AN IMPROVED CHEMILUMINESCENCE ASSAY SUGGESTS NON NITRIC OXIDE-MEDIATED ACTION OF LYSOPHOSPHATIDYLCHOLINE AND ACETYLCHOLINE

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Abstract. Using a new nitric oxide analyser, we have developed a sensitive chemiluminescence assay to detect trace quantities of NO in aqueous solutions. This improved technique in combination with the bioassay has been employed to verify the theory that NO released by vascular endothelium, accounts for the relaxation produced by acetylcholine, lysophosphatidylcholine and calcium ionophore A23187. Our results show that while calcium ionophore A23187 continuously released NO, acetylcholine and lysophosphatidylcholine relaxed vascular strips without releasing NO over the basal level.

Introduction. Nitric oxide has been implicated as the endothelium-derived relaxing factor (EDRF), which mediates the vascular relaxation produced by acetylcholine and calcium ionophore A23187 (1,2). Recently we have demonstrated that lysophosphatidylcholine (LPC) also produces endothelium-dependent relaxation of rabbit thoracic aorta as well as bovine intrapulmonary artery and vein (3-6). The nature of the relaxing substance produced by LPC has not been determined. The idea that EDRF could be NO emerged from analagous findings with nitrovasodilators, acidification of inorganic nitrite which generates NO, and the vascular relaxing effects of aqueous solutions of NO gas in superfusion cascade bioassays (7-9). The chemical assays for the detection NO have utilized the chemiluminescence reaction of NO with ozone to form NO_2, or the diazotization of sulphanilic acid (1,2). Both these techniques require the acidification of the effluents from cultured endothelial cells or arterial vessels, and hence do not conclusively identify the actual vascular smooth muscle relaxant as NO, since under acidic conditions nitroso compounds, inorganic and alkyl nitrites, as well as nitrosamines can liberate NO (10,11). In this paper we describe a sensitive chemiluminescence assay to detect trace quantities of NO (3×10^{-10} M) in aqueous biological solutions in the presence and absence of acid. In combination with bioassay superfusion experiments this technique is more direct and sensitive for the detection of NO than those previously described.

Materials and Methods. Detection of nitric oxide. Nitric Oxide Analyser (NOA) model 270 was purchased from Sievers Research, Inc., Boulder, CO. Detection of NO is based on the chemiluminescence reaction of NO with ozone.

$$NO + O_3 \rightarrow NO_2 + O_2 + h\nu.$$

The basic apparatus is shown in figure 1. The apparatus consists of a special purge and trap device (J. Pirolo, Alhambra, CA) interfaced to the NOA. The leak-proof purge and trap device consists of a purging unit made of borosilicate glass (7.5 cm X 1.27 cm) with a glass bulb (3.7 cm) at the top and a medium porosity glass frit at the bottom. This is connected by a U-tube and an oblique high vacuum cup stopcock to an inlet of helium. The port at the top permits introduction and removal of samples. The side arm on the bulb is connected by a high vacuum stopcock to the NOA which is maintained under reduced pressure (0-1 Torr) by a vacuum pump (Edwards Model E2M-1). Gases from the purge device pass through a needle valve that regulates the flow upstream and enters the detector. Aqueous samples can be injected through a gas-tight septum (Hewlett-Packard, PA) at the top port. The purge vessel is degreased with organic solvents and rinsed copiously with distilled water. Ozone is generated in situ by electrical discharge of air drawn in by the vacuum pump and is mixed with the effluent gases from the purge vessel in a 4 ml chemiluminescence cell located directly in front of a cooled, red-sensitive photomultiplier tube (Hamamatsu R712-PHA). The light from the reaction is measured by photon counting technique and is recorded on a chart (Soltec Corporation, Sun Valley, CA).

In a typical experiment, the purge vessel is evacuated to 0-1 Torr. With the outlet and inlet stopcocks closed, 2 ml of the sample is injected into the vessel. Helium is introduced at the rate of 40 ml/min for 15 seconds and the gases are purged into the chemiluminescence cell by opening the outlet stopcock. The maximum signal is recorded as a measure of the absolute amount of NO formed.

Bioassay for EDRF. The superfusion cascade experiments have been described in detail elsewhere (12,9,6). Bovine lungs were obtained from Shamrock Co., Vernon, CA. A segment of the intrapulmonary artery (12 cm long) was perfused with Krebs-Henseleit (3) and the perfusate was allowed to superfuse an endothelium-deprived detector strip. The perfusion and superfusion media contained 10^{-5}M indomethacin. Changes in the isometric tension of the detector were recorded after preconstriction with 10^{-5}M histamine. In a typical experiment, bioassays were

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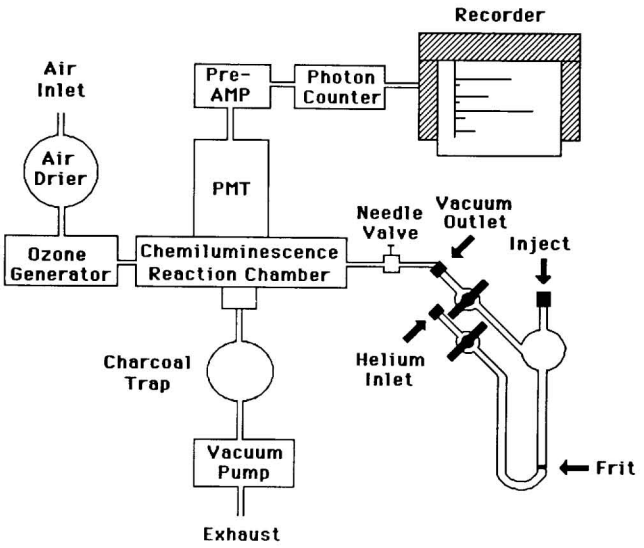

Figure 1. Schematic representation of the nitric oxide analyzer model 270 with the purge and trap device.

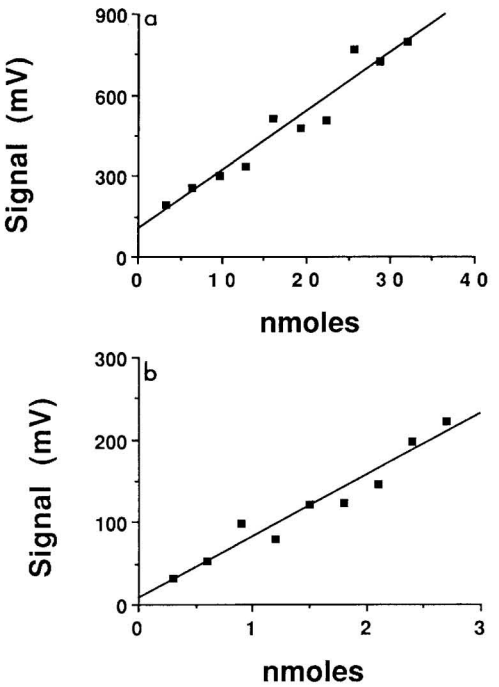

Figure 2. (a) Chemiluminescence responses obtained with higher concentrations of aqueous NO solution. (b) Chemiluminescence responses obtained with lower concentrations of aqueous NO solution.

carried out first. Following this, relaxing substances to be tested, 10^{-6}M acetylcholine (ACh), 10^{-5}M lysophosphatidylcholine (LPC), and 10^{-6}M calcium ionophore A23187 were added to the generator and the relaxation of the detector was recorded. Subsequently, NO determination was carried out in 2 ml of the effluent collected directly from the generator in a pressure-lok gas syringe (Dynatech Precision Sampling Corp., Baton Rouge, LA) in an atmosphere of N_2. The procedure was repeated by collecting the effluent in 10% v/v of 4N HCL. In experiments involving ACh, arterial strips were pretreated with 10^{-8}M atropine sulphate to prevent the direct contractile effects of ACh (15).

Preparation of Standard Solutions of NO. To prepare a saturated solution of NO at 25°C, 10 ml of Krebs or water were deoxygenated by flushing with N_2. NO gas (99.99% Matheson Gas Co., Cucamonga, CA) was bubbled through at a rate of one bubble per second for 20 minutes, while the gas above was flushed away with N_2. The tube was quickly closed with a serum cap. The solubility of NO in water is 7.34 cm^3/100 ml and assuming saturation (13), the maximal concentration of NO in the solution is 3.3 mM which is equivalent to 3 nmoles of NO/μl of sample. Dilute solutions were prepared similarly by bubbling pure NO through 100 ml of solution for 20 minutes and 10 minutes respectively.

Drugs. Histamine, acetylcholine chloride indomethacin and lysophosphatidylcholine from egg yolk were obtained from Sigma Chemical Co., St. Louis, MO. Atropine sulphate was from Lypomed, Inc., Rosemont, IL.

Results. The chemiluminescence responses obtained from aqueous solutions of NO were linear between 300 picomoles and 35 nmoles (Fig. 2a and b). Higher concentrations overloaded the detector. The initial response upon purging was very rapid as was the decay from the peak value. Tailing of the peaks was not noticed. For integration times above 0.25 seconds, the noise level increased. Standard solutions prepared in water as well as Krebs gave identical responses in the ranges studied.

Figures 3a and b represent the relaxant responses and the concomitant release of NO from the effluent of the generator after perfusing with 10^{-5}M histamine (HA), 10^{-5}M LPC, 10^{-6}M ACh and 10^{-6}M A23187. Perfusate from the generator produced relaxation of the detector after precontraction with histamine indicating basal release of EDRF. Perfusion with ACh and LPC caused further relaxation, but the NO signal was very low. In most cases the signal was not greater than the basal release. On the other hand, A23187 generated substantial amounts of NO continuously for over 90 minutes. Upon acidification of the effluent all NO signals increased by seven to thirteen fold.

The chemiluminescence response obtained from sodium nitrite in solution was a thousand times less when compared to NO solution. 2900 nmoles of sodium nitrite produced the chracteristic signal of 3 nmoles of aqueous solution. Acidification enhanced the signal by four fold.

Discussion. We have described here a sensitive chemiluminescence assay to detect NO up to 300 picomoles directly in aqueous biological samples. In a similar chemiluminescence technique to determine NO from seawater, Zafiriou and McFarland have established that the signal-causing gas is NO (14). In the present method NO can be generated under reduced pressure, thus eliminating the acidification step employed previously. Using this technique we have attempted to identify the nature of the endothelium-derived relaxing factor, released from bovine intrapulmonary artery upon stimulation with ACh, LPC and the calcium ionophore A23187. Our observations provide pharmacological evidence of the release of EDRF after perfusion with ACh and LPC, but the chemical evidence does not reveal the release of NO above basal level without acid hydrolysis. On the other hand, upon perfusion

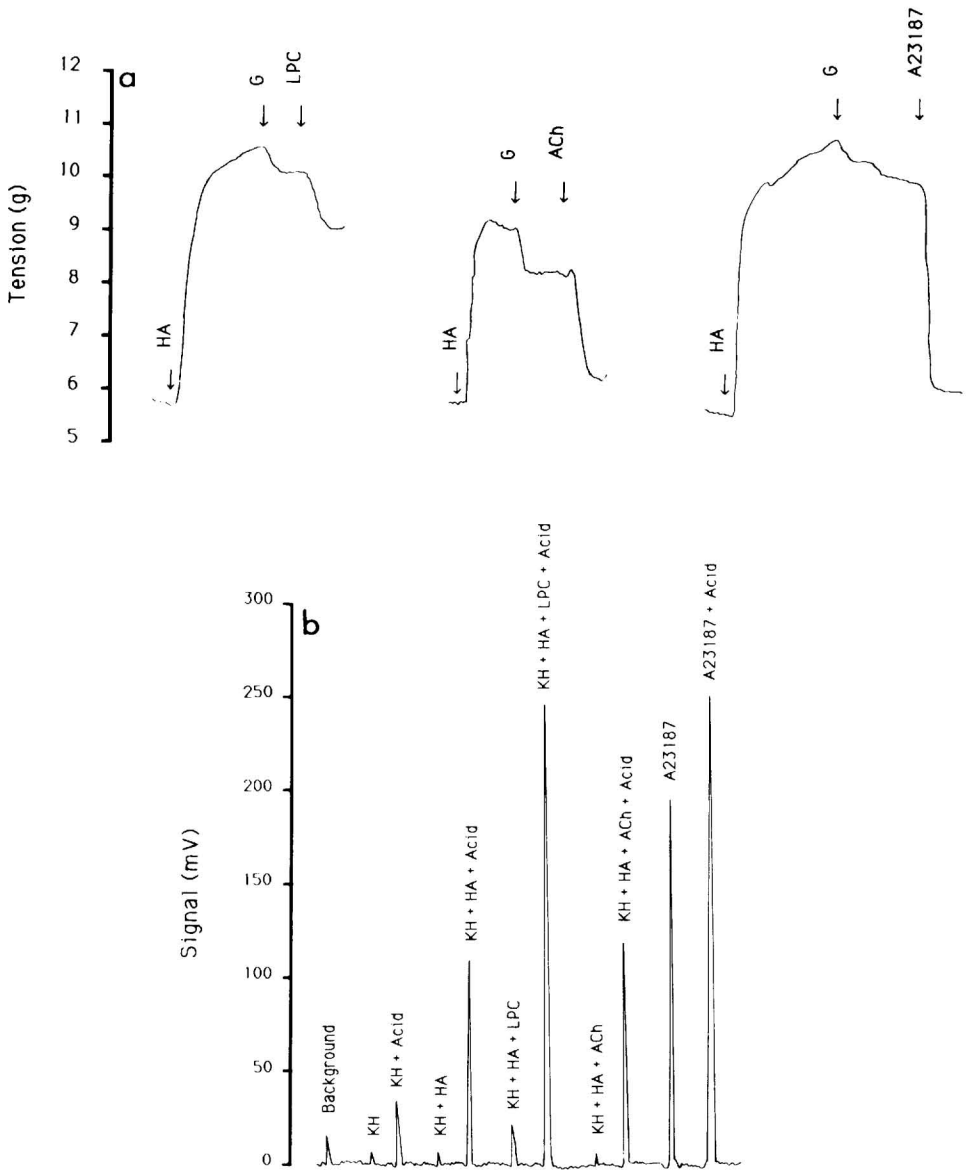

Figure 3. (a) Superfusion bioassay for EDRF. A segment of the bovine intrapulmonary artery was perfused with Krebs-Henseleit containing 10^{-5} *M* indomethacin. The perfusate superfused an endothelium-deprived detector strip from the third branch. Changes in isometric tension of the detector were recorded after preconstriction with 10^{-5} *M* histamine. G, effluent from the generator: 10^{-5} *M* LPC, 10^{-6} *M* ACh, and 10^{-6} *M* calcium ionophore A23187 were delivered by superfusion. (b) Chemiluminescence response for NO obtained from effluents of bovine intrapulmonary artery. Following the bioassay, the detector was disconnected and 2 ml of the effluent were collected directly for NO determination in the presence and absence of 10% *v/v* of 4 *N* HCl. KH (Krebs-Henseleit), with 10^{-5} *M* indomethacin; HA, 10^{-5} *M* histamine; LPC, 10^{-5} *M* lysophosphatidylcholine; ACh, 10^{-6} *M* acetylcholine; A23187, 10^{-6} *M* calcium ionophore.

with calcium ionophore A23187 there was substantial release of NO which persisted over 90 minutes. Acidification of the effluent enhanced the NO signal from all the relaxing agents as well as the basal release.

In summary, our results suggest that while the relaxation produced by calcium ionophore A23187 may be mediated through NO, it is unlikely that the generation of NO is required for the action of ACh and LPC. The endothelial precursors responsible for the enhanced release of NO upon acidification could be organic or inorganic nitrite, nitroso compounds and nitrosamines.

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