Nitric oxide synthase in cultured endocardial cells of the pig

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1 Endocardial cells release factors which regulate myocardial contractility and guanosine 3':5'-cyclic monophosphate (cyclic GMP) levels. One of these factors is indistinguishable from endothelium-derived relaxing factor (EDRF).

2 The effluent from pig heart endocardial cells cultured on microcarrier beads caused the relaxation of a pig coronary artery ring denuded of endothelium. This relaxation was enhanced by a combination of superoxide dismutase and catalase and was attenuated by haemoglobin, which binds nitric oxide (NO), and by inhibitors of NO synthase, N^G-monomethyl-L-arginine (L-NMMA) or N^G-nitro-L-arginine.

3 A Ca²⁺-, L-arginine- and NADPH-dependent enzyme activity which generated NO was detected by a specific spectrophotometric assay in cytosol prepared from endocardial cells. The formation of NO was inhibited in a concentration-dependent manner by L-NMMA (but not D-NMMA) and this could be partially reversed upon addition of excess L-arginine.

4 Like endothelial cells from the blood vessels, the endocardial cells possess the ability to synthesize NO, which may act to regulate myocardial contractility.

Keywords: Endocardium; endothelium-derived relaxing factor (EDRF); nitric oxide; cell culture

Introduction

Vascular endothelial cells generate nitric oxide (NO) from the terminal guanidino nitrogen atom(s) of L-arginine (Palmer *et al.*, 1987; 1988a). This NO, which causes the relaxation of vascular smooth muscle (Palmer *et al.*, 1987; Hutchinson *et al.*, 1987) and inhibits platelet aggregation (Radomski *et al.*, 1987; Furlong *et al.*, 1987), explains the biological actions of endothelium-derived relaxing factor (EDRF, Furchgott & Zawadzki, 1980). An NADPH-dependent enzyme, now known as NO synthase, was found in the cytosolic fraction of endothelial cells (Palmer & Moncada, 1989; Mülsch *et al.*, 1989; Mayer *et al.*, 1989). This enzyme catalyses the formation of NO from L-arginine, yielding L-citrulline as the co-product (Palmer & Moncada, 1989). The L-arginine to NO pathway has now been shown to exist in a variety of cells (Moncada & Higgs, 1990) where it serves as the transduction mechanism for the stimulation of the soluble guanylate cyclase (Moncada *et al.*, 1989).

The endocardial cells are ontogenetically similar to the endothelium. Analogous to the role of the endothelium in the regulation of vascular tone, it has been shown that the endocardium plays a role in modulating the contractility of cardiac muscle (Lewis *et al.*, 1990; Smith *et al.*, 1991). Cultured endocardial cells release a factor indistinguishable from EDRF as well as a factor(s), putatively termed endocardin, which exerts a positive inotropic action in isolated papillary muscle segments selectively denuded of endocardium (Smith *et al.*, 1991). In the present study we have identified and partially characterized a NO synthesizing enzyme present in the cytosol of cultured endocardial cells.

Methods

Endocardial cell culture

Endocardial cells were isolated from freshly obtained pig hearts as previously described (Smith *et al.*, 1991). Briefly, cells were harvested by exposure of the right ventricle to 0.2% collagenase in medium E199 and cultured in complete medium (medium E199 supplemented with 10% foetal bovine serum, 10% newborn bovine serum, glutamine (6 mM), benzyl-

penicillin (200 u ml⁻¹), streptomycin (200 µg ml⁻¹), and kanamycin (100 µg ml⁻¹) in fibronectin-coated 75 cm² plastic tissue culture flasks. Flasks were coated with fibronectin by exposure to a sterile solution of fibronectin (10 µg ml⁻¹) in 10 mM HEPES (pH 7.3) for 1 h. Confluent cells were subcultured by brief exposure (3–5 min) to a 0.85% saline solution containing 0.05% trypsin and 0.02% EDTA. Newborn calf serum was added to inactivate the trypsin and the cell suspension was centrifuged (200 *g* for 5 min) and resuspended in complete medium. Cells used in this study were either primary cultures or those in first passage, exhibited cobblestone morphology, and incorporated fluorescent labelled acetylated low density lipoprotein (Smith *et al.*, 1991).

Determination of EDRF release by bioassay

Endocardial cells were grown on microcarrier culture beads as described (Gryglewski *et al.*, 1986a; Smith *et al.*, 1991); 0.5–0.8 ml of cell-covered beads (approximately 7.5 × 10⁶ cells ml⁻¹ beads) were washed in saline and placed in a 2 ml polypropylene syringe and superfused at a flow rate of 3.5 ml min⁻¹ with oxygenated Krebs-Ringer solution at 35°C containing indomethacin (1 µM). The effluent of the cell column was used to superfuse an endothelium-denuded pig coronary artery ring preparation mounted as previously described (Christie & Lewis, 1988). The ring was preconstricted submaximally with prostaglandin F_{2α} (1 µM) and 5-hydroxytryptamine (5-HT, 1 µM) infused distal to the cells and changes in isometric tension were recorded.

Preparation of endocardial cytosol

Culture medium was removed and the cells were washed three times with Dulbecco's phosphate buffered saline (PBS). Cells were removed from the flasks by trypsinization, placed in an equal volume of culture medium and centrifuged (200 *g* for 10 min). The cell pellet was washed twice with PBS before resuspending the cells at a concentration of approximately 30–70 × 10⁶ cells ml⁻¹ in ice-cold homogenization buffer containing 320 mM sucrose, 10 mM HEPES, 0.1 mM EDTA, 1 mM DL-dithiothreitol, 100 µg ml⁻¹ phenylmethylsulphonyl fluoride, 10 µg ml⁻¹ leupeptin, 10 µg ml⁻¹ soybean trypsin inhibitor, and 2 µg ml⁻¹ aprotinin (adjusted to pH 7.2 at 20°C with HCl). The cells were homogenized by sonicating twice for 3 s at 100 W with an ultrasonic probe (Soniprep, MSE). The homogenate was centrifuged at 150,000 *g* for 35 min at 4°C

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and the supernatant was incubated with cation-exchange resin (AG 50W-X8) for 5 min at 4°C to deplete endogenous L-arginine. Upon settling of the resin, the supernatant was kept on ice and used within 2 h for determination of NO synthase activity.

Spectrophotometric determination of nitric oxide synthase activity

The formation of NO in cytosol was measured spectrophotometrically by the method of Feelisch & Noack (1987) based upon the rapid and stoichiometric reaction of NO with oxyhaemoglobin to yield methaemoglobin and nitrate. Cytosol was added in a 1:1 ratio (vol/vol) to the assay buffer (50 mM phosphate buffer, 1 mM MgCl₂, 10 μ M oxyhaemoglobin, pH 7.2) at 37°C into a prewarmed cuvette. L-Arginine (30 μ M), CaCl₂ (200 μ M) and NADPH (100 μ M) were added to initiate the reaction and the change in the difference in the absorbance at 401 and 421 nm (extinction coefficient 77,200 M⁻¹ cm⁻¹) was monitored at 37°C with a double beam dual wavelength spectrophotometer (Shimadzu UV-3000) at a band width of 5 nm. The rate of NO synthesis was linear for 10 min. The Ca²⁺-dependency of NO synthase was assayed in the presence of 1 mM EGTA and free Ca²⁺ was controlled by the addition of CaCl₂ solution to the reaction mixture (Knowles *et al.*, 1989). The protein content of the cytosol was determined with bicinchoninic acid protein assay reagent, using bovine serum albumin as a standard.

Chemicals

Cell culture materials (Flow and Sigma), N^G-monomethyl-L-arginine (L-NMMA) and D-NMMA acetate salts (Wellcome), cation exchange resin (AG 50W-X8, 200–400 mesh; Bio-Rad), bicinchoninic acid protein assay reagent (Pierce) and NADPH tetrasodium salt (Boehringer Mannheim) were obtained as indicated. Human haemoglobin was prepared as described (Paterson *et al.*, 1976). All other reagents were from BDH or Sigma.

Statistics

All values are expressed as mean $\pm$ s.e.mean of *n* experiments and were compared by Student's *t* test for paired or unpaired data, as appropriate, with *P* < 0.05 considered as statistically significant.

Results

Detection of EDRF by bioassay

Effluent from the perfused column of endocardial cells contained a diffusible factor which induced a relaxation of the bioassay detector tissue. This relaxation, measured as the percentage decrease of the initial tension, was enhanced in the presence of superoxide dismutase and catalase (73.9 $\pm$ 14.2 versus 36.9 $\pm$ 9.4%; *P* < 0.01; *n* = 8) and was completely abolished in the presence of haemoglobin (0.5 μ M, *n* = 6) (Figure 1). Furthermore, the relaxation to the effluent from the cell column was completely reversed by addition of inhibitors of NO synthase, L-NMMA (50 μ M, *n* = 12), or N^G-nitro-L-arginine (20 μ M, *n* = 4) to the solution perfusing the cell column (data not shown).

Detection of nitric oxide by spectrophotometry

Addition of L-arginine (0.3–30 μ M) to the cytosol, in the presence of NADPH (100 μ M) and CaCl₂ (200 μ M), caused a concentration-dependent increase in the rate of formation of NO to a maximum at 30 μ M of 5.67 $\pm$ 0.80 pmol min⁻¹ mg⁻¹ protein (*n* = 3; data not shown). In the absence of L-arginine or NADPH, or if D-arginine (30 μ M) was substituted for L-arginine, the rate of NO synthesis was below the limit of detection (<0.1 pmol min⁻¹ mg⁻¹ protein; *n* = 3 for each).

Figure 1 Bioassay of endothelium-derived relaxing factor (EDRF) release from a perfused column of cultured endocardial cells, recorded as the change in tension of the detector tissue, a preconstricted ring of coronary artery of the pig denuded of endothelium. Exposure to the effluent from the endocardial cells caused the detector tissue to relax. This effect was enhanced by superoxide dismutase (SOD, 30 u ml⁻¹) and catalase (CAT, 100 u ml⁻¹) infused through the column (t.c.) and was reversed by oxyhaemoglobin (Hb, 0.5 μ M) infused over the bioassay tissue (o.t.). The tracing was redrawn and shortened by a 20 min period, represented by the double bar, and is representative of 6 experiments.

In the presence of L-arginine (30 μ M), the synthesis of NO was inhibited in a concentration-dependent manner by L-NMMA (*n* = 3; Figure 2). L-NMMA (30 μ M) abolished the formation of NO, an effect that was partially reversible upon addition of a ten fold molar excess of L-arginine (300 μ M; *n* = 3). In the presence of D-NMMA (30 μ M) there was a slight reduction in the rate of NO synthesis from 3.74 $\pm$ 0.72 to 2.92 $\pm$ 0.81 pmol min⁻¹ mg⁻¹ protein (*n* = 3, data not shown) which was not statistically significant.

In the absence of free Ca²⁺ (1 mM EGTA), there was no measurable formation of NO by endocardial cell cytosol (*n* = 3). The addition of Ca²⁺ (resultant free Ca²⁺ concentration 0.1–71 μ M) resulted in a concentration-dependent rise in the rate of NO synthesis with an EC₅₀ value of 0.65 $\pm$ 0.10 μ M (*n* = 3; Figure 3).

Discussion

We have demonstrated the release from endocardial cells of a factor with the characteristics of EDRF. It caused the relax-

Figure 2 Effect of N^G-monomethyl-L-arginine (L-NMMA) on the formation of NO in endocardial cell cytosol. Addition of L-arginine (L-Arg, 30 μ M) initiated the formation of NO which was inhibited by L-NMMA (3 and 30 μ M). The formation of NO by L-Arg (30 μ M) was abolished with L-NMMA (30 μ M) and could be partially reversed by addition of a ten fold molar excess of L-Arg (300 μ M). Each column is the mean of 3 separate experiments; vertical bars show s.e.mean.

Figure 3 Ca^{2+} -dependence of NO synthesis in endocardial cell cytosol. The rate of synthesis of NO from L-arginine ($30 \mu\text{M}$) at free Ca^{2+} concentrations defined by Ca^{2+} /EGTA buffers is shown. Each point is the mean of 3 separate experiments; vertical bars show s.e.mean.

ation of vascular smooth muscle (Furchgott & Zawadzki, 1980) and its effect was potentiated by superoxide dismutase which prevents the breakdown of EDRF by superoxide anion (Gryglewski *et al.*, 1986b), and inhibited by haemoglobin (Martin *et al.*, 1985), which avidly binds NO (Gibson & Roughton, 1957). Furthermore the release of EDRF from endocardial cells was inhibited by L-NMMA and N^G -nitro-L-arginine (Palmer *et al.*, 1988b; Rees *et al.*, 1990a; Moore *et al.*, 1990), specific inhibitors of the NO synthase. It has also been demonstrated that substance P provokes an endocardium-dependent increase in guanosine 3':5'-cyclic monophosphate (cyclic GMP) levels in isolated papillary muscles of the ferret which is abolished by haemoglobin (Smith *et al.*, 1991). These results suggest that endocardial cells have the ability to synthesize NO, which accounts for the biological actions of EDRF (Palmer *et al.*, 1987).

Using a spectrophotometric assay specific for the detection of NO (Feelisch & Noack, 1987), we have now demonstrated that the cytosol of endocardial cells contains a Ca^{2+} -, L-arginine- and NADPH-dependent enzyme which synthesizes NO. The formation of NO in the cytosol was inhibited in an enantiomeric specific fashion by L-NMMA, but not D-NMMA, an effect which was reversible upon addition of excess L-arginine. These results, along with the strict Ca^{2+} -dependency of NO synthesis in the cytosol, suggest that the enzyme present in endocardial cells is similar to that found in endothelial cells (Palmer & Moncada, 1989; Mülsch *et al.*, 1989; Mayer *et al.*, 1989; Rees *et al.*, 1990a), the brain (Knowles *et al.*, 1989; Bredt & Snyder, 1990), platelets (Radomski *et al.*, 1990a) and the adrenal gland (Palacios *et al.*, 1989).

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