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DETERMINATION OF IMMUNOREACTIVE ENDOTHELIN IN MEDIUM FROM CULTURED ENDOTHELIAL CELLS AND HUMAN PLASMA

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We have developed a sensitive and selective radioimmunoassay for porcine/human endothelin (ET_1). The assay has a detection limit of 0.62 pg/tube and exhibits no cross-reactivity to atrial natriuretic peptide, arginine vasopressin, or angiotensin II. Procedures were developed for extraction of endothelin from human plasma samples and samples of buffer from endothelial cell incubations using C_{18} Sep-Pak extraction cartridges. The mean recovery following extraction was approximately 80%. Both bovine and porcine aortic endothelial cells were found to produce immunoreactive endothelin (IR-ET) with porcine cells producing 4.7 ± 1.1 ng of IR-ET/mg cell protein after 6 hours. Human plasma samples were extracted, assayed and found to contain a mean concentration of 2.0 ± 0.4 pg/ml of IR-ET.

Recently, it has been found that endothelial cells synthesize and release endothelin, a potent vasoactive peptide (1,2) This peptide constricts coronary artery strips and renal arterioles (3,4), stimulates atrial natriuretic peptide (ANP) release (5), increases systemic blood pressure (1,4), inhibits renin release (6,7) and is thought to be important in regulating systemic and/or local blood flow. While the mechanisms of action of endothelin are not completely clear, it appears that it binds to a specific receptor on smooth muscle cells, activates phospholipase C and elevates intracellular Ca^{2+} levels (3,8-10). Interestingly, we have found that the endothelin-stimulated, sustained elevation in intracellular Ca^{2+} is regulated by an interaction between Ca^{2+} influx through the voltage sensitive Ca^{2+} channel and prolonged inositol phosphate formation (10).

Very little data exists regarding the relationship between endothelin levels in vivo and vascular function partly due to the lack of simple, reliable, quantitative techniques for quantitating this peptide. Furthermore, understanding mechanisms which regulate endothelin synthesis by endothelial cells also depends on reliable assay procedures. In both cases very low levels of endothelin present a major analytical problem. The aim of the current report is to describe a simple sensitive radioimmunoassay for endothelin which can

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be applied to both media from cultured cells and human plasma. In addition, we have developed procedures to extract and concentrate endothelin from samples prior to analysis.

MATERIALS AND METHODS

Materials. Synthetic endothelin and endothelin antiserum (porcine, human) were purchased from Peninsula Laboratories, Inc. (Belmont, CA). C_{18} μBondapak HPLC columns and Sep-Pak C_{18} cartridges were from Waters, Inc. (Milford, Mass.). Iodo-gen was purchased from Pierce (Rockfod, IL).

Endothelin iodination and purification. Synthetic endothelin was iodinated with ^{125}I using Iodo-gen. To accomplish this, 3.0 μg of endothelin, 60 μl of 0.25 M sodium phosphate (pH 7.4) and 1 mCi of $Na^{125}I$ (New England Nuclear) were added to a tube precoated with 3 μg of Iodo-gen. Following a 10 min incubation, the reaction mixture was directly loaded on a C_{18} reverse phase HPLC column (0.39 x 30 cm μBondapak) which had been equilibrated with 20% acetonitrile in 0.1% trifluoroacetic acid. The bound ^{125}I-endothelin was eluted with a linear gradient of 20-70% acetonitrile in 0.1% trifluoroacetic acid over 30 minutes and collected at a flow rate of 1 ml/min/fraction. Aliquots of each fraction were counted in a scintillation counter and assayed by RIA to determine the specific binding activity. The fraction containing the highest binding activity was dried and stored at -20°C.

Endothelin radioimmunoassay (RIA). Rabbit antisera specific for endothelin was diluted with buffer A containing 0.15 M NaCl, 0.02 M sodium phosphate, 0.01% BSA, 0.1% gelatin and 0.01% thimerosal (pH 7.4). ^{125}I-ET, cold ET and unknown sample were dissolved in buffer B (buffer A plus 0.1% Triton X-100), ^{125}I-ET (9,000 cpm) was mixed with endothelin antiserum (final dilution, 60,000) and unlabelled endothelin (or unknown samples) in a final volume of 250 μl. Binding of ^{125}I-ET in the absence of any added unlabeled ET (Bo) was also determined. The mixtures were incubated at 4°C for 20-24 hours. Bound and free ^{125}I-ET was separated by the addition of 0.25 ml charcoal solution (buffer A plus 2.5% charcoal and 0.5% BSA) and centrifuged. The resulting supernatants were collected and the amount of bound ^{125}I determined. Standard curves were analyzed by Scatchard and log/logit analysis. Endothelin values are reported as immunoreactive endothelin (IR-ET).

Extraction Procedures. Human blood bank plasma was incubated at 37°C for 24 hours and found by RIA to contain no endothelin. It was used as a blank to determine recoveries of both unlabelled and labelled endothelin through the extraction procedure using a C_{18} Sep-Pak extraction cartridge. The Sep-Pak C_{18} cartridge was preconditioned by treatment with 3 ml of acetonitrile followed by 6 ml of water. To determine the recovery of ^{125}I-ET, through the extraction procedure, 1 ml of the blank plasma containing ^{125}I-ET and 250 pg of cold ET was passed through the conditioned cartridge. The cartridge was then washed with 4 x 1 ml of 10 mM Tris-HCl (pH 7.4) and eluted with 3 x 1 ml of elution buffer (60% acetonitrile/4 mM Tris-HCl solution). Each fraction was collected and the amount of ^{125}I determined. To examine unlabelled ET recovery, the treated cartridge was loaded with 125 pg of synthetic ET/ml plasma, washed and eluted with the elution buffer as described above. Blank plasma was compared to blank plasma containing added synthetic ET. As stated above, blank plasma was found to contain no endothelin. The resulting eluates were dried under vacuum, and reconstituted in the buffer B prior to storage or analysis by RIA.

Preparation of samples. Blood samples (usually 10 ml) were collected in ice-cold polypropylene tubes containing 1mM EDTA and proteolytic enzyme inhibitors, soybean trypsin inhibitor (25 μg/ml), and bacitracin (25 μg/ml). After centrifugation, the plasma was collected and 1-2 ml were extracted as described above. The eluates were dried under vacuum, dissolved in 100-200 μl of buffer B and subjected to RIA.

Samples from endothelial cells were either assayed directly (50 μl to the RIA) or were concentrated by the extraction procedure described above. Recoveries of labelled and

unlabelled endothelin from the medium collected from cells was similar to the recoveries from plasma.

Cells Culture. Bovine or porcine aortic endothelial cells were prepared and cultured as previously described (11). To measure the production of endothelin, confluent monolayers of endothelial cells in T-25 cm^2 flasks were incubated with 3 ml of Hanks' balanced salt solution (HBSS) containing 0.25% BSA plus 10 mM HEPES (pH 7.4). After incubation, the media were removed and subjected to ET RIA directly or concentrated as described above.

RESULTS

Iodination using Iodo-gen followed by HPLC purification gave a high yield of endothelin with a specific activity of 700 Ci/mmol. As shown in Fig. 1, the peak of radioiodinated ET was shifted two fractions relative to the peak of unlabelled synthetic endothelin (sET). Those fractions containing the highest level of ^{125}I were also found to exhibit the highest binding to the ET-antisera giving approximately 40% binding at a 1:60,000 dilution of the antibody. Non-specific binding of freshly prepared ^{125}I-ET tracer did not exceed 6% of the total radioactivity. After lyophilization, the ^{125}I-ET was dissolved in buffer B and stored at -20°C. Fig. 2A depicts a typical standard curve for endothelin binding which was done with a final 1:60,000 dilution of antibody yielding a maximal binding of 35% of the amount of ^{125}I-ET (9,000 cpm/tube) added. Half maximal inhibition of binding of labelled ET by unlabelled ET was 18 pg/tube and the detection limit of the assay was 0.62 pg/tube. No cross-reactivity was seen with atrial natriuretic peptide (ANP), arginine vasopressin (AVP) or angiotensin II (AgII). Fig. 2 B is a log/logit analysis of data

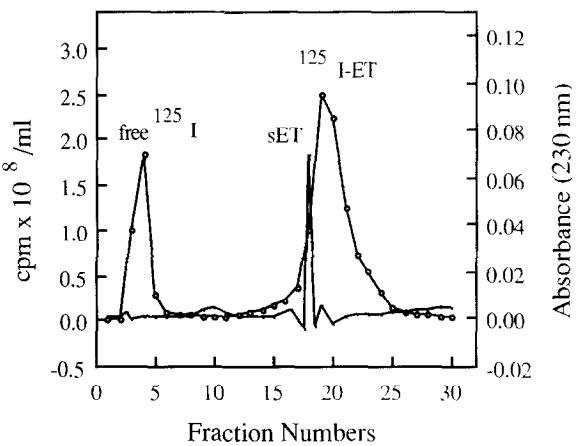


Figure 1. HPLC separation of synthetic ET and ^{125}I-ET. Chromatography was performed on a μBondapak C_{18} reverse phase HPLC column. Elution was done with a linear gradient of 20-70% acetonitrile in 0.1% TFA over 30 minutes at a flow rate of 1 ml/fraction/min. The solid line with points represents the absorption at 230 nm while the solid line with open circles represents ^{125}I-radioactivity from an Iodo-gen iodination reaction. Alqiuots of each fraction were removed for determination of binding activity using the RIA.

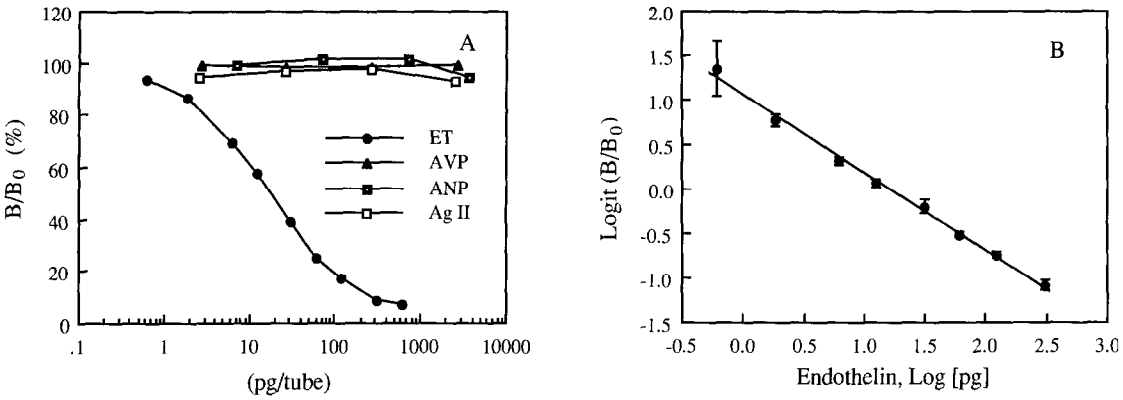


Figure 2A. Inhibition of binding of ^{125}I-ET to anti-ET serum by serial dilution of synthetic ET. Measurable inhibition of binding was detected at 0.62 pg/tube, with a half-maximal inhibition occuring at 18 pg/tube. There was no cross-reactivity to AVP, ANP and Ag II.

B. Log/Logit analysis of binding data. The standard curve is linear from 0.6 pg to 300 pg/tube. The slope is -0.995 with y = 0.98, n=4 on separate days.

from several standard curves run on different days. The regression coefferient is 0.98, the slope is -0.995 (n=4).

To examine the recovery of ^{125}I-ET from human plasma, 1 ml of blank plasma containing ^{125}I-ET (18,800 cpm) and 250 pg cold ET was added to pretreated C_{18} Sep-Pak cartridge for extraction. As shown in Fig. 3, 87 ± 1.7% of the total radioactivity added was absorbed on the cartridges. Absorbed ^{125}I-ET was eluted with 2 ml of elution buffer with an overall recovery of 78 ± 4.7%. C_{18} cartridges from other manufactures were found to be much less efficient in binding endothelin (62 ± 4.1% for Bond-Elut C_{18}). To

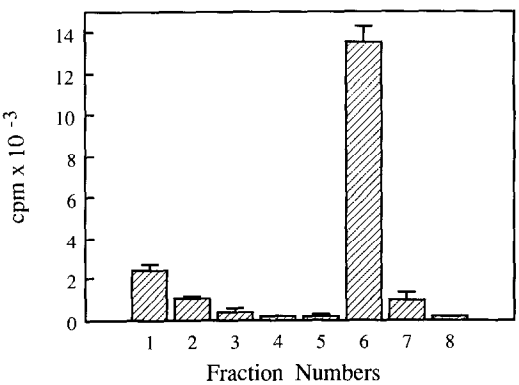
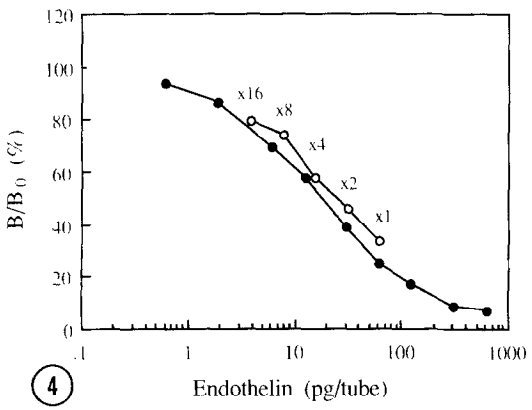


Figure 3. Adsorption and elution of ^{125}I-ET from Sep-Pak C_{18} cartridges. Each fraction represents the radioactivity recovered in each of 1 ml collected from the column. Fraction 1 represents the radioactivity not bound when plasma was added, fractions 2-5 represent radioactivity washed from the column with 10 mM Tris-HCl, pH 7.4; fractions 6-8 represent radioactivity removed by elution with 60% acetonitrile in 4 mM Tris-HCl, pH 7.4. Each bar represents the mean ± SD (CPM/fraction), n=14.

determine whether the extracted endothelin remained intact, 125 pg/ml of unlabelled endothelin alone was added to each of 1 ml of blank plasma and extracted by the same procedures. The eluates were lyophilized, and dissolved in buffer B and analyzed by RIA. As shown in Fig. 4, serial dilution of the plasma extracts inhibited the binding of ^{125}I-ET to antibody in parallel with the curve for synthetic unextracted ET. After calculation, the recovery was 85 ± 5%. Using these procedures on freshly collected human plasma, we found the mean concentration of IR-ET to be 2.0 ± 0.4 pg/ml of plasma (n=12).

Recovery of endothelin was also investigated from buffers collected from endothelial cells incubated in HBSS containing 10 mM HEPES (pH 7.4) and 0.25% BSA. After incubation (6 hours), 9200 cpm of ^{125}I-ET was added to 3 ml of buffer and the buffer extracted as above. Binding of ET to the C_{18} resin was 86 ± 2% of the total and the overall recovery in the eluate was 79 ± 4% (n=3). After analysis by RIA, values obtained from buffer assayed directly was comparable to those obtained after analysis of appropriate dilutions of extracted samples.

Using these procedures, we found that porcine endothelial cells progressively synthesize and release IR-ET into the buffer bathing the cells (Fig. 5). The production rate was approximately 0.8 ng/mg cell protein/hr and was completely blocked by 5 μg/ml of cycloheximide (data not shown). IR-ET could be directly measured in 50 μl of the buffer when the incubations were carried out in 3 ml total volume in a T-25 cm^2 flask of confluent cells. Importantly 0.25% BSA was required to prevent excessive binding of ET to the polypropylene tubes used for collection and storage. Under these conditions IR-ET was



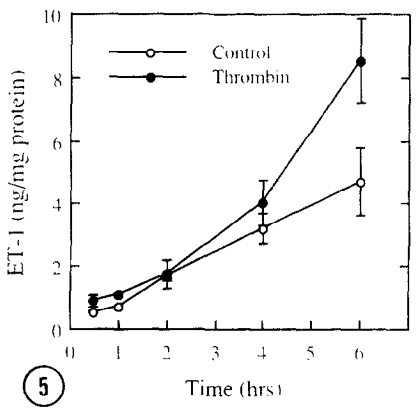


Figure 4. Inhibition of binding of ^{125}I-ET to anti-ET serum by serial dilution of extracts from human plasma containing synthetic ET. The dilutions are given opposite the open circles (X16 = 16 fold dilution). Closed circles are bindng data from Fig. 2A above.

Figure 5. Synthesis of ET by porcine aortic endothelial cells. Cells at confluence were incubated at 37°C in Hank's balanced salt solution containing 10 mM HEPES (pH 7.4) and 0.25% BSA or the same buffer containing 2 U/ml of thrombin. Samples are collected at the times indicated and analyzed by RIA. Data given as mean ± SEM, n=4.

stable for at least one month at -20° C. Finally, as reported by others, thrombin (2 U/ml) stimulated ET production approximately 2-fold after 6 hours (Fig. 5).

DISCUSSION

We have developed a sensitive and reliable radioimmunoassay for porcine/human ET_1 using a commercially available polyclonal antibody and ^{125}I-ET labelled in our laboratory. Initial attempts to iodinate ET_1 using chloramine T produced an unreliable product with variable yields. On the other hand, iodination of ET_1 using Iodo-gen gave a high yield of labelled product which bound efficiently to the antibody. The ^{125}I-ET was stable for at least 3 weeks when resuspended in buffer B and stored at -20°C after lyophilization, and exhibited a low degree of non-specific binding which did not exceed 6% of the total counts.

The assay had a lower limit of detection was approximately 0.6 pg and was useful in determining ET levels in human plasma and in buffers collected from vascular endothelial cells. After incubation of porcine aortic endothelial cells for approximately 1 hour ($2x10^6$ cells/3 ml buffer) The level of ET produced was 50 pg/ml so that 50 μl of sample could be assayed directly and reproducibly without extraction and concentration. However, the levels of ET_1 in human plasma was quite low (~2.0 pg/ml) and required extraction and concentration prior to analysis. To accomplish this we have developed efficient extraction techniques using C_{18} resins. We observed variability between C_{18} resins from various manufacturers, so binding to the cartridges must be investigated before using.

These procedures have been applied to porcine aortic endothelial cells in culture. We have found, as have others (12,13), that IR-ET is produced progressively over 6 hours through a process which is primarily dependent on protein synthesis. The synthesis of ET is stimulated by thrombin with the major effect occuring between 2 and 6 hours. These data are consistent with those showing increases in ET-mRNA levels in response to thrombin (1).

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