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Simultaneous measurement of endothelium-derived relaxing factor by bioassay and guanylate cyclase stimulation

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1 Endothelium-derived relaxing factor (EDRF) released by cultured endothelial cells (EC) from bovine aortae was measured by bioassay using pre-contracted strips of rabbit aorta and by radioimmunoassay of guanosine 3′:5′-cyclic monophosphate (cyclic GMP) produced by stimulation of bovine lung soluble guanylate cyclase.

2 Bradykinin (Bk, 3 and 30 pmol) injected through a column of EC caused release of EDRF as detected by bioassay and increased cyclic GMP concentrations. Superoxide dismutase (SOD, 15 u ml^{-1}) increased the amount of EDRF detected by the activation of soluble guanylate cyclase.

3 In the absence of endothelial cells, nitric oxide (NO, 1–2 μM), arachidonic acid (AA, 3–30 μM) or sodium nitroprusside (SNP, 1–100 μM) stimulated guanylate cyclase. Superoxide dismutase strongly increased the stimulation of guanylate cyclase induced by NO, but had little effect on the stimulation induced by SNP and no effect on the stimulation induced by AA.

4 Oxyhaemoglobin (10–300 μM) abolished the stimulation of guanylate cyclase by EDRF, NO or SNP but was much less effective as an inhibitor of AA-induced stimulation of guanylate cyclase.

5 These results demonstrate that measurement of guanylate cyclase stimulation by radioimmunoassay is a viable method for detecting EDRF release, especially useful when the drugs used interfere with bioassay tissues.

Introduction

The mechanism of release of endothelium-derived relaxing factor (EDRF) has been studied mainly by bioassay using either isolated vascular strips (Furchgott & Zawadzki, 1980) or columns of cultured endothelial cells (EC; Cocks *et al.*, 1985; Gryglewski *et al.*, 1986a; de Nucci *et al.*, 1988a). This latter method made it possible to separate the EDRF-generating cells from the detecting tissues.

The use of bioassay to detect EDRF is limited because some drugs or procedures interfere with the responses of the bioassay tissues. It is also difficult to study the kinetics of EDRF release by bioassay for the tissue relaxations have a slow time course, taking minutes to wear off after the removal of the stimulus.

We have, therefore, developed another method to measure EDRF release from cultured bovine aortic EC. EDRF activates guanylate cyclase (Rapoport & Murad, 1983) and Mulsch *et al.* (1987) showed that EDRF activates purified soluble guanylate cyclase from bovine lung but did not report on the effects of other endogenous activators of guanylate cyclase such as arachidonic acid (AA), lipid peroxides or hydrogen peroxide.

We have compared activation of guanylate cyclase from bovine lungs with measurement of EDRF by bioassay tissues. The guanylate cyclase method has been used to elucidate the dynamics of EDRF release.

Methods

Bovine aortic endothelial cells

Endothelial cells were isolated by treatment of bovine aorta with 0.2% (w/v) collagenase. Cells were

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grown to confluence in plastic vessels, then removed by treatment with 0.05% (w/v) trypsin, and seeded onto Cytodex 3 microcarrier beads (Pharmacia). The beads were stirred for 3–7 days, until the cells became confluent, and then packed into columns of 1.5–2 ml containing 15–20 × 10^6 EC (de Nucci *et al.*, 1988a).

Preparation of guanylate cyclase from bovine lungs

The following procedures were carried out in an ice bath or refrigerator. Pieces (100 g) of bovine lungs were chopped and homogenized in a Polytron homogenizer with a buffer at pH 7.4 [Tris/HCl 10 mM, 300 ml, containing sucrose (0.25 M), ethylenediaminetetra-acetic acid (EDTA, 1 mM) and dithiothreitol (DTT, 2 mM)]. The homogenates were centrifuged at 500 *g* for 10 min, the supernatant collected and further centrifuged at 105,000 *g* for 1 h. The high speed supernatant (cytosol fraction) was applied to a column (4 × 15 cm) of DEAE cellulose (DE-52, Whatman) which was pre-washed (2 l) with buffer (Tris/HCl, 10 mM) containing DTT (2 mM) at pH 7.4 (buffer A). The column was further washed with the same buffer (1 l) followed by washing (1 l) with buffer A containing NaCl (0.1 M). For elution, the column was finally washed with the same buffer containing NaCl (0.5 M) and 10 ml fractions were collected. Fractions containing enzymatic activity were collected and dialysed overnight against buffer A. The dialysate was applied to a column (1.5 × 19 cm) of Blue-Sepharose CL-6B (Pharmacia) previously equilibrated with buffer A and the column washed with 1 l of buffer A containing NaCl (0.1 M). The enzyme was eluted with buffer A containing NaCl (0.5 M) and 3 ml fractions were collected. The main active fractions (absorbance at 280 nm above 0.1) were pooled and dialysed overnight against buffer A. This procedure removed most of the haemoglobin from the eluate of the column of DE-52. The dialysate was frozen in aliquots at −70°C, thawed before use and not refrozen. In SDS-polyacrylamide gel electrophoresis about 50% of the protein migrated at 72 kD, the molecular weight reported for the soluble guanylate cyclase subunit of bovine lung (Gerzer *et al.*, 1981). This method is a modified version of that described by Gerzer *et al.* (1981).

Enzyme assay

The activity of the enzyme was monitored by incubating the enzyme for 5 min at 37°C with buffer B at pH 7.4 containing Tris/HCl (50 mM), $MgCl_2$ (5 mM), ethyleneglycol-*bis*-(α-aminoethyl ether)N,N,N,N, tetraacetic acid (EGTA; 10 mM) and guanosine-5′-triphosphate (GTP, 0.2 mM). The reaction was terminated by adding two volumes of EDTA (70 mM; pH 7.4) which chelates Mg^{2+}, an essential cofactor of guanylate cyclase and guanosine 3′:5′-cyclic monophosphate (cyclic GMP) formation measured by a radioimmunoassay kit using [^{3}H]-cyclic GMP (Amersham).

Simultaneous detection of EDRF by bioassay and guanylate cyclase activation

EDRF was measured by bioassay using a cascade of pre-contracted rabbit aortic strips (RbAs) which were denuded of endothelium and superfused with the effluent from a column of EC (Gryglewski *et al.*, 1986a). For the simultaneous detection of EDRF by bioassay and guanylate cyclase activation the effluent of the column was split into two. One half superfused the assay tissues and the other was collected in 20 s fractions in test tubes containing the same volume of buffer B (twice concentrated). The guanylate cyclase was added to the test tube just before the collection of the effluent. After collection of the effluent the reaction was continued for another 10 min at room temperature and terminated as described above.

Detection of EDRF release from a mini-column with guanylate cyclase activation

A mini-column packed with 0.5 ml of beads carrying 2.5–5 × 10^6 cells was perfused at 1 ml min^{-1} with oxygenated (95% O_2/5% CO_2) and warmed (37°C) Krebs solution. The column was immersed in a water bath at 37°C and left to stabilize for 20 min before collecting the first fraction into test tubes as described above. Bradykinin (1–10 pmol) was injected through the mini-column and the effluent collected every 15 s into tubes containing the enzyme preparation. The reaction was continued for 5 min at 37°C and terminated as described above.

Preparation of oxyhaemoglobin

Oxyhaemoglobin was prepared by reduction of bovine haemoglobin with sodium hydrosulphite followed by gel filtration with a prepacked disposable column PD-10 (Pharmacia) previously equilibrated with Tris/HCl (50 mM) at pH 7.4. The concentration of oxyhaemoglobin was determined by a spectrophotometric method (Shimadzu UV-160; E_{576nm} = 15.99 mM^{-1} cm^{-1}).

Preparation of nitric oxide

Nitric oxide solution was prepared by injecting 1 ml of NO gas into 40 ml of helium-deoxygenated water

(de Nucci et al., 1988b). The concentration of NO was calculated assuming that all the NO was dissolved (solubility of NO is 4.6 ml per 100 ml at 20°C).

Materials

Superoxide dismutase (SOD, from bovine erythrocytes) was dissolved in saline before use and kept at room temperature. Sodium arachidonate (AA, from porcine liver) was dissolved in Tris/HCl (50 mM) pH 7.4 and protected from light until used. Bradykinin, SOD, AA, atriopeptin (II) (rat sequence), ADP, sodium nitroprusside, Trizma, sodium hydrosulphite, DL-dithiothreitol, haemoglobin (from bovine blood) and EGTA were obtained from Sigma (Poole, UK). EDTA and NO cylinders were purchased from BDH (Dagenham, UK). GTP (disodium salt) was obtained from Boehringer Mannheim (West Germany) and 3-isobutyl-1-methylxanthine (IBMX) from Aldrich (Poole, UK). U46619 was a gift from Dr J. Pike (Upjohn, Kalamazoo).

Statistics

Results are shown as mean values ± s.e.mean for n experiments. Student's unpaired t test was used to determine the significance of differences between means and a P value of <0.05 was taken as significant.

Results

Simultaneous detection of EDRF by bioassay and cyclic GMP-radioimmunoassay

Bradykinin (3 and 30 pmol) injected through the column (TC) of EC caused release of EDRF, as detected by the relaxation of the RbAs (Figure 1a) and radioimmunoassay for cyclic GMP (Figure 1b). Note that the amounts of cyclic GMP reached a maximum before the relaxation of the bioassay tissues was complete. Glyceryl trinitrate (GTN, 1–10 μM) did not affect the enzyme preparation. Significant correlation ($P < 0.01$) was observed between relaxation of RbA and activation of soluble guanylate cyclase (Figure 2).

Experiments with mini-columns of endothelial cells

Bradykinin (1–10 pmol) injected through mini-columns of EC induced EDRF release in a dose-dependent manner, as detected by stimulation of soluble guanylate cyclase (Figure 3). The amounts of basal and stimulated EDRF release were significantly ($P < 0.05$) increased by infusion of SOD (15 u ml^{-1}) through the column (Figure 3a). Subsequent addition of catalase (100 u ml^{-1}, $n = 3$) did not affect bradykinin-induced release of EDRF (not shown). Oxyhaemoglobin (10 nM) inhibited the stimulation of soluble guanylate cyclase induced by EDRF (Figure 3b).

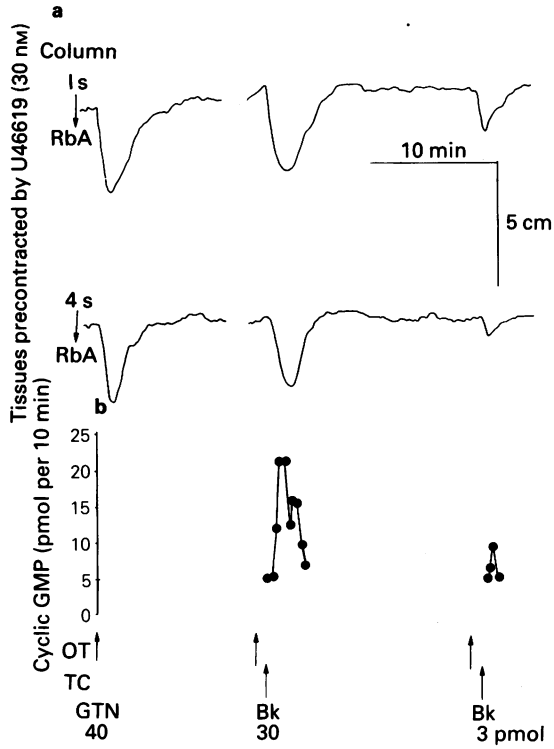

Figure 1 Endothelium-derived relaxing factor (EDRF) release measured by bioassay and guanylate cyclase assay. (a) Relaxation of rabbit aortae (RbAs) after injection of bradykinin (Bk; 3 and 30 pmol) through the column of endothelial cells (EC). EDRF released by Bk (30 pmol) caused relaxation of RbAs equivalent to that of glyceryl trinitrate (GTN, 40 pmol) U46619 = 11,9 epoxymethano-PGH_2 (b): Guanylate cyclase activation. Column effluent (0.88 ml) was collected for 20 s into each test tube containing the same volume of buffer B (twice concentrated) before and after injection of Bk. Guanylate cyclase (17 μg) was added just before collection of the effluent. Superoxide dismutase (10 u ml^{-1}) was infused through the column of EC throughout the experiment. Results of a typical experiment (one of three) are shown. OT = over the assay tissues; TC = through the column of EC.

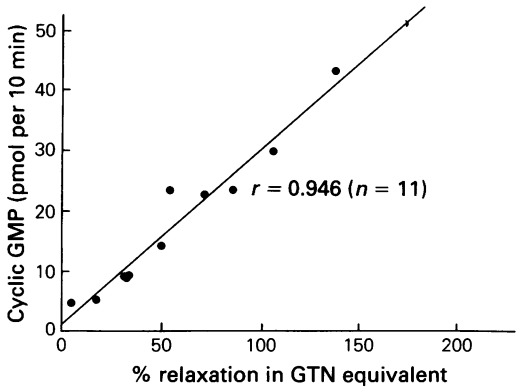

Figure 2 Correlation between bioassay and radioimmunoassay for cyclic GMP as detector methods for endothelium-derived relaxing factor (EDRF) release. The release of EDRF from a column of endothelial cells (EC) was induced by ADP (2 nmol) or bradykinin (3–100 pmol). The experiments were carried out under the same conditions as in Figure 1. The same preparation of soluble guanylate cyclase was used for all determinations. Each point represents assay of EDRF after injections of ADP or bradykinin in 4 experiments. EDRF release was measured on the first rabbit aorta and expressed as equivalents of glyceryl trinitrate (GTN).

Experiments with soluble guanylate cyclase

Nitric oxide (1–2 μM), sodium nitroprusside (SNP, 0.3–100 μM) or arachidonic acid (AA, 3–30 μM) activated soluble guanylate cyclase in the absence of endothelial cells (Figure 4). Superoxide dismutase (15 u ml^{-1}) strongly potentiated the stimulation of the enzyme induced by NO but it was less effective when guanylate cyclase was activated by SNP (Figures 4a and b). However, SOD had little or no effect on AA-induced guanylate cyclase activation (Figure 4c). When all the nine basal values (with no addition of NO, SNP or AA) were compared, there was a significant increase in guanylate cyclase activity in the presence of SOD (15 u ml^{-1}) from 4.4 $\pm$ 0.4 to 5.9 $\pm$ 0.4 pmol per 5 min ($n = 9$, $P < 0.05$). The basal activity of soluble guanylate cyclase was not affected by pre-incubation with IBMX (0.01–1 mM, $n = 3$, results not shown) or rat atrial natriuretic factor (ANF, 10–100 nM, $n = 3$, results not shown).

Oxyhaemoglobin (10–300 nM) prevented stimulation of the enzyme by SNP or NO but not that by AA (Figure 5).

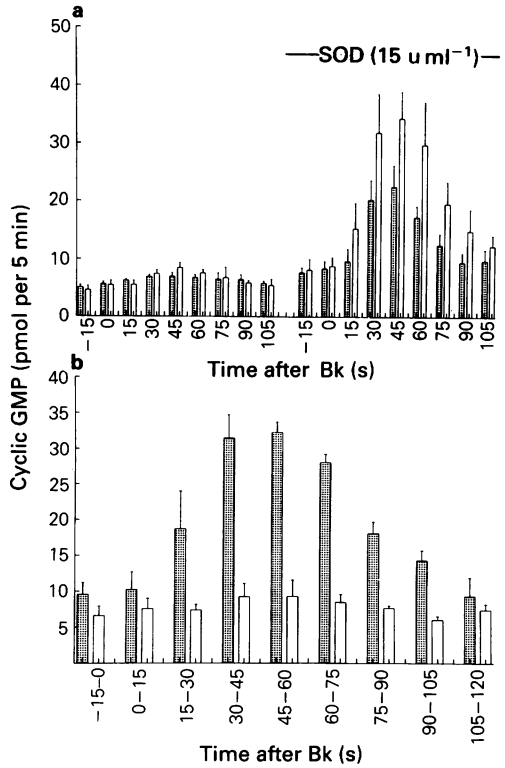

Figure 3 (a) Superoxide dismutase (SOD, 15 u ml^{-1}) increased the activation of guanylate cyclase by the effluent from a mini-column of endothelial cells (EC) stimulated by bradykinin (Bk, 1 or 10 pmol). Ten minutes after the start of infusion of SOD, Bk (1 or 10 pmol) was injected through the column of EC. Fractions (250 μl for 15 s) were collected into each test tube containing 250 μl of buffer B (twice concentrated). Guanylate cyclase (9 μg) was added to each test tube just before collection of the effluent. Each column represents mean with s.e.mean shown by vertical bars ($n = 3$). Stippled column, Bk 1 pmol; open column, Bk 10 pmol. (b) Suppression by oxyhaemoglobin of guanylate cyclase activation by the effluent from a mini-column of EC. Oxyhaemoglobin (10 nM final concentration) was added to each test tube containing 250 μl of buffer B (twice concentrated). Guanylate cyclase (9 μg) was added to the test tube just before collection of the effluent. Samples (250 μl) were collected for 15 s. Superoxide dismutase (15 u ml^{-1}) was infused through the column of EC throughout the experiment. The release of EDRF was induced by Bk (10 pmol). The guanylate cyclase activation after Bk injection was abolished by oxyhaemoglobin. Each column represents mean with s.e.mean shown by vertical bars ($n = 3$). Control, stippled column; with oxyhaemoglobin, open column.

Discussion

We describe here an alternative method using soluble guanylate cyclase for detection of EDRF release from bovine cultured EC. Simultaneous measurement of EDRF release by bioassay and activation of guanylate cyclase showed a good correlation

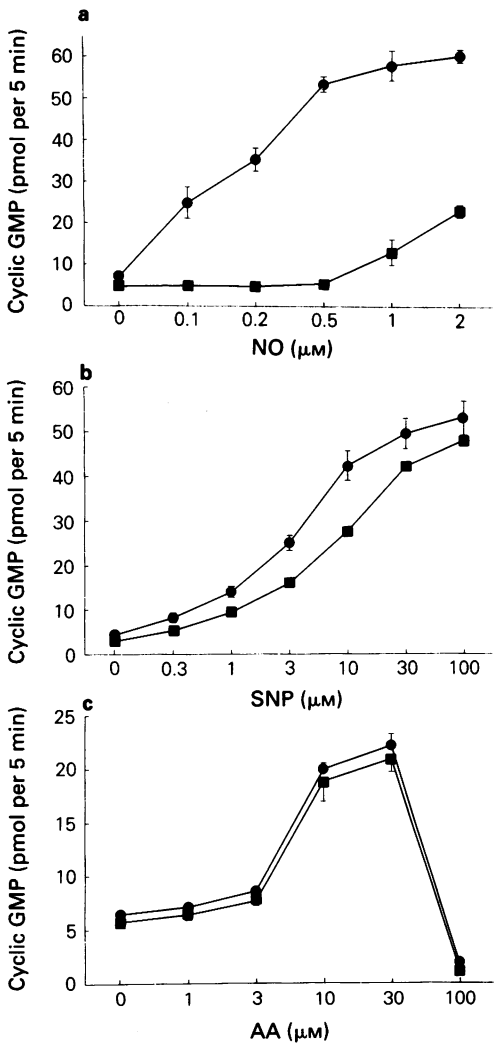

Figure 4 (a) Superoxide dismutase (SOD, 15 u ml^{-1}) strongly potentiated the activation of guanylate cyclase in test tubes by nitric oxide (NO). To imitate the conditions in experiments with columns, 250 μl of oxygenated Krebs solution was added to 235 μl of buffer B (twice concentrated) containing SOD (15 u ml^{-1}) or saline (control). Reaction mixtures were incubated at 37°C for 2 min before addition of 15 μl of guanylate cyclase (9 μg). The reaction was allowed to proceed for 5 min. Nitric oxide was added 10 s after addition of guanylate cyclase. (b) SOD (15 u ml^{-1}) also increased guanylate cyclase activation by sodium nitroprusside (SNP). The reaction was carried out under the same conditions as described in (a). (c) SOD (15 u ml^{-1}) had little or no effect on the activation of guanylate cyclase by arachidonic acid (AA). The reaction was carried out under the same conditions as described in (a). Each point represents mean of 3 determinations with s.e.mean shown by vertical bars. Control (■); SOD 15 u ml^{-1} (●).

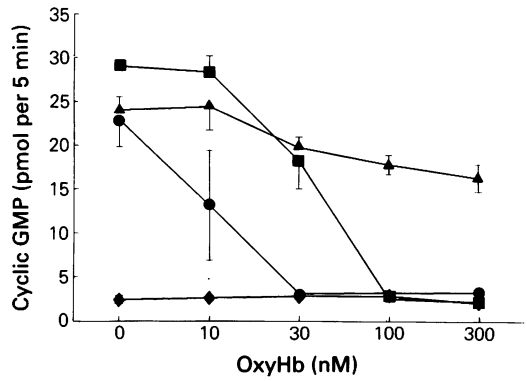

Figure 5 Oxyhaemoglobin (OxyHb) suppressed activation of guanylate cyclase by nitric oxide (1 μM, ●) and sodium nitroprusside (10 μM, ■) but not by arachidonic acid (30 μM, ▲). The reactions were started by the addition of guanylate cyclase (9 μg) to the reaction mixtures as in Figure 4. Oxyhaemoglobin was added 2 min before addition of enzyme. The basal activity of guanylate cyclase is represented by (◆). Each point is the mean of three determinations with s.e.mean shown by vertical bars.

between these two methods. The guanylate cyclase method has several advantages as compared to bioassay using columns of EC. It is sensitive enough to allow the use of smaller numbers of EC (packed in mini-columns). It allows the study of the kinetics of EDRF release, since the biological effect of EDRF on the assay tissues usually outlasts its presence. Some substances (e.g. ANF or GTN) interfere with the baseline and responses of the bioassay tissues and the guanylate cyclase method can be used to study the effect on EDRF release by these substances. Furthermore, the advantage of using several minicolumns at the same time will facilitate the study of biochemical changes occurring in the EC during EDRF release.

Although our enzyme preparation is not homogeneous and only about 50% pure, the lack of effect of IBMX or ANF indicates that there is no contamination by phosphodiesterases or by particulate guanylate cyclase.

Substances other than EDRF or NO which stimulate soluble guanylate cyclase may compromise the specificity of the assay. For instance, AA is released from EC (Moncada *et al.*, 1976) and stimulates guanylate cyclase from platelets (Glass *et al.*, 1977; Gerzer *et al.*, 1983) and spleen (Graff *et al.*, 1978). Thus, it is important to distinguish the guanylate cyclase activation by EDRF from that induced by AA or its metabolites. In our experiments, it is clear that the activation of guanylate cyclase observed following stimulation of EC by bradykinin or ADP is

due to release of EDRF and not of AA, since this activation was strongly potentiated by SOD, which had little or no effect on AA-induced activation of guanylate cyclase. Thus, AA may activate guanylate cyclase by a different mechanism from that exerted by EDRF. At higher concentrations of AA (100 μM) the guanylate cyclase appeared to be denatured as both the stimulated and basal activity were strongly inhibited. Whether the mechanism by which AA stimulates guanylate cyclase is due to metabolites of AA or AA itself is currently under investigation.

Oxyhaemoglobin inhibits NO- and SNP-induced guanylate cyclase activation by oxidising NO to NO_3^- (Doyle & Hoekstra, 1981). The finding that oxyhaemoglobin did not prevent AA-induced activation of guanylate cyclase reinforces the conclusion that a different mechanism of activation of guanylate cyclase is involved.

Superoxide dismutase increased the basal activity of guanylate cyclase, an effect also observed with hepatic guanylate cyclase (Mittal & Murad, 1977). Although this could be a direct effect of SOD, it is more likely to be due to the removal of superoxide anions (O_2^-) present in the Krebs solution (de Nucci *et al.*, 1988b). This is further supported by the finding that although SOD directly stimulated guanylate cyclase, a much stronger potentiation was observed when SOD was infused through the EC. By dismutation of O_2^-, hydrogen peroxide (H_2O_2) should be generated and H_2O_2 activates splenic guanylate cyclase (Graff *et al.*, 1978). However, it is unlikely that H_2O_2 is responsible for guanylate cyclase activation in our experiments, since catalase had no effect when infused through the EC. These data suggest that in our experiments guanylate cyclase activation by the effluent of EC only reflects EDRF release. It is interesting to note that the enzyme has basal activity in the absence of any known stimulator such as NO or AA. The finding that SOD potentiated stimulation of guanylate cyclase by NO far more than that by SNP is probably because NO free in solution is rapidly destroyed by O_2^- (Gryglewski *et al.*, 1986b; Warner *et al.*, 1989) whereas SNP provides a stable carrier for NO.

Comparison of tissue relaxation with guanylate cyclase activation showed that guanylate cyclase activation preceded tissue relaxation. Thus, the method with guanylate cyclase will make it possible to detect biochemical changes in EC during EDRF release more precisely than the bioassay method.

In conclusion, the method with guanylate cyclase will facilitate studies on the effects of vasoactive compounds on EDRF release and allow correlation of the biochemical changes in EC with EDRF release.

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