

Short Communication

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# NO-generating Systems

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## Introduction

Nitric oxide (NO) produced by nitric oxide synthases (NOS) *in vivo* plays multiple roles in the body. There are three NOS isozymes whose tissue-distribution and regulation are variable. Effects of NO produced by NOS, which is expressed constitutively or induced by various stimuli, can be examined *in vitro* and *in vivo*. Precise control of NO production by NOS *in vivo* or in culture is, however, not possible. Because NO is such a simple molecule and a fairly stable radical, NO gas and NO donors are available for various experiments. If high concentrations are required, NO gas can be used by simply flushing target cells or tissues. Various NO donors, such as nitrosocysteine, nitrosoglutathione, S-nitroso-N-acetyl-D,L-penicillamine (SNAP), 3-morpholinomethylsyndonimine (SIN-1) (produces both NO and superoxide), which spontaneously release NO species, are also commercially available.

## Protocol

### NO generation by peritoneal macrophages

1. Collect macrophages from the peritoneal cavity with Hank's balanced salt solution (without calcium and magnesium but with heparin (10 units/mL).
2. Centrifuge at 250 g for 10 min at 4°C.
3. Isolate the supernatant by aspiration.
4. Add an appropriate volume of supplemented Eagle's minimum essential medium (SMEM) without phenol red and supplemented with sodium bicarbonate (2.0 g/L); sodium pyruvate (110 mg/L); glucose (3.5 g/L); L-glutamine (584 mg/L); penicillin (5000 units/L); streptomycin (50 µg/L); Hepes (15 mM); and 10% heat-inactivated foetal calf serum
5. Repeat twice.
6. Adjust number of macrophages to  $1 \times 10^6$ /mL.
7. Incubate macrophages to adhere at 37°C for more than 1 h.
8. Isolate the supernatant by aspiration.
9. Add an appropriate volume of SMEM containing lipopolysaccharide + interferon- $\gamma$ .
10. After several hours the macrophages produce nitrite and nitrate.

### NO gas

1. Displace  $O_2$  in pipework with flowing inert gas.
2. Introduce NO gas for reaction with reactant. (A mixture of 10% NO in  $N_2$  gas is commercially available).
3. After the reaction NO must be displaced with flowing inert gas.

### NO donors

Various NO donors are available and applicable for *in vivo* and *in vitro* experiments. They generally release reactive nitrogen species spontaneously when dissolved in culture medium or administered to animals.

## Results

The results are illustrated in Figure 1.

## Comments

### NO generation by peritoneal macrophages

A mouse-derived cell line such as RAW264.7 (commercially available from ATCC) is also often used.

Bloodletting of animals should be conducted to avoid contamination with blood cells. After injection of Hank's balanced salt solution into the peritoneal cavity, the abdomen should be massaged vigorously for ca 1 min to free adhered macrophages. Take care not to aspirate the internal organs or fatty tissue when the intraperitoneal fluid is collected. It is better to use young rats.

In this system, NO produced can be reacted with oxygen and spontaneously converted to  $NO_2$ ,  $N_2O_3$ ,  $N_2O_4$ , and finally  $NO_2^-$  and  $NO_3^-$ .  $NO_2^-$  and  $NO_3^-$  can be easily measured by use of an automated Griess method [1]. It is important to know whether or not the sample dose contains inhibitors of the reduction of  $NO_3^-$  to  $NO_2^-$  [2,3].

### NO gas

Stainless steel gas regulators should be used on NO gas tanks to avoid leaks resulting from corrosion. High concentrations of NO react with oxygen producing  $NO_2$  and the solution becomes acidic. To avoid

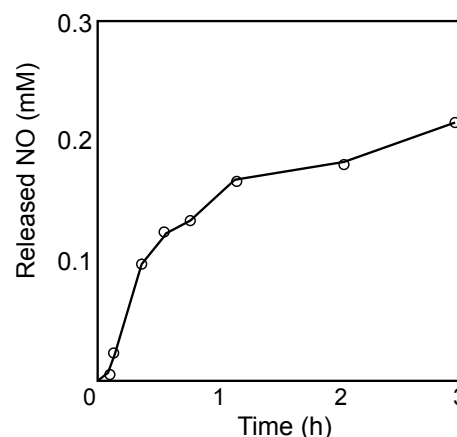


Figure 1: Time course of NO released from 0.5 mM SNAP.

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this, sample solutions should be freed from oxygen by purging with inactive gas (N<sub>2</sub>, Ar, He).

Because NO and NO<sub>2</sub> are harmful, all experiments should be conducted in a fume cupboard. An outlet pipe from equipment should be vented into the fume cupboard.

The concentration of NO can be measured by use of a thermal energy analyser.

### NO donors

Because each NO donor releases specific NO species with different time courses and different efficiencies, special attention should be paid

to distinguish whether results arise as a result of NO species or as a side-effect of the donor molecule.

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