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Two different forms of endothelin receptors in rat lung

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We provided evidence for the presence of two distinct types of rat endothelin (ET) receptors having different molecular masses by affinity labelling of rat lung membranes with ^{125}I-ET-1, ET-2 and ET-3 and SDS-PAGE followed by autoradiography. One type, which is 44 kDa has a higher affinity for ET-1 and -2 than ET-3, whereas the other type, which is 32 kDa, preferably interacts with ET-3 rather than ET-1 and -2.

Endothelin; Endothelin receptor; Receptor subtype; Affinity labelling

1. INTRODUCTION

Endothelin (ET), originally identified in culture media of porcine aortic endothelial cells, is one of the most potent vasoactive peptides and consists of 21 amino acids [1-3]. Recently, 3 isopeptides of ET, named ET-1, -2 and -3, have been identified by the analysis of a human genomic library [4]. It has also been found that other mammals, including mouse, have three ET isopeptides [5,6].

ET is now believed to exert its biological activity by interaction with its specific receptors on the surface of target cells [7,8]. We have identified, using chick cardiac membranes, the existence of multiple ET receptor subtypes, which could be tentatively classified into two families based upon respective affinities for 3 types of ET and their molecular masses [8]; the 50 kDa species exhibits higher affinity for ET-1 and -2 than ET-3 while 43 kDa and 31 kDa species have a preference for ET-3 rather than ET-1 and -2. On the other hand, intravenously injected ET-1 has been found to be distributed predominantly to the lung, kidney and liver in rat [9]. Furthermore, Nucci et al. have suggested that, in isolated lungs from guinea pig or rat, ET-1 causes the release of prostacyclin and thromboxane A_2 from the guinea pig or rat isolated lung [10]. This information indicates that rat lung can be a suitable tissue for characterization of ET receptors.

In this study, to investigate the molecular mass of rat ET receptors and whether ET receptor subtypes are also present in rat tissues, we have performed covalent cross-linking of rat lung membranes with ^{125}I-ET-1, -2 and -3.

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2. MATERIALS AND METHODS

2.1. *Materials*

Male Sprague-Dawley rats (200-250 g) were purchased from CLEA Co., Tokyo, Japan. ^{125}I-ET-1, -2 and -3 were obtained from Amersham Co. Antipain, leupeptin, ET-1, -2, -3 and human atrial natriuretic peptide (ANP) were purchased from Protein Research Foundation, Osaka, Japan. Disuccinimidyl tartarate (DST) was obtained from Pierce Chemical Co.

2.2. *Membrane preparation and cross-linking*

Rat lung membranes prepared by exactly the same method as described [8] were cross-linked with ^{125}I-ET-1, -2 or -3 using the cross-linking agent DST in the presence and absence of unlabelled ET-1, -2 or -3 as described [8], except that instead of 150 μg, 300 μg of membrane proteins were incubated in 500 μl of assay buffer.

2.3. *SDS-PAGE*

SDS-PAGE was performed according to the method of Laemmli [11]. Solubilized samples were applied to 2.0-mm thick gradient gels (3.5-13%). After electrophoresis, the gels were stained with Coomassie brilliant blue. Autoradiograms were obtained from dried gels after exposure to Fuji medical X-ray film with intensifying screen at -70°C.

3. RESULTS AND DISCUSSION

Affinity labelling of rat lung membranes by cross-linking with ^{125}I-ET-1 via disuccinimidyl tartarate (DST) indicated the presence of two ET receptor forms (fig.1). SDS-PAGE and autoradiographic analysis of the ^{125}I-ET-1-labelled materials, in the absence of the disulfide reducing agent, 2-mercaptoethanol (2-ME), revealed two major bands with molecular masses of 45 and 35 kDa (lane a). The presence of a 100 nM concentration of unlabelled ET-1 during the incubation of membranes with ^{125}I-ET-1 effectively abolished the appearance of the two bands (lanes a and b). Unlike nonradioactive ET-1, an unrelated peptide, 100 nM atrial natriuretic peptide, was ineffective in diminishing

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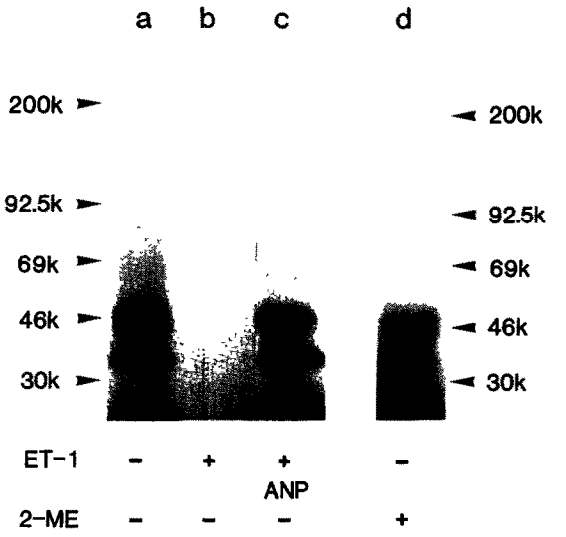

Fig.1. Affinity labelling of rat lung membranes with ^{125}I-ET-1. Membranes of rat lung were incubated with ^{125}I-ET-1 in the absence (−) (lanes a and d) or presence (+) of a 100 nM concentration of unlabelled ET-1 (lane b) or ANP (lane c). Samples were solubilized in SDS sample buffer in the absence (−) (lanes a–c) or presence (+) (lane d) of 2-ME.

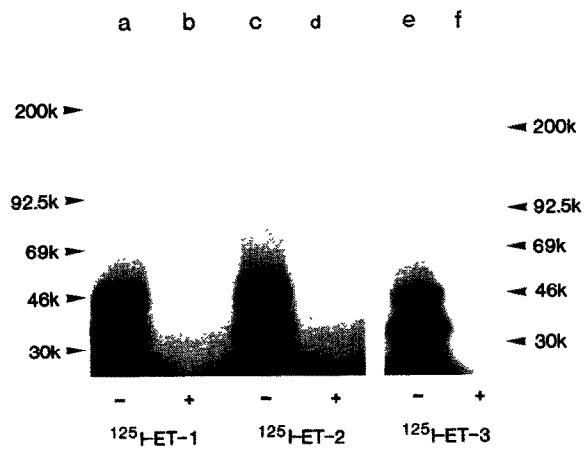

Fig.2. Cross-linking of ^{125}I-ET-1, -2 and -3 to rat lung membranes. Rat lung membranes were incubated with ^{125}I-ET-1, -2 and -3 in the absence (−) (lanes a,c and e) or presence (+) (lanes b,d and f) of 100 nM of unlabelled ET-1, -2 and -3, respectively. Following cross-linking with 1 mM DST, the samples were subjected to SDS-PAGE under nonreducing conditions.

the labelling of the two bands (lane c). Although the appearance of some minor labelled species was also observed, their intensities were different from experiment to experiment and always much less than those of the major specific bands. These results suggest that the 45 and 35 kDa bands were due to specific binding of the ligand. Furthermore, the labelling pattern obtained under both reducing and nonreducing conditions was almost the same except that the mobility of the 45 kDa band was changed to the position at 47 kDa upon reduction, demonstrating that the two specific bands observed here are not disulfide-linked to any other protein(s) in the membranes (lane d). Taken into consideration the molecular masses of ^{125}I-ET-1 and DST, the 47 and 35 kDa bands correspond to proteins with molecular masses of 44 kDa and 32 kDa, respectively. Together, these data indicate that the 44 and 32 kDa proteins represent native ET receptor components.

To examine further the nature of these ET receptor subtypes, lung membranes were cross-linked via DST with each ^{125}I-ET-1, -2 and -3, respectively (fig.2). Autoradiography after SDS-PAGE of cross-linked materials revealed that, although the 45 and 35 kDa bands were always reproducibly observed in all the cases using ^{125}I-ET-1, -2 and -3, the ratio of intensity of the two bands was different between the labelling with ^{125}I-ET-1 and -2, and that with ^{125}I-ET-3 (cf. lanes a, c and e): i.e. the two bands had approximately the same amounts of radioactivity when the cross-linking was performed with ^{125}I-ET-1 and -2, whereas the intensity of the 35 kDa band was much greater than that of the 45 kDa band in the case of ^{125}I-ET-3. This result raises the possibility that, although all the radioiodinated isopeptides of ET could bind to the two forms of ET receptors, ^{125}I-ET-3 binds to the 32 kDa component with higher affinity than ^{125}I-ET-1 and -2.

To test this possibility, replacement of ^{125}I-ET-1 binding by 3 types of ET was examined (fig.3). When ET-1 and -2 were used as competitors for binding of ^{125}I-ET-1, the 45 kDa band disappeared more effectively compared to the band of 35 kDa (lanes a–e); in the presence of 10 nM ET-1 and -2, the intensity of the 45 kDa band slightly decreased without a significant change in the intensity of the 35 kDa band (lanes b and d). In contrast, unlabelled ET-3 blocked the appearance of the 35 kDa band more effectively than that of the 45 kDa band (lanes f and g); the appearance of the 35 kDa band was totally inhibited by 100 nM unlabell-

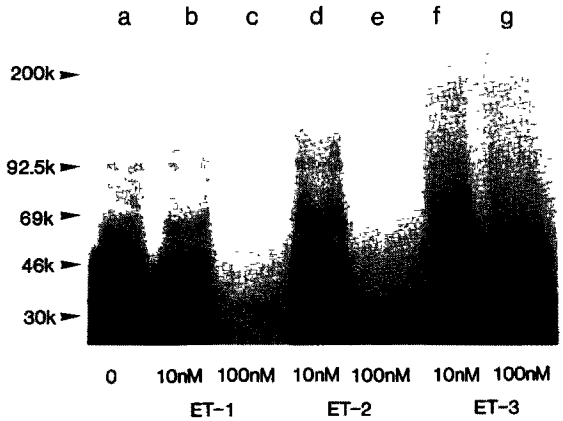

Fig.3. Competitive displacement of affinity labelling of rat lung ^{125}I-ET-1 binding proteins by unlabelled ET-1, -2 and -3. Rat lung membranes labelled with ^{125}I-ET-1 in the absence (lane a) or presence of increasing concentrations of unlabelled ET-1 (lanes b and c), -2 (lanes d and e) and -3 (lanes f and g) were cross-linked with 1 mM DST and subjected to SDS-PAGE under non-reducing conditions.

ed ET-3, but at the same concentration of ET-3 the 45 kDa band had not disappeared. These data demonstrate that ET-1 and -2 have a higher affinity for the 44 kDa species and ET-3 has conversely a preference for the 32 kDa species.

We have previously identified two distinct types of ET receptors on chick cardiac membranes. One type, the 50 kDa type, has a higher affinity for ET-1 and -2 than ET-3. The other type represents proteins of 43 and 31 kDa which have a higher affinity for ET-3 [8]. Apparently, rat lung membranes lack the 50 kDa species which is found in chick heart, suggesting that ET receptor subtypes are expressed differentially from tissue to tissue. It is not clear at present whether the 44 kDa species in rat lung is comparable to the 43 kDa species in chick heart. If both species are equivalent, the results from the cross-linking studies using two different tissues account for the contradiction; the 44 kDa species in rat lung has a higher affinity for ET-1, -2 than ET-3 whereas the 43 kDa species in chick heart prefers ET-3. It is possible to speculate that the 43–44 kDa species has loose specificity and available for all 3 types of ET depending on the presence of the 50 kDa species which is presumably the most ET-1 and -2 preferring component. There remains to be investigated the further kinetics of interaction between ETs and their receptor subtypes.

In summary, this study provides the first biochemical evidence for the presence of two ET receptor subtypes in rat. Although both the types can interact with each ET isopeptide, the 44 kDa species has a slightly higher affinity for ET-1 and -2, and conversely, the 32 kDa species has a preference for ET-3.

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