Real Time Measurement of Nitric Oxide Released from Cultured Endothelial Cells

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Direct detection of nitric oxide (NO) is essential for understanding the precise mechanism of its production from endothelial cells. Previously, we developed an NO detection system based on the chemiluminescence reaction between NO and luminol-H₂O₂. Here, we have applied this system to cultured endothelial cells for the direct and on-time measurement of NO. The perfusate from cultured endothelial cells was continuously mixed with luminol-H₂O₂, NO−monomethyl-l-arginine (l-NMMA) (10⁻⁴ m), and the chemiluminescence signal of NO, suggesting the existence of basal NO release. Bradykinin (10⁻⁸ m—10⁻⁶ m) increased the NO signal (10⁻⁴ m; 5.1±0.4 fmol/min, corresponding to 1.7 pm in the perfusate), and this was inhibited by 10⁻⁵ m l-NMMA (1.8±0.3 fmol/min). These results corresponded to the changes in cGMP levels in RFL-6 cells, which provide an NO bioassay system. We conclude that the luminol-H₂O₂ system is useful for the direct and continuous measurement of NO from cultured endothelial cells.

Key words  nitric oxide; endothelium; bradykinin; chemiluminescence; luminol

In 1980, Furchgott et al. showed that acetylcholine evoked the release of a vasodilating mediator from the endothelium, and called this mediator endothelin-derived relaxing factor (EDRF). Later, EDRF has been identified as nitric oxide (NO) or its related compounds synthesized from l-arginine. NO has been shown to play an important role as a messenger molecule, not only in the vasculature, but also in central and peripheral neurons and phagocytic cells. Studies on NO have been carried out using mainly NO synthase inhibitors such as N⁶-nitro-l-arginine and N⁶-nitro-l-arginine (l-NMMA), although several direct NO detection systems have also been reported. These systems are based on the reaction between NO and ozone; the product of a nitrite azo-coupling reaction, oxidation of HBO₂; GC/MS; EPR assay; fluorometric assay; and other methods. However, there has been a technical problem on the real-time measurement of NO since the concentration of NO is very low and its half-life is very short (<(6 s) in physiological solutions. Although the real-time detection of NO using an NO electrode has been reported, this method has not been applied to a perfusion system. Previously, we developed a highly sensitive NO detection system based on the reaction between NO and luminol-H₂O₂, and thus achieved the continuous detection of NO from isolated rat kidney. In the present study, we applied this system to cultured endothelial cells, which are more suitable for studying the cellular mechanisms of NO production. A column was filled with microcarrier beads containing endothelial cells. The perfusate and the chemiluminescence probe (composed mainly of luminol and H₂O₂) were continuously mixed and the resulting chemiluminescence was detected. At the same time, the production of NO from cells was confirmed by a bioassay based on elevation of the cGMP level in RFL-6 cells. Bradykinin was used to stimulate NO release because it shows high NO releasing ability in cultured cells.

MATERIALS AND METHODS

Endothelial Cell Culture  Primary cultures of endothelial cells were prepared from bovine carotid artery according to Gimbrone et al., with a slight modification. Cells were grown on 75 cm² tissue culture flasks in MEM medium supplemented with l-glutamine and 10% fetal calf serum (FCS). Human umbilical vein endothelial cells were purchased from Kurabow (Tokyo, Japan). One gram of Cytox 3 microcarrier beads was swollen by phosphate-buffered saline deficient in Ca²⁺ and Mg²⁺ (PBS−) and then equilibrated with MEM supplemented with 10% FCS. 5×10⁵ cells were removed from the culture flasks with 0.5% trypsin–0.2% EDTA in PBS−. The cells and the beads were incubated at 37°C for 8 cycles, each consisting of 3 min with stirring at 40 rpm and 30 min at rest, before being continuously stirred at 80 rpm in 500 ml of culture medium on a microcarrier stirrer system (Iwaki Glass, Chiba, Japan). After 3 to 4 d in culture, the cells reached confluence on the beads, as confirmed by phase-contrast microscopy.

Real Time NO Assay Procedure  A column containing 1 ml of beads covered with confluent endothelial cells (3×10⁵ cells) was continuously perfused with Krebs-Henseleit buffer gassed with a mixture of 95% O₂ and 5% CO₂ at 37°C. The composition of Krebs-Henseleit buffer (mM) was as follows: 118 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, 25.0 NaHCO₃, and 10 glucose. The perfusion rate was 3 ml/min. A three-way cock was set at the end of the column to divide the perfusate into two portions. For the chemiluminescence assay, one portion of the perfusate was adjusted to a rate of 2 ml/min, and the rest was used for the cGMP assay. An on-line filter was placed before the plunger pump to prevent endothelial debris from flowing into the system. The column effluent was mixed with a 0.5 ml/min chemiluminescence probe (luminol 33 μM, desferrioxamine 350 μM, H₂O₂ 15 mM and potassium carbonate 6.6 mM) using double plunger headed pumps (PU980, JASCO Corporation).
Tokyo, Japan) and a rotating flow mixer (KZS-1, Kyowa Seimitsu, Tokyo, Japan) for thorough mixing. The mixer was connected to a chemiluminescence detector (825-CI, JASCO Corporation, Tokyo, Japan) (Fig. 1). After 2 to 3 h for equilibration, the endothelial cell beads were exposed to bradykinin and/or l-NMMA by infusion using a syringe pump (Model 11 Harvard Apparatus, South Natick, U.S.A.) at a rate of 0.3 ml/min.

A standard curve of NO concentration was obtained as described previously. The chemiluminescence of NO standard solutions was recorded by the injection of NO standard solutions instead of endothelial cell perfusates into the perfusion system. All data for the NO amount are shown as the difference in values before and after drug application. Since the absolute intensity of chemiluminescence may contain some background signal which is not derived from NO, the basal level of the chemiluminescence could not be converted to the absolute NO value. In the present study, however, a possible basal release of NO was estimated by means of measuring chemiluminescence which was decreased by an NO synthase inhibitor, l-NMMA.

**cGMP Measurement** Rat fetal lung fibroblasts (RFL-6 cells) were purchased from Dainippon Pharmaceutical (Osaka, Japan), and maintained in F12 medium supplemented with 10% FCS. A bioassay for NO was performed based on the elevation of cGMP in RFL-6 cells.

**Chemicals** The following chemicals were used: bradykinin (Peptide Institute, Inc., Osaka, Japan), luminol, H_{2}O_{2} (Wako Pure Chemical, Osaka, Japan), desferrioxamine (Ciba-Geigy-Japan, Takarazuka, Japan), minimum essential medium (MEM) (Nissui Pharmaceutical, Tokyo, Japan), fetal calf serum (FCS) ( Irvine Scientific, California, U.S.A.), F12 medium, trypsin–EDTA solution ( Gibco BRL, Maryland, U.S.A.), cGMP assay kit (Yumasa Shouyu, Tokyo, Japan), Cytodex 3 microcarrier beads (Pharmacia, Sweden), l-NMMA was kindly provided by Tanabe Seiyaku Co., Ltd. (Saitama, Japan). Luminol was purified by recrystallization to a monosodium salt form using the method of Stott with slight modification.

**Statistics** Data are shown as means±S.E.M. Statistical analysis between two groups was performed using Student's t-test. Statistical tests among three groups were performed using analysis of variance (ANOVA) followed by Bonferroni’s test. p values of less than 0.05 were considered significant.

**RESULTS**

The perfusate which was passed through endothelial cell-free columns did not increase in chemiluminescence based on NO. Similarly, 10^{-6} M bradykinin had no significant effect on the signal. When using the bovine endothelial cell perfusate, 10^{-5} M to 10^{-4} M bradykinin rapidly increased the NO signal (Fig. 2A). The increase in the signal was gradually abolished after the termination of the bradykinin infusion. Peak values of NO release from 5 batches of endothelial cell beads are summarized in Fig. 2B and Table 1. In the presence of 10^{-6} M bradykinin, the NO amount reached a maximum value (5.1±0.4 fmol/min). This value corresponded to 1.7 pm in the perfusate. The NO amount in response to 10^{-6} M bradykinin was significantly larger than 10^{-5} M or 10^{-4} M bradykinin (p<0.05, ANOVA). An NO synthase inhibitor, l-NMMA (10^{-4} M), decreased the bovine endothelial cell NO signal by 4.2±0.6 fmol/min. As shown in Table 1, the bradykinin (10^{-6} M)-induced NO amount was significantly inhibited by 10^{-4} M l-NMMA (p<0.05, t-test; Table 1).

The perfusate from bovine endothelial cells did not alter the amount of cGMP in RFL-6 cells significantly (p>0.05, ANOVA; Fig. 3). The infusion of 10^{-6} M bradykinin caused a significant increase in the amount of cGMP in comparison with the endothelial cell perfusate in the absence of bradykinin (p<0.05, ANOVA).
Fig. 2. A: Typical Recordings of the Chemiluminescence Signal Evoked by $10^{-7}$M and $10^{-8}$M Bradykinin. B: The Average Peak Height of the NO Signal from Bovine Endothelial Cells on Microcarrier Beads in 5 Separate Experiments in Response to Various Concentrations of Bradykinin.

Each bar represents the mean±S.E.M.

<table>
<thead>
<tr>
<th>Sources of cell</th>
<th>Cell number</th>
<th>NO Release ($\Delta$fmol/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Bradykinin (10^{-7} M) + bradykinin</td>
</tr>
<tr>
<td>Bovine carotid artery</td>
<td>3.0±10^2</td>
<td>2.7±0.5 0.4±0.3*</td>
</tr>
<tr>
<td></td>
<td>10^{-8}M</td>
<td>5.1±0.4 1.8±0.3*</td>
</tr>
<tr>
<td>Human umbilical vein</td>
<td>1.3±10^0</td>
<td>3.9±0.3 2.4±0.3</td>
</tr>
</tbody>
</table>

Each data point represents mean±S.E.M. *p<0.05 compared with bradykinin-treated group without L-NMMA.

The amount of NO released from human umbilical vein endothelial cells was compared to that from bovine aortic endothelial cells (Table 1). In the perfusate passed through human umbilical vein endothelial cells, almost the same amount of NO was detected, in spite of using fewer cells. In human endothelial cells, L-NMMA did not inhibit the NO signal significantly ($p>0.05$, t-test).

**DISCUSSION**

Since bioactive substances derived from cultured endothelial cells are usually present in small amounts, a perfusion system of microcarrier beads has been developed, whereby many cells can be used.21,22 Cocks et al.23 and Gryglewski et al.24 reported an adaptation of this method to the bioassay of EDRF. In the present study, we employed this system for real-time NO detection. Since NO is unstable, especially in a physiological solution, it is important to consider the time required for the solution to flow from the end of the column to the detector in this perfusion system. We previously reported that the half-life of NO in oxygenated Krebs-Henseleit buffer was approximately 6.4 s.17 Because 17 s was needed for the flow from the end of the column to the detector, the amount of NO at the end of the column was calculated to be about 6 times higher than the detected level.

Vascular endothelial cells possess constitutive NO synthase (eNOS) and also inducible NO synthase (iNOS) when immunologically stimulated.4 To induce iNOS in endothelial cells, incubation with interferon-γ or lipopolysaccharide for more than 2 h is needed.4 Here, we studied the amount of NO synthesized by eNOS, which is known to be regulated by intracellular Ca^{2+} and plays an important role in the control of vascular tone and blood pressure.7 Several agonists, including acetylcholine, bradykinin and histamine, are known to produce NO through the activation of eNOS. As shown in Table 1, the bradykinin-induced chemiluminescence signal of NO was detected with endothelial cells from both bovine carotid artery and human umbilical vein. The two higher doses of bradykinin represented rough dose-dependency, although the lowest dose seemed to produce a less clear dose-related response, suggesting that there might be a limitation to measuring the NO amount quantitatively with a lower dose of bradykinin.

Endothelial cells have been reported to synthesize NO continuously without stimulation by agonists.25,26 In endothelial cells from the bovine carotid artery, the NO signal decreased rapidly when L-NMMA was infused. This decrease
in signal was not observed in the perfusate from the cell-free column. Therefore, this decrease reflects the inhibition of basal NO release. The basal release of NO has been reported to be stimulated by shear stress.²⁵⁻²⁶ In the present perfusion system, endothelial cells would receive shear stress and release NO. During the treatment of endothelial cells with bradykinin, the actual amount of NO would be higher than the level shown in Table 1, since the basal NO release was not considered in the difference value before and after bradykinin.

To confirm NO release from endothelial cells, we assessed NO using an RFL cell-based bioassay system. This method has been reported to be an easy technique and to have relatively high sensitivity. However, since it is not useful for online and continuous assay, part of the perfusate was added to the RFL-6 cells for 40 s, after which cGMP was measured. This bioassay confirmed an increase in NO induced by bradykinin. Ishii et al.¹⁹ reported that they could detect 100 to 200 fmol of NO using this system. Considering the time from the end of the column to the chemiluminescence detector, the amount of NO just at the end of the column, which is estimated from the data obtained with the luminol-H₂O₂ system, was approximately 32 fmol after stimulation with 10⁻⁶ M bradykinin. Therefore, the amount of NO released by bradykinin in the present study would be almost at the detection limit of the RFL-6 cell-based bioassay.

The amount of bioactive substance released from cultured endothelial cells depends upon many factors such as the sources of the cells, the passage number, and culture density. As shown in Table 1, almost the same amount of NO was detected from the endothelial cells of human umbilical vein as from those of bovine carotid artery, in spite of employing fewer cells. Therefore, the total amount of NO released from human umbilical vein endothelial cells could be greater than that from bovine carotid arterial cells. It is surprising, however, that L-NMMA inhibited NO production less in the umbilical vein than in the carotid artery. The concentration used in the present study may not have been high enough to inhibit NO synthesis completely in this type of endothelial cell.

In conclusion, the luminol-H₂O₂ system is useful for the direct and continuous measurement of NO from cultured endothelial cells.

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