Improved detection of nitric oxide radical (NO•) production in an activated macrophage culture with a radical scavenger, carboxy PTIO, and Griess reagent

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Abstract An improved method for the detection of nitric oxide radicals (NO•♥ in cultures of activated macrophages was developed, involving a nitric oxide radical scavenger, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl (carboxy PTIO) and Griess reagent. A murine macrophage-like cell line, J774.1, was activated with interferon-γ (IFN-γ) and bacterial lipopolysaccharide (LPS), which induced the production and secretion of NO_2^- into the culture supernatant. Addition of carboxy PTIO to the activated macrophages increased the amount of NO_2^- to 4- to 5-fold without cell damages, probably because carboxy PTIO rapidly reacted with NO• to form NO_2^-, which was finally assayed by the Griess reaction.

Key words: Murine macrophage-like cell; Nitric oxide; Macrophage activation; Carboxy PTIO

1. Introduction

The production of nitric oxide radicals (NO•) by a number of cells, including brain cells, endothelial cells and macrophages, which have distinct types of nitric oxide synthases, respectively, has been reported [1]. In macrophages, NOS is produced in the process of macrophage activation by IFN-γ and LPS, and the activities of these enzymes have usually been determined as the amount of NO_2^-, a stable metabolite of NO•, in a culture with Griess reagent [2], as the shift in electron spin resonance (ESR) on the conversion of carbonyl hemoglobin (CO-Hb) to nitroso hemoglobin (NO-Hb) [3], or as the formation of L-citrulline from L-arginine, a substrate of NOS [4]. The most convenient and widespread of these methods for determination of the amount of NO• is the Griess reaction for detection of NO_2^-, because activated macrophages produce more than several nanomols per liter of NO_2^- in the culture supernatant, and the NO_2^- easily, rapidly and specifically reacts with Griess reagent, showing a colorimetric change [2]. However, NO_2^- is one of the terminal derivatives of NO• in biological systems, because NO• is a very reactive gas and readilly trapped by heme [3], reactive oxygen [5] or other biological materials, such as glutathione, sulfhydryl (SH-) groups or unsaturated fatty acids [6]. To determine accurately the amount of NO• produced by activated macrophages, we tried to use carboxy PTIO as an oxidizing reagent for NO•, which directly oxidizes NO• to form $NO_2^•$ [7], which readily reacts with another NO• to form N_2O_3 [$NO_2^• + NO^• \rightleftarrows N_2O_3$], and then N_2O_3 reacts with H_2O to generate NO_2^- [$N_2O_3 + H_2O \rightleftarrows 2NO_2^- + 2H^+$] [8]. As a whole, carboxy PTIO mediates the conversion of NO• to NO_2^- in a molar to a molar ratio [7]. The resultant product, NO_2^-, of NO• and carboxy PTIO, together with NO_2^- automatically produced from NO• in biological systems (see Fig. 1 for a model) should be detected by the Griess reagent. In this paper, we present results showing that activated macrophages produce 4- to 5-fold higher levels of NO_2^- in the presence of carboxy PTIO than in its absence, and suggest that the addition of carboxy PTIO to macrophage cultures will improve detection of NO• produced by activated macrophages.

2. Materials and methods

2.1. Materials

Carboxy PTIO and carboxy PTI were either purchased from Dojin Chemicals (Kumamoto, Japan) or donated by Dr. Katayama (the Research Center of Dojin Chemicals Co.). Sodium nitrite and Griess Roijin reagent were obtained from Wako Pure Chemical Industries (Tokyo, Japan), and LPS, L-NMMA and NADH were from Sigma (St. Louis, MO). Murine recombinant IFN-γ was a gift from Toray (Tokyo, Japan).

2.2. Cell culture

Culture of the murine macrophage-like cell line, J774.1, was performed as described previously [9]. In brief, a subline of J774.1, the JA-4 cell line, which was established in our laboratory [9], was seeded at 2×10^5 cells/well/0.5 ml of Ham's F-12 medium (Flow Laboratories, McLean, Va), supplemented with 10% of heat-inactivated fetal bovine serum (Gibco, Grand Island, NY), and 10 μg/ml of penicillin and 10 U/ml of streptomycin (Flow Laboratories) in 24-well tissue culture clusters (Costar #3524, Cambridge, MA), and then incubated at 37°C overnight in a humidified CO_2 incubator (5% CO_2/95% air). After replacing the medium with 0.5 ml/well of fresh medium, 0.1 μg/ml of LPS and 10 U/ml of IFN-γ were added to each culture, and then the cells were incubated further at 37°C for 8–24 h. Carboxy PTIO was usually dissolved in 0.1 M sodium phosphate buffer, pH. 9.0, at 10 mg/ml as a stock solution, and 5 μl of this solution was added to the culture. Dilution of this reagent was performed just before the addition. L-NMMA was dissolved at 100 mM in distilled water as a stock solution and added at 1 mM to the culture.

2.3. Assaying of NO_2^-

To determine the amount of NO_2^-, supernatants were collected from the macrophage cultures, and 100 μl of each of them was placed, in duplicate, in a 96 well-microplate for ELISA (Sumitomo Bakelite, Tokyo). A standard solution of a known concentration of $NaNO_2$, usually 0–200 μM, was also placed in the wells of the same microplate. To quantitate NO_2^-, Griess reagent for the nitrite assay was dissolved at 6 mg/ml, and 100 μl/well of the reagent was added to both the standards and the samples on the microplate, followed by mixing with a microplate mixer (Toyoshima, Tokyo) for about 10 seconds. The

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Abbreviations: carboxy PTI, 2-(4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl; carboxy PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl; IFN-γ, interferon-γ; LPS, lipopolysaccharide; L-NMMA, N^G-monomethyl-L-arginine; NOS, nitric oxide synthase.

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reaction products were colorimetrically quantitated at 550 nm with background subtraction at 630 nm, using a Multiscan plus (Falcon, Beckton Dickenson) microplate reader. The concentration of NO_2^- was determined from the calibration curve for the standards.

2.4. Other assays

Cytotoxicity was examined as the release of lactate dehydrogenase (LDH), a cytoplasmic enzyme of macrophages [10]. Assaying of LDH was performed according to Pesce et al. [11]. For assaying of cell protein, the cells were washed with phosphate-buffered saline and then treated with 5% trichloroacetic acid. Protein concentrations were determined by the method of Lowry et al. [12] with bovine serum albumin (Fraction V., Sigma) as a standard.

3. Results

3.1. Dose-dependent effect of carboxy PTIO

To determine the effect of carboxy PTIO, i.e. whether or not it increases the level of NO_2^- produced by activated macrophages, we first determined the effective doses of carboxy PTIO in macrophage cultures (Fig. 2a). During the incubation of activated macrophages pretreated with LPS and IFN-γ, considerable amounts of NO_2^- were released and carboxy PTIO increased the level to 4- to 5-fold higher at more than 100 μM. The effect of carboxy PTIO was maximum at 300 μM, but it was less effective at 1 mM. In control macrophage culture, which had not been treated with LPS or IFN-γ, carboxy PTIO showed little effect up to 300 μM, but increased the NO_2^- level at 1 mM. Under these conditions, carboxy PTIO showed some effect on the cell viability of non-activated macrophages (Fig. 2b). During the incubation of macrophages pretreated with or without LPS + IFN-γ, a cytoplasmic enzyme, LDH, was released by the control macrophages with 100 μM and more of carboxy PTIO, although the leakage of LDH was not observed in a culture of activated macrophages even with 300 μM carboxy PTIO. To optimize the enhancing effect of carboxy PTIO on NO_2^- production by activated macrophages and also to minimize the cell damage caused by this reagent, we selected 100 μM carboxy PTIO for use in this study.

3.2. Involvement of NOS in carboxy-PTIO-elevated production of NO_2^-

It was believed that the production of NO_2^- by activated macrophages is mediated by NOS [2], but it seemed necessary to confirm the involvement of this enzyme in the elevated production of NO_2^- due to carboxy PTIO in our experiments. The addition of L-NMMA, an analog of L-arginine and also one of the strongest inhibitors of NOS [1], inhibited the production of NO_2^- by activated macrophages pretreated with LPS + IFN-γ to the basal level in both the presence and the absence of carboxy PTIO (Fig. 3). These results suggest that the elevated production of NO_2^- caused by carboxy PTIO in an activated macrophage culture was not due to an artefact of the reagent but enhancement of the conversion of NO$^\bullet$, initially produced by NOS, to NO_2^-, as proposed in Fig. 1.

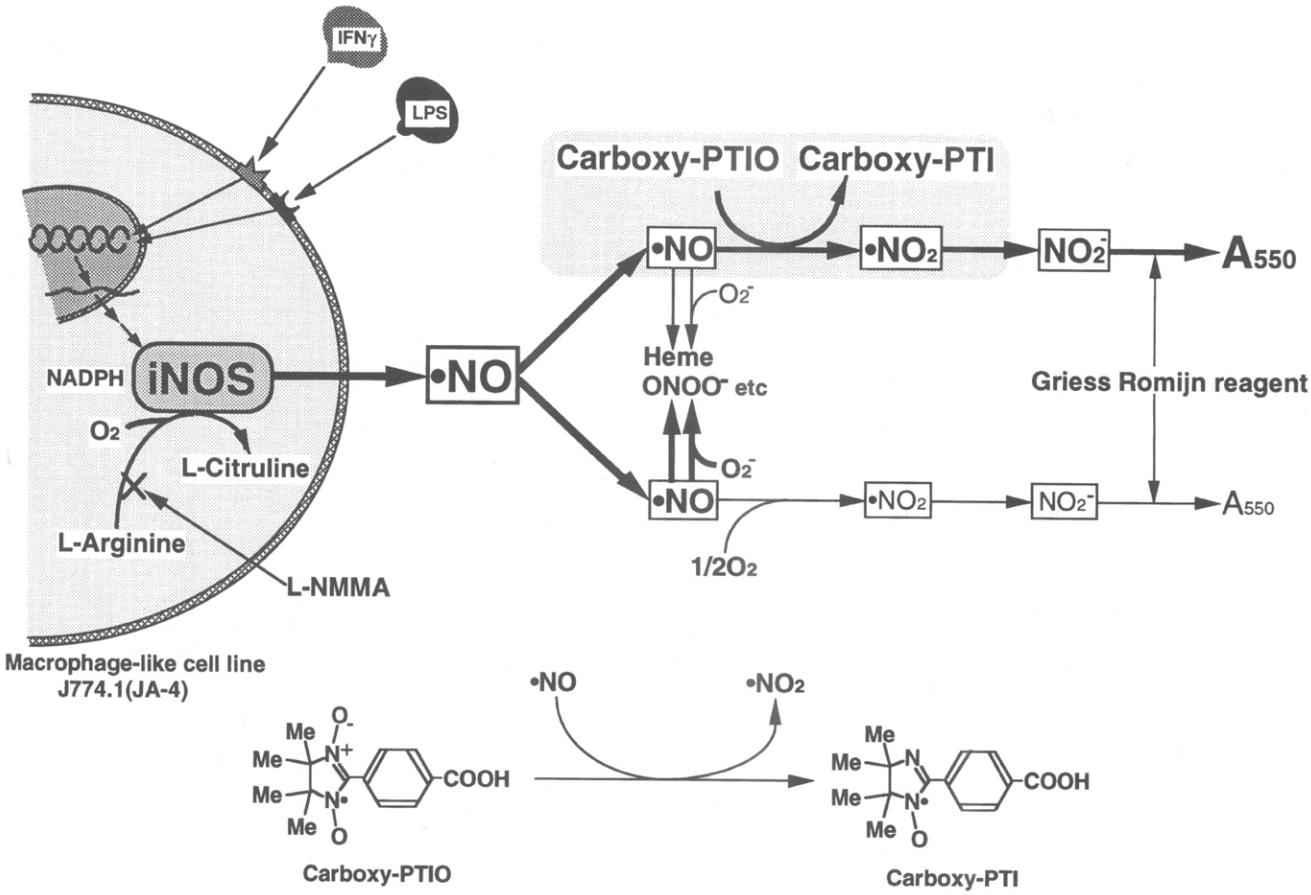

Fig. 1. A model of the enhancing effect of carboxy PTIO on the production of NO_2^- by an activated macrophage culture, and improvement of the detection of NO$^\bullet$ by the combined use of carboxy PTIO and Griess reagent.

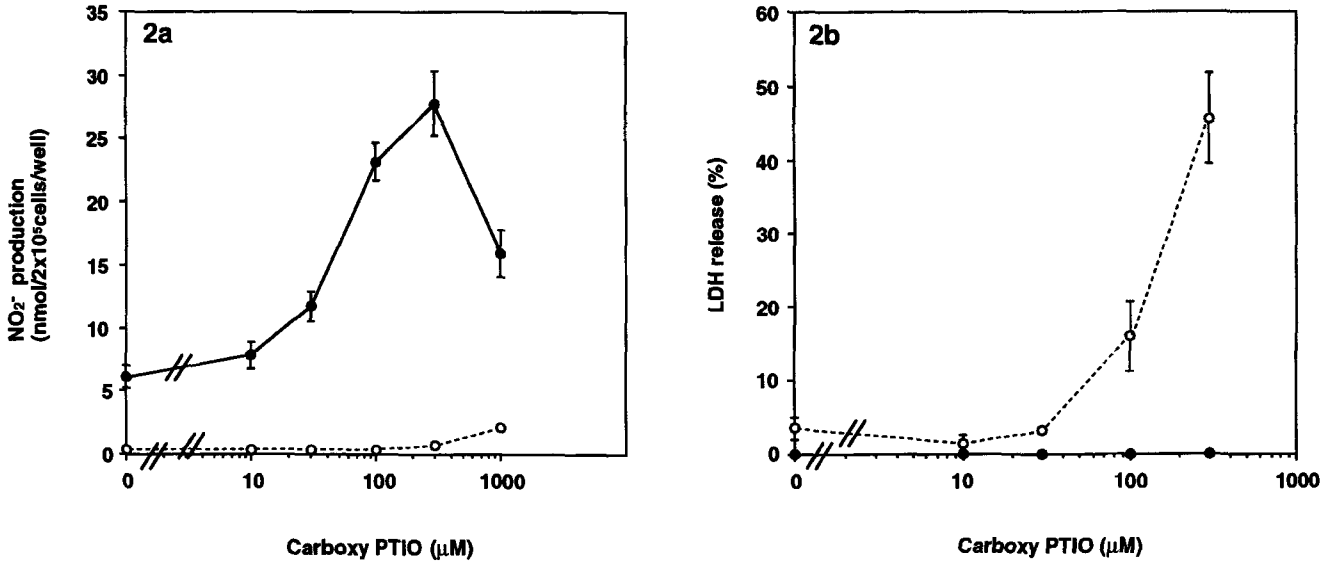

Fig. 2 (a) Dose-dependent increase in NO_2^- in macrophage culture supernatants. The cells were preincubated overnight and treated with (●) or without (○) 0.1 μg/ml of LPS + 10 U/ml of IFN-γ in fresh medium at 37°C for 8 h. Then the culture medium was replaced with fresh medium containing various concentrations of carboxy PTIO, as shown on the abscissa. After 16 h reincubation at 37°C, the culture supernatants were collected and assayed for NO_2^- with Griess reagent as shown in the text. The results are presented as the means ± S.E. for three different experiments. (b) Effect of carboxy PTIO on the cell viability of macrophages. Incubation of the macrophages, reincubation of the cells in fresh medium containing carboxy PTIO, and collection of the culture supernatants were performed as described in Fig. 2a. To examine the cell viability, LDH was assayed in the homogenates of cells as well as in the culture supernatants, as described in the text. The leakage of LDH was estimated as a percentage of the released LDH in the supernatants on the basis of the total recovered LDH in both the cell homogenates and culture supernatants. Symbols in Fig. 2b are the same as in Fig. 2a.

3.3. *Specificity of carboxy PTIO*

To confirm that the elevated production of NO_2^- caused by carboxy PTIO in an activated macrophage culture is due to the conversion of NO• to NO_2^- through the oxygenation reaction of carboxy PTIO, we examined carboxy PTI, which has no oxygen at the 3-position of the tetramethylimidazoline (Fig. 1), as to whether or not it can elevate the production of NO_2^-. As shown in Fig. 4, carboxy PTI caused little elevation of the NO_2^- level in an activated macrophage culture. This suggests that the increase in NO_2^- during the incubation of activated macrophages with carboxy PTIO is mainly due to the reaction between carboxy PTIO and NO•, and that the reactive oxygen at the 3-position of the tetramethylimidazoline is responsible for this reaction.

We next tried to use carboxy PTIO for analysis of the first order kinetics of the production of NO• by an activated macrophage culture. In the absence of carboxy PTIO, the initial rate of NO_2^- production by activated macrophages, pretreated with both LPS and IFN-γ, was too low to determine during the first 1 or 2 h, however, the addition of carboxy PTIO increased the NO_2^- level high enough for evaluation in this period (Fig. 5). The combination of L-NMMA and carboxy PTIO abolished the elevated production of NO_2^-, suggesting the specific reaction of NOS in such cultures. These results suggest that the addition of carboxy PTIO enables us to analyze the first order kinetics of NO• production by activated macrophages in culture.

Discussion

There are several methods for assaying the level of NO• in the biological systems of activated macrophages, endothelial cells, and brain and nerve cells [1]. To elucidate the regulatory mechanisms for NO• production by activated macrophages, rapid and easy methods for the detection of NO• are necessary. To date, the most convenient and widespread way of assessing the level of NO• is to measure NO_2^- with Griess reagent [2]. However, NO_2^- is only one of the terminal derivatives of NO•

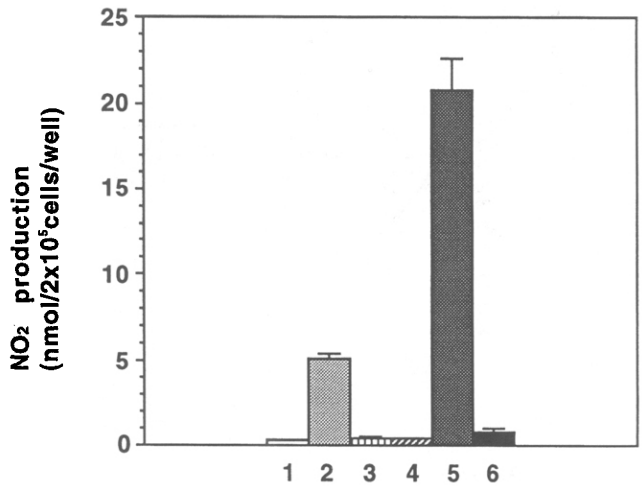

Fig. 3. Effect of L-NMMA on the elevated production of NO_2^- by activated macrophage cultures in the presence of carboxy PTIO. The experiments were performed as described in Fig. 2a, but 1 mM L-NMMA was added to the reincubation medium together with or without carboxy PTIO. The number under each bar corresponds to the sample from 1, + nothing; 2, + LPS + IFN-γ; 3, + LPS + IFN-γ + L-NMMA; 4, + carboxy PTIO; 5, + LPS + IFN-γ + carboxy PTIO; and 6, + LPS + IFN-γ + L-NMMA + carboxy PTIO. The results are means ± S.E. for three different experiments.

and thus does not actually correspond to the net amount of NO• produced by activated macrophages. In this paper, we presented an improved method for the assaying of NO• in an activated macrophage culture involving a specific NO• radical scavenger, carboxy PTIO [7], and Griess reagent (Fig. 1). Carboxy PTIO, but not carboxy PTI, an analog of carboxy PTIO lacking the reactive oxygen donor at the 3-position of the tetramethylimidazoline, increased the level of NO_2^- to 4- to 5-fold higher, without cell damage (Figs. 2a,b and 4). In addition, this enhancement of NO_2^- was shown to be due to NOS by experiments involving a specific inhibitor, L-NMMA (Fig. 3). The results suggest that carboxy PTIO reacts with NO• rapidly enough to trap this radical to form NO_2^- efficiently. As carboxy PTIO is soluble in culture medium, it will trap NO• gas before escaping into the atomosphere. Furthermore, $NO_2^•$, one of the primary reaction products of NO• and carboxy PTIO (Fig. 1), rapidly reacts with another NO• to form N_2O_3 in the solution [8]. These chain reactions convert NO• into more stable intermediates, such as N_2O_3, and finally into NO_2^-, which now reacts with Griess reagent. Apparently, the addition of 100 μM carboxy PTIO improves the detection of NO• as NO_2^- with Griess reagent 4- to 5-fold, without cell damage. This new system will be advantageous for studying on NOS in activated macrophages with a smaller number of cells, a smaller scale culture, and also a weaker activator of macrophages for NOS induction. Knowing the more accurate amounts of NO•, produced in the activated macrophage culture, will help us to understand novel regulatory mechanisms which are involved in activation or inhibition of NOS activity in in vitro assays such as citrulline formation [4].

Furthermore, as shown in Fig. 5, application of this system enabled us to analyze the first order kinetics of NO• production by activated macrophages in vivo. Using this system, we are currently searching for a novel factor and mechanisms for the regulation of NO• production by activated macrophages.

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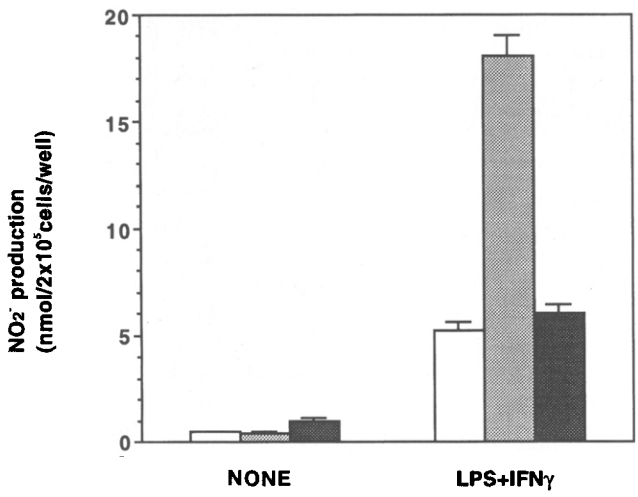

Fig. 4. Structure–activity relationship of carboxy PTIO in the elevation of NO_2^- in activated macrophage cultures. Either carboxy PTIO (lightly shaded columns) or carboxy PTI (dark shaded columns) at 100 μM, or nothing (open columns) was added to activated macrophages, followed by incubation as described in Fig. 2a. The results are means ± SE for three different experiments.

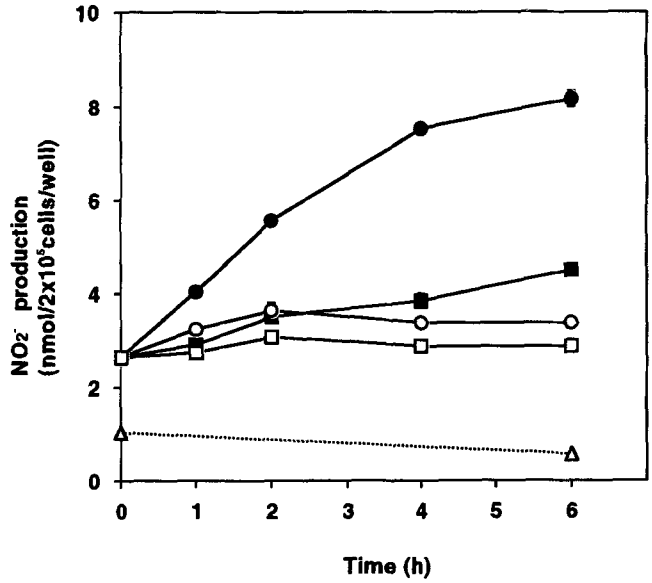

Fig. 5. Analysis of the initial rates of NO_2^- production by activated macrophages in the presence and absence of carboxy PTIO. Cells were pretreated with both 0.1 μg/ml of LPS and 10 U/ml of IFN-γ at 37°C for 8h, and then the medium was replaced with fresh medium, containing nothing (■), 100 μM carboxy PTIO (●), 1mM L-NMMA (□), or 100 μM carboxy PTIO and 1 mM L-NMMA (○). The cells were reincubated at 37°C for 1–6 h, and then the culture supernatants were collected and analyzed with Griess reagent for NO_2^- as described in the text. Cells without pretreatment were also incubated with the medium alone (△), and shown in the figure as a negative control. Representative results of repeated experiments are shown.

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