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Level of ET_B receptor mRNA is down-regulated by endothelins through decreasing the intracellular stability of mRNA molecules

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Using ROS17/2 rat osteosarcoma cells as a model system, we examined the possibility that endothelin (ET)-induced down-regulation of ET_B receptor was accompanied by a decrease in levels of ET_B receptor mRNA. Northern blot analysis showed that low doses of ET-1 and ET-3 caused a transient decrease in ET_B receptor mRNA in the cells. The maximum decrease in the levels of ET_B receptor mRNA (80 %) occurred after 2 ~4 h of exposure of the cells to ETs and was followed by a gradual recovery to control levels by 24 h. The effects were dose-dependent (EC_{50} ~1 nM), and ET-1 and ET-3 were almost equipotent in eliciting the response. The addition of either ionomycin, a Ca^{2+} ionophore, or phorbol dibutyrate, a protein kinase C activator, mimicked the effect of ETs. These results suggested that ETs-induced down-regulation of ET_B receptor mRNA was mediated by the activation of ET_B receptor and that it may have involved ET_B receptor coupled second messenger pathways. We also showed that ET_B receptor mRNA had a long intracellular life span which suggested that ETs-induced down-regulation of ET_B receptor mRNA may have been due to a decrease in the stability of mRNA, rather than inactivation of the transcription of mRNA.

Endothelin-1 is an endothelium-derived 21 amino acid peptide known to be the most potent vasopressor compound yet characterized (1). ETs were subsequently found to have a wide variety of effects on both vascular and non-vascular tissues. The discovery that the three isopeptides of ET, ET-1, ET-2 and ET-3, each possessed a diverse set of pharmacological activities with different potency rank orders, suggested the existence of multiple ET receptor subtypes. Recently, we and other groups have isolated cDNA clones encoding ET receptors by employing expression cloning techniques (2-4). According to the relative binding affinities of the three ET isopeptides to the receptors which were expressed in the transfected mammalian cells, these

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receptors can be classified into two groups: The affinity order of the ETs to the first receptor type, designated ET_A, was ET-1 ≥ ET-2 » ET-3 (ET-1 binded about 100 times more potently than did ET-3) (2, 4). The second receptor type, designated ET_B, showed similar affinities for all of the three ETs (3). The ET_B receptor was recently demonstrated to exist on the human and porcine aortic endothelial cells and to mediate ET-induced production of endothelium-derived relaxing factor (EDRF). The expression level of ET_B receptor in the endothelial cells may have affected this ET-induced EDRF release (5-7).

Exposure to ETs for periods in excess of several hours resulted in a decrease in the density of ET receptors (8). The recent availability of cDNA probes for ET receptors has now allowed us to examine the molecular mechanisms of homologous down-regulation of ET receptors. As a first step toward this goal, we examined ETs-induced change in the levels of ET_B receptor mRNA by using ROS17/2 rat osteosarcoma cells as a model system.

MATERIALS AND METHODS

Cell Culture. ROS17/2 cells were kindly donated by Dr. Y. Ohtawara. Stock cultures of ROS17/2 cells were maintained in DMEM (Dulbecco's modified Eagle medium) supplemented with 10 % fetal calf serum in a humidified atmosphere containing 5 % CO_2.

Reagents. Stock solutions used were ET-1 and ET-3 (Peptide Institute, Osaka, Japan); 10^{-6} M in H_2O, ionomycin (Calbiochem, La Jolla, CA, U.S.A.) ; 10 mM in ethanol, actinomycin D (Sigma); 2 mg/ml in 80 % (v/v) ethanol, Phorbol dibutyrate (PDBu) (Sigma); 10 mM in dimethyl-sulfoxide.

Preparation of RNA. Cells were subcultured in 60 mm dishes 3 days before, and allowed to grow into confluent monolayers. 12 h prior to the experiments, the cells were fed with serum-free DMEM medium containing 0.3 % bovine serum albumin (BSA). Appropriate amounts of reagent stock solutions were directly added to the medium. At the time of cell harvest, the medium was thoroughly aspirated and cells were immediately lysed by the direct addition to the dishes of 3 ml of 3 M LiCl / 6 M urea. Cell layers were scraped off with a rubber policeman, transferred to a microcentrifuge tube and incubated at 4 °C for 8 h. Precipitated RNA was collected by centrifugation at 12,000 *g* for 20 min., and pellet was resuspended in 400 µl of 10 mM Tris-HCl (pH 8.0) / 1 mM EDTA / 0.5 % SDS / 200 µg ml^{-1} proteinase K. After incubation at 37 °C for 4 h, RNA was purified by a phenol / chloroform extraction and an ethanol precipitation.

Northern Blot Analysis. Total cellular RNA (10µg) from ROS17/2 cells were separated by formaldehyde/1.1 % agarose gel electrophoresis, and transferred to an HyBond-N^+ membrane (Amersham). The insert (2 kb) of prETR-7, which included the entire coding region of ET_B receptor (3). was labelled to a specific activity of $8x10^8$ c.p.m./µg with [α-^{32}P]dCTP (3000Ci/mmol, New England Nuclear) by the random priming method, and hybridization was conducted at 42 °C in 1M NaCl / 50% formamide / 1% SDS / 250µg/ml salmon sperm DNA. The membrane was washed finally in 0.1 x SSC / 0.1% SDS at 50°C, and autoradiographed with intensifying screens at -80 °C for 10 h. The same blots were re-hybridized by cDNA probe for β-actin as an internal control.

RESULTS AND DISCUSSION

Effects of ET-1 and ET-3 on the Levels of ET_B receptor mRNA. A 5.0-kb ET_B receptor mRNA was detected by Northern hybridization of RNA from ROS17/2 cells with a cDNA probe. The incubation of ROS17/2 cells with 10^{-8} M of ET-1 or ET-3 at various time periods caused a time-dependent decrease in the levels of ET_B receptor mRNA, as measured by Northern blot hybridization (Fig. 1 a). The maximum decrease in the levels of ET_B receptor mRNA (80%) occurred after 2~4 h of exposure to endothelins and was followed by a gradual recovery to control levels by 24 h (Fig. 1 b). Addition of ETs (10^{-8} M) to the cells at points of 4, 8, 16 and 24 h did not prevent the recovery of mRNA levels at 8 or 24 h (data not shown) which indicated that the increase in ET_B receptor mRNA after 4 h was not simply due to degradation of

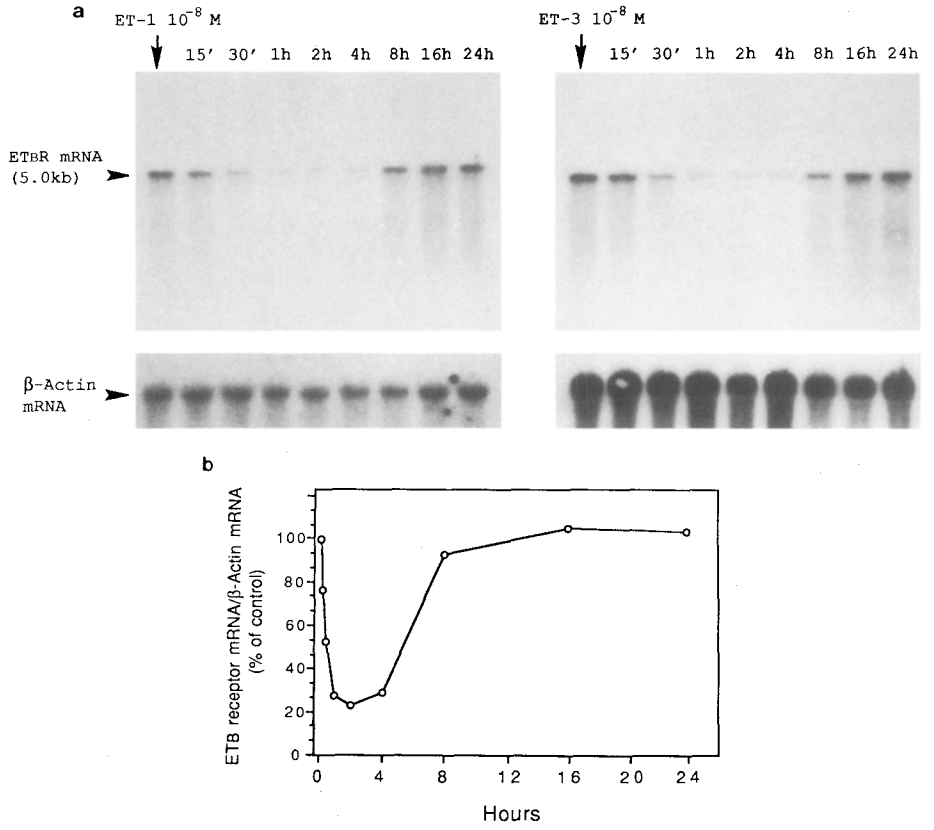

Fig. 1. Time course of the effect of ETs on the level of ET_B receptor mRNA as measured by Northern analysis. a. ROS17/2 cells were incubated with ET-1 or ET-3 (10^{-8} M) for the designated times. Total RNA was extracted and subjected to Northern blot hybridization with a ^{32}P-labelled cDNA probe for ET_B receptor or for β-actin as an internal control. We obtained similar results from 7 and 4 independent experiments (respectively with ET-1 and ET-3.) b. The autoradiogram of an experiment for ET-1 was scanned by a densitometer, and the ratio between ET_B receptor and β-actin mRNA was calculated, and plotted for the indicated times.

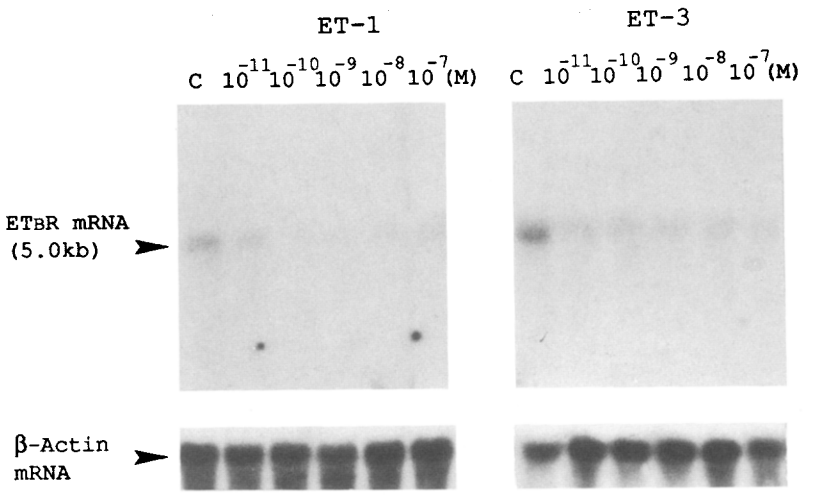

Fig. 2. Concentration dependence of the ETs-induced decrease in the levels of ET_B receptor mRNA. ROS17/2 cells were incubated with indicated doses of ET-1 or ET-3 (10^{-8} M) for 2 h. Total RNA was extracted and subjected to Northern blot hybridization with a ^{32}P-labelled cDNA probe for ET_B receptor or for β-actin as an internal control.

ETs in the incubation medium. ET-1 and ET-3 had almost identical effects on the ET_B receptor mRNA levels in terms of both the time course and the amplitude of the response (Fig. 1 a).

To determine the pharmacological properties of the effects of ETs on the levels of ET_B receptor mRNA, ROS17/2 cells were treated with various doses of ET-1 and ET-3 (Fig. 2). The effects of ETs were concentration dependent with EC_{50} value of about 1 nM, and ET-1 and ET-3 were almost equipotent which suggested that this effect may have probably been caused through the activation of ET_B receptor.

Effects of Ionomycin and Phorbor Ester on the Levels of ET_B Receptor mRNA. One of the consequences of the interaction between ETs and ET_B receptor was an activation of phospholipase C (PLC) via G protein (3). The hydrolysis of phosphoinositides by PLC resulted in the activation of protein kinase C (PKC) via the formation of 1,2-diacylglycerol, and in mobilization of Ca^{2+} through the production of inositol-1,4,5-trisphosphate. We examined the effects of the incubation with ionomycin (a Ca^{2+} ionophore) and phorbol dibutyrate (PDBu ; a potent PKC activator) on the levels of ET_B receptor mRNA in ROS17/2 cells. As anticipated, the incubation of ROS17/2 cells with either 0.3 μM ionomycin or 0.3 μM PDBu for 2 h both markedly down-regulated the ET_B receptor mRNA (Fig. 3). This observation suggested that the down-regulation of ET_B receptor mRNA by ETs may have been mediated by the actions of second messengers from the phosphoinositide turnover pathway.

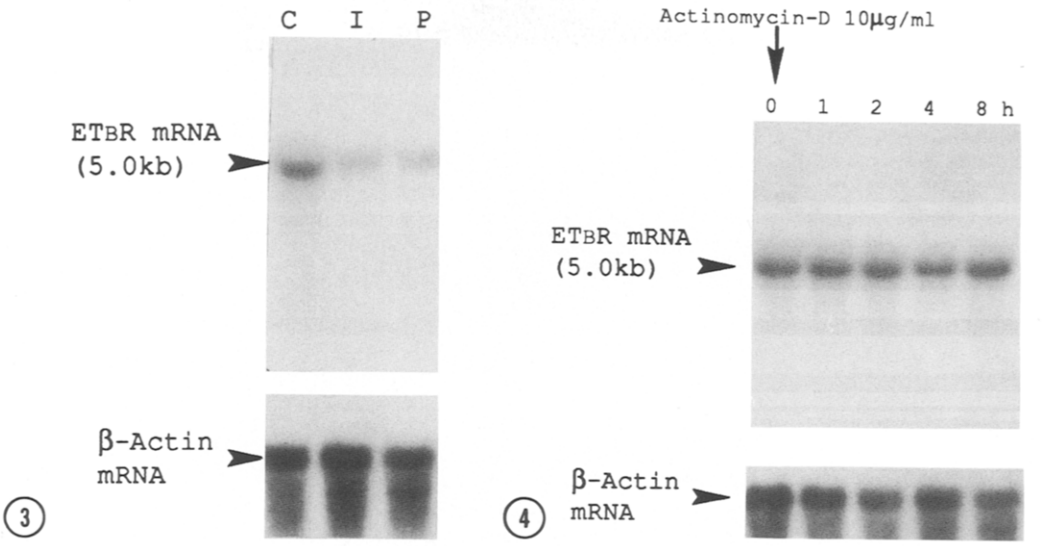

Fig. 3. Effects of ionomycin and phorbor dibutyrate (PDBu) on the levels of ET_B receptor mRNA. ROS17/2 cells were incubated with ionomycin (0.3 μM) or PDBu (0.3 μM) for 2 h. We obtained similar results from 5 independent experiments.

Fig. 4. Effects of Actinomycin D on the levels of ET_B receptor mRNA. We obtained the same results from 5 independent experiments.

Intracellular Stability of ET_B Receptor mRNA. As an initial approach for assessing the mechanism by which ETs decrease the levels of ET_B receptor mRNA, we estimated the life-span of ET_B receptor mRNA by monitoring the rate of disappearance of the mRNA after the addition of an RNA synthesis inhibitor, actinomycin D, to the culture medium. Treatment of the cells with a high concentration of actinomycin D (10 μg/ml for 8 h) caused inhibition of [^{3}H] uridine incorporation by >95 % (data not shown). However, this treatment did not cause a significant decrease in the level of ET_B receptor mRNA (Fig. 4). This finding indicated that the effects of ETs on the levels of ET_B receptor mRNA could not have been accounted for by the inhibition of transcription, and that ETs may have acted by decreasing the intracellular stability of the ET_B receptor mRNA, because ETs induced a markedly higher decrease in the level of ET_B receptor mRNA, as compared with that of actinomycin D. Interestingly, ETs did not cause a decrease in the level of ET_B receptor mRNA in the presence of actinomycin D (10 μg/ml) (Data not shown). These results suggested that the effects of ETs on the levels of ET_B receptor mRNA may have been dependent on the transcription of *trans*-acting genes which were activated by signals from the phosphoinositide turnover pathway.

It has been reported that the stability of α_1- and β_2-adrenergic receptor mRNA levels were also decreased as the results of prolonged agonist stimulations (9-11). Our observations suggested the possibility that similar mechanisms, *i.e.* decreasing the intracellular stability of the mRNA molecules, may also have been operating in the ET-induced down-regulation of ET_B receptor mRNA.

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