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Modulation of the β-adrenergic receptor system of vascular smooth muscle cells *in vitro* and *in vivo* by chronically elevated endothelin-1 levels

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Abstract

Endothelin-1 (ET-1) levels are chronically elevated in several cardiovascular diseases and correlate with an increased mortality. However, in contrast to acute biological activities such as vasoconstriction, little is known about long-term effects of ET-1. In this study we determined the effects of ET-1 on the β_2-adrenergic receptor (AR) system. Incubation of smooth muscle cells with ET-1 for 72 hr led to increased β_2AR density as determined by radioligand binding. Experiments with inhibitors of protein and RNA synthesis as well as RT-PCR revealed that β_2AR upregulation required *de novo* synthesis. In addition, protein kinase C but neither NO nor prostaglandin metabolism were involved in this effect. The enhanced expression of β_2AR was associated with an increased expression of its stimulatory G-protein and the receptor's ability to stimulate adenylyl cyclase. To study chronic effects of ET-1 *in vivo*, rats were infused with ET-1 for 3 weeks. Similarly as in cultured cells, prolonged ET-1 exposure led to increased βAR expression *in vivo*. As a consequence, β_2AR-induced vasodilatation was increased in aortic rings from ET-1-treated animals. Our results therefore suggest that chronically elevated ET-1 levels *in vitro* and *in vivo* induce counterregulatory mechanisms by increasing βARs that attenuate the vasoconstrictive effects of ET-1.

Keywords: Endothelin-1; Smooth muscle; Receptors; β-Adrenergic; Signal transduction; Rat

1. Introduction

ET-1 is the predominant form of peptides of the endothelin family that also includes ET-2 and ET-3. ET-1 has been originally identified as a potent vasoconstrictor in porcine vascular endothelial cells. The biological effects of ET-1 are transduced through two types of receptors, termed ET_A and ET_B. Both ET receptors exert their effects through guanine nucleotide-binding proteins (G-proteins) leading to the activation phospholipase C, protein kinase C (PKC) and other second messenger systems [1,2]. Activation of PKC regulates the sensitivity of receptor-mediated responses either by direct posttranslational modification (phosphorylation) of the receptor or indirectly by inducing receptor gene expression [3].

Although the molecular functions of ET-1, in particular its acute and long lasting vasoconstricting effects, have been intensively studied, chronic adaptive changes mediated by long-term exposure to ET-1 are less well understood. Interestingly, it has been found that ET-1 transgenic mice develop glomerulosclerosis and interstitial fibrosis but, unexpectedly, not hypertension [4]. The reasons for these seemingly contradictory findings in acute effects on blood pressure of intravenous ET-1 infusion vs. chronic overexpression of the ET-1 gene remain unclear. The absence of an increased blood pressure in the ET-1 transgenic mouse model implies that the ET-1 system is tightly controlled and that chronically elevated ET-1 levels

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Abbreviations: ET-1, endothelin-1; PKC, protein kinase C; βAR, β-adrenergic receptor; ^{125}ICYP, [^{125}I]iodocyanopindolol; G-protein, guanine nucleotide-binding protein; CHX, cycloheximide; ET_A, endothelin$_A$ receptor; ET_B, endothelin$_B$ receptor.

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induce counterregulatory mechanisms which limit ET-1-mediated vasoconstriction.

The aim of the present study was to analyze the effects of chronically elevated ET-1 levels on the regulation of β adrenoreceptor complex, another G-protein-coupled receptor system that plays an important role in the regulation of the vascular tone and other cardiovascular functions.

2. Materials and methods

2.1. Reagents

[^{125}I]Iodocyanopindolol (^{125}ICYP, 81.4 Tbq/mmol), [^{125}I]endothelin-1 (81.4 Tbq/mmol) and α[^{32}P]-ATP (111 Tbq/mmol) were obtained from NEN. ET-1 was from Roche Molecular Biochemicals. Bosentan, a non-selective ET_A and ET_B receptor antagonist, was from Roche. An antiserum (RM/1) specific for the α-subunit of the stimulatory G-protein [5,6] was obtained from DuPont. The antibody detected two splice variants at 54 and 48 kDa. All other chemicals were purchased from Sigma Chemicals.

2.2. Cell culture

A-10 cells, derived from embryonic rat thoracic aortal smooth muscle, were obtained from the Deutsche Sammlung für Zellkultur and maintained in Dulbeccos's modified Eagle's medium containing 100 unit/mL penicillin, 100 μg/mL streptomycin and 10% heat-inactivated newborn calf serum. A-10 cells express only the β_2AR and ET_A receptor subclasses, respectively. Cells were treated with ET-1 (10^{-10} to 10^{-7} mol/L) for 6–72 hr. When A-10 cells were incubated with actinomycin D or cycloheximide, experiments were performed with 10^{-7} mol/L ET-1.

2.3. Animal model

In order to investigate the effects of a prolonged intravenous ET-1 application, male Wistar Kyoto rats were used. All surgical procedures were performed according to the guidelines of the ethics committee of the University of Tübingen. Under a deep anesthesia with ketamine (100 mg/kg body weight) and xylazine (5 mg/kg body weight) the internal jugular vein was prepared. A catheter was then introduced into the vessel and ended in the *superior vena cava*. ET-1 (7.5 ng/kg/min) was infused *via* this catheter using an osmotic mini pump (Alzet, 2ML4, Charles River). In order to obtain a steady-state ET-1 level, the application lasted for 3 weeks, while controls received the vehicle 0.9% NaCl in the absence of ET-1. Following this treatment, serum ET-1 concentrations increased from 0.47 ± 0.06 to 2.20 ± 0.05 fmol ($N = 10$, $P < 0.01$). This ET-1 dose was used, since in patients with generalized atherosclerosis or severe heart failure a two- to four-fold increase of ET-1 has been found [7,8]. Blood pressure was recorded from conscious animals by tail plethysmography. In order to study a physiological parameter for changed βAR density and function, isoproterenol-mediated vasorelaxation was measured. The experiments were performed with deendothelialized aortic rings from the thoracic aorta where connective tissue and fat had been trimmed off [9]. Deendothelialized aortic rings were placed in an organ bath (thyrode, 37°, pH 7.4). Contraction was induced by 10^{-6} mol/L epinephrine, and maximal contraction was normalized to 100%. Afterwards 3×10^{-10} to 10^{-4} mol/L isoproterenol was added [9]. For radioligand binding studies only the isolated media of the aorta was used.

2.4. Radioligand binding

Radioligand binding experiments were performed in 50 mmol/L Tris–HCl pH 7.4. For βAR binding membranes from aortas were incubated with increasing concentrations of [^{125}I]iodocyanopindolol. Nonspecific binding was determined with 5×10^{-6} mol/L (−)-alprenolol. After incubation for 60 min at 37°, the reaction was terminated by vacuum filtration through Whatman GF/C filters and four washing steps with ice-cold incubation buffer. Remaining radioactivity on the filter was measured in a γ-radiation counter [10,11]. All experiments were performed in duplicates. β_1- and β_2AR subtypes were determined using CGP 207.12A as a β_1-selective antagonist and ICI 118.551 as a β_2-selective antagonist in the presence of 50 mmol/L [^{125}I]iodocyanopindolol [10,11]. In line with previous data [11], the experiments revealed that aortal smooth muscle cells almost exclusively possess β_2AR.

2.5. Western blot analysis

Plasma membranes (10 μg) were fractionated by SDS-polyacrylamide electrophoresis and transferred to nitrocellulose membranes (Amersham) using a electrophoretic transfer cell (Hoefer) at 100 V for 0.5 hr (2.5 mA/cm^2). Membranes were blocked for 12 hr with 5% non-fat dry milk powder in TBS and then immunoblotted for 2 hr with the primary antibody RM/1 directed against the α-subunit of the stimulatory G-protein. Bound primary antibody was reacted with anti-rabbit peroxidase-conjugated IgG for 1 hr. Following extensive washing, the reaction was developed by enhanced chemiluminescent staining (Amersham). The quantity of the specific proteins was determined by densitometric analysis using the Imagemaster software (Pharmacia).

2.6. RNA preparation and reverse transcription PCR

Expression of β_2AR transcripts was determined by RT-PCR. Total cellular RNA was prepared using the RNAzol B

kit (Wack Chemie). Prior to reverse transcription a DNase digestion step was carried out 60 min at 25°, and the RNA was precipitated again with 75% ethanol. First strand cDNA synthesis from 250 ng total RNA was performed in RT buffer (20 mmol/L Tris–HCl, pH 8.3, 50 mmol/L KCl, 3 mmol/L $MgCl_2$, 0.1 mmol/L dithiothreitol) with 20 U RNAsin (Pharmacia), 50 pmol antisense primer, 0.02 mmol/L dNTPs and 100 U Moloney Murine Leukemia Virus reverse transcriptase (Gibco/BRL). The samples were incubated at 42° for 30 min, before they were heated to 95° for 10 min and then cooled to 4°. For cDNA amplification, the reaction volume was increased by adding 25 pmol sense primer, 2.5 μL PCR buffer (750 mmol/L Tris–HCl pH 9.0, 200 mmol/L $(NH_4)_2SO_4$, 0.1% (w/v) Tween-20), 3 μL 25 mmol/L $MgCl_2$, 3 μL [^{3}H]-TTP (130 Ci/mmol) and 1 U Taq-DNA polymerase (Eurogentec). The amplification procedure involved denaturation at 94° for 4 min and 95° for 1 min, followed by primer annealing at 56° for 1 min, and primer extension at 74° for 1 min. For quantification the PCR conditions were optimized to obtain the exponential phase of amplification. Twenty-six cycles were necessary for GAPDH, and 30 cycles were required for β_2AR [12,13]. An aliquot that served as an internal control for each experiment was fractionated on 1.2% agarose gels containing 0.01% ethidium bromide and visualized by UV irradiation at 320 nm. The remainder was quantified using the Quant-Amp® scintillation proximity assay (SPA) according to the manufacturer's recommendations (Amersham). An aliquot of 10 μL was incubated with 20 pmol of the biotinylated internal oligonucleotide before heating for 15 min to 95°, cooling to room temperature and measurement in a scintillation counter. The antisense primer for β_2AR was d(TTGACGACACACTTCTGGAGG) and the sense primer d(GACGTTAGGCATCATC-ATGG) [12]. GAPDH was used as an internal standard with the antisense primer d(ACTCCACG-ACATACTCAGCACCA) and the sense primer d(GTGAAGGTCGGTGT-GAACGGA). The biotinylated oligonucleotides for the Quant-Amp SPA® assay were d(GATTGCCTTCC-AGGAGCTTC) for β_2AR and d(TGGTGCTGAGTATGTCGTGGA GT) for GAPDH [14].

2.7. Miscellaneous

Adenylyl cyclase activity was determined as described [15–17]. Protein was measured by the Bradford procedure using bovine serum albumin as a standard [18]. RNA concentration was determined at 260 nm using an UV spectrophotometer.

2.8. Data analysis

Saturation curves were analyzed by computer software using non-linear least square curve fitting based on the law of mass action; the software was originally developed by De Lean and co-workers [19,20]. The proportion of receptors able to form the agonist-promoted "high affinity state" was determined using a "two-site one-ligand fit" according to the mass action law. Dilator responses were given as percentage dilation relative to the precontraction level. Statistical analyses were performed using analysis of variance and Student's Newman Keuls test for the assessment of significance. *P*- values <0.05 were considered to denote statistical significant differences. Data are expressed as the mean ± SEM of 3 to 13 sets of experiments.

3. Results

3.1. Increase in β_2AR density after prolonged ET-1 incubation in vitro

Incubation with 10^{-7} mol/L ET-1 for a period of 72 hr led to a 52% increase in β_2AR density in the plasma membranes of smooth muscle cells (Control: 35.4 ± 3.5; ET-1: 56.3 ± 4.2 fmol/mg protein, $P < 0.01$, $N = 5$). The affinity of the β_2AR for the radioligand remained unchanged (Control: K_D 36 ± 4 pmol/L; ET-1: K_D 44 ± 7 pmol/L) (Fig. 1A). The ability of β_2AR to form the agonist-promoted "high affinity state" was determined in agonist competition experiments (Fig. 1B). In smooth muscle cells grown without ET-1, 58% of the receptors bound the β-agonist with high affinity ($K_{D_H} = 2.8\pm$ 0.5 nmol/L, $N = 3$), while 42% of the β_2AR population bound the β-agonist with low affinity ($K_{D_L} = 88\pm$ 12 nmol/L). After addition of the non-hydrolyzable GTP-analog Gpp(NH)p (10^{-4} mol/L) the curve was steepened and shifted rightward, which indicated that all receptors bound the βAR-agonist with low affinity (Fig. 1B). In ET-1 treated cells 54% of the β_2ARs were able to form the agonist-promoted "high affinity state", indicating that β_2ARs were not uncoupled from the G-protein. When the $ET_{A/B}$ receptor antagonist bosentan was added to the growth medium prior to ET-1 incubation, the increase in β_2AR density was inhibited (42.0 ± 2.3 fmol/mg protein, data not shown). Bosentan alone did not alter β_2AR density.

3.2. Dose response and time course of the increase in β_2AR after chronic ET-1 incubation

Incubation of smooth muscle cells for 72 hr with various ET-1 concentrations (10^{-10} to 10^{-7} mol/L) resulted in a dose-dependent increase in β_2AR binding sites (data not shown). Incubation with 10^{-9} mol/L ET-1 was sufficient to evoke a significant increase in β_2AR density (Control: 33.0 ± 1.4 vs. ET-1: 42.1 ± 2.0 fmol/mg protein, $P < 0.01$, $N = 4$). Further experiments were performed with 10^{-7} mol/L ET-1, since a maximal receptor increase (53.8 ± 5.2 fmol/mg protein) was induced at this concentration.

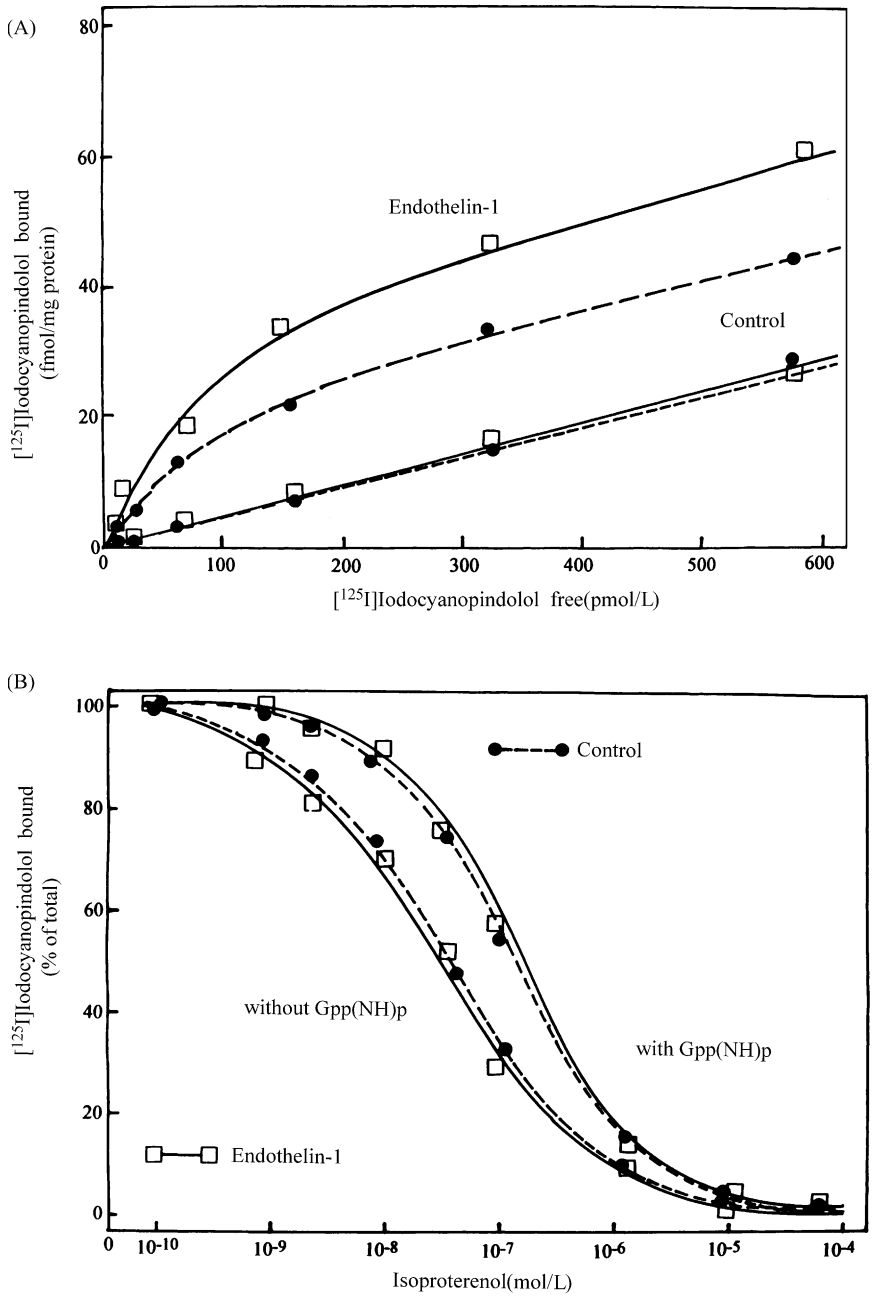

Fig. 1. Effects of ET-1 on β_2AR density and G-protein coupling. (A) Increase of β_2ARs in smooth muscle cells after 72 hr of ET-1 incubation. Cells were either left untreated (control) or grown in the presence of 10^{-7} mol/L ET-1 for 3 days (dashed lines). The density of β_2ARs in crude plasma membranes was determined using the radiolabeled βAR antagonist [^{125}I]ICYP in saturation isotherms. Non-specific binding was defined as the residual binding in the presence of alprenolol (5×10^{-6} mol/L, straight line). The average of five different sets of experiments is shown in which least square curve fitting techniques based on the mass law action were applied. The affinities of the receptor for the radioligand remained unchanged after ET-1 incubation ($N = 5$, $^{*}P < 0.01$). (B) Coupling of βARs. The agonist-competition curves in the absence or presence of the non-hydrolyzable GTP analog Gpp(NH)p are shown for each group. Duplicate determinations were made for each concentration of the β-agonist isoproterenol and plotted as means of three series of experiments. After ET-1 incubation β_2ARs were not uncoupled from the G-protein.

Treatment with ET-1 (10^{-7} mol/L) for various time points (6, 12, 24, 36 and 48 hr) showed an initially non-significant decrease in receptor density after 6 hr followed by an increase after 24 hr (Fig. 2). A maximal increase occurred after 48 hr (ET-1: 53.8 ± 5.0, $P < 0.05$, $N = 4$). Incubation for 72 hr did not result in a further increase in β_2AR density.

3.3. Mechanism responsible for the regulation of β_2AR density

In order to determine whether the increase of β_2ARs was caused by *de novo* synthesis or enhanced receptor externalization, ET-1 (10^{-7} mol/L, 48 hr) was added to cells in the presence of cycloheximide or actinomycin D. The

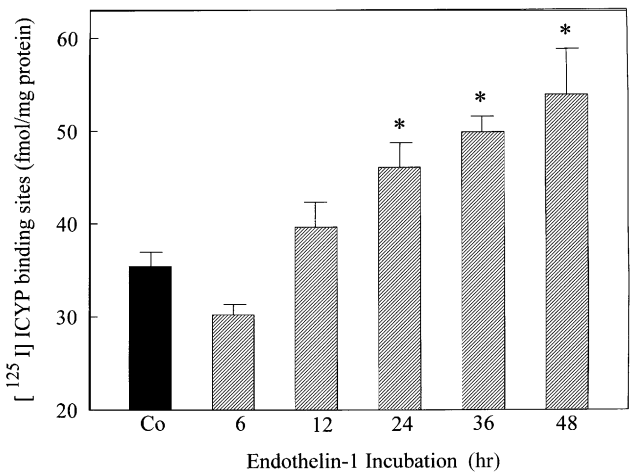

Fig. 2. Time course of β_2AR expression during ET-1 incubation. Cells were incubated with ET-1 (10^{-7} mol/L) for the indicated time points. The increase of β_2AR started after 24 hr and reached a maximum after 48 hr. After 6 hr of ET-1 incubation a transient decrease in β_2AR number occurred that was not longer detectable after 12 hr. The average ± SEM of three sets of experiments are shown and least square curve fitting techniques were applied ($N = 4$, $^*P < 0.05$).

enhancement in β_2AR density was inhibited by 10^{-6} mol/L CHX (ET-1: 53.0 ± 4.9 vs. ET-1 + CHX: 37.5 ± 3.8 fmol/mg protein, $N = 4$, $P < 0.05$), suggesting that the increase was due to *de novo* synthesis (Fig. 3A). Furthermore, 10^{-6} mol/L actinomycin D inhibited receptor upregulation (36.8 ± 3.7 fmol/mg protein, $N = 4$, $P < 0.05$), which indicated that mRNA synthesis was required for ET-1-induced increase of β_2AR.

To determine the effects of chronic ET-1 incubation on β_2AR mRNA levels, semiquantitative RT-PCR was performed. Similar to the receptor binding experiments (Fig. 2), after 2 hr of ET-1 incubation a decrease of 50 ± 7.5% was observed which persisted for 6 hr. After 8 hr of ET-1 incubation β_2ARs-specific mRNA expression increased to control levels and after 12 hr, a 153 ± 8.8% increase was detected ($N = 3$, $P < 0.05$, Fig. 3B). GAPDH transcription served as an internal control but remained unchanged during ET-1 incubation.

The contribution of NO, prostaglandins and PKC to ET-1-induced β_2ARs upregulation was investigated using the pharmacological inhibitors N:(G)-monomethyl-L-arginine (L-NMMA), indomethacin and calphostin C, respectively. As shown in Fig. 4, in the presence of increasing concentrations of calphostin C, a PKC inhibitor, ET-1-mediated increase in β_2AR density was dose-dependently suppressed (ET-1 + 10^{-6} mol/L calphostin C 33.0 ± 6.0 fmol/mg protein, $P < 0.05$, $N = 4$). In contrast, ET-1 cotreatment with either L-NMMA or indomethacin did not affect β_2AR upregulation. This indicated that PKC but neither NO nor prostaglandin metabolites were involved in the ET-1 effect.

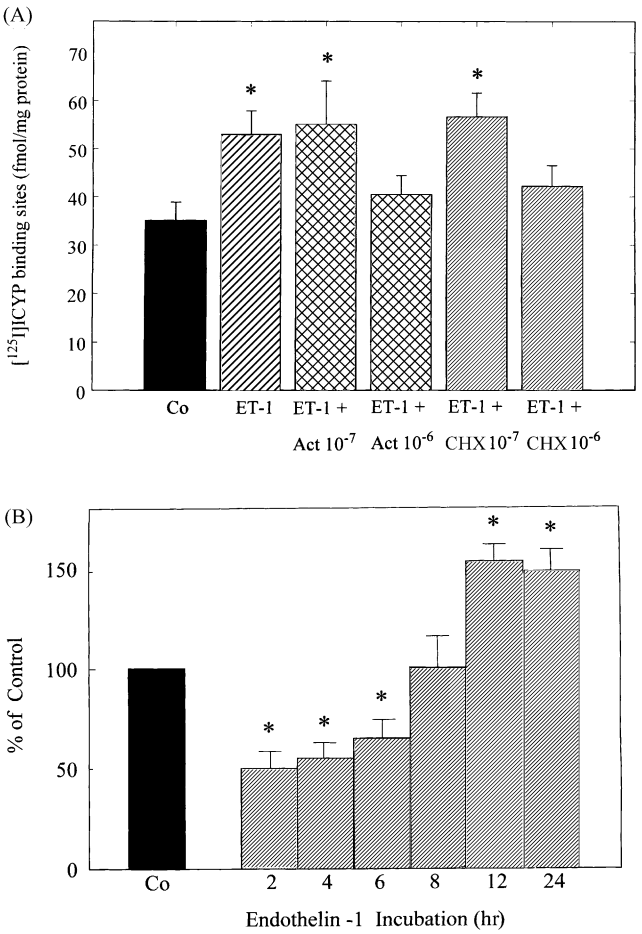

Fig. 3. Requirement of *de novo* synthesis for ET-1-mediated upregulation of βARs. (A) Effect of inhibitors of macromolecular synthesis. To analyze whether protein or mRNA synthesis was required for βAR upregulation, smooth muscle cells were incubated with 10^{-7} mol/L ET-1 in the presence of 10^{-7} or 10^{-6} mol/L of the protein synthesis inhibitor cycloheximide (CHX) or actinomycin D (Act), an inhibitor of mRNA synthesis. The average ± SEM of four sets of experiments are shown in which least square fitting techniques were applied ($N = 4$, $^*P < 0.05$ vs. Co = Control). (B) Time-dependent increase of β_2AR mRNA expression after ET-1 incubation. Total RNA was isolated from ET-1-treated or control smooth muscle cells. mRNA levels were quantified by semiquantitative RT-PCR using biotinylated oligonucleotides followed by an incubation with SPA® elements as described in Section 2. The values are expressed as percentage ± SEM relative to the control without ET-1 incubation ($N = 3$, $^*P < 0.05$).

3.4. Increase of the α-subunit of the stimulatory G-protein after ET-1 incubation

For determining long-term effects of ET-1 incubation on expression of the α-subunit of the stimulatory G-protein, cells were incubated for various time points up to 72 hr with ET-1. As demonstrated by immunoblot analysis (Fig. 5), the density of the 54 kDa band corresponding to the α-subunit increased after 24 hr to 260 ± 26% of control ($N = 7$, $P < 0.01$). The expression of the 48 kDa splice variant was also time-dependently increased, while the intensity of tubulin, which served as a non-related protein control, remained unchanged (data not shown).

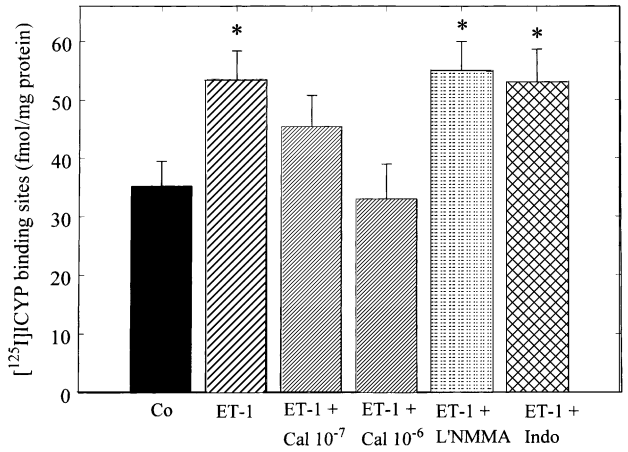

Fig. 4. Effects of calphostin C, L-NMMA and indomethacin on ET-1-mediated transregulation of β_2ARs. Cells were incubated in the absence or presence of ET-1 together with either calphostin C (Cal, 10^{-7} or 10^{-6} mol/L), L-NMMA or indomethacin (Indo). Radioligand binding was measured after 48 hr of incubation. Data are expressed as mean ± SEM ($N = 4$). $^{*}P < 0.05$ between treatment and control.

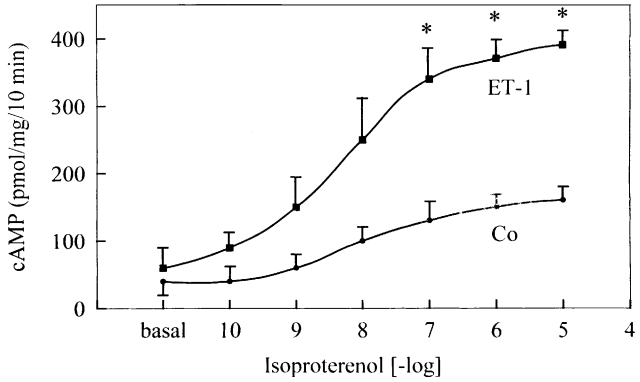

Fig. 6. Effect of ET-1 on adenylyl cyclase activity. Smooth muscle cells were grown for 3 days either in the presence or absence of ET-1 (10^{-7} mol/L), before plasma membranes were prepared and analyzed for adenylyl cyclase activity. Stimulation of βARs with eisoproterenol induced a higher increase of cAMP synthesis in ET-1-treated cultures than in controls ($N = 3$, $^{*}P < 0.01$).

When bosentan (10^{-5} mol/L) was added to the growth medium prior to ET-1 incubation, the increase of both splice variants was inhibited by approximately 28%. Bosentan alone did not alter $G_{s\alpha}$-protein expression.

3.5. Increased isoproterenol-mediated adenylyl cyclase activity after ET-1 incubation

To assess the functional relevance of the increased β_2AR density, the responsiveness of adenylyl cyclase after β_2AR activation was determined. Isoproterenol-stimulated adenylyl cyclase activity was increased after 72 hr of incubation with 10^{-7} mol/L ET-1 (Fig. 6). Compared to cells grown in the absence of ET-1, a concentration of 10^{-7} mol/L isoproterenol caused an 228% increase of adenylyl cyclase activity (Control: 128 ± 28, ET-1 treated cells: 292 ± 40 pmol/mg/10 min cAMP, $N = 3$, $P < 0.01$).

3.6. Chronic ET-1 infusion in rats

Since the previous experiments demonstrated a transregulation of the ET-1 and β_2AR system in cultured smooth muscle cells, we asked whether this observation might represent a more general mechanism. To address this question, male rats were intravenously infused with ET-1 (7.5 ng/kg/min) for 3 weeks. The systolic blood pressure was elevated after 3 weeks of ET-1 infusion (Controls: 95 ± 2.7 mm Hg; ET-1-treated rats: 104 ± 2.97 mm Hg,

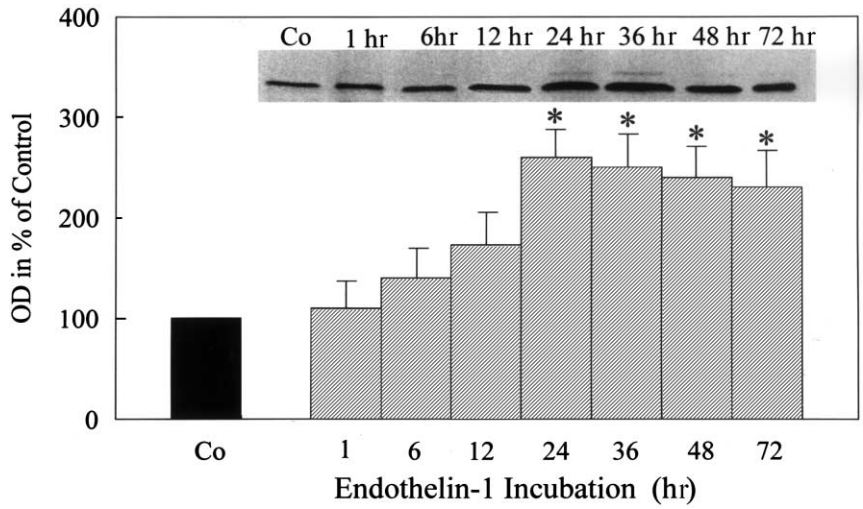

Fig. 5. Effect of ET-1 on the expression of the stimulatory G-protein α-subunit. After the indicated times, crude plasma membranes were prepared from ET-1-treated smooth muscle cells, separated by SDS polyacrylamide gel electrophoresis and immunoblotted with an antiserum against the α-subunit. The intensity of the specific 54 kDa protein band was determined by densitometric analysis and is indicated as the ratio relative to the control. Following ET-1 incubation, a continuous increase of α-subunit expression was detected. The upper panel shows one representative immunoblot of the 54 kDa band from seven different experiments ($N = 7$, $^{*}P < 0.01$).

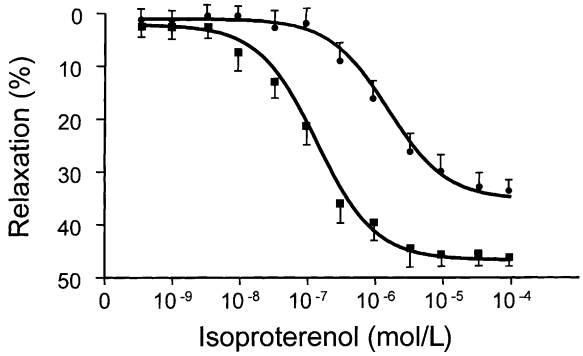

Fig. 7. βAR-mediated vasorelaxation after chronic ET-1 infusion. Male rats were infused with ET-1 (7.5 ng/kg/min) for 3 weeks. Denuded aortic rings were placed in an organ bath and contracted with epinephrine (10^{-6} mol/L). To study the βAR-mediated vasodilation, isoproterenol was added at the indicated concentrations. Isoproterenol-mediated vasodilation was significantly increased in aortic rings from ET-1-treated rats compared to those from controls ($N = 4$, $^{*}P < 0.01$).

$N = 10$, $P < 0.05$). Myocardial βAR density increased from 84 ± 8.5 to 222 ± 12.6 fmol/mg after chronic ET-1 infusion. In rat lung the expression of βAR after ET-1 infusion was increased by 43% (Controls: 556 ± 28, ET-1 treated rats: 797 ± 14 fmol/mg protein, $N = 4$, $P < 0.01$). Similarly, in rat aorta the receptor density was enhanced by 62% (Controls: 23.2 ± 1.2 vs. ET-1-treated rats: 37.7 ± 1.6 fmol/mg protein, $N = 4$, $P < 0.01$).

In order to analyze whether the increase in β_2AR expression had a functional relevance *in vivo*, vasorelaxation was measured in denuded aortic rings. Maximal vasoconstriction induced by epinephrine was 15.5 ± 0.6 mN in controls, but was reduced to 12.7 ± 0.7 mN ($P < 0.01, N = 13$) after ET-1 treatment. Moreover, as shown in Fig. 7, isoproterenol-mediated vasorelaxation was increased ($N = 4$, $P < 0.01$) after chronic ET-1 infusion. Thus, these results demonstrate that a chronic elevation of ET-1 leads to a counterregulatory transactivation of the βAR system both *in vitro* and *in vivo*.

4. Discussion

The present study showed that chronic activation of ET receptors resulted in the upregulation of βAR by a mechanism requiring gene expression. The $ET_{A/B}$ receptor antagonist bosentan inhibited this transregulation *in vitro*. Inhibition of PKC prevented the increase in β_2AR density. Furthermore, expression of the $G_{s\alpha}$-subunit was induced after chronic ET-1 incubation. This transregulation was of functional relevance, since β_2AR-mediated adenylyl cyclase activity increased. Importantly, a similar upregulation of βAR density was observed in lung, heart and aorta of rats chronically exposed to ET-1. This upregulation of βAR resulted in increased isoproterenol-mediated vasodilation of aortic rings. Thus, the increase in βAR density may represent a counterregulatory adaptive mechanism limiting the vasoconstrictive action of ET-1.

Our present results are corroborated by recently published data. Simonson and Dunn [21] showed that ET-1 amplified βAR-stimulated cAMP accumulation by a PGE_2-dependent mechanism. Transgenic overexpression of ET-1 was associated with glomerulosclerosis and interstitial fibrosis, but did not lead to hypertension [4]. Furthermore, the knockout of ET-1 in mice by homologous recombination resulted in slightly higher blood pressure but not, as expected, in hypotension [22]. The exact reasons for these apparently contradictory findings in the acute effects on blood pressure of intravenous ET-1 infusion vs. overexpression or chronic administration of ET are largely unclear. It can be assumed that long-term activation of the ET system results in strong activation of adaptive compensatory mechanisms.

The counterregulation of ET-1 action could involve either downregulation of ET receptors, inhibition of downstream signaling or unrelated mechanisms. The activation of the endothelial ET_B receptor has been shown to trigger increased synthesis of NO and prostacyclin, both of which have vasodilating effects and may counteract the vasoconstrictive activity of ET-1 [23]. Increased levels of PGE_2 may further cause increased cAMP levels [24] which may serve as an additional negative regulator for vasoconstriction.

In our experiments, we could demonstrate an involvement of neither NO nor prostaglandin synthesis in the counterregulatory response leading to β_2AR expression, as pharmacological inhibition of both pathways by L-NMMA or indomethacin did not abolish the effect. Instead, the increase in β_2ARs was PKC-dependent, since the PKC inhibitor calphostin C inhibited ET-1-induced β_2AR upregulation. Previously, it was reported that agonists of the phosphoinositol cascade could potentiate βAR-stimulated cAMP accumulation by a PKC-dependent pathway [25,26]. In vascular smooth muscle cells, angiotensin II amplified cAMP accumulation stimulated by isoproterenol and vasoactive intestinal peptide by a PKC-dependent mechanism [27–29]. This might be due to a differential activation of PKC isoenzymes by physiologically relevant agonists such as ET-1, ATP and phenylephrine [30]. Whether PKC up- or downregulates the βAR has not been conclusively demonstrated. Reupcke *et al.* [31] found that a short activation of PKC by phorbolesters resulted in a downregulation of βAR in myocytes. In contrast, Yonemochi *et al.* showed that captopril-mediated upregulation of βARs was PKC-dependent [32], while a prolonged PKC activation induced the transcriptional expression of α_{1B}AR in smooth muscle cells [33]. Recently, Bin *et al.* characterized an effect showing that chronic elevated interleukin-1β concentrations upregulates βAR by a PKC-dependent mechanism [34]. In these experiments chronic incubation with interleukin-1β increased β_2AR receptor density to 213%. This effect was inhibited by inhibition of PKC but also by cycloheximide [35]. Interleukin-1β increased β_2AR mRNA and protein over time (maximum 36 hr)

and in biphasic fashion with increasing dose [35]. Furthermore, phospholipase A_2 expression and activity was increased by chronic ET-1 incubation in mesangial cells [36].

ET-1 may also interact with other vasoconstrictor systems such as angiotensin II [37]. It was also shown that incubation of human mammary artery rings with low concentrations of ET-1 potentiated serotonin- and norepinephrine-mediated vasoconstriction [38]. In contrast to these vasoconstrictive actions, our *in vitro* and *in vivo* results as well as the transgenic and knockout animal models clearly show that, unlike the acute effects, a chronic exposure to ET-1 changes the balance between vasoconstrictor and vasodilating responses by inducing adaptive feedback mechanisms. Since ET-1 is chronically elevated rather early during development of heart failure [7,8], it may prevent β_2AR downregulation. This might stabilize myocardial function in the initial phase of myocardial insufficiency and prevent apoptosis [39]. Future experiments will have to define the exact molecular mechanisms and signaling cascades involved in ET-1 induced upregulation of the β_2AR system as well as its pathophysiological relevance.

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