Brief Communications

Functional and Autoradiographic Evidence for Endothelin 1 Receptors on Human and Rat Cardiac Myocytes
Comparison With Single Smooth Muscle Cells


This study aimed to determine whether receptors for endothelin were present on the cardiac myocyte as well as on vascular smooth muscle cells. Low- and high-resolution autoradiography was performed using 125I-endothelin 1 on intact rat myocardium and samples of human ventricle obtained from explanted hearts at the time of transplant. In addition to specific binding to the smooth muscle of the blood vessel lumen, there was considerable binding associated with cardiac myocytes. To discover whether there was any functional correlate for this binding, muscle cells were isolated enzymatically from human and rat ventricle and from rat femoral artery, and their contractile characteristics were studied. Single cardiac cells were superfused with physiological saline at 32°C, and their length change was displayed continuously on a chart recorder. Endothelin 1 had a pronounced effect on shortening in both rat and human myocytes. The contraction amplitude was approximately doubled in both cases, from 4.1±0.8% cell length to 8.1±1.3% for rat (mean±SEM, n=9, p < 0.001), and from 2.1±0.5% to 4.0±0.5% in human (n=10, p < 0.001). In rat, the magnitude of the effect was comparable to that of the α-adrenoceptor agonist phenylephrine. The maximum contraction amplitude of the human cells, produced by raising extracellular calcium to greater than 10 mM, was 11.4±1.1% cell length (n=9), significantly greater than that produced by endothelin (p < 0.001). The threshold for the effect of endothelin 1 was around 0.3 nM, and maximum effects were attained at 30 nM, compared with 1 and 100 μM, respectively, for the α-adrenoceptor effect. Endothelin 1 had a potent action on single vascular smooth muscle cells. The EC50 for endothelin 1 was 36±4 pM (n=8) compared with 3.0±1.6 μM (n=7) for phenylephrine. Maximum contraction was attained by depolarization with 80 mM KCl, and was 26±3% (n=11) of resting length. The response to endothelin 1 in single smooth muscle cells was quantitatively similar to the response to phenylephrine (85±9% of KCl contracture [n=12] versus 83±7% [n=7] for phenylephrine). We conclude that endothelin 1 binding has a significant functional correlate in the direct effects on cardiac myocyte contraction for both rat and human, but that concentrations required for cardiac effects are greater than those for smooth muscle activation. (Circulation Research 1990;67:764–769)

Endothelin, a 21-residue peptide isolated from the supernatant of cultured porcine endothelial cells, has been shown to have powerful effects on smooth muscle. The presence of 125I-endothelin binding sites has been demonstrated in human coronary vessels using in vitro autoradiography. This binding is associated mainly with the smooth muscle, with some binding to perivascular structures. There is recent evidence that, apart from its constrictor action, endothelin has both positive inotropic and chronotropic effects on the heart. The purpose of this study was to use high- and low-resolution autoradiography to determine whether 125I-endothelin 1 binding sites are present on cardiomyocytes and to establish the functional relevance of such sites using isolated myocytes prepared from human and rat heart. The contractile properties of...
cardiac muscle cells exposed to endothelin 1 were compared with those of single arterial wall smooth muscle cells. The effects of α-adrenoceptor agonists, which also combine vasoconstrictor and positive inotropic actions, were determined in parallel with those of endothelin 1 on both cell types.

Methods

In Vitro Autoradiography

The in vitro autoradiographic technique used for this study was essentially that described by Young and Kuhar, with modifications for vascular tissue. Ventricular tissue, obtained from patients undergoing heart transplantation (n = 7), was frozen in isopentane or on solid CO₂ (Distillers Mg. Ltd., Surrey, U.K.) and stored at −70°C. Hearts were removed from male rats (200 g) killed by cervical dislocation and were frozen on solid CO₂. Ten- and 20-μm frozen sections (48–144 per rat or patient) were cut in a cryostat at −20°C, thaw-mounted onto gelatinized microscope slides, and stored at −20°C until incubations were carried out. Tissue was preincubated for 15 minutes at 20°C in 50 mM Tris buffer, pH 7.4, containing 0.2% bovine serum albumin (BDH, Poole, U.K.), 100 kalikrein inhibiting units/ml aprotinin (Sigma Chemical Co., Poole, U.K.), and 5 mM MgCl₂ to reduce levels of endogenous peptide. After preincubation, tissue was transferred to buffer containing 20–50 pM [³¹²]endothelin 1 (specific activity, 1,800 Ci/mmol, Amersham Int., Amersham, U.K.). The degree of nonspecific binding was established by incubating paired slides as above, but in the presence of 500 nM unlabeled endothelin 1 (Bachem Fine Chemicals, Essex, U.K.). Sections were then washed twice for 5 minutes at 4°C in buffer before being blotted and dried in a stream of cold air. After these incubation conditions, both high- and low-resolution autoradiography were performed.

Low-Resolution Studies

Slide-mounted sections (20 μm) were placed in 24×30 cm radiographic cassettes and apposed to Hyperfilm 3H (Amersham) for 15 to 21 days, stored at 4°C. Films were developed in D19 (Kodak, Middlesex, U.K.; undiluted) for 5 minutes at 20°C, fixed in IF 23 (Ilford, Cheshire, U.K.; diluted 1:4 with glass-distilled water) for 5 minutes at 20°C, washed for at least 20 minutes in running tap water, and dried at room temperature. Autoradiographs were then examined and photographed on a Nikon macro system.

High-Resolution Studies

Slide-mounted sections (10 μm) were postfixed by exposure to paraformaldehyde vapor (80°C for 2 hours) in an evacuated desiccator. Sections were then defatted by two Histoclear (National Diagnostics, Inc., Bucks, U.K.) washes and a series of aqueous ethanol (100–70%) washes followed by washing in glass-distilled water. Slides were then dipped individually into molten nuclear emulsion (Ilford, K2 diluted 1:1 with 2% glycerol in glass-distilled water) and allowed to dry for several hours at room temperature. Slides were stored in lightproof boxes containing silica gel for 10–21 days at 4°C before being developed in D19 (Kodak, undiluted) for 5 minutes at 20°C, dipped in 1% acetic acid, and fixed in HYPAM (Ilford, diluted 1:4 with glass-distilled water) for 2 minutes. Sections were then washed in four changes of glass-distilled water for 30 minutes, stained with Mayer’s hematoxylin, and coverslipped. Sections and autoradiographs were viewed under dark and bright field illumination on an Olympus Vanox microscope and photographed where appropriate. In some instances, tissue used for low-resolution autoradiography (i.e., that exposed to Hyperfilm) was postfixed and reprocessed for high-resolution autoradiography as described above.

Isolation of Cardiac Myocytes

Myocytes were isolated from Langendorff-perfused rat heart as previously described. Tissue was obtained from explanted human heart at the time of transplant, cut into chunks of approximately 1 mm³ usingrazors, and shaken in 25–50 ml of a low-calcium medium composed of (mM) NaCl 120, KCl 5.4, MgSO₄ 5, pyruvate 5, glucose 20, taurine 20, HEPES 10, and nitriilotriacetic acid 5, pH 6.95 (measured calcium, 2.5–3 μM), at 35°C for 12 minutes. The solution was bubbled with 100% O₂ and was changed at 3-minute intervals. The chunks were drained and transferred to 10–15 ml low-calcium solution with nitriilotriacetic acid omitted and 50 μM calcium added, containing protease (Sigma type XXIV, 4 IU/ml), and were shaken gently for 45 minutes under a 100% O₂ atmosphere. The enzyme was then exchanged for collagenase (BCL, Lewes, U.K.; 400 IU/ml) at the same concentration. Ventricular cells were collected after 45 minutes of this second digestion. The contraction amplitude of the myocytes, superfused with Krebs-Henseleit solution containing 1.3 mM calcium at 32°C and electrically stimulated (0.5 Hz, rat; 0.2 Hz, human), was measured as previously described. Concentration-response curves to human or porcine endothelin 1, phenylephrine (in the presence of 10 μM propranolol to block β-adrenoceptor effects), or calcium were performed cumulatively until responses were stable or toxic effects occurred.

Isolation of Smooth Muscle Cells

Heparinized (5,000 IU/kg) male Sprague-Dawley rats were killed by cervical dislocation, and both femoral arteries (combined wet weight, 10–20 mg) were dissected free of fatty connective tissue and cut into approximately 1-mm² pieces with a razor blade. Smooth muscle cell isolation procedures were all performed at 36°C and under a 100% O₂ atmosphere. The pieces were transferred to 15 ml low-calcium solution for a total of 12 minutes with a change of solution after 3 minutes. Excess solution was aspirated and the tissue shaken gently in silanized tubes containing 1 mg/ml CLS II collagenase (Worthington...
Biochemical Corp., Freehold, N.J.) dissolved in enzyme solution (as above, but with nitrotriacetic acid omitted, pH 7.4, containing 235 μM Ca²⁺). After 1 hour, the collagenase solution was renewed for a further 30-minute incubation. Cells were dispersed into the collagenase solution by gentle trituration with a 1-mm–diameter bore plastic Pasteur pipette and bovine serum albumin (predialyzed against enzyme solution) added to 2 g/100 ml. Warmed enzyme solution was added to 10 ml, and cells were sieved through 300-μm nylon mesh into plastic centrifuge tubes containing 1 ml silicone oil (BDH DCFS-1265), which were spun in a Sorvall RT 6000B bench centrifuge (Huntingdon, U.K.) at 37 g for 5 minutes. The supernatant was replaced with fresh solution, and the cells were resedimented (conditions as above). Cells were gently resuspended in 1 ml supernatant, stored in sealed tubes at room temperature, and used within 5 hours of isolation. Cells were allowed to settle (10 minutes) in a glass-bottomed perspex chamber 10×4×6.25 mm (length by width by depth) on the stage of a Zeiss 1M inverted microscope (Carl Zeiss, Inc., Oberkochen, FRG) and were superfused with 2.5 mM Ca²⁺ Tyrode’s solution composed of (mM) NaCl 150, KCl 5.4, MgCl₂ 1.2, HEPES 5, glucose 10, pH 7.4, at 36°C and 2 ml/min. Cumulative concentrations of agonist (phenylephrine or endothelin 1) were added for 10-minute equilibration periods until maximal cell shortening was observed. This protocol was devised because isolated smooth muscle cells show incomplete relaxation on washout of contractile agents, and because endothelin responses in other tissues are only reversed by prolonged washing. Finally, cells were challenged with high-potassium Tyrode’s solution (as above, except containing 5.4 mM NaCl and 80 mM KCl). Experiments were recorded using a video camera and VCR coupled to the inverted microscope. Hard copies were obtained of live or recorded images with a video printer (Sony Corp., Japan), and cell length was measured with a computer-linked digitizing tablet. A range of cell lengths was obtained from each preparation, from which the most relaxed cells were chosen. For standardization of data, agonist-induced responses were either normalized to the maximum response to that agonist or expressed as a percentage of the response to KCl depolarization in the same cell.

Statistics

Data are expressed as mean±SEM. Comparisons were made on grouped or paired data using the Student’s t test, where n is the number of cells. Number of animals or patients is indicated where relevant.

Results

Autoradiography

There was dense binding of ¹²⁵I–endothelin 1 to the human ventricular myocardium, which was displaced by approximately 80% in those sections incubated in the presence of 500 nM unlabeled endothelin 1 (Figure 1). The pattern of binding was similar in all the sections of the human tissue studied (48–144 sections per patient). Autoradiography carried out on longitudinal sections of rat heart (n=5, 48–144 sections per animal) revealed similar displaceable ¹²⁵I–endothelin 1 binding to the myocardium, with a
higher density associated with atrial tissue. High-resolution autoradiographs of human ventricular tissue revealed that specific binding sites were associated with intramyocardial vessels (diameters approximately 20–200 μm) and myocytes (Figure 2). A similar pattern was seen with rat myocardium (results not shown).

Isolated Cardiac Myocytes

Myocytes from rat heart had an average length of 119±3.1 μm and sarcomere length of 1.84±0.02 μm (n=16, eight animals), compared with 127±10 μm (n=10) and 1.79±0.03 μm (n=9), respectively, for human cells (four patients). The contraction amplitude of rhythmically contracting cells was expressed as percent cell shortening with each beat. Addition of a maximally effective concentration of endothelin 1 doubled the contraction amplitude of both rat and human myocytes in 1.3 mM calcium (Figure 3). Each of the nine rat cells responded to endothelin with an increase in contraction amplitude, as did eight of the 10 human cells. It should be emphasized that the nonresponding cells were included in all statistical analyses. The distribution of data is consistent with the nonresponding cells representing the extreme of a normal distribution and falling below the limits of detection, rather than representing a separate population. The average increase in amplitude was similar to that produced in rat cells by the α-adrenoceptor agonist phenylephrine in the presence of propranolol (10 μM). The threshold concentration for the effect of endothelin 1 in rat was 0.3 nM, and the maximum was attained at around 30 nM. Corresponding values for phenylephrine were 1 and 100 μM. Maximum shortening in these cells, obtained by increasing the calcium concentration of the bathing solution to 10 mM or more, was 11.4±1.1% in human (n=10) and 16.3±0.7% (n=15) in rat, significantly greater than that produced by either endothelin 1 or phenylephrine (p<0.001). Because of limitations of resolution, it was not possible to obtain accurate EC50 values for either endothelin 1 or phenylephrine in cardiac myocytes. Only left ventricular cells from rat were tested, but both left and right ventricular cells from human tissue responded to endothelin 1 with increases in contraction amplitude of a similar magnitude.
Neither endothelin 1 nor phenylephrine increased the normalized velocity of contraction and relaxation significantly (results not shown). This is in contrast to isoproterenol, which accelerates the change in length with each beat. Reversal of the effect of endothelin 1 was slow; in four rat cells, $t_{1/2}$ for washout of endothelin 1 was $32.3\pm11.4$ minutes compared with $0.53\pm0.13$ minutes ($n=3$) for reversal of the effects of maximum calcium.

**Isolated Smooth Muscle Cells**

Concentration-response curves to phenylephrine and endothelin 1 (six animals for each) are shown in Figure 4. EC$_{50}$ values were $3.0\pm1.6$ μM (phenylephrine, $n=7$) and $36\pm4$ pM (endothelin 1, $n=8$). The maximum decrease in resting length with endothelin 1 was $15\pm3\%$ ($n=12$). Maximum contraction was attained by depolarization with 80 mM KCl and was $26\pm3\%$ ($n=11$) of resting length. The response to endothelin 1 in single smooth muscle cells was quantitatively similar to the response to phenylephrine (85±9% of KCl contracture, $n=12$ versus $83\pm7\%, n=7$ for phenylephrine). There was no occasion on which a smooth muscle cell failed to respond to endothelin but subsequently contracted with 80 mM KCl.

**Discussion**

Various studies have demonstrated binding of 125I-endothelin 1 that is displaceable by unlabeled endothelin but not by other vasoactive peptides, suggesting the existence of specific endothelin receptors in these tissues. The low-resolution autoradiographic data from this study revealed a dense, displaceable binding of 125I-endothelin to both human and rat myocardium. High-resolution autoradiographs showed a variable distribution of 125I-endothelin binding sites within myocardial tissue. We have previously reported dense endothelin binding to human epicardial vessels, but the present results revealed strong binding also to small-diameter (20–200 μm) intramyocardial vessels. This binding correlates well with function, endothelin being a potent vasoconstrictor in the blood vessels so far tested.

Endothelin also produced decreases in length of the isolated smooth muscle cells in this study at concentrations as low as 10 pM. The extreme sensitivity of these cells to endothelin suggests that the potency in intact smooth muscle is due to receptors on the muscle cell and not to an endothelium-dependent modulation of endothelin's effects.

The pattern of 125I-endothelin binding also indicated that a large proportion of binding to the myocardium was associated with cardiac myocytes. Recent reports suggest that endothelin has a positive inotropic effect on whole atrial and ventricular muscle. Cultures of neonatal rat myocytes have been shown to have specific endothelin binding sites and to respond to endothelin with an increased calcium transient, but no contractile studies were performed. The studies reported here on isolated ventricular myocytes confirm the inotropic action of endothelin on cardiac muscle cells.
muscle and show that, as with the smooth muscle, the effect is not endothelium dependent.

The percentage changes in contraction amplitude induced by endothelin in the single cardiac cells are considerably greater than those of tension in whole muscle. Amplitude was doubled in both rat and human cells, whereas the reported increases in papillary muscle tension were only 17.1% and 9.9%, respectively. Concentrations used were similar—50 nM on whole muscle compared with the 30 nM maximum in this study. It has been noted that removal of endothelin can increase the apparent effect of endothelin in rabbit femoral arteries, suggesting that the endothelium may form a barrier to the passage of exogenously added endothelin in isolated whole muscle preparations. It may be that the sheath of endothelium surrounding papillary muscles also limits the action of endothelin, causing the discrepancy in effectiveness between cells and whole muscle. Alternatively, the sheath of endothelium may be releasing endothelin tonically, obscuring the effect of the exogenous addition. The differences could also be due to the fact that the whole muscle was contracting isometrically, whereas the single cells were unloaded.

The response of rat myocytes to endothelin was similar in magnitude to that produced by the α-adrenoceptor agonist, phenylephrine, and considerably smaller than that to high calcium. We have previously shown that β-adrenoceptor agonists produce maximum contraction amplitudes approximately equivalent to those of calcium in rat, rabbit, and human myocytes. Endothelin and phenylephrine are also alike in that they do not accelerate shortening or relaxation. In contrast, β-adrenoceptor agonists speed these parameters in both rat and human cells. These results are consistent with suggestions that both endothelin and phenylephrine produce their results through an increase in inositol trisphosphate rather than cyclic AMP.

Is the positive inotropic effect of endothelin likely to contribute significantly to heart force under physiological conditions? Endothelin does not begin to have an effect on the cardiac myocyte until a concentration of 0.3 nM is reached, 10-fold higher than its EC50 on vascular muscle. It is unknown whether sufficiently high levels would be reached in the vicinity of the cardiac muscle cells in vivo. Furthermore, the magnitude of the effect of endothelin is small. However, it has been suggested that the similarly sized effect of α-agonists can become important in maintaining heart force when β-adrenoceptors are desensitized. Endothelin may therefore make a contribution to heart force under circumstances in which other inotropic mechanisms are inoperative. It is possible that the small, irreversible decrease in force that occurs when isolated papillary muscle endothelium is selectively damaged may be due to a reduction in the endothelin concentration at the cardiac myocyte.

References

**Key Words** • endothelin • isolated smooth muscle cells • cardiomyocytes • autoradiography
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