Chronic elevated endothelin-1 concentrations regulate mitogen-activated protein kinases ERK 1 and ERK 2 in vascular smooth muscle cells

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ABSTRACT
Increased endothelin-1 (ET-1) levels are found in patients with atherosclerosis. ET-1 is known to increase the mitotic response of different growth factors already at threshold concentrations. The aim of this study was to investigate the influence of ET-1 on the mitogen-activated protein (MAP) kinases, extracellular signal-regulated protein kinase (ERK) 1 and ERK 2. Smooth muscle cells were incubated with ET-1 at a concentration of $10^{-7}$ M for 1–120 h. ERK 1 and ERK 2 were determined in cell homogenates by electrophoresis. Specific antibodies were used to investigate the amount of ERK 1 or ERK 2 in the homogenate. The functional activity of ERK 1 and ERK 2 was determined. Immunofluorescence microscopy was performed to analyse the translocation of the MAP kinases into the nucleus. ET-1 incubation for 12 h decreased ERK 1 concentration by $\approx 51\%$. After 36 h of ET-1 application the concentration of ERK 1 increased to control levels again. When the cells were incubated for 120 h ERK 1 rose by +65% above control. The incubation with ET-1 in the presence of an ET$_A$ receptor antagonist inhibited the increase of ERK 1. ERK 2 showed a comparable time course with an initial decrease in the protein concentration followed by an increase after 120 h. Incubation with an ET$_A$ receptor antagonist inhibited the increase in protein concentration after 120 h. However, the functional activity of both MAP kinases remained unchanged between 1 and 120 h. Especially, after 120 h of ET-1 incubation no translocation into the nucleus was observed. However, an additional stimulus with angiotensin II resulted in translocation of ERK into the nucleus. These data show that ET-1 increases the protein concentration of MAP kinases ERK 1 and ERK 2 but not their basal activity. Only an additional stimulation with angiotensin II leads to the translocation of ERK into the cell nucleus.

INTRODUCTION
The role of endothelin-1 (ET-1) in cardiovascular diseases with dysregulation of vascular tone, such as congestive heart failure, pulmonary and systemic hypertension, and cerebrovascular spasms after subarachnoid haemorrhage, is well documented. Stimulation of ET receptors leads to coupling of G-proteins, inducing an activation of protein kinase C and mitogen-activated protein (MAP) kinases. ET-1 is known to be a potent co-mitogen and pro-

Key words: endothelin-1, mitogen-activated protein kinase, vascular smooth muscle cell.
Abbreviations: ET, endothelin; ERK, extracellular signal-regulated protein kinase; MAP kinase, mitogen-activated protein kinase; PDGF, platelet-derived growth factor.
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liferation promoting factor in smooth muscle cells and fibroblasts [1,2]. In atherosclerotic lesions ET-1 was found in high concentrations [3,4]. Inhibition of ET₄ receptors reduces the induction of fibromuscular plaque formation [5].

Different studies using animal or human tissue indicate that ET-1 contributes to vascular structural changes in proliferative cardiovascular disease [6,7]. However, ET-1 alone is not able to induce cell proliferation [8]. MAP kinase activation alone is necessary, but not sufficient, to induce cell proliferation [9]. Additionally, crosstalk of G-protein-coupled receptors, such as ET receptors, with other receptor pathways has been documented [10]. The aim of this study was to investigate the regulation of the MAP kinases extracellular signal-related protein kinase (ERK) 1 and ERK 2 after chronic ET-1 incubation.

**METHODS**

**Cell culture**
A-10 cells, derived from embryonic rat thoracic aortal smooth muscle, were obtained from the Deutsche Sammlung für Zellkultur (Wiesbaden, Germany) and maintained in Dulbecco’s modified Eagle’s medium containing 100 units/ml penicillin, 100 μg/ml streptomycin and 10% (v/v) heat-inactivated newborn calf serum. A-10 cells express only the ET₄ receptor subclass. Experiments were performed in serum-free tissue medium containing transferrin (10 μg/ml) and thyroglobin (10 μg/ml). Cells were treated with ET-1 (10⁻¹⁰ to 10⁻¹² M) for 1 h to 120 h.

**Western blot analysis**
Plasma membranes (10 μg) were fractionated by SDS/12% (w/v) polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Amersham, Germany) using an electrophoretic transfer cell (Hoefer, Freiburg, Germany) at 100 V for 0.5 h (2.5 mA/cm²). Membranes were blocked for 12 h with 5% (w/v) non-fat dry milk powder in Tris-buffered saline and then immunoblotted for 2 h with the primary antibody directed against ERK 1 or ERK 2. Bound primary antibody was reacted with anti-rabbit peroxidase-conjugated IgG for 1 h. 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, Freiburg, Germany). Immunohistological detection of total ERK was performed with the antibody described above. Bound primary antibody was detected with FITC-conjugated anti-rabbit IgG. Statistical tests were performed using ANOVA and Student’s Newman Keuls test for the assessment of significance. P values < 0.05 were considered to denote statistically significant differences. Data are expressed as the mean ± S.E.M. for three to seven sets of experiments.

**RESULTS**

**Increased expression of ERK 1 after chronic ET-1 incubation**
Smooth muscle cells were incubated under serum-free conditions with ET-1 for 1–120 h. In the cell homogenate a biphasic regulation of ERK 1 could be demonstrated. In the presence of ET-1, ERK 1 decreased by 15% after 1 h of incubation. During the first 12 h ERK 1 protein level was reduced by 51%. But after 36 h a constant increase was shown. A maximum increase of +65% above control could be found after 120 h of ET-1 incubation (P < 0.05) (Figure 1).

**Changed expression of ERK 2 after chronic ET-1 incubation**
During the first 12 h of ET-1 incubation ERK 2 concentration decreased by 39%. Afterwards ERK 2 gradually increased to control levels after 36 h and a maximum of +38% above control was reached after 96 h of ET-1 incubation (P < 0.05; results not shown).

**Influence of ET receptor antagonists on ERK 1 and ERK 2**
In order to show a specific ET receptor-dependent mechanism, smooth muscle cells were incubated with ET-1 for 120 h in the presence of the ET₄ receptor antagonist LU 302146 (1 μM, Knoll, Ludwigshafen, Germany). After 120 h of ET-1 incubation ERK 1 and ERK 2 were increased. The co-incubation with the ET receptor
Regulation of mitogen-activated protein kinases by endothelin

Figure 2 MAP kinase activity
After 1–120 h of incubation with ET-1 the kinase activity was not significantly changed.

antagonist blocked ERK 1 and ERK 2 upregulation (results not shown).

Activity of MAP kinase after ET-1 incubation
Since ET-1 activates MAP kinases, their activity was investigated. During the time course of ET-1 incubation the MAP kinase was not activated (Figure 2), although the protein amount was increased. These data show that chronic ET-1 incubation did not induce a permanent activation of MAP kinases.

Influence of angiotensin II on MAP kinase after chronic ET-1 incubation
In order to find out whether ERK can be activated again after 120 h of ET-1 stimulation, smooth muscle cells were incubated with angiotensin II for 30 min. Immunofluorescence microscopy was performed to analyse the translocation of the MAP kinases into the nucleus of the cells as an indirect marker of activation. After addition of angiotensin II, MAP kinases translocated into the nucleus (results not shown).

DISCUSSION

These data show that chronic incubation of smooth muscle cells with ET-1 results in an increase of protein concentration of the MAP kinases ERK 1 and ERK 2. After 120 h of incubation ERKs were not activated. However, the addition of angiotensin II induced a rapid activation of MAP kinases with a translocation into the nucleus of the smooth muscle cells. This shows that a chronic incubation of smooth muscle cells with ET-1 increases the protein level of ERK 1 and ERK 2, which then become activated by an additional stimulus. This additional stimulus activates the increased amount of MAP kinases and might lead to an augmented activity.

Recent studies using animal models indicate that ET-1 contributes to vascular changes in cardiovascular disease [11,12]. It is interesting to note that in vitro experiments with cultured smooth muscle cells did not always find mitogenic effects of ET-1. These differences were attributed to varying culture conditions, especially whether serum was present or absent [6,7]. In our experiments cells did not grow under serum-free conditions. Cell proliferation was not found and MAP kinases ERK 1 as well as ERK 2 were not activated.

Yang et al. could demonstrate that ET-1 alone was not able to increase smooth muscle cell proliferation [8]. The ineffectiveness of ET-1 to stimulate smooth muscle cell proliferation was further confirmed by the results that ET-1 was not able to activate cyclin-dependent kinase 2. Furthermore, the cdk inhibitor, p27kip1, was downregulated and the retinoblastoma protein, pRb, was not hyperphosphorylated, being crucial for cell cycle progression. The addition of platelet-derived growth factor (PDGF)-BB, however, potentiated [3H]thymidine incorporation. These effects were inhibited by the ET\textsubscript{A} receptor antagonist LU135252. The potentiating effects of ET-1 on cell growth were not due to an overexpression of PDGF receptors in those experiments.

In our experiments ET-1 did not activate MAP kinases ERK 1 and ERK 2 but increased their protein levels. ERK 1 and ERK 2 were not activated after 120 h of ET-1 incubation. Additionally a translocation into the nucleus was not present. Only the additional stimulation with angiotensin II activated the ERKs.

These data show that ET-1 induces adaptive changes in the second messenger cascade that provide a potentiated activation of other receptor systems (as shown for PDGF-BB [8]). This increase of ERKs was inhibited by ET\textsubscript{A} receptor blockade. This indicates that stimulation of ET\textsubscript{A} receptors is responsible for the potentiating effects of ET-1. These regulatory mechanisms of ET-1 potentiate the responses to other receptor stimuli that might be important in the development of functional and structural changes in patients with atherosclerosis or restenosis. These data provide support that an early use of ET receptor antagonists might prevent the adaptive changes due to ET-1 in other receptor pathways.

REFERENCES