in endothelial cell permeability evokes cholesterol synthesis induced by reactive oxygen species (Kozar et al., 1994) which could act to 'fill in' membrane defects. Such a mechanism is unlikely in the present study however, since Kozar and co-workers showed that bovine pulmonary artery endothelial cells needed to be exposed to $H_2O_2$ for at least 24 h for cholesterol synthesis to be significantly enhanced.

Having defined the parameters of our model of acute endothelial dysfunction, we next investigated the effects of

100 μM sodium nitroprusside and $H_2O_2$ in combination. Nitroprusside has been reported to 'spontaneously' release NO in biological systems (Feelisch and Noack, 1987; Lopez-Belmonte et al., 1993) though this is now thought to occur via a photochemical reaction (Butler et al., 1995). We first indirectly established that 100 μM sodium nitroprusside could release NO in our system by measuring nitrite release from cells incubated with sodium nitroprusside. We next showed that sodium nitroprusside alone did not affect trypan blue-labelled albumin transfer at 100 μM. Finally the combination of 100 μM sodium nitroprusside and $H_2O_2$ increased permeability to a greater extent than had been the case with $H_2O_2$ alone. We do not feel that this effect was due to cyanide release from sodium nitroprusside as equimolar potassium ferricyanide failed to alter the $H_2O_2$-induced effect.

Having observed an apparent potentiation of the $H_2O_2$ effect by 100 μM sodium nitroprusside, we next investigated the effects of another NO donor, glyceryl trinitrate on permeability. Biotransformation of glyceryl trinitrate is more tightly regulated by vascular endothelial enzymatic pathways (Feelisch and Kelm, 1991) and significantly less nitrite was released from endothelial cells treated with 100 μM glyceryl trinitrate compared to sodium nitroprusside (Table 2). Furthermore while this concentration of glyceryl trinitrate did not itself affect trypan blue-labelled albumin transfer, in contrast to 100 μM sodium nitroprusside it gave a small degree of cytoprotection against the $H_2O_2$ effect. The illustration of this cytoprotective effect in Fig. 7 requires some comment. The apparent overlap of error bars in the figure was due to a broad spread of data within each treatment group. This is because the data were derived from two sets of measurements with very different basal ($111 \pm 6$ and $225 \pm 13$ μg/h) and $H_2O_2$-stimulated ($332 \pm 20$ and $914 \pm 30$ μg/h) transfer rates. However when analysed by block design analysis of variance followed by an $F$ test, the reduction in the $H_2O_2$ effect seen with glyceryl trinitrate proved to be significant.

We also found evidence for cytoprotection with 10 μM sodium nitroprusside which (i) released similar amounts of nitrite to 100 μM glyceryl trinitrate and (ii) significantly reduced the $H_2O_2$-mediated increase in albumin transfer. At 1 μM, sodium nitroprusside neither released detectable nitrite nor affected the $H_2O_2$ response. Considering the results of experiments with sodium nitroprusside and glyceryl trinitrate together, it can be concluded that larger amounts of NO contribute to endothelial dysfunction while smaller amounts are cytoprotective. This conclusion has also been drawn from a study by Lopez-Belmonte et al. (1993) who found that NO donors could prevent or induce rat gastric mucosal injury in a manner dependent on the amount of NO to which the microvascular endothelium was exposed.

Finally the question arises as to the mechanism by which NO released from sodium nitroprusside or glyceryl trinitrate modulates the action of $H_2O_2$. In the case of 100 μM sodium nitroprusside, an interaction may occur with $H_2O_2$ to yield the potentially cytotoxic singlet oxygen species (as has been demonstrated in vitro, see Noronha-Dutra et al., 1993), though the exact nature of the injury which this species would produce can only be a matter for speculation. The likelihood that peroxynitrite is responsible for the combination effect is small, since the latter is produced by combination of NO with superoxide (Beckman et al., 1990). It is however theoretically possible that superoxide could be produced from $H_2O_2$ since a combination of phorbol ester and calcium ionophore have been shown to cause superoxide release from human endothelial cells (Matsubara and Ziff, 1986) and as mentioned above $H_2O_2$ can increase cellular $[Ca^{2+}]$ and activate protein kinase C. Finally higher amount of sodium nitroprusside and $H_2O_2$ may interact at the level of the glutathione redox cycle. This cycle is used to detoxify cells or tissues exposed to $H_2O_2$ and has been proposed to be more important than catalase in this respect (Suttorp et al., 1986). Glutathione being an antioxidant also protects against the injurious effects of large amounts of NO (Walker et al., 1995). If cells are simultaneously exposed to large amounts of $H_2O_2$ and NO, the effect may be to overwhelm the defensive capacity of the glutathione redox system resulting in increased toxicity of $H_2O_2$ and/or sodium nitroprusside.

The small amount of cytoprotection afforded by 100 μM glyceryl trinitrate and 10 μM nitroprusside in offsetting the effect of $H_2O_2$ may have been due to release of NO followed by stimulation of endothelial cGMP production. Cyclic GMP has been shown previously to reduce permeability increases stimulated (i) by agents which elevate endothelial cell $[Ca^{2+}]$ (Draijer et al., 1995) and protein kinase C activity (Buchan and Martin, 1992) or (ii) by oxidants (Lofton et al., 1991). Such a cytoprotective effect would presumably be overridden in conditions where there are larger amounts of NO in the presence of $H_2O_2$.

In summary, this study has shown that the NO donor drugs sodium nitroprusside and glyceryl trinitrate modulate pulmonary endothelial barrier dysfunction caused by acute exposure to the oxidant $H_2O_2$, in a manner consistent with the amounts of NO released by these agents. We propose that the cytotoxic or cytoprotective nature of NO's action on oxidant-injured vascular endothelium is therefore dependent on the quantity of NO released. In the clinical context low concentrations of inhaled NO may therefore serve to protect the pulmonary vascular endothelium in acute inflammatory conditions associated with the release of reactive oxygen species.

## Acknowledgements

This work was supported by the Wellcome Trust (project grant no. 36702/Z/92/Z). We also wish to acknowl-

edge Mr. Cormac O'Connell's assistance with the electron micrographs.

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