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# Self-limiting enhancement by nitric oxide of oxygen free radical-induced endothelial cell injury: evidence against the dual action of NO as hydroxyl radical donor/scavenger

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**1** The effects of oxygen free radical scavengers and endothelial cell-derived nitric oxide (EDNO) on the death of porcine cultured aortic endothelial cells exposed to exogenous superoxide- [xanthine (0.4 mM)/xanthine oxidase (0.04 unit $ml^{-1}$) + diethylenetriaminepentaacetic acid (DTPA, 10 $\mu$M)] or hydroxyl radical-generating system(s) [superoxide generating system + ferric iron ($Fe^{3+}$, 0.1 mM) or peroxynitrite (0-100 $\mu$M)] have been evaluated.

**2** Spin trapping studies using 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) with electron paramagnetic resonance spectrometry were also conducted to determine qualitatively the oxidant species generated by the oxidant generating systems.

**3** Endothelial cell injury provoked by the exogenous superoxide generating system was inhibited by catalase, DTPA and a hydroxyl radical scavenger (dimethyl sulphoxide, DMSO), but not by superoxide dismutase (SOD). Addition of $Fe^{3+}$ to the superoxide generating system enhanced the cell injury. These suggested that the direct cytotoxicity of exogenous superoxide is limited, and that endogenous transition metal-dependent hydroxyl radical formation is involved in the cell injury.

**4** An inhibitor of the constitutive NO-pathway, $N^G$-monomethyl-L-arginine, did not influence cell injury induced by the superoxide generating system, suggesting that basal NO production is not responsible for the cytotoxicity.

**5** Stimulation of endothelial cells with bradykinin enhanced cell injury provoked by the exogenous superoxide generating system, but not by the exogenous hydroxyl radical generating system. The enhancement by bradykinin was inhibited by $N^G$-monomethyl-L-arginine and bradykinin $B_2$-receptor antagonist, D-Arg-[$Hyp^3$, $Thi^{5,8}$, D-$Phe^7$] bradykinin, suggesting that an interaction of NO with superoxide is involved in the enhanced cytotoxicity. A possible intermediate of this reaction, peroxynitrite, also caused endothelial cell injury in a concentration-dependent manner.

**6** The modulatory effects of NO on hydroxyl radical-like activity (= formaldehyde production) from the superoxide generating system was also evaluated in a cell-free superoxide/NO generating system, consisting of xanthine/xanthine oxidase, DTPA, DMSO, and various amounts of a spontaneous NO generator, sodium nitroprusside (SNP) and were compared with those of $Fe^{3+}$. At doses up to 10 $\mu$M, SNP concentration-dependently increased the formaldehyde production while the higher concentrations of SNP decreased. The maximum amount of formaldehyde produced by SNP was 5 fold less than that produced by $Fe^{3+}$ (0.1 mM). Peroxynitrite-induced formaldehyde formation was concentration-dependently inhibited by SNP.

**7** We conclude that agonist-stimulated but not basal NO production acts as cytotoxic hydroxyl radical donor as well as the endogenous transition metal when endothelial cells are exposed to exogenous superoxide anion, while the modulatory effect of EDNO is limited by a secondary reaction with hydroxyl radicals.

**Keywords:** Endothelial cells; xanthine oxidase; cytotoxicity; superoxide; hydroxyl radical; nitric oxide; peroxynitrite; bradykinin; sodium nitroprusside; ferric ion; electron paramagnetic resonance spectrometry

## Introduction

Reactive oxygen metabolites have been implicated in the pathogenesis of endothelial cell injury provoked during acute lung injury (Freeman & Crapo, 1981; Ward *et al.*, 1983; Fox, 1984; Kuroda *et al.*, 1987), ischaemia/reperfusion (Zweier *et al.*, 1988; 1994a,b), or atherosclerosis (White *et al.*, 1994). Of these oxidant species, superoxide and $H_2O_2$ are produced through the electron transfer reaction of NADPH oxidase by phagocytes in a response to several stimuli, such as phorbol myristate acetate (Britigan *et al.*, 1986a,b; Pou *et al.*, 1989), opsonized zymosan (Britigan *et al.*, 1986a; 1990a; Samuni *et al.*, 1988), tumour necrosis factor, and complement (C5a) (Hardy *et al.*, 1994). Similarly, the reaction between xanthine (or hypoxanthine) and xanthine oxidase also contributes to the oxidant generation during ischaemia/reperfusion (Zweier *et al.*, 1988; 1994a,b). While the reactivity of such oxidant species with several biological molecules is limited, mechanisms generating far more toxic oxidant species, e.g. hydroxyl radicals, are likely to induce endothelial cell injury. The most recognised mechanism involved in the formation of hydroxyl radicals is the transition metal catalysed Haber-Weiss reaction (Britigan *et al.*, 1986b; 1990a,b):

$$O_2^- + Fe^{3+} \rightarrow Fe^{2+} + O_2$$

$$\underline{H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH + OH^-}$$

$$O_2^- + H_2O_2 \rightarrow OH + OH^- + O_2$$

Recently, another mechanism, that involving the interaction of superoxide with nitric oxide (NO), has also been suggested to

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form hydroxyl radicals (Saran *et al.*, 1990; Beckman *et al.*, 1990):

$$O_2^- + NO \rightarrow ONOO^-$$
$$ONOO^- + H^+ \rightarrow ONOOH \rightarrow NO_2 + OH$$

Since the intermediate of this reaction (peroxynitrite; $ONOO^-$) has a p*K*a of 7.5 at 37°C (Beckham *et al.*, 1990), approximately 50% of the compound will be protonated at physiological pH. Once protonated, the resultant peroxynitrous acid (ONOOH) is very unstable (half life <2 s; Beckman *et al.*, 1990), and decomposes by homolytic fission, giving rise to oxidant species, hydroxyl radicals (Augusto *et al.*, 1994).

Several investigators have demonstrated the formation of peroxynitrite in endothelial cells or phagocytes through such reaction (Wang *et al.*, 1991; Ischiropoulos *et al.*, 1992; Kooy & Royall, 1994; Carreras *et al.*, 1994). However, it is not yet clear whether the NO-pathway is implicated in endothelial cell injury provoked by exposure to exogenous oxygen free radicals, i.e. endothelial cell derived-NO (EDNO) acts as a potentially cytotoxic hydroxyl radical donor.

In the present study, we evaluated the modulatory effects of EDNO and the transition metal on endothelial cell injury provoked by xanthine/xanthine oxidase. The dydroxyl radical-like activities in the cell-free superoxide/NO, peroxynitrate/NO, or iron-catalysed hydroxyl radical generating system(s) were also assessed by formaldehyde production.

## Methods

### *Preparation of endothelial cells*

Isolation of endothelial cells from porcine thoracic aortae was performed as previously described (Az-ma *et al.*, 1995a). The porcine aortic endothelial (PAE) cells obtained were grown to passage 3 (split ratio, 1:3) in RD medium [1:1 (v/v) RPMI 1640 medium Dulbecco's modified Eagle's medium (DMEM)] supplemented with bicarbonate (2 mg $ml^{-1}$), HEPES (15 mM), ampicillin (90 mg $ml^{-1}$), kanamycin (90 mg $ml^{-1}$), heparin (10 $\mu$g $ml^{-1}$), and 10% (v/v) foetal bovine serum under humidified atmosphere of 5% $CO_2$/95% air at 37°C. The culture medium was replaced every day. Studies were carried out with passage 3 PAE cell monolayers grown to a density of $0.8-1.2 \times 10^5$ cells/$cm^2$ in collagen-coated 24-well multiplates at 1–2 days postconfluence.

### *Exogenous oxygen free radical(s) generating system*

Superoxide generation was achieved by adding xanthine oxidase (0.04 unit $ml^{-1}$ at final concentration) to xanthine (0.4 mM). The hydroxyl radical generating system consisted of ferric ammonium sulphate ($Fe^{3+}$, 0.1 mM) added to the above reaction mixture.

Unless otherwise noted, experiments were performed at 37°C in physiological salt solution (PSS) containing (in mM): NaCl 117, KCl 4.7, $CaCl_2$ 1.7, $MgSO_4$ 1.2, $KH_2PO_4$ 1.2, D-glucose 11, HEPES 3 and diethylenetriominepentaacetic acid (DTPA), 0.01 (pH 7.4). The PSS contained an iron chelator (DTPA) which eliminated an unexpected transition metal dependent hydroxyl radical production (Britigan *et al.*, 1990b). The minimum but effective amount of DTPA (10 $\mu$M) was determined by electron paramagnetic resonance (e.p.m.r.) spin trapping study (see below).

### *Endogenous NO generating system*

EDNO was produced by incubation of PAE cells with various amounts of bradykinin. The bradykinin concentration-dependent increase in the intracellular calcium ($EC_{50} \cong 1$ nM; $EC_{100} \cong 100$ nM) was confirmed by fura-2 fluorescence spectrometry as described previously (Az-ma *et al.*, 1995a). EDNO release from the cells was also examined by the inhibition of collagen-induced platelet aggregation as described by Az-ma *et al.* (1995b): addition of the incubation buffer from bradykinin-stimulated PAE cells to platelets caused anti-aggregation, which was inhibited by the co-treatment of the cells with $N^G$-methyl-L-arginine.

### *Assay of endothelial cell injury*

Endothelial cell injury was assessed by counting the number of viable cells in culture wells exposed to xanthine/xanthine oxidase or peroxynitrite. PAE cells were washed and pre-equilibrated with PSS containing xanthine for 10 min. Various inhibitors of oxidants/EDNO or $Fe^{3+}$ were co-incubated with xanthine 10 min prior to the addition of xanthine oxidase, while bradykinin was loaded just before the addition of xanthine oxidase. When peroxynitrite was used, the cells were pre-incubated with catalase (500 unit $ml^{-1}$) and DTPA (1.0 mM) for 10 min to eliminate contamination with $H_2O_2$ by the peroxynitrite synthesis. Following 15 min incubation with xanthine oxidase or peroxynitrite at 37°C, PAE cell monolayers were gently washed three times with the culture medium to remove xanthine/xanthine oxidase and the incubation continued for 30 min. The adherent cells were then dispersed with trypsin/EDTA (0.125%/0.04%) in Dulbecco's phosphate buffered saline. The cells were stained for 2 min with 0.02% trypan blue to determine cell viability by the exclusion of the stain, and the number of viable cells was counted using a haemocytometer. Endothelial cell injury was assessed according to the following equation:

$$\text{endothelial cell injury(\%)} = \frac{(\text{total cell count} - \text{survival cell count}) \times 100}{\text{total cell count}}$$

where the total cell count represented the number of viable cells in control culture wells without exposure to xanthine/xanthine oxidase, and the survival cell count represented that of culture wells exposed to the oxidant generating system in the presence or absence of various agents.

### *Spin trapping*

The incubation buffers of PAE cell monolayers containing a spin trap, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO, 90 mM) and dimethylsulphoxide (DMSO, 0.14 M) were aspirated into a glass capillary tube [1.0 mm (i.d.) × 75 mm length] at pre-selected incubation periods. The tube was immediately inserted into a narrow quartz cell which was in turn fitted into the cavity of an e.p.m.r. spectrometer (JEOL JES-FE1X, Tokyo, Japan) and the e.p.m.r. spectra were recorded. Unless otherwise noted, e.p.m.r. spectra were obtained at 25°C, under the following spectrometer settings: microwave power, 10 mW; modulation frequency, 100 kHz; modulation amplitude, 1 G; response time, 0.3 s; sweep rate, 50 G $min^{-1}$; and apparatus gain, $2 \times 10^3$.

### *Influence of NO and ferric salt on hydroxyl radical generation in cell-free system*

To compare the influence of NO on hydroxyl radical formation from the mixture of xanthine/xanthine oxidase, with that of $Fe^{3+}$, we used photolysis of sodium nitroprusside (SNP) as a NO generator (Butler & Glidewell, 1987; Joseph *et al.*, 1993). Various concentrations of SNP or $Fe^{3+}$ were added to 100 mM sodium phosphate buffer (pH 7.4) containing xanthine (0.4 mM), DTPA (10 $\mu$M–1.0 mM) and DMSO (0.14 M). Superoxide generation commenced by the addition of xanthine oxidase (0.04 unit $ml^{-1}$), followed by incubation for 15 min at 25°C. The enzyme reaction was terminated by the addition of 0.76 M trichloroacetic acid to the reaction mixture [2:3 (v/v)]. Non-enzymatic production of hydroxyl radical was achieved by adding peroxynitrite to phosphate buffer, containing catalase (500 units $ml^{-1}$),

DTPA (1.0 mM), DMSO (0.14 M) and various concentrations of SNP at 25°C.

The hydroxyl radical-like activity was assessed by formaldehyde production (Beckman *et al.*, 1990), measured by the Nash reaction and absorbance reading at 412 nm (Werringloer, 1987). Briefly, Nash solution, containing ammonium acetate (6 M), acetylacetone (60 mM) and acetic acid (0.15 M) in distilled water, was added to the sample material [1:2 (v/v)], followed by incubation in a 60°C water bath for 10 min. The absorbance at 412 nm above background was fitted to a calibration curve obtained from formaldehyde standard solution, and the concentration of formaldehyde was determined.

### *Determination of NO in the cell-free system*

The concentration of NO generated from SNP solution was determined using a NO trap 2-phenyl-4,4,5,5-tetramethylimidazolene-1-oxyl 3-oxide (PTIO). PTIO reacts with NO according to the following equation (Akaike *et al.*, 1993):

$$PTIO + NO \rightarrow PTI + NO_2$$

Therefore, PTIO (10–500 $\mu$M) was added to the NO generator and the PTI yielding rate in 5–60 s was measured by the e.p.m.r. spectrometer. The concentration of NO was calculated from the reaction rate constant of NO and PTIO ($5.15 \times 10^3$ $M^{-1}$ $s^{-1}$). The hyperfine splitting constant(s) for PTIO was $a_N^{1,3} = 8.2$ G and for PTI were $a_N^1 = 9.8$ G and $a_N^3 = 4.4$ G, respectively. The e.p.m.r. spectra of the reaction mixtures were recorded using the same spectrometer setting described for spin trapping study, except for a microwave power of 5 mW.

### *Pre-formed DMPO-OH and DMPO-$CH_3$*

DMPO-OH was generated by irradiating a glass test tube, containing 1.0 ml solution of DMPO (90 mM) and $H_2O_2$ (0.3%) in 100 mM sodium phosphate buffer (pH 7.4), placed 15 cm from an ultraviolet (u.v.) light source (15 W) for 15 min. For DMPO-$CH_3$, DMSO (0.14 M) was added to the reaction mixture prior to the u.v. irradiation.

### *Reagents*

DMEM, RPMI 1640, HEPES, ampicillin, kanamycin, DTPA, heparin, trypsin, superoxide dismutase (SOD), catalase, $N^G$-methyl-L-arginine, bradykinin, and SNP were purchased from Sigma (St. Louis, MO, U.S.A.). Des-Arg$^9$-[Leu$^8$] bradykinin and D-Arg-[Hyp$^3$, Thi$^{5,8}$, D-Phe$^7$] bradykinin were obtained from Peptide Institute (Osaka, Japan); high purity DMPO from Mitsui toatsu (Tokyo, Japan); PTIO from Wako pure chemical (Osaka); NO gas from Nippon sanso (Tochigi, Japan); and foetal bovine serum from JRH Biosciences (Lenaxa, KS, U.S.A.). DMSO, ferric ammonium sulphate, and disodium EDTA were purchased from Katayama (Osaka). All other chemicals were of analytical quality.

Peroxynitrite was synthesized in a quenched flow reactor according to the method described by Beckman *et al.* (1990), and stocked at −20°C. Peroxynitrite at desired concentrations was prepared by diluting in 0.1 N NaOH just before use, and the concentration was determined by absorbance reading at 302 nm ($\varepsilon = 1670$ $M^{-1}$ $cm^{-1}$). NO was prepared as a saturated solution according to the method described previously (Az-ma *et al.*, 1994).

### *Statistical analysis*

Data are expressed as mean ± s.e.mean. Multiple comparisons were performed by analysis of variance followed by the Dunn's test ($P < 0.05$).

## Results

### *Qualitative detection of oxidant species in the free radical generating systems*

The supernatant of PAE cells incubated with PSS containing xanthine (0.4 mM)/xanthine oxidase (0.04 unit $ml^{-1}$), DMPO (90 mM) and DMSO (0.14 M) was placed in the cavity of an e.p.m.r. spectrometer and scanned for the spectra (Figure 1a). The predominant spin adduct species was the superoxide adduct (DMPO-OOH; $a_N = 14.3$ G, $a_H = 11.7$ G, $a_H^g = 1.3$ G), accompanied by a small amount of hydroxyl adduct (DMPO-OH; $a_{N,H} = 14.9$ G) (Britigan *et al.*, 1986a; 1990b; Samuni *et al.*, 1988). The hydroxyl adduct, DMPO-OH, is known to be generated by the rapid decomposition of DMPO-OOH, suggesting that the detection of this spin adduct did not confirm hydroxyl radical generation (Britigan *et al.*, 1986a). Indeed, SOD (15–300 units $ml^{-1}$) caused a complete inhibition of spin trapping for at least 30 min (Figure 1b), while catalase (15–300 units $ml^{-1}$) did not block the generation of DMPO-OOH and -OH (Figure 1c), suggesting that xanthine/xanthine oxidase yielded superoxide without a significant hydroxyl radical production.

Ferric ammonium sulphate ($Fe^{3+}$, 0.1 mM) was added to the above superoxide generating system to produce hydroxyl radicals. Figure 2a shows e.p.m.r. spectra obtained from the supernatant of PAE cells incubated with xanthine/xanthine oxidase + $Fe^{3+}$. The methyl radical adduct (DMPO-$CH_3$; $a_N = 15.3$ G, $a_H = 22.0$ G), representing a direct evidence against hydroxyl radical formation, increased with time. In contrast to the result obtained from the superoxide generating system, SOD (15–300 units $ml^{-1}$) enhanced the production of

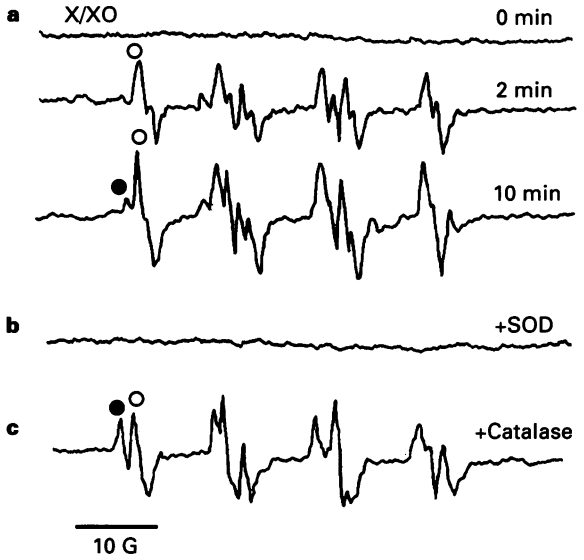


**Figure 1** (a) E.p.m.r. spectra obtained from the supernatant of endothelial cell monolayers incubated with xanthine (0.4 mM)/xanthine oxidase (0.04 units $ml^{-1}$) (X/XO), DMPO (90 mM) and DMSO (0.14 M) at 37°C. Note that the incubation buffer also contained 10 $\mu$M DTPA. DMPO-OOH accompanied by small amounts of -OH increased with time. (○) First low field peak of DMPO-OOH; (●) first low field peak of DMPO-OH. (b) E.p.m.r. spectra obtained 10 min after the addition of xanthine oxidase under the same conditions as in (a) except that the cells were pretreated with SOD (15 units $ml^{-1}$). No significant e.p.m.r. spectra were obtained. (c) E.p.m.r. spectra obtained 10 min after the addition of xanthine oxidase under the same conditions as in (a) except that the cells were pretreated with catalase (15 units $ml^{-1}$). Catalase did not change DMPO-OOH production. Experimental conditions: microwave power, 10 mW; modulation frequency, 100 kHz; modulation amplitude, 1 G; response time, 0.3 s; sweep rate, 50 G $min^{-1}$; and apparatus gain, $2 \times 10^3$. Spectra shown are representative of 3–5 separate experiments.

DMPO-$CH_3$ (Figure 2b), while catalase (15–300 units $ml^{-1}$) markedly decreased the signal intensity of DMPO-$CH_3$ (Figure 2c). In the presence of DTPA (1.0 mM), the predominant species was DMPO-OOH together with a small amount of DMPO-OH, while the generation of DMPO-$CH_3$ was blocked (Figure 2d). Since SOD enhances the conversion of superoxide to $H_2O_2$ while the latter is the substrate for the iron-catalysed hydroxyl radical formation, it is likely that SOD enhances hydroxyl radical production in the presence of $Fe^{3+}$.

### *Effects of oxygen free radicals and various inhibitors on endothelial cell injury*

Endothelial cell injury provoked by exogenous oxygen free radical generating system(s) was assessed by the viable cell counts (Table 1). Incubation of PAE cells with xanthine did not cause cell injury for up to 60 min, while 15 min incubation in the presence of xanthine oxidase caused a significant cell injury.

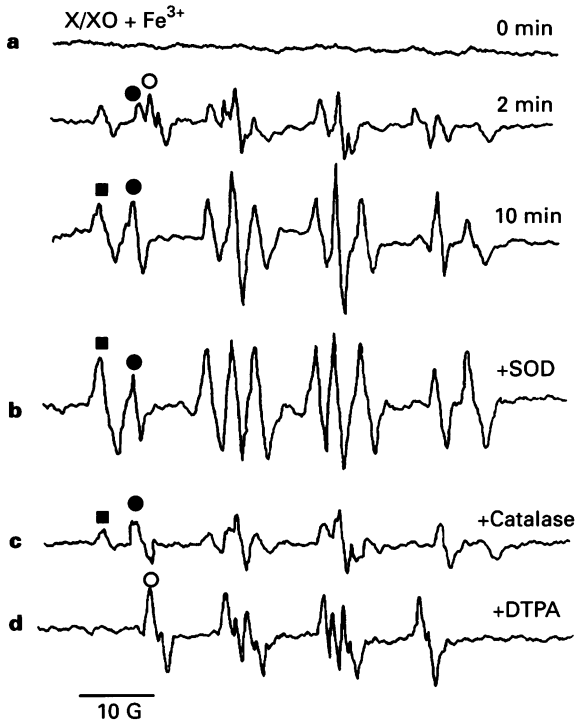


**Figure 2** (a) E.p.m.r. spectra obtained from the supernatant of endothelial cell monolayers incubated with xanthine (0.4 mM)/xanthine oxidase (0.04 units $ml^{-1}$) (X/XO), DMPO (90 mM), DMSO (0.14 M), and ferric ammonium sulphate ($Fe^{3+}$, 0.1 mM) at 37°C. Note that the incubation buffer also contained 10 $\mu$M DTPA. DMPO-$CH_3$ accompanied by -OH increased with time. (○) First low field peak of DMPO-OOH; (●) first low field peak of DMPO-OH; (■) first low field peak of DMPO-$CH_3$. (b) E.p.m.r. spectra obtained 10 min after the addition of xanthine oxidase under the same conditions as in (a) except that the cells were pretreated with SOD (15 units $ml^{-1}$). SOD increased the signal intensity of DMPO-$CH_3$. (c) E.p.m.r. spectra obtained 10 min after the addition of xanthine oxidase under the same conditions as in (a) except that the cells were pretreated with catalase (15 units $ml^{-1}$). Catalase markedly decreased the signal intensity of DMPO-OH and -$CH_3$. (d) E.p.m.r. spectra obtained 10 min after the addition of xanthine oxidase under the same conditions as in (a) except that the amount of DTPA was increased to 1.0 mM. DMPO-$CH_3$ was decreased behind the background noise level, while -OOH was generated as a predominant spin adduct accompanied by -OH. Experimental conditions: microwave power, 10 mW; modulation frequency, 100 kHz; modulation amplitude, 1 G; response time, 0.3 s; sweep rate, 50 G $min^{-1}$; and apparatus gain, $2 \times 10^3$. Spectra shown are representative of 3–5 separate experiments.

**Table 1** Effects of ferric salt and various oxidants inhibitors on endothelial cell injury provoked by exposure to xanthine/xanthine oxidase

| | *Endothelial cell injury (%)* | |
|---|---|---|
| Xanthine (0.4 mM) | 1% | |
| + xanthine oxidase (0.04 units $ml^{-1}$) | 52% | (±6.0)* |
| + SOD (150 units $ml^{-1}$) | 60% | (±6.2) |
| (300 units $ml^{-1}$) | 65% | (±4.4) |
| + Catalase (150 units $ml^{-1}$) | 20% | (± 12)** |
| (300 units $ml^{-1}$) | 9% | (±6.9)** |
| + DTPA (0.5 mM) | 34% | (±3.5)** |
| (1.0 mM) | 43% | (±3.1)** |
| + DMSO (0.07 M) | 33% | (±0.8)** |
| (0.14 M) | 30% | (±2.9)** |
| + $Fe^{3+}$ (0.1 mM) | 63% | (±3.8)** |

Monolayers of endothelial cells were incubated with 0.4 mM xanthine for 10 min, followed by another 15 min incubation with 0.04 units $ml^{-1}$ xanthine oxidase at 37°C. Data represent mean ± s.e.mean of 4–7 separate experiments; *Significant difference from results without xanthine oxidase; **Significant difference from results without inhibitors.

In the next step we evaluated the effects of various oxidants inhibitors on endothelial cell injury. SOD (up to 300 units $ml^{-1}$) did not inhibit cell injury although the predominant exogenous oxygen free radical species yielded by xanthine/xanthine oxidase was superoxide as validated by the spin trapping study. In contrast, a marked inhibition of endothelial cell injury was observed following the addition of catalase (150, 300 units $ml^{-1}$), which is the inhibitor of another oxidant species, $H_2O_2$, yielded by xanthine/xanthine oxidase. Treatment of cells with an iron chelator (DTPA; 0.5, 1.0 mM) or a hydroxyl radical scavenger (DMSO; 0.07, 0.14 M) also decreased cell injury. On the other hand, the addition of $Fe^{3+}$ enhanced endothelial cell injury, while the modulatory effects of inhibitors on cell injury were also similar to those without $Fe^{3+}$ (data not shown). These results suggested that the endogenous transition metal-dependent hydroxyl radical formation was involved in the endothelial cell injury provoked by exogenous superoxide generating system.

### *Modulatory effects of basal and bradykinin-enhanced NO production on endothelial cell injury provoked by exposure to oxygen free radicals*

To investigate the influence of EDNO on the cytotoxicity provoked by the exogenous superoxide generating system, PAE cells were loaded with xanthine and bradykinin ± $N^G$-monomethyl-L-arginine prior to the addition of xanthine oxidase. Priming of PAE cells with bradykinin (100 nM) produced a slight but significant enhancement of cell injury, while pre-incubation of the cells with $N^G$-monomethyl-L-arginine blocked the effect of bradykinin. Bradykinin-dependent enhancement of cell injury was also inhibited by pre-incubation with bradykinin $B_2$ receptor antagonist (D-Arg-[Hyp$^3$, Thi$^{5,8}$, D-Phe$^7$] bradykinin), but not with $B_1$ receptor antagonist (Des-Arg$^9$-[Leu$^8$] bradykinin) (Figure 3). In contrast, endothelial cell injury provoked by exposure to the exogenous hydroxyl radical generating system (xanthine/xanthine oxidase with $Fe^{3+}$) was not enhanced by bradykinin priming of the cells (Figure 4). Because a single in-

cubation of endothelial cells with up to 100 nM bradykinin did not cause cell injury (data not shown), it is likely that the enhanced cell injury was dependent on the interaction of NO with superoxide to yield peroxynitrate.

We also evaluated the effect of peroxynitrate on endothelial cell injury. The addition of peroxynitrite to PAE cells caused a concentration-dependent cell injury (Table 2). The peroxynitrite-induced endothelial cell injury was potently blocked by DMSO (0.14 M). DMPO-OH and -$CH_3$ were observed from the reaction mixture of peroxynitrite (10 $\mu$M), catalase (500 units $ml^{-1}$), DTPA (1.0 mM), DMSO (0.14 M), and DMPO (90 mM) by spin trapping study (data not shown; and Augusto *et al.*, 1994). These results indicated that peroxynitrite produced hydroxyl radicals.

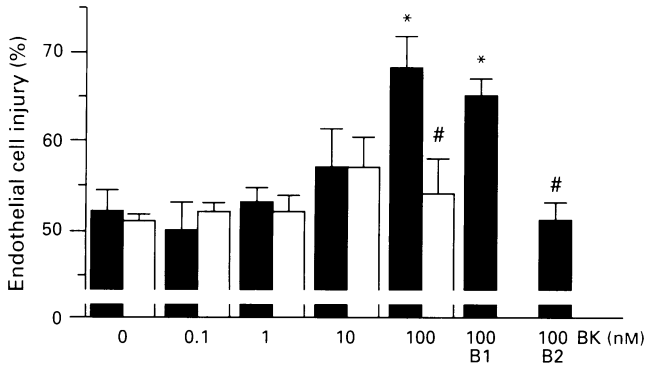


**Figure 3** Effects of bradykinin (BK) and/or various inhibitors of NO-pathway on endothelial cell injury provoked by the exogenous superoxide generating system. Monolayers of endothelial cells were incubated at 37°C with xanthine (0.4 mM), DTPA (10 $\mu$M) and bradykinin (0–100 nM) in the presence (open columns) or absence (closed columns) of $N^G$-monomethyl-L-arginine, Des-Arg$^9$-[Leu$^8$] bradykinin (bradykinin $B_1$ receptor antagonist, $B_1$), or D-Arg-[Hyp$^3$, Thi$^{5,8}$, D-Phe$^7$] bradykinin ($B_2$ receptor antagonist, $B_2$), followed by the addition of xanthine oxidase (0.04 units $ml^{-1}$). Endothelial cell injury was assayed 15 min after the addition of xanthine oxidase. Bradykinin (100 nM) enhanced cell injury provoked by xanthine/xanthine oxidase, while $N^G$-monomethyl-L-arginine and the $B_2$ receptor antagonist blocked this effect. *Significant difference from without bradykinin; #Significant difference from 100 nM bradykinin. Data represent mean±s.e.mean of 6 separate experiments.

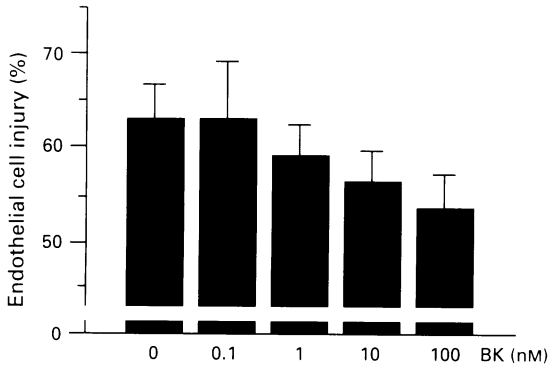


**Figure 4** Effect of bradykinin (BK) on endothelial cell injury provoked by the exogenous hydroxyl radical generating system. Monolayers of endothelial cells were incubated at 37°C with xanthine (0.4 mM), DTPA (10 $\mu$M), bradykinin (0–100 nM) in the presence of ferric ammonium sulphate ($Fe^{3+}$, 0.1 mM), followed by the addition of xanthine oxidase (0.04 units $ml^{-1}$). Endothelial cell injury was assayed 15 min after the addition of xanthine oxidase. Bradykinin did not significantly influence cell injury provoked by xanthine/xanthine oxidase with $Fe^{3+}$. Data represent mean±s.e.mean of 6 separate experiments.

**Table 2** Endothelial cell injury provoked by exposure to peroxynitrite anion

| | *Endothelial cell injury (%)* | |
|---|---|---|
| *Peroxynitrite* | | |
| 0 $\mu$M | 1% | (±0.9) |
| 10 $\mu$M | 20% | (±4.6) |
| 30 $\mu$M | 31% | (±6.6) |
| 50 $\mu$M | 63% | (±18) |
| 70 $\mu$M | 58% | (±15) |
| 100 $\mu$M | 63% | (±7.7) |
| *Peroxynitrite (30 $\mu$M) + DMSO* | | |
| (0.07 M) | 10% | (±1.4) |
| (0.14 M) | 0% | (±4.9)* |

Various amounts of peroxynitrite were added to monolayers of endothelial cells containing catalase (500 units $ml^{-1}$) and DTPA (1.0 mM), followed by 15 min incubation at 37°C. Peroxynitrite increased endothelial cell injury in a concentration-dependent manner, while a hydroxyl radical scavenger (DMSO) blocked this effect. Data represent the mean±s.e.mean of 3–4 experiments; *Significant difference from 30 $\mu$M peroxynitrite.

### *Hydroxyl radical-like activity in concomitant superoxide/NO generating system*

The possible role of NO in modulating oxidant-induced endothelial cell injury was extensively investigated using a cell-free superoxide/NO generating system, and was compared with that of the Haber-Weiss reaction. SNP is known to form NO photolytically even at room light intensity (Butler & Glidewell, 1987). The NO yeilding rate from SNP was constant at least for 15 min (data not shown). A linear relationship between the concentration of SNP and NO was observed ($r^2=0.981$, $n=14$, inset of Figure 5a) although the concentrations of NO generated at SNP levels less than $10^{-4}$ M were below the detection limit. Thus, xanthine oxidase was added to a solution containing xanthine in the presence of SNP/DTPA or $Fe^{3+}$/DTPA. The maximum level of formaldehyde production was observed at $10^{-5}$–$10^{-4}$ M SNP, which was 5 fold less than that by 0.1 mM $Fe^{3+}$ (Figure 5a,b). Interestingly, higher concentrations of SNP reduced the production of formaldehyde (Figure 5a).

The effect of NO on peroxynitrite induced non-enzymatic yielding of hydroxyl radical-like activity was further evaluated. Peroxynitrite was added to a solution containing DTPA (1.0 mM), catalase (500 units $ml^{-1}$) and various amounts of SNP (Figure 6). SNP caused a concentration-dependent inhibition of hydroxyl radical-like activity. The u.v. irradiation of the reaction mixture, conducted to increase NO generation from SNP, enhanced the inhibitory effect of SNP on formaldehyde formation. These results suggested that NO acts as a hydroxyl radical scavenger, as well as a hydroxyl radical donor.

### *Skin trapping of oxygen free radicals in the presence of NO*

Our attempt to obtain direct spin trap evidence for NO-mediated hydroxyl radical generation in endothelial cells exposed to superoxide generating system was, unfortunately, not successful due to the reasons discussed below. The supernatant of PAE cells incubated with xanthine (0.4 mM)/xanthine oxidase (0.04 units $ml^{-1}$), bradykinin (100 nM), DMPO (90 mM), and DMSO (0.14 M) was scanned for e.p.m.r. spectra. The obtained spin adduct was DMPO-OOH, while DMPO-OH and -$CH_3$ were not observed (Figure 7a). However, we confirmed that pre-formed DMPO-OH was decomposed by incubation of endothelial cells with bradykinin (Figure 7b).

Furthermore, both SNP (1.0 mM) and authentic NO (40 $\mu$M) decreased the amount of pre-formed DMPO-OH (Figure 7c,d) and DMPO-$CH_3$ (data not shown) below the background noise, suggesting that such spin adducts were rapidly decomposed by NO.

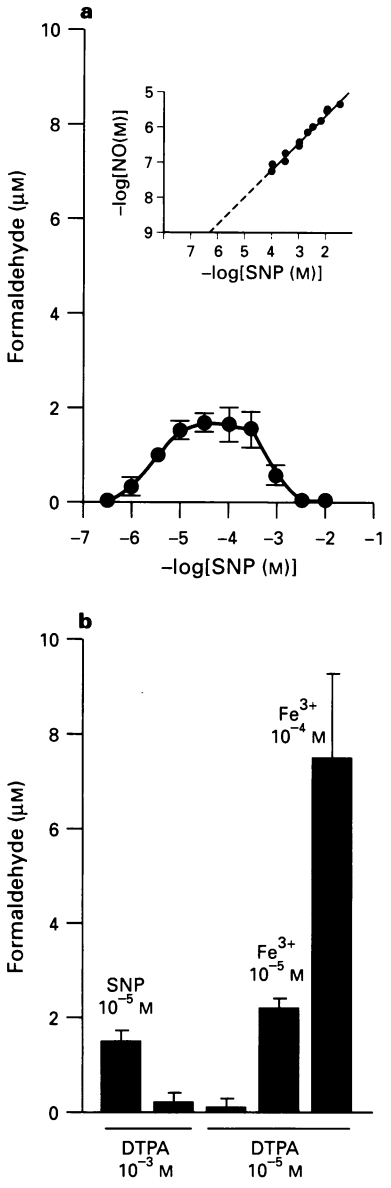


**Figure 5** (a) Modulatory effect of sodium nitroprusside (SNP) on hydroxyl radical-like activity (formaldehyde production) produced by xanthine/xanthine oxidase. Xanthine oxidase (0.04 units $ml^{-1}$) was incubated with xanthine (0.4 mM), DTPA (1.0 mM), DMSO (0.14 M), and various amounts of SNP at 25°C. The enzyme reaction was terminated 15 min after the commencement of the reaction by the addition of 0.76 M trichloroacetic acid to the reaction mixture [2:3 (v/v)]. The concentration of formaldehyde was determined by the Nash reaction. Data represent mean ± s.e.mean ($n=4$). (inset of a) Concentrations of NO generated from SNP. The concentration of NO was determined by the amount of PTI produced through the reaction of NO with PTIO (10–500 $\mu$M). A linear relationship between the concentration of SNP and NO was observed ($r^2=0.981$, $n=14$). Note that the concentration of NO yielded from less than $10^{-4}$ M SNP was below the detection limit of the assay. (b) Hydroxyl radical-like activity produced by xanthine/xanthine oxidase in the presence or absence of SNP ($10^{-5}$ M)/DTPA ($10^{-3}$ M) or ferric ammonium sulphate ($10^{-5}$, $10^{-4}$ M)/DTPA ($10^{-5}$ M) ($n=4$). The production of formaldehyde in the presence of $Fe^{3+}$ ($10^{-4}$ M) was 5 fold higher than that in the presence of SNP ($10^{-5}$ M).

## Discussion

The reaction of xanthine/xanthine oxidase provides a simple and reliable tool to investigate the effects of controlled fluxes of superoxide and $H_2O_2$. However, the iron content of commercially available xanthine oxidase preparations and the well recognised problem of adventitious iron contamination in experimental systems may induce unexpected transition metal-dependent hydroxyl radical production from such a superoxide generating system (Britigan *et al.*, 1990b). Therefore, we carefully determined the experimental settings for exogenous superoxide/hydroxyl radical generating systems through qualitative assessments by spin trapping studies (Figures 1 and 2).

### *Influence of endogenous transition metal on endothelial cell injury*

Our results indicated initially that the intracellular transition metal-catalysed hydroxyl radical formation is implicated in the underlying mechanism for the cytotoxicity provoked by exogenous superoxide (and $H_2O_2$) generating system (Table 1). This conclusion is based on the following observations. (1) Both a transition metal chelator (DTPA) and a hydroxyl radical scavenger (DMSO) inhibited endothelial cell injury provoked by exogenous xanthine/xanthine oxidase. (2) Catalase, which decreases the substrate of transition metal-catalyzed hydroxyl radical yielding reaction ($=H_2O_2$), markedly blocked cell injury. (3) Finally, addition of $Fe^{3+}$ to the superoxide generating system enhanced cell injury, confirming that the hydroxyl radical is a more toxic oxidant species than superoxide or $H_2O_2$, generated directly from xanthine/xanthine oxidase.

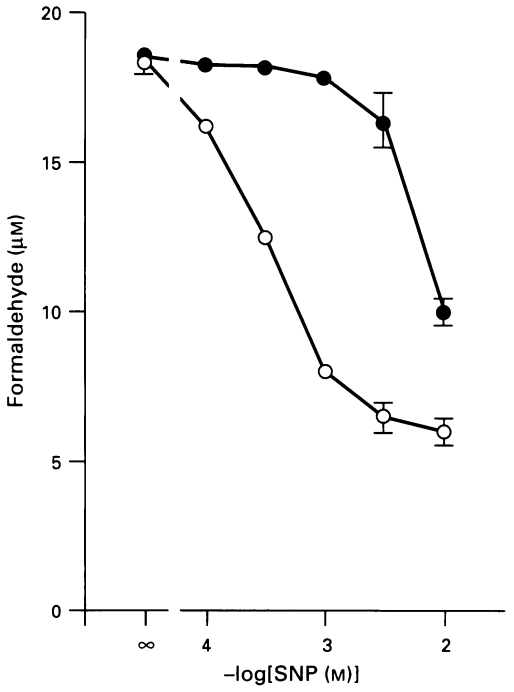


**Figure 6** Inhibition of peroxynitrite-induced hydroxyl radical-like activity (formaldehyde production) by sodium nitroprusside (SNP). Peroxynitrite (150 $\mu$M) was added to catalase (500 units $ml^{-1}$), DTPA (1.0 mM), DMSO (0.14 M), and various amounts of SNP. After 15 min incubation period, the concentration of formaldehyde was determined by the Nash reaction. SNP inhibited formaldehyde formation from peroxynitrite in a concentration-dependent manner (●). Ultraviolet-irradiation of the reaction mixture during the incubation period enhanced the effect of SNP (○). Data represent mean ± s.e.mean ($n=3$).

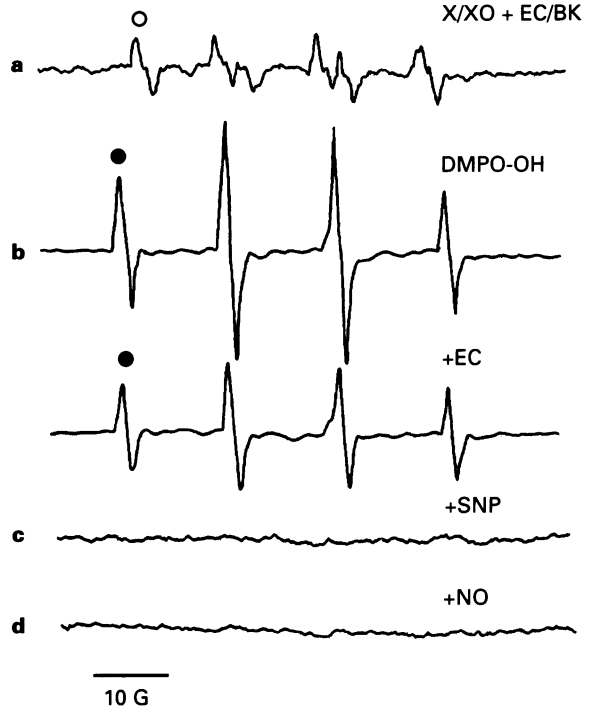


**Figure 7** (a) E.p.m.r. spectra obtained from the supernatant of endothelial cell monolayers (EC) incubated with xanthine (0.4 mM)/xanthine oxidase (0.04 units $ml^{-1}$) (X/XO), bradykinin (BK 100 nM), DTPA (10 $\mu$M), DMPO (90 mM), and DMSO (0.14 M) at 37°C. At 10 min after the addition of xanthine oxidase, the supernatant was scanned for the spectra. DMPO-OOH, but not -OH or $-CH_3$ was observed. (○) First low field peak of DMPO-OOH. (b) E.p.m.r. spectra of pre-formed DMPO-OH in the absence (top scan) or presence of co-incubation with 100 nM bradykinin-treated endothelial cells for 15 min (second scan). Bradykinin-treated endothelial cells reduced the signal intensity of pre-formed DMPO-OH. (●) First low field peak of DMPO-OH. (c) E.p.m.r. spectra obtained from the reaction mixture of SNP (1.0 mM) and pre-formed DMPO-OH at the same amount as in (b). E.p.m.r. spectra of pre-formed DMPO-OH was reduced below the background noise level. (d) Addition of authentic NO (40 $\mu$M) caused similar results as with SNP. Experimental conditions: microwave power, 10 mW; modulation frequency, 100 kHz; modulation amplitude, 1 G; response time, 0.3 s; sweep rate, 50 G $min^{-1}$; and apparatus gain, $2 \times 10^3$ for (a, c, d) and $2 \times 10^2$ for (b). Spectra shown are representative of 3–5 separate experiments.

The direct cytotoxicity of superoxide was also indicated from the significant, but incomplete effects of these inhibitors on the xanthine/xanthine oxidase-induced cell injury because superoxide generation was not inhibited by the above inhibitors (Figures 1 and 2). On the other hand, SOD failed to inhibit the cell injury (Table 1) in contrast to the spin trapping evidence indicating the significant inhibitory effects of SOD on superoxide generation (Figure 1). If the transition metal exists, however, SOD increases the hydroxyl radical formation through the enhanced generation of $H_2O_2$, as confirmed by our spin trapping study (Figure 2) and early observations by other investigators (Britigan *et al.*, 1986a). It is likely therefore that superoxide can be directly toxic although its oxidant reactivity is limited and that endothelial cell injury provoked by the exogenous superoxide generating system was not inhibited by SOD in the presence of endogenous transition metals.

### *Involvement of peroxynitrite-mediated hydroxyl radical formation on endothelial cell injury*

We tested the influence of EDNO on endothelial cell injury provoked by the exogenous superoxide generating system. NO is formed through the oxidative deamination of L-arginine by a calcium/calmodulin-dependent NADPH oxidase (constitutive-NO synthase; Pollock *et al.*, 1991), which is enchanced by several stimuli (e.g., acetylcholine, bradykinin, A23187, or shear stress) through calcium signalling (for review, see Moncada *et al.*, 1991). Thus, we examined the influence of bradykinin on endothelial cell injury exposed to the superoxide generating system in the presence or absence of NO-pathway antagonists (Figure 3). In the absence of bradykinin, $N^G$-monomethyl-L-arginine did not influence cytotoxicity, suggesting that basal NO production from endothelial cells is not responsible for endothelial cell injury. In contrast, stimulation of endothelial cells with bradykinin increased cell injury provoked by exogenous superoxide generating system. This enhancement of cell injury was inhibited by $N^G$-monomethyl-L-arginine and bradykinin $B_2$-receptor antagonist, D-Arg-[Hyp$^3$, Thi$^{5,8}$, D-Phe$^7$] bradykinin. We have previously reported that PAE cells are stimulated by bradykinin through the $B_2$-receptor (Az-ma *et al.*, 1995a), thus suggesting that the agonist-enhanced EDNO production was responsible for cytotoxicity when cells were exposed to exogenous superoxide.

### *Limitation of NO enhancement of endothelial cell injury exposed to exogenous superoxide generating system*

As indicated in the present study, NO released from agonist-stimulated endothelial cells may be the critical target of exogenous superoxide anion to enhance cell injury. This is partly supported by our observation that peroxynitrite, a possible intermediate of the reaction between superoxide/NO, caused endothelial cell injury in a concentration-dependent manner (Table 2), and by others demonstrating the peroxynitrite-related chemiluminescence detection from agonist(s)-stimulated endothelial cells (Kooy & Royall, 1994).

However, the influence of NO-pathway agonist and/or inhibitors of endothelial cell injury provoked by exogenous superoxide anion appeared not to be potent as compared with that of catalase, a substrate scavenger for the Haber-Weiss reaction (Figure 5 and Table 1). We also confirmed that the maximum hydroxyl radical-like activity yielded by the superoxide/NO interaction is smaller than that by the transition metal catalysed reaction (Figure 5).

These findings may be explained by the other observations that at least $10^{-7}-10^{-6}$ M or higher concentrations of NO decreased the hydroxyl radical-like activity of superoxide/NO generating system (Figure 5a) or peroxynitrite (Figure 6), implicating the secondary reaction of NO with the hydroxyl radical (or peroxynitrite) to scavenge cytotoxicities induced by the latter. Because agonist-stimulated endothelial cells have been reported to release NO maximally at the range close to $10^{-7}-10^{-6}$ M (Malinski & Taha, 1992; Malinski *et al.*, 1993), it is likely that the cytotoxicity induced by the reaction of NO with superoxide to form hydroxyl radical-like activity is limited.

In summary, we conclude that agonist-stimulated but not basal NO production as well as the intracellular transition metal acts as a cytotoxic hydroxyl radical donor when endothelial cells are exposed to the exogenous superoxide anion, while the modulatory effect of NO is limited due to its secondary reaction with hydroxyl radicals.

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