Specific hepatocyte endothelin receptors and the direct effect of endothelin on protein synthesis

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Summary

High affinity binding sites for endothelin (ET)-1 were identified on mouse parenchymal hepatocytes. The binding of ¹²⁵I-ET-1 at its site was specific, saturable, and time dependent, but dissociation of the receptor-bound ligand was minimal. Scatchard analysis of the binding data revealed the presence of a non-interacting, single class of high-affinity binding sites on hepatocytes. The dissociation binding constant (Kd) and the maximal binding capacity (Bmax) were determined as 2.1 x 10⁻¹⁰ M and 3.0 x 10² sites/cell, respectively. Unlabeled ET-1 competitively inhibited the binding of ¹²⁵I-ET-1 to primary cultured mouse hepatocytes with a concentration for half-maximal inhibition (IC₅₀) of 2.0 x 10⁻¹⁰ M.

ET-1 apparently enhanced total protein synthesis in primary cultured mouse hepatocytes concentration ranging from 3 x 10⁻¹¹ M to 3 x 10⁻⁸ M in a dose-dependent manner.

These data indicate that specific receptor for ET-1 is present in the mouse liver and suggest for the first time that hepatocyte protein synthesis is involved in its direct, agonistic effect.

Key words: endothelin, receptor assay, binding affinity, mouse hepatocyte, protein synthesis

Introduction

Endothelin (ET) is a potent vasoconstrictive peptide originally isolated from the culture supernatant of porcine aortic endothelial cells¹. It consists of 21 amino acid residues with two intramolecular disulfide linkages, and is now regarded as a local regulator of vascular tonus. In addition to its potent, long lasting vasoconstrictor and pressor actions², ET has been revealed in extravascular tissues to have a wide range of biological effects, most of which are elicited physiologically by the constriction of smooth muscle. Furthermore, recent extensive studies have produced the evidence that ET also has bioactivities even in noncontractile tissues such as fibroblast cells, adrenals, mesangial cells, and central nervous systems³. The pharmacological and biological effects of ET on the liver, however, have not been widely demonstrated⁴. We report here that ET-1 binds with a high affinity to mouse hepatocytes at its specific ET receptor site, and demonstrate that ET-1 stimulates primary cultured hepatocyte protein synthesis directly. It therefore has the characteristics of a local regulatory agonist of liver function.
Materials and methods

(1) Mouse hepatocyte isolation

Hepatocytes were isolated from 2-3 month-old female Balb/C mice weighing 20g using a modification of the collagenase perfusion technique of Seglen5).

In brief, after the mice were anesthetized with intraperitoneal pentobarbital sodium, the vena cava was cannulated and retrograde perfusion was begun with a solution containing 142 mM of sodium chloride, 6.7 mM of potassium chloride, 10 mM of HEPES buffer (Gibco, #380-5630), and 2.5 mM of EGTA (Sigma, E-4378) maintained at 37°C. The portal vein was immediately cut and the perfusion was continued at a flow rate of 8 ml/min for five minutes in situ. The solution was then changed to a collagenase perfusate containing 67 mM of sodium chloride, 6.7 mM of potassium chloride, 100 mM of HEPES, 4.8 mM of calcium chloride, 1% of bovine serum albumin (Sigma), and 0.05% type-I collagenase (Sigma, C-0130). The second perfusion was continued for 15 minutes at the same flow rate. The liver was then removed from the animal and combed to disperse the hepatocytes. The cells were collected by centrifugation at 50g and washed three times with minimal essential medium (Gibco). The cells were then resuspended in minimal essential medium for enumeration and determination of viability by trypan blue exclusion.

Hepatocyte preparations were further purified after the second wash by centrifugation through a polyvinylpyrrolidone colloidal silica (Percoll, #17-0891-01, Pharmacia Fine Chemicals) cushion. The cell suspensions were layered over a 40% solution of Percoll in minimal essential medium and centrifuged at 250g for five minutes. The cellular pellet, which was enriched in viable hepatocytes, was subjected to a final wash before counting. This procedure usually yields 15 to 20 × 10⁶ hepatocytes of greater than 90% viability6).

(2) Cell culture

The isolated, enumerated hepatocytes were inoculated into collagen (Collaborative Res., rat tail type-I, #40236) precoated 24-well culture dishes (Falcon) at a density of 3.5 × 10⁴/cm² in Williams E (Gibco, #320-2551) medium containing 15 mM HEPES, 1.0 μM of regular insulin, 10,000 u/l of penicillin G potassium, 100 mg/l of streptomycin sulfate, and 10% newborn calf serum (Sigma).

The plates were incubated for 4 hours at 37°C in an atmosphere of 5% carbon dioxide and 95% air. After this attachment period, the medium was replaced with serum free Williams E medium containing 0.1% defatted BSA (Sigma) and monolayer cultures were incubated at 37°C for an additional period of 20 hours.

(3) Binding assay

At the end of the 20-hour culture, the growth medium was removed from the dishes 15 to 30 minutes before the beginning of the binding assay, and then the monolayers were rinsed and replaced with binding buffer (Hanks' balanced salt solution (Gibco #310-4065) containing 1% BSA and 20 mM HEPES, pH 7.4). The cells were counted in randomly selected plates and cell viability was checked by trypan blue dye exclusion. Binding studies were performed essentially in the same manner as ¹²⁵I-ET-1 binding to vascular smooth muscle cells as described7).

1) Specific binding of ¹²⁵I-ET-1 to hepatocyte as functions of time and temperature

After being washed, the monolayers were incubated with 2.5 × 10⁻¹¹ M ¹²⁵I-ET-1 (Amersham
International, #IM.233, specific activity: 2,000 Ci/mmol) at 37°C or 4°C for 15, 30, 60, 90, and 120 min in the absence or presence of unlabeled ET. The binding reaction was terminated by removing the medium and rinsing the monolayers rapidly four times with ice-cold binding buffer. The cells were then dissolved in 1 ml 1 N NaOH for 2 hours at room temperature. The radioactivity in the dissolved cells was quantitated in an autogamma counter (Beckman, Gamma 5500). Specific binding was obtained by subtracting nonspecific binding activity in the presence of an excess (1.25 × 10^{-7} M) unlabeled ET-1 (Calbiochem, #324765) from the total binding.

2) Dissociation of cell-bound 125I-ET-1 from cultured hepatocytes

After incubation with 2.5 × 10^{-11} M 125I-ET-1 at 37°C for 120 min, the cells were washed and further incubated in fresh binding buffer. At 0, 15, 30, 60, 90, and 120 min the cell-bound radioactivity was determined.

3) Saturable binding of 125ET-1 to cultured hepatocytes

Hepatocyte monolayers were incubated at 37°C for 120 min with various concentrations of 125I-ET-1 (4, 2, 1, 0.5, 0.25, 0.125, 0.063, 0.032, 0.016 × 10^{-9} M) to study the affinity of the iodinated ligand for the receptor. Specific binding was obtained by subtracting nonspecific binding in the presence of the 5,000-fold excess of unlabeled ET-1 from total binding.

4) Competitive binding assay

After being washed, the hepatocytes were further incubated with 2.5 × 10^{-11} M 125I-ET-1 in the presence of increasing concentrations of 10^{-12} M to 10^{-6} M unlabeled ET-1.

The dissociation constant (K_d) and the maximal binding capacity (B_{max}) were obtained through this series of experiments.

(4) Assay for hepatocyte protein synthesis

Isolated hepatocytes were plated in 96 microwells (2.5 × 10^4 cells/well) and cultured for 20 hours in the presence of various concentrations of ET-1 in Williams E medium containing 0.1% BSA. After being washed with leucine-free minimal essential medium (Gibco, #320-1890), the cultures were incubated for 4 hours at 37°C in the minimal essential medium containing 10 μCi/ml of ^3H-leucine (New England Nuclear, NET-135L, specific activity: 5 Ci/mmol). Then the cells were lysed with 2 N NaOH containing 0.025% Triton-X. Trichloroacetic acid (40%) precipitable materials from the cells and medium were then harvested on glass-fiber filter paper with a semiautomatic cell hervester (Skatron) and their radioactivity levels were determined by liquid scintillation counting (Beckman).

Results

1. ET-1 binding assay on hepatocyte

Specific bindings of 125I-ET-1 to primary cultured mouse hepatocytes was a time- and temperature-dependent process (Fig. 1). Binding at 37°C was rapid, reaching an apparent equilibrium between 120–180 min, while at 4°C it was slower and showed one-third that of 37°C. Therefore subsequent binding studies were performed at 37°C for 120 min.

To determine the dissociation of cell-bound 125I-ET-1 hepatocytes were incubated with 125I-ET-1 at 37°C for 120 min, followed by the removal of the medium and extensive washing. The cells were then further incubated at 37°C in fresh medium without radioligand. As shown in Fig. 2, the cell-bound 125I-ET-1 decreased only slightly during the subsequent incubation; 90% of
Fig. 1 Specific binding of $^{125}$I-ET-1 to primary cultured mouse hepatocytes as functions of time and temperature. Monolayers were incubated with $2.5 \times 10^{-11}$ M $^{125}$I-ET-1 at 37°C or 4°C for the times indicated. Specific binding was obtained by subtracting nonspecific binding in the presence of $1.25 \times 10^{-7}$ M unlabeled ET-1 from the total binding. Each point represents the mean ± SEM of three experiments.

Fig. 2 Dissociation of cell-bound $^{125}$I-ET-1 from primary cultured mouse hepatocytes. After incubation with $2.5 \times 10^{-11}$ M $^{125}$I-ET-1 at 37°C for 120 min, the cells were washed (time 0) and further incubated at 37°C in fresh medium. At the indicated times, the cell-bound radioactivity was determined. Each point represents the mean ± SEM of three experiments.

Fig. 3 Saturable binding of $^{125}$I-ET-1 to primary cultured mouse hepatocytes. Monolayers were incubated at 37°C for 120 min with various concentrations of $^{125}$I-ET-1 as indicated. Specific binding was obtained by subtracting nonspecific binding in the presence of the 5,000-fold excess of unlabeled ET-1 from the total binding. Each point represents the mean ± SEM of three experiments. (Inset) Scatchard plot of binding data. The ratio of bound to free ET-1 is plotted against the concentrations of bound ET-1. Scatchard plots were calculated by linear curve fitting.

Fig. 4 Competitive binding of $^{125}$I-ET-1 to primary cultured mouse hepatocytes. Monolayers were incubated with $2.5 \times 10^{-11}$ M $^{125}$I-ET-1 in the absence and presence of unlabeled ET-1 at concentrations indicated. Each point represents the mean of two experiments.
the initial cell-bound radioactivity remained even after 180 min.

Binding of $^{125}$I-ET-1 to primary cultured mouse hepatocytes was saturable and showed high affinity (Fig. 3). Scatchard analysis revealed the presence of a non-interacting, single class of high-affinity binding sites (Fig. 3, Inset); the apparent dissociation constant ($K_d$) was $2.1 \times 10^{-10}$ M and the maximal binding capacity ($B_{\text{max}}$) was $3.0 \times 10^2$ sites/cell.

Competitive binding of $^{125}$I-ET-1 to mouse hepatocytes by unlabeled ET-1 is shown in Fig. 4. Unlabeled ET-1 inhibited the binding of $^{125}$I-ET-1 to its binding sites with a concentration for half-maximal inhibition (IC$_{50}$) of $2.0 \times 10^{-10}$ M. The inhibition constant for ET-1 ($K_i$) was determined as $1.8 \times 10^{-10}$ M according to the equation $K_i = IC_{50}/(1 + L/K_d)$, where $L$ is the concentration of $^{125}$I-ET-1. The calculated $K_i$ value is almost identical to the IC$_{50}$ value.

2. Effect of ET-1 on hepatocyte protein synthesis

As shown in Fig. 5, ET-1 apparently stimulated total protein synthesis in primary cultured hepatocytes ranging from $3 \times 10^{-11}$ M to $3 \times 10^{-8}$ M in a dose-dependent manner. At $3 \times 10^{-8}$ M, protein synthesis was already maximally increased and $10^{-7}$ M ET-1 did not further stimulate its synthesis. In three independent experiments, $3 \times 10^{-8}$ M ET-1 enhanced the protein synthesis by 33% (p<0.005 by Student t-test) above the control level.

Discussion

The newly recognized vasoactive peptide, endothelin (ET) is the most potent vasoconstrictor reported to date$^{11}$. It is synthesized as a precursor peptide of 203 amino acids containing a signal sequence, which is proteolytically cleaved to produce the 38 (human) or 39 (porcine) amino acid intermediate, big endothelin-1. This is subsequently processed to mature ET-1 (formerly human and porcine ET) by a putative endothelin-converting enzyme$^{11}$. ET-1 was at first believed to play an important role in the local paracrine regulation of vascular smooth muscular tonus. However, ET-1 has recently been shown to have a specific action on the cardiovascular system as well as a wide variety of biological activity in general. Activity attributed to ET-1 includes cardiac effects such as positive inotropic and chronotropic actions and stimulation of atrial natriuretic factor release, non-vascular renal effects such as the inhibition of Na+/K+ ATPase and renin release, mitogenic effects on vascular smooth muscle cells, mesangial cells and fibroblasts in vitro, modulation of catecholamine release from sympathetic nerve termini, stimulation of aldosterone production in zona glomerulosa cells, as well as central nervous system effects such as potent...
pressor action, and release of eicosanoids (PGI₂, TXA₂) and endothelium-derived relaxing factor (EDRF)⁵,⁹. In vivo tissue autoradiographic studies with ¹²⁵I-ET-1 demonstrate the distribution of specific high affinity binding sites for ET-1, not only in the vascular system, but also in other organs such as the intestines, heart, lungs, kidneys, adrenal glands and the brain which support these observations¹⁰⁻¹²).

Though little is known about the influence of ET-1 on the liver, it was noted that there was some uptake of infused radioiodinated ET-1 in the liver on autoradiography¹⁰,¹³ which could not be attributed exclusively to vascular smooth muscle uptake. Thus hepatic tissue itself was considered to be a site of action of this peptide. Using Northern blotting analysis for ET-1 receptor mRNA, Hosoda et al.¹⁴ could not detect cDNA in human liver tissue. But, other researchers have demonstrated the presence of cDNA encoding ET-1 receptors in bovine and rat liver¹⁵,¹⁶. This discrepancy may be explained in part by taxonomical differences and/or the binding assay method employed. The characterization of specific and functionally active receptors has yet to be elucidated. The actual receptor binding process for ET-1 and the action of ET-1 on hepatocytes has not been clearly demonstrated either.

The existence of specific receptors for ET-1 in mouse liver and the fact that ET-1 is a stimulator of protein synthesis is reported there for the first time. ET-1 receptors found comprised of a single class of high-affinity binding sites and the apparent Kᵦ was 2.1 × 10⁻¹⁰ M. The IC₅₀ was 2.0 × 10⁻¹⁰ M. These values obtained for ET-1 in mouse liver correlate well with those reported from binding studies in other tissues⁷,¹²,¹⁷, and place mouse liver into the high affinity tissue group¹¹,¹⁸. In contrast to the Kᵦ result, Bₘₐₓ was 3.0 × 10² sites/cell for mouse hepatocytes which was lower than Bₘₐₓ for other tissues. Differences in profiles of Bₘₐₓ among mouse strains have been observed in other binding studies using ET-1 in mouse fibroblasts (unpublished data in our laboratory). The lower Bₘₐₓ value in this case may be attributed to strain specificity of Balb/C rather than to tissue differences.

The present study also showed that ET-1 caused a pronounced increase in total protein synthesis in primary cultured hepatocytes in a dose-dependent manner at concentrations ranging from 3 × 10⁻¹¹ M to 3 × 10⁻⁸ M with a maximal 1.33-fold enhancement being attained at 3 × 10⁻⁸ M.

Few reports on the action of ET-1 on the liver exist in the literature⁴,¹⁹. Gandhi et al.¹⁹ demonstrated that ET-1 perfused via the portal vein modified liver glycogenolysis and hepatic oxygen consumption and concluded that the effect of ET-1 was due to vasoconstriction of portal vasculature. Research on its hemodynamic effects by other authors in earlier studies demonstrated that the pressor response to ET-1 was caused mainly by vasoconstriction of the splanchnic vascular bed, most profoundly in the mesenteric vessels, and that in the liver, ET-1 reduced hepatic vascular resistance and increased liver blood flow. They assumed that an increase in resistance to portal flow resulted in decreased hepatic arterial resistancehores, which in turn affected hepatocellular function. However, the present study demonstrates that ET-1 directly stimulates protein synthesis in primary cultured mouse hepatocytes without microvasculature and that it has an agonistic effect on protein synthesis. Serradeil-Le Gal et al.⁶ also observed that ET directly activated glycogenolysis in rat hepatocytes. On the basis of the above findings, the liver ought to be considered as one of the primary target organs for the action of ET-1.
and as such warrants further study in this regard.

The mechanism by which ET-1 augments protein synthesis remains unclear. Many studies have proved that phosphokinase-C dependent mobilization of cytosolic calcium ([Ca^{2+}]) is the most important component of the signal transduction pathway by which ET-1 elicits its effects\(^1,2,21,22\). An increase in [Ca^{2+}] also seems likely to trigger a sequence of early biochemical events in hepatocytes and modulates cellular metabolism\(^4\). The mechanism by which ET-1 stimulated hepatocellular protein synthesis, however, needs to be elucidated and awaits investigation.

It may be postulated that under traumatic conditions such as shock and/or other forms of liver injury which lead to liver vessel endothelial cell damage, the endothelial cells in the major blood vessels and microvasculature are stimulated to synthesize and secrete endothelin and that as a result, hepatic function is altered\(^4\). Liver sinusoidal cells play a major role in endotoxin detoxification, and interestingly, endotoxin has been observed to stimulate endothelin synthesis both in vitro and in vivo, suggesting that ET-1 acts in mediating the pathological effects of endotoxin which may be associated with endothelial cell damage\(^23,24\). Moreover, the secretion of ET-1 has been shown to be stimulated by interleukin-1\(^25\), and tumor necrotizing factor\(^26\). IL-1 has also been found to modify the stimulatory effect of ET-1 on protein synthesis in hepatocytes in a dose-dependent manner (data not shown). These facts suggest that some cytokines and ET-1 are involved closely in the control of hepatocyte metabolism. Further investigation is necessary in this regard.

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References


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