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INTRACELLULAR DISTRIBUTION OF ENDOTHELIN-1 RECEPTORS IN RAT LIVER CELLS

Berthold Hocher#, Christoph Rubens, Johannes Hensen, Peter Gross and Christian Bauer*

Abteilung für Innere Medizin, Klinikum Steglitz and *Institut für Biochemie and Molekularbiologie, Freie Universität Berlin
Hindenburgdamm 30, D-1000 Berlin 45, Germany

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We studied the binding of (^{125}I)-endothelin-1 as well as that of the vasopressin analogue (^{125}I)-[8-phenylpropionyl]-LVP to purified plasma membranes, Golgi cisternae and cell nuclei from rat liver. Cell organelles were isolated by differential centrifugation and discontinuous sucrose gradients. Endothelin-1 exhibited specific binding to plasma membranes, Golgi cisternae and nuclei, while the binding of (^{125}I)-[8-phenylpropionyl]-LVP was restricted to the plasma membranes. The number of receptors (B_{max}) and the binding constants (K_d) were determined by Scatchard analysis of competition binding studies. In all cases only one class of Et-1 binding sites could be detected. The presence of Et-1 receptors on the Golgi complex either indicates that the receptor is glycosylated within the cisternae or alternatively, there exists a recycling pathway. The unexpected finding of Et-1 receptors on highly purified nuclei suggests that this peptide may exert part of its biological functions intracellularly via the nucleus.

Endothelin-1 stimulates hepatocytes to increase the intracellular concentration of calcium, glycolysis is likewise stimulated (1). In perfused rat liver, Et-1 leads to an increase in portal blood pressure (2). Typically the biological effects of peptide hormones are mediated by plasma membrane-bound receptors on target cells. Recently, however, there have been indications of an intracellular binding of peptide hormones as well. Binding sites for angiotensin 2, insulin, EGF and prolactin have been detected in isolated hepatocytes (3,4). The biological significance of such intracellular binding sites of peptide hormones is generally not understood. Yet, for prolactin it has been demonstrated that binding of the hormone to isolated nuclei from hepatocytes activates a nucleus-bound protein kinase C (5). Our interest has been focused on endothelin and the aim of this study presented here was to analyze the intracellular distribution of Et-1 receptors in rat liver cells.

To whom correspondence should be addressed.

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MATERIALS AND METHODS

- Male Wistar rats, weighing about 190 g each, were fed on commercial diet Altromin (Altromin GmbH, Germany) and water given ad libitum. (^{125}I)-Endothelin-1 (human, 2000 Ci/mmol) was obtained from Du Pont, Germany. (^{125}I)-[Phenylpropionyl]-LVP (2000 Ci/mmol) was prepared in our laboratory (6). Unlabeled Et-1 and Lys^{8}-vasopressin (LVP) was from Peninsula Laboratories, Inc. (Germany). Unless otherwise stated, all reagents were of analytical grade and were purchased from Merck (Darmstadt, Germany) and Sigma (München, Germany).
- Isolation of the Golgi apparatus and cell nuclei was performed according to reported methods (7,8). Plasma membranes were prepared as described by Neville (9) with some modifications (10). The purity of all fractions was checked by electron microscopy and by assay of the following marker enzymes: glucose-6-phosphatase, 5'nucleotidase, galactosyl-transferase and succinate-INT-reductase (11).
- Assay buffer for binding studies contained 1 mg/ml bacitracin, 100 mM Tris-HCl, 5 mM $MgCl_2$, and 0.1 g% BSA, pH 7.4 in a total volume of 150 μl. Unless otherwise stated, binding studies were performed at room temperature for 90 min. The tracer concentration was kept constant at 40000 cpm/tube, while the concentration of unlabeled Et-1 or LVP was increased from 0 to 50 nM (competition studies with "cold saturation"). Samples from nuclei and Golgi apparatus were used at a concentration of 0.8 mg protein/ml; those from plasma membrane at a concentration of 0.2 mg protein/ml. Nonspecific binding was assessed in the presence of excess Et-1 (5 μM) or LVP (5 μM). Free and receptor-bound radioactivity were separated by centrifugation at 30000 x g (4°C) for 20 min after addition of 1 ml of cold binding buffer and the pellets thus obtained were washed two additional times with 1 ml of cold binding buffer. (^{125}I) was counted in a Packard Gamma Counter (78% counting efficiency for ^{125}I).

RESULTS

Validity and significance of the data presented in this paper depend directly on the quality of the preparation of the cell nuclei, Golgi apparatus and plasma membranes. The purity of the fractions was checked by both electron microscopy and biochemical markers. In comparison with data from the literature (for ref. see 7) the Golgi fractions were contaminated to a lesser extent by plasma membranes and one of the best-known marker enzyme of the Golgi, the UDP-galactose: glycoprotein galactosyltransferase was enriched 150 fold (Table 1). Most important the nuclear fraction was essentially free of any contaminants when analyzing the electron micrographs from different preparations. Besides, when compared to an enriched plasma membrane fraction, only 0.4% of the specific activity of 5'-nucleotidase was associated with the nuclei. The occurrence of glucose-6-phosphatase activity is not surprising, because there exists a structural continuity between the endoplasmatic reticulum and the outer nuclear membrane.

Binding of ^{125}I-Et-1 at 22°C to rat liver subcellular fractions was time dependent (plasma membrane, $t_{1/2}$: 19 min, Golgi apparatus, $t_{1/2}$: 22 min, cell nucleus, $t_{1/2}$: 27 min, n = 3 in each case) . Dissociation of receptor bound ligand was minimal after the addition of 2.5

Table 1. Specific activities of marker enzymes in the homogenate and subcellular fractions of rat liver

FRACTION	5'Nucleotidase	Glucose-6-phosphatase	Succinate-INT-reductase	Galactosyltransferase
	μmol/h/mg protein			nmol/h/mg protein
HOMOGENATE	4.1+/-1.2	4.0+/-0.6	2.0+/-0.7	4.9+/-2.5
NUCLEUS	0.35+/-0.03	2.0+/-0.3	0.03+/-0.01	0.7+/-0.2
GOLGI CISTERNAE	3.3+/-0.6	5.1+/-0.6	0.06+/-0.01	488.3+/-54.8
PLASMA MEMBRANE	77.8+/-4.8	5.5+/-0.5	0.75+/-0.01	2.0+/-0.4

All enzymes were assayed at 37°C. Values are means from 6 individual determinations +/- S.D.

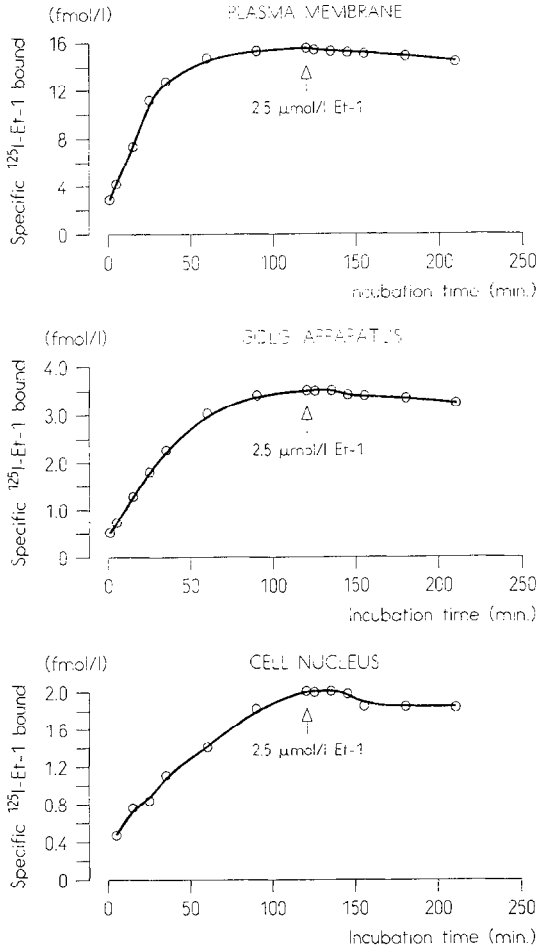

Figure 1. Time course of specific binding of ^{125}I-Et-1 (using 20 pM ^{125}I-Et-1 per test tube). Values are means from 3 independent experiments.

Table 2. Specific binding of ^{125}I-Et-1 and (^{125}I)-[8-phenylpropionyl]-LVP to liver plasma membrane, Golgi apparatus and cell nuclei

FRACTION	(^{125}I)-[8-phenylpropionl]-LVP		(^{125})I-Et-1	
	K_d (nmol/l)	B_{max} (fmol/mg)	K_d (nmol/l)	B_{max} (fmol/mg)
NUCLEUS	--	--	0.28+/-0.07	23+/-8
GOLGI CISTERNAE	--	--	0.22+/-0.02	238+/-13
PLASMA MEMBRANE	0.41+/-0.02	106+/-21	0.23+/-0.03	2161+/-364

Data from Scatchard plots of (^{125}I)-Et-1 or (^{125}I)-[Phenylpropionyl]-LVP binding studies. Nonspecific binding was assessed in the presence of unlabeled Et-1 or LVP. It ranges between 5-10% (Et-1) or 15-25% (LVP). Samples from nuclei and Golgi apparatus were used at a concentration of 0.8 mg protein/ml; those from plasma membrane at a concentration of 0.2 mg protein/ml. Values are means +/- SEM of 4 separate experiments.

μmol/l unlabeled Et-1 (Fig. 1). The number of receptors (B_{max}) and the binding constant (K_d) were determined by competition studies ("cold saturation"). Et-1 bound with very high affinity to the plasma membrane, Golgi apparatus and nucleus (Table 2). Scatchard analysis revealed only one type of Et-1 receptor on each of the isolated organelles (Fig. 2). The vasopressin analogue ((^{125}I)-[Phenylpropionyl]-LVP) concomitantly examined for comparison showed no binding to the Golgi apparatus or the nucleus. Even in the presence of a five fold increase in the amount of cell organelles (based on protein) no binding of the vasopressin analogue to the Golgi or nuclear fraction could be detected.

DISCUSSION

We have identified high-affinity binding sites for Et-1 on rat liver plasma membranes, Golgi cisternae and cell nuclei. Scatchard analysis revealed a single class of Et-1 receptors. The high degree of affinity of Et-1 to its receptor is also reflected by the very low reversibility of Et-1 binding. ^{125}I-Et-1 binding kinetics with slow or minimal dissociation of endothelin from its plasma membrane receptor have been reported in various tissues including liver (1), aorta and lung (12), kidney (13) and vascular smooth muscle cells (14), and could explain the long lasting effect of elevation of intracellular free Ca^{2+} (1,15).

Rat, bovine and human Et-1 receptors have been cloned (16-18). The receptor possess 7 transmembrane domains and the amino-acid sequences are quite similar. N-glycosylation sites were identified in the outer cell domain and there are indications that the C-terminal tail may be phosphorylated by protein kinases. (17,18).

The detection of Et-1 receptors on the Golgi apparatus could either indicate a posttranslational glycosylation of the Et-1 receptor or, alternatively, a recycling.

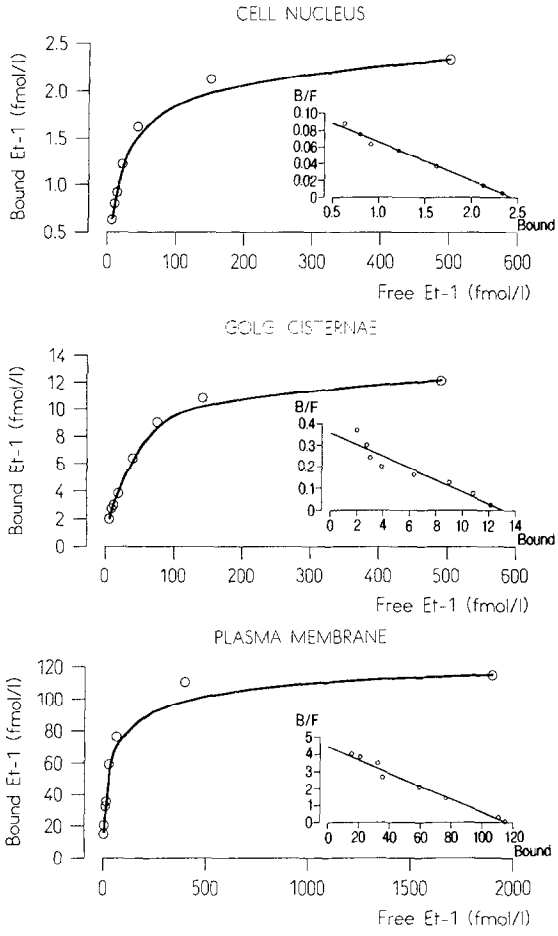

Figure 2. Specific binding of ^{125}I-Et-1 to liver plasma membrane, Golgi apparatus and nucleus at varying but defined concentrations of Et-1 and Scatchard plots of binding data of representative experiments (inserts).

Posttranslational glycosylation is compatible with the above described data from the cloned Et-1 receptors and with recently described findings that incubation of a crude rat brain plasma membrane fraction with neuraminidase leads to a drastic decrease of Et-1 binding (18). The detection of Et-1 receptors on the hepatic nucleus could be the first indication of an intracellular target of endothelin. The nuclear binding sites may play an important role in mediating the intracellular action of Et-1 and one might assume, as has been discussed for the epidermal growth factor (3), that the occurence of the nuclear endothelin receptor is not dependent on a transport from the plasma membrane to the center of the cell.

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REFERENCES

1. Serradeil-Le Gal, C., Jouneaux, C., Sanchez-Bueno, A.,Raufaste, D.,Roche, B.,Preaux, A.M., Maffrand, J.P., Cobbold, P.H., Hanounce, J. and Lotersztajn, S. (1991) J. Clin. Invest., 87, 133-138 .

2. Gandhi, C.R., Stephenson, K. and Olson, S. (1990) J. Biol.Chem., 265, 17432-17435.

3. Marti, U., Burwen, S.J., Wells, A., Barker, M.E., Huling, S., Feren, A.M. and Jones A.L. (1991) Hepatology, 13, 15-20.

4. Kovacs, P., Csaba, G. and Toeroek O. (1990) Histochemistry, 93, 429-431.

5. Buckley, A.R. and Crowe P.D. (1988) Proc. Natl. Acad. Sci. USA, 85, 8649-8653.

6. Hocher, B., Hensen, J., Lindschau, C., Broser, M., Haller, H., Koppenhagen, K., Meinhold, H., Gross, P. und Dürr, J. (1991) Acta Endocrinologica, 124 (Suppl. 1), 115.

7. Bauer, Ch., Lukaschek, R. and Reutter, W. (1974) Biochem. J., 142, 221-230.

8. Blobel, G. and Potter, van R. (1966) Science 154, 1662-1665.

9. Neville, D.M. (1960) J. Biophys. Biochem. Cytol. 8, 415-422

10 Hartel-Schenk, S., Loch, N., Zimmermann, M. and Reutter, W. (1991) Eur. J. Biochem. 196, 349-355

11. Tauber, R., Schenck, I., Josic, D., Gross, W., Heinrich, P.C., Gerok, W. and Reutter, W. (1986) EMBO J., 5, 2109-2114.

12. Kanse, S.M., Ghatel, M.A. and Bloom, S.R. (1988) Eur. J. Biochem., 182, 175-179.

13. Martin, E.P., Marsden, P.A., Brenner, B.M. and Ballermann, B.J. (1989) Biochem. Biophys. Res. Commun., 161, 130-137.

14. Clozel, M. Fischli, W. and Guilly, C. (1989) J. Clin. Invest., 83, 1758-1761.

15. Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K. and Masaki, T. (1988) Nature, (Lond.) 332, 411-415.

16. Arai, H., Hori, S., Aramori, I., Ohkubo, H. and Nakanishi, S. (1990) Nature, 348, 730-732.

17. Saurai, T., Yanagisawa, M., Takuwa, Y., Miyazaki, H., Kimura, S., Goto, K. and Masaki, T. (1990) Nature, 348, 732-735.

18. Adachi, M., Yang, Y.Y., Furuichi, Y. and Miyamoto, C. (1991) Biochem. Biophys. Res. Commun., 180, 1265-1272

19. Bousso-Mittler, Galron, R. and Sokolovsky M. (1991) Biochem Biophys. Res. Commun., 178, 921-926.