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Reaction of nitric oxide with hydrogen peroxide to produce potentially cytotoxic singlet oxygen as a model for nitric oxide-mediated killing

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Nitric oxide, as well as being a major regulator of vascular reactivity, has been shown to be one of the mediators of cytotoxicity in macrophages. This cytotoxic effect seems to be due to the interaction between nitric oxide and oxygen-related free radicals. This study shows that, in vitro, nitric oxide reacts with hydrogen peroxide to release large amounts of chemiluminescence with the characteristics of the highly cytotoxic species, singlet oxygen. This is supported by the observation that when nitric oxide was added to a superoxide generating system, catalase inhibited the production of singlet oxygen while superoxide dismutase enhanced it.

Nitric oxide; Hydrogen peroxide; Singlet oxygen; Superoxide; Cytotoxicity

1. INTRODUCTION

Nitric oxide (NO) is the major determinant of vascular tone [1,2]. It is constitutively produced by endothelial cells, catalysed by the calcium-dependent enzyme, NO synthase. In many cells there is also an inducible enzyme for NO production which is calcium independent [3]. NO production via the inducible pathway has been shown to be one of the mediators of cytotoxicity in macrophages activated by microorganisms in vivo or by cytokinins in vitro [4–6].

It has been suggested that NO may exert its cytotoxic function directly through the inhibition of mitochondrial respiration and inhibition of the citric acid cycle [7], or indirectly by reacting with superoxide. The latter view is supported by Beckman et al. [8] who have suggested that the mechanism for such cytotoxicity might be the production of peroxynitrite from the reaction between superoxide and NO. Peroxynitrite decomposition generates the hydroxyl radical, a strong oxidant. It was also hypothesized that this mechanism may play a role in ischemia-reperfusion injury.

We have studied the interactions between NO and superoxide, using chemiluminescence to identify the presence of oxygen-based free radicals, and, as a result wish to suggest an alternative: that NO reacts with hydrogen peroxide to produce singlet oxygen, a highly reactive form of oxygen.

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2. EXPERIMENTAL

Saturated NO solutions were prepared by bubbling 20 ml of argon-deoxygenated water with NO gas (BDH). The solutions were kept at 4°C and were stable for at least 3 h. Aliquots (200 μl) of NO solution were injected, with a gas-tight syringe, directly into the samples while chemiluminescence was being measured.

Chemiluminescence was measured in a luminometer designed by A.A. Dutra, which incorporates a gallium arsenide photomultiplier tube with a response above 10% of quantum efficiency in the wavelength range 200–900 nm. The samples tested (1 ml in 35 mm petri dishes covered with clingfilm) were kept at 37°C in a light-tight rotating chamber above the photomultiplier tube. This chamber was deoxygenated by purging with argon throughout the experiments. The selection of wavelengths was achieved by placing bandpass interference filters (Ealing Electro-Optics) in the chamber over the photomultiplier tube.

All sample solutions were in 100 mM potassium phosphate buffer (pH 7.0–7.4). The samples of hydrogen peroxide (10 mM) and potassium superoxide (1 mM from a stock in crown ether (Aldrich, UK) dissolved in deoxygenated DMSO or acetonitrile) were made up in buffer deoxygenated with argon. Superoxide dismutase (2.5 U/ml) and catalase (10 U/ml) were added to a 0.1 U/ml xanthine oxidase/1 mM hypoxanthine solution in 100 mM phosphate buffer (pH 7.2) before the injection of the NO-saturated solution.

All chemicals were from Sigma (UK) unless otherwise stated. The experiments were repeated at least three times and the results are expressed as average counts/s.

3. RESULTS AND DISCUSSION

In our studies superoxide was produced either from potassium superoxide in crown ether, or from a xanthine oxidase/hypoxanthine system. When a deoxygenated solution of NO was added to either of these superoxide generating systems, a sharp increase in chemiluminescence was observed at a wavelength compatible with that of singlet oxygen (Fig. 1a). In the course of these experiments we noted that the longer the interval

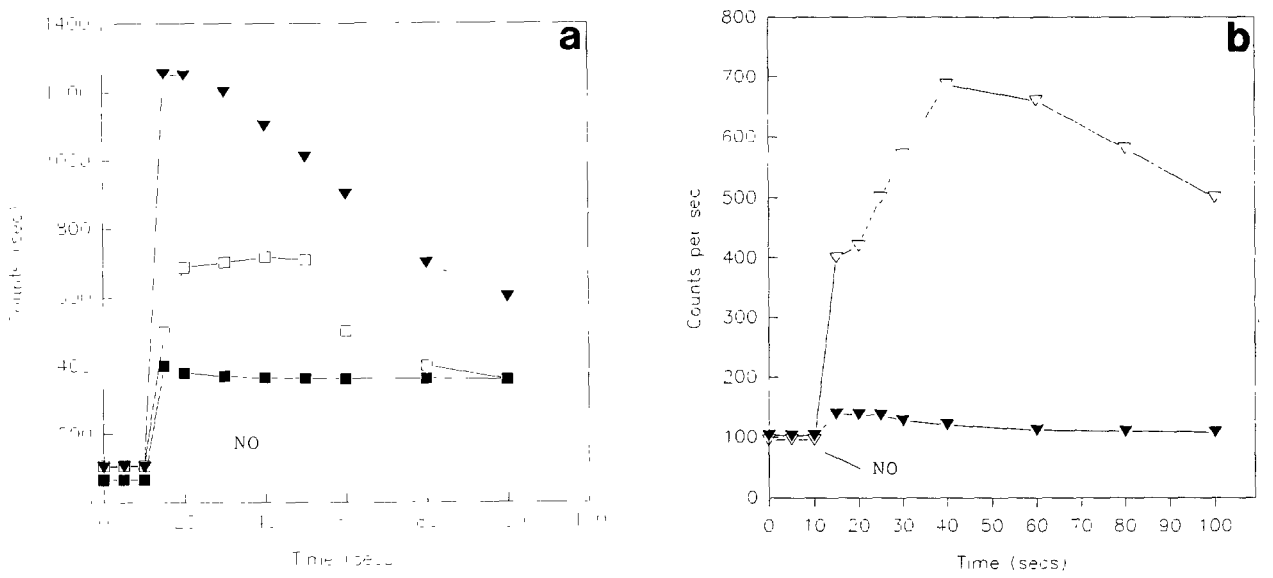

Fig. 1. (a) Chemiluminescence resulting from the addition of NO to hydrogen peroxide (▼), potassium superoxide in crown ether (□), or hypoxanthine/xanthine oxidase (■). (b) The effect of superoxide dismutase (▽) and catalase (▼) on the chemiluminescence produced by the addition of NO to a solution of xanthine oxidase/hypoxanthine.

allowed to elapse before adding NO to the system, the greater the amount of chemiluminescence produced. At physiological pH (7.0–7.4) superoxide is very rapidly dismutated to hydrogen peroxide ($K_2 = 5 \times 10^5$ $M^{-1} \cdot s^{-1}$) and this led us to investigate the possibility that the chemiluminescence we had observed was due to the interaction of NO with hydrogen peroxide.

The addition of catalase to the xanthine oxidase/hypoxanthine system before adding NO was followed by marked inhibition of light emission while the addition of superoxide dismutase was associated with an increase in light emission (Fig. 1b). The addition of albumin (as a protein control) at the same molar concentration as the enzymes used had no effect on light emission. These results lend support to the suggestion that it is hydrogen peroxide rather than superoxide which reacts with the NO in this system.

The following experiments confirmed that the chemiluminescence we detected was due to singlet oxygen formation.

(i) NO injected into a deoxygenated solution of hydrogen peroxide in buffer produced chemiluminescence at wavelengths of 633 and 703 nm but not at 670 nm (Fig. 2). This is the region where the luminescence of dimol collision, characteristic of singlet oxygen, would be expected [9]. The same was true when the organic peroxides, butyl hydroperoxide and cumene hydroperoxide, were used instead of hydrogen peroxide (data not shown). Neither hydrogen peroxide nor NO by themselves showed chemiluminescence.

(ii) In experiments using sodium nitroprusside as the source of NO, much more chemiluminescence was observed after the addition of hydrogen peroxide, when enhancers of the chemiluminescence associated with singlet oxygen were used. When 5-methoxy-vinylpyrene was added before the addition of hydrogen peroxide, there was an increase in chemiluminescence of the order of at least 1,000 times (200,000 cps compared with 200 cps). This degree of enhancement was at least 100-times greater than that which occurred when another enhancer, 7-8 bi-pyridine-dihydrodiol was used (2,000 cps) (Fig. 3). This is consistent with the results obtained by Posner et al. [10] using the same chemiluminescent probes for singlet oxygen.

(iii) Further to confirm the involvement of singlet oxygen in the chemiluminescence resulting from the reaction between NO and hydrogen peroxide, we used the well-known quenchers of singlet oxygen, histidine and sodium azide, and found that they caused a decrease in the amount of chemiluminescence produced (Table I). Also, when deuterium oxide was used in making up the

Table I

The effect of various compounds on the chemiluminescence produced by the reaction between NO and hydrogen peroxide

Treatment	Chemiluminescence (cps)
Control	1,398 ± 11.2
Histidine (10 mM)	775 ± 9.8
Sodium azide (10 mM)	760 ± 9.1
Mannitol (1 mM)	1,369 ± 7.8
DMSO (10%)	1,401 ± 9.1
Desferal (1 mM)	1,392 ± 7.6

Results are expressed as average counts per second (cps) (recorded over periods of 100 s) ± S.D

buffer, the degree of chemiluminescence was increased. This is consistent with the known effect of deuterium oxide in increasing the chemiluminescence due to the presence of singlet oxygen [11].

The possibility that the chemiluminescence was due to the presence of free hydroxyl radicals was eliminated by using dimethyl sulphoxide at a concentration of 10% in the buffer or mannitol at a concentration of 1 mmolar. Both of these quench chemiluminescence due to free hydroxyl production, but no such effect was obtained when either of these compounds were added to the systems described above. Similarly, desferal, an inhibitor of iron-dependent free hydroxyl formation, did not inhibit chemiluminescence when NO was added to a hydrogen peroxide solution in buffer (Table I).

Recently Murphy and Sies [12] have suggested that superoxide dismutase could effect the process by which NO^- (which could be formed by NO-synthesizing enzymes) is converted to NO. In our experiments using superoxide-generating systems and NO-purged water, superoxide dismutase increased light emission by about 50%, however, it had no effect in the experiments in which hydrogen peroxide was used instead of a superoxide-generating system. This suggests that in this case, superoxide dismutase is acting only on superoxide, leading to its faster dismutation to hydrogen peroxide.

We have thus shown that NO can react with hydrogen peroxide, giving rise to singlet oxygen, a highly reactive oxygen species. In conditions of cell injury or inflammation, not only NO is produced, but also a large amount of superoxide dismutase (present in abundance in all human tissue) is released leading to a quick dismutation of superoxide to hydrogen peroxide. Therefore, at any given time the concentration of hydrogen peroxide in the tissue is likely to be much higher than that of superoxide, favouring the reaction described above. The idea that the reaction above may be involved in the cytotoxicity of macrophages is supported by the findings of Li et al. [13], showing that the killing of *Leishmania* by macrophages, known to be mediated by NO, is inhibited by catalase, and is thus dependent on the presence of hydrogen peroxide.

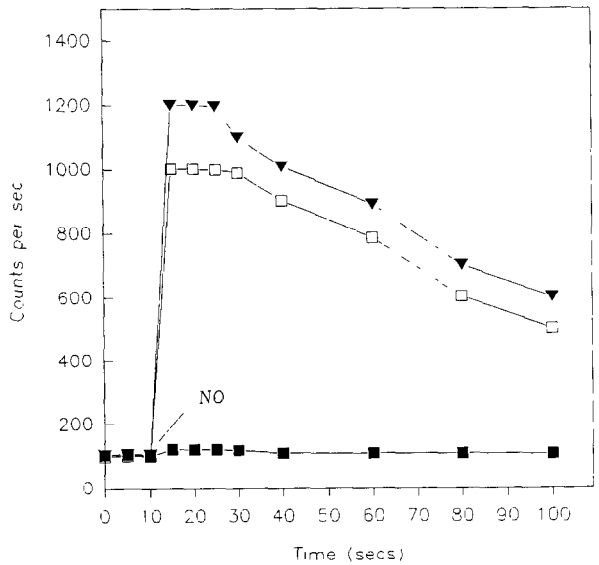

Fig. 2. Chemiluminescence produced during the reaction of NO with hydrogen peroxide measured through interference filters at 703 nm (▼), 633 nm (□), and 670 nm (■)

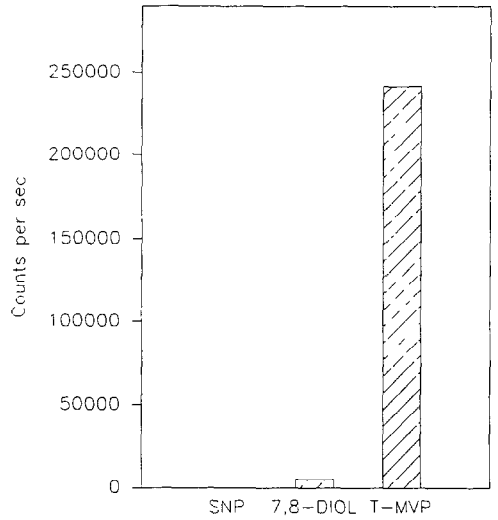

Fig. 3. The effect of singlet oxygen enhancers on the chemiluminescence produced after the addition of hydrogen peroxide to a sodium nitroprusside solution Bars show the maximum chemiluminescence produced after the injection of 10 mM hydrogen peroxide to 1 mM sodium nitroprusside in 10 mM potassium phosphate buffer, pH 7.2, (200 cps), and the effect of adding 13 nM 7,8 bipyridine-dihydrodiol (7,8 diol) (2,000 cps) or 13 nM 5-trans-methoxy vinylpyrene (T-MVP) (> 200,000 cps) to the same system. The enhancers were kept in stock solutions in methanol and were added to 1 ml samples of sodium nitroprusside before the addition of hydrogen peroxide.

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