

Nitric Oxide Production by Human Peripheral Blood Mononuclear Cells

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Abstract

Introduction: There are conflicting data on the ability of human mononuclear cells to produce nitric oxide (NO). We investigated nitric oxide production from peripheral blood mononuclear cells (PBMs) by using a new sensitive fluorescent indicator. **Materials and Methods:** PBMs from healthy volunteers were collected, plated in 96-well microplates, and loaded with the fluorescent nitric oxide probe, 4,5-diaminofluorescein diacetate (DAF-2DA). Experiments were performed in normal control and endotoxin-stimulated PBMs, with and without exogenous L-arginine. The exogenous nitric oxide donor S-nitroso-N-acetyl-penicillamine (SNAP) was used as a positive control. Fluorescence intensity was measured with a fluorescence microplate reader. **Results:** Nitric oxide production by human PBMs can be demonstrated by the use of the fluorescent indicator, DAF-2DA, in both control and endotoxin-stimulated conditions. Nitric oxide production was independent of the concentration of exogenous L-arginine. The addition of endotoxin did not change nitric oxide production. PBMs treated with SNAP showed a concentration dependent increase in fluorescence. Nitric oxide production over 5 hours was constant and identical in both control and stimulated groups. **Conclusion:** This fluorescent indicator technique is useful for the study of NO production by human PBMs. Nitric oxide production by PBMs was independent of exogenous L-arginine concentration and was not affected by endotoxin.

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Key words: Endotoxin, Fluorescence, Human mononuclear cells, L-arginine, Nitric oxide production, SNAP

Introduction

The discovery of nitric oxide as a biomolecule with important physiological function has led to the development of various analytical methods¹⁻⁶ for its detection and quantification. To measure nitric oxide generated under physiological conditions several different fluorimetric methods which were originally developed for nitrite (NO₂⁻) and nitrate (NO₃⁻) determination have been employed.^{7,8} These methods have used the aromatic diamino compound 2,3-diaminonaphthalene (DAN) as an indicator of nitric oxide formation. Although the DAN assay is more sensitive than the Griess reaction, it is not sensitive enough to quantify nitric oxide production in cells expressing only the constitutive nitric oxide synthase (cNOS) and it cannot be used with intact cells because it leaks through cell membranes after the loading.⁹

The newer generation of fluorescent indicators, 4,5-diaminofluoresceins (DAF-2), has been developed for real-time and continuous measurements of nitric oxide production from living cells.^{10,11} They have been used

successfully in both bovine aortic endothelial cells and a rodent macrophage cell line (RAW 264.7) to quantify nitric oxide production.¹² The diacetate form (DAF-2DA) was prepared to improve cellular loading of the probe as it can readily permeate into cells, where it is hydrolysed by intracellular esterases to generate DAF-2. The relatively non-fluorescent DAF-2 reacts rapidly with nitric oxide in oxygen-containing solution to yield its highly fluorescent product triazolofluorescein (DAF-2T).

There are conflicting data on the ability of human blood mononuclear cells to produce nitric oxide.^{3,13-16} We investigated nitric oxide production by human peripheral blood mononuclear cells, under normal physiological and endotoxin-stimulated conditions, using DAF-2DA, the new sensitive fluorescent indicator technique.

Materials and Methods

Materials

4,5-diaminofluorescein diacetate (DAF-2DA) was purchased from Calbiochem-Novabiochem (UK) Ltd.

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(Nottingham, UK). Lipopolysaccharide (LPS) (*Escherichia coli* serotype O55: B5), S-Nitroso-N-Acetyl-Penicillamine (SNAP), and L-arginine were purchased from Sigma Chemical (Poole, Dorset, UK). COSTAR flat bottom 96 well microplates with low evaporation lids were purchased from Fisher Scientific UK (Loughborough, Leicestershire, UK). Krebs' Ringer solution consists of 110 mM NaCl, 5 mM KCl, 1.25 mM MgSO_4 , 16 mM Na_2HPO_4 , 4 mM NaH_2PO_4 , 0.5 mM CaCl_2 , and 5 mM D-Glucose (pH 7.4).

Methods

Human peripheral blood mononuclear cells (PBMs) were used to detect the nitric oxide production with DAF-2DA. Cells were prepared by a density centrifugation technique with Histopaque (Sigma, Poole, UK) from the heparinised blood of normal volunteers, and from heparinised blood incubated with 0.5 $\mu\text{g}/\text{mL}$ *Escherichia coli* lipopolysaccharide for 12 hours. PBMs were washed twice with Krebs' solution, and contaminated red blood cells were lysed with sterile water. After cell counting and viability assessment with trypan blue, both LPS-stimulated and non-stimulated human PBMs were plated on a 96 well microplate at the concentration of 1×10^6 cells per well, (2×10^5 cells per well for experiments with SNAP), for the detection of the fluorescent response, and kept at 37°C in a 95% O_2 /5% CO_2 atmosphere for 2 hours to make the cells adherent to the wells. The cells were subsequently washed twice with Krebs solution to remove non-adherent cells, then 200 μL of Krebs solution with DAF-2DA (10 μM), and a range of concentrations of L-arginine were added to the wells.

Endogenous nitric oxide production was measured by

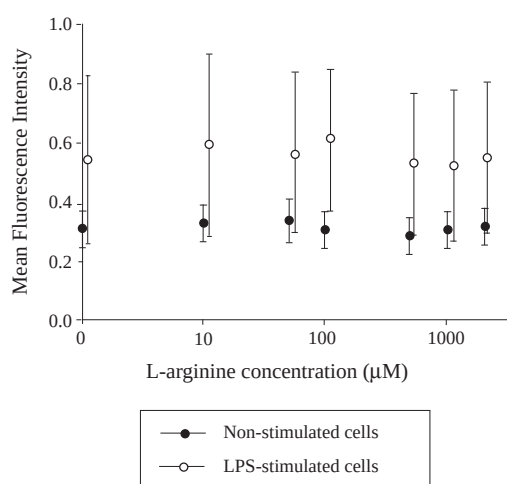


Fig. 1. Nitric oxide-induced fluorescence in human PBMs, with various concentrations of extracellular L-arginine. Results from non-stimulated, and 12-hour LPS-stimulated cells are shown. Fluorescence intensity was measured after 60 min incubation with 10 μM DAF-2DA. All data are mean $\pm$ SD ($n = 6$).

the fluorescent intensity response by the human PBMs without the addition of extracellular L-arginine. The nitric oxide production after the addition of extracellular L-arginine was measured by the fluorescence intensity after the addition of 10 μM to 2 mM L-arginine. The response of human PBMs to exogenous SNAP (6.25 to 250 μM) was also measured as a positive control.

The fluorescence was measured one hour after incubation with DAF-2DA with a fluorescence microplate reader (Titertek Fluoroskan II, Labsystems Group UK Ltd, Basingstoke, Hampshire, UK), calibrated for excitation at 485 nm and emission at 538 nm.

The study of effects of time on nitric oxide production was performed with both LPS-stimulated and non-stimulated human PBMs. Cells were prepared as before, and DAF-2DA was added to a final concentration of 10 μM with or without L-arginine (1 mM) to each well with one hour interval period. The plates were read with the Fluoroskan II at one hour intervals.

Results

Figure 1 shows the relationship between nitric oxide production and the exogenous concentration of L-arginine. Nitric oxide production from both control and stimulated cells was independent of exogenous L-arginine concentration. Although the mean value of mean fluorescence intensity (MFI) from LPS-stimulated cells was found to be higher than that of control cells at every concentration of L-arginine tested, no statistically significant difference was found between the groups because of the variability of the stimulated cell group.

Figure 2 shows the relationship between fluorescence

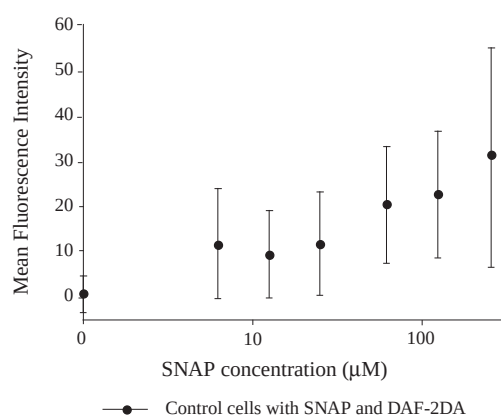


Fig. 2. Effects of various concentrations of SNAP on fluorescence generation. Fluorescence intensity was measured after 60 min incubation with 10 μM DAF-2DA. All data are mean $\pm$ SD ($n = 6$).

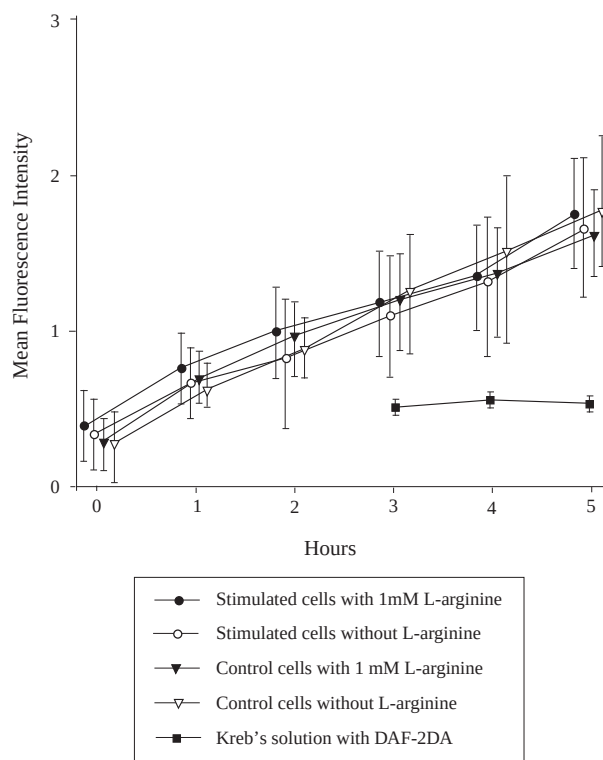


Fig. 3. Time course of fluorescence intensity of DAF-2DA by NO release from human PBMs, and Krebs' solution with DAF-2DA. All data are mean $\pm$ SD ($n = 6$).

intensity from PBMs (2×10^5 cells/well) and SNAP.

Figure 3 shows the effect of time on nitric oxide production in control and endotoxin-stimulated cells. Mean fluorescence intensity increased linearly with time in both cell groups, while the response of Krebs' solution with DAF-2DA did not change with time.

Discussion

Nitric oxide production by human PBMs was clearly demonstrated in this study. The nitric oxide production appears to be independent of extracellular L-arginine concentrations, and is unchanged by prior 12-hour incubation with *Escherichia coli* lipopolysaccharide. The SNAP assay was included in this study to ensure that fluorescence expressed from human PBMs could be mimicked with an exogenous nitric oxide donor.

Kojima et al¹⁰ have used DAF-2DA to demonstrate nitric oxide production in activated rat aortic smooth muscle cells using fluorescence microscopy. They demonstrated that fluorescence intensity in endotoxin- and cytokine-activated cells increased due to DAF-2T production from DAF-2 by reaction with nitric oxide, and was suppressed by the addition of N-monomethyl-L-arginine. Nakatsubo et al¹² also used the technique to demonstrate an endotoxin-induced increase in nitric oxide production in the mouse

macrophage cell line RAW 264.7. However, rodent cells are known to produce considerably more nitric oxide in response to lipopolysaccharide stimulation than human cells.

The nitric oxide production from human PBMs has been investigated using a range of techniques (reviewed in detail by Weinberg¹³). In studies using mononuclear cells or purified monocytes, nitric oxide production measured by nitrite release into the culture medium or a variety of bioassays have produced conflicting results, with the majority demonstrating nitric oxide production,^{14,15} but several studies fail to demonstrate any production.^{3,16} This partially may be due to inadequacies of the available analytic techniques coupled with a low level of basal and induced nitric oxide production in human monocytes. The commonly used Griess assay has a limited sensitivity and measures only nitrite, underestimating the total nitric oxide metabolites which in oxygen-rich environments include nitrate. Unless a nitrate to nitrite conversion is used, nitric oxide production will be underestimated.

The fluorescence technique appears to be sensitive, detecting basal nitric oxide production in non-stimulated PBMs. Although absolute quantification of nitric oxide cannot be made using this technique, the use of micromolar concentrations of SNAP as a positive control suggests the technique is sensitive.

In this study *E. coli* lipopolysaccharide stimulation of human PBMs did not cause an increase in nitric oxide production compared to non-stimulated PBMs. Since the induction of inducible nitric oxide synthase (iNOS) has not been universally documented in monocytes,¹³ the control of expression and subsequent regulation of iNOS activity in human monocytes is unclear. However, constitutive endothelium nitric oxide synthase (cNOS) has been documented in human monocytes¹⁷ and this may explain the detection of nitric oxide production from control non-stimulated human mononuclear cell group. Therefore, elevated nitric oxide production in septic shock may come from other sources, not from human mononuclear cells.

Human nitric oxide production has been recently investigated in patients with septic shock from cellulitis. This study has shown increased iNOS activity from infected tissues but not in adjacent normal tissues.¹⁸ It may imply that iNOS expression is not widespread and is restricted to the nidus of infection. The mechanism to produce sepsis-like condition in *in vitro* cell studies, by using lipopolysaccharide from gram-negative bacteria, may not be responsible for the overall host response during endotoxic shock.¹⁹ Therefore, further studies in this area from clinical human septic patients are still needed. This study used monocytes, but there is no reason why the same technique cannot be used on any tissue types that can be obtained at

operation or by biopsy. This would allow study of nitric oxide production in tissues remote from the infection and allow the source of the excess nitric oxide metabolites found in sepsis to be located. Once the tissues that produce the excess nitric oxide have been located, the exact mechanism underlying the increase can be determined.

In summary, this fluorescent indicator technique is useful for the study of nitric oxide production by human peripheral blood mononuclear cells. Nitric oxide production by human peripheral blood mononuclear cells was independent of exogenous L-arginine concentration and was not affected by endotoxin. This technique could also be used to quantify nitric oxide concentration in cell culture medium and to localise nitric oxide production within cells.

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