Mechanosensitive Transcription Factors Involved in Endothelin B Receptor Expression

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Mechanosensitive endothelin B receptor expression

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Summary

Growing evidence suggests an involvement of the endothelin B receptor (ET\textsubscript{B}-R) in blood pressure-dependent arterial remodeling. To study the molecular mechanisms leading to deformation-induced ET\textsubscript{B}-R expression, we have cultured rat aortic smooth muscle cells (SMC) on flexible elastomers and, when grown to 70% confluence, periodically stretched them for 6 h (15% elongation, 0.5 Hz). The cells responded with an increase both in ET\textsubscript{B}-R mRNA (12-fold compared to control) and protein (4-fold). According to nuclear run-on analysis this increase in ET\textsubscript{B}-R expression occurred at the level of transcription. Among various kinase pathways, rho kinase (ROCK) and p38 mitogen-activated protein kinase (p38 MAPK) mediated part of the deformation-induced increase in ET\textsubscript{B}-R expression, as judged by the inhibitory effect of Y27632 (1 \( \mu \)M, 38% inhibition) and SB202190 (10 \( \mu \)M, 44% inhibition), respectively. Gel shift assaying of the 3 transcription factors principally activated by these kinases, revealed a transient deformation-induced activation of activator protein-1 (AP-1) and CCAAT enhancer binding protein (C/EBP), but not activating transcription factor (ATF) that was sensitive to both Y27632 and SB212190. The potential role of AP-1 and C/EBP in deformation-induced ET\textsubscript{B}-R expression was verified both by using decoy oligodeoxynucleotides (dODN) mimicking the DNA-binding sites of these transcription factors and a nuclear run-on based assay employing specific antibodies directed against AP-1 and C/EBP. Both techniques unequivocally demonstrated that activation of these transcription factors, namely that of C/EBP\textsubscript{β}, contribute to the increase of ET\textsubscript{B}-R gene expression in response to cyclic stretch.
Introduction

One of the most potent stimuli for the synthesis of endothelin-1 (ET-1) is a high level of circumferential stretch to which preferentially SMC but also endothelial cells are exposed to by an increase in blood pressure (1). Besides its vasoconstrictor effect primarily mediated by the A type receptor (ET$_A$-R), ET-1 has also been shown to be a potent co-mitogen for vascular smooth muscle cells (SMC, 2). Significant expression of the B type receptor (ET$_B$-R) in these cells is only detectable upon exposure to cyclic stretch (3,4). However, activation of this receptor seems to contribute to changes in medial SMC phenotype that are typical for the early phase of pressure-dependent remodeling of the vessel wall (3,4).

The endothelin system may thus act as a molecular switch between maintenance of vascular tone through ET$_A$-R-mediated vasoconstriction and, in conditions of a prolonged supra-physiological increase in blood pressure, ET$_B$-R-mediated adaptive remodeling. This process, when exaggerated, may play an important role e.g., in the manifestation of arterial hypertension (5), vein graft disease (6) or restenosis following angioplasty (7) that often are resistant to therapeutic interventions.

Preventing deformation-induced ET$_B$-R expression in vascular SMC may thus constitute a feasible therapeutic option at the onset of these cardiovascular complications. To this end we have investigated the transcriptional control of ET$_B$-R expression in vascular SMC exposed to cyclic stretch by using e.g., a novel nuclear run-on-based technique that directly analyzes the functional significance of a given transcription factor in expression of the gene product of interest (run on based regulation of transcription analysis, ROBERTA).
Experimental procedures

Materials

Antibodies against AP-1 (mouse monoclonal, 2 mg/mL), C/EBPβ (mouse monoclonal, 2 mg/mL) and the signal transducer and activator of transcription (STAT-1, mouse monoclonal, 2 mg/mL) were from Santa Cruz, Heidelberg, Germany. The antibody against rat fibronectin (mouse monoclonal, 1 mg/mL) was from BD Biosciences, Heidelberg, Germany. The antibody against β-actin (mouse monoclonal), secondary antibodies (goat anti-mouse, HRP-coupled) as well as laboratory chemicals were from Sigma, Deisenhofen, Germany. 4-(4-Fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole (SB212190) was from Calbiochem, Bad Soden, Germany. (+)-(R)-trans-4-(1-Aminoethyl)-N-(4-pyridyl)-cyclohexanecarboxamide (Y27632) was a kind gift of Yoshitomi Pharmaceutical Industries, Osaka, Japan.

Cell culture

Isolation and culture of SMC derived from the rat aorta was done as previously described (3).

RT-PCR analysis

Isolation of RNA, reverse transcription and subsequent analysis of relative amounts of cDNA by PCR was done as described previously (3). The range of cycles to be in the exponential phase of the PCR reaction was newly established for each set of samples.

Gel shift analysis

Gel shift analyses were performed as described previously (8). The custom made palindromic oligodeoxynucleotide (NAPS, Goettingen, Germany) containing the consensus binding site for ATF had the following sequence: 5'
GACTTGACGTC-3’. It was hybridized to the double-stranded form by standard procedures (9).

**Decoy oligodeoxynucleotide (dODN) technique**

Incubation of SMC with the double-stranded dODN (10 µM final concentration) for 4 h was done as described previously (10). Exposure of the cells to 2 different dODN was done consecutively with an exchange of the medium in-between. The sequence for the ATF dODN was exactly the same as that used for gel shift analysis except that the terminal 4 bases on either site of the molecule were linked by phosphorothioate esters for added stability.

**Nuclear run-on analysis and ROBERTA**

The RT-PCR-based nuclear run-on analysis was done as described previously (8). Besides the de novo transcription of the ETB-R gene, transcription of GAPDH as an internal standard was analyzed, too. The run-on-based regulation of transcription assay (ROBERTA) is a modified nuclear run-on-analysis. Prior to incubation with nucleotides (CTP, GTP and UTP, 2 mmol/L each) and the energy-regenerating system (creatinine-phosphate plus creatine-kinase) for specific RNA synthesis, nuclei (150 µL of suspension) were incubated at 0°C for 15 min with 0.5% Triton X-100, ATP (3 mmol/L) and the antibody against the transcription factor of interest. Thereafter, the nuclear run-on procedure was started by the addition of the three ribonucleotides, creatinine-phosphate and creatine kinase, exactly as described (8). The amount as well as the type of antibody for effective inhibition of transcription had to be established for every transcription factor and antibody. In this study the final antibody concentrations were as follows: anti-c-Jun (20 µg/mL); anti-C/EBPβ (20 µg/mL); anti-STAT-1 (20 µg/mL) and anti-fibronectin (50 µg/mL).
Western blot analysis

Western blot analysis was performed according to standard procedures (10) using PVDF membranes (PALL Gelman, Dreieich, Germany). For detection of ET$_B$-R protein, a primary rabbit polyclonal antibody derived against a C-terminal peptide of the receptor (11) was used together with a secondary HRP-conjugated anti-rabbit antibody and the Super Signal Blaze™ chemiluminescent reagent (Pierce, St. Augustin, Germany). Loading and transfer of equal amounts of protein in each lane was verified by re-probing the membrane (5 min incubation in 200 mmol/L sodium hydroxide and consecutive neutralization by several washes with distilled water) with a monoclonal anti-β-actin antibody and visualization with a secondary HRP-conjugated anti-mouse antibody as described (9).

Statistical analysis

Unless indicated otherwise, results are expressed as means ± SEM of n observations with cells obtained from different animals. One sample t test with two-sided P value or one-way analysis of variance followed by Dunnett post test were used where appropriate to determine statistical significant differences between the means and/or the means and control with \( P < 0.05 \) considered significant.
Results

Stretch-induced ET<sub>B</sub>-R expression is regulated at the transcriptional level

ET<sub>B</sub>-R mRNA and protein abundance were both enhanced in the cultured SMC after 6 h exposure to a level of cyclic stretch similar to the in vivo situation (12, Fig. 1). To evaluate whether the increase in ET<sub>B</sub>-R mRNA was due to transcriptional activation or enhanced mRNA stability, SMC were incubated with the transcriptional inhibitor actinomycin D (Fig. 2A). In addition, a RT-PCR-based nuclear run-on assay was performed, demonstrating enhanced de novo synthesis of ET<sub>B</sub>-R mRNA in nuclei isolated from stretched SMC as compared to nuclei from SMC incubated under static conditions (Fig. 2B).

Signal transduction pathways involved in stretch-induced ET<sub>B</sub>-R expression

Signal transduction pathways thought to be activated upon mechanical stimulation in vascular cells were targeted by using specific protein kinase inhibitors. Neither PD98059 (1 µM, 1 h pre-incubation), a specific inhibitor of MEK1 located up-stream of ERK/1/2, nor the protein kinase C inhibitor, Ro 31-8220 (1 µM, 1 h pre-incubation), affected deformation-induced ET<sub>B</sub>-R expression (n=4, not shown). In contrast, the ROCK inhibitor Y27632 as well as SB 212190, an inhibitor of p38 MAPK, significantly attenuated ET<sub>B</sub>-R expression in response to cyclic stretch both at the mRNA and protein level (Fig. 3). Co- incubation of the SMC with both inhibitors, however, did not result in a more pronounced inhibition (Fig. 3A), indicating that both kinases are part of a common mechanotransduction pathway.

Transcription factors involved in stretch-induced ET<sub>B</sub>-R expression

According to TRANSFAC analysis (13), the promoter of the human ET<sub>B</sub>-R gene (GenBank accession no. AL139002) contains several consensus binding sites for the
transcription factors ATF, AP-1 and C/EBP, the activation of which has been linked to
the ROCK and/or p38 MAPK signaling pathways (14-17). Therefore, nuclear translocation of these transcription factors in SMC exposed to cyclic stretch was monitored by gel shift analysis. Whereas the transcription factor ATF appeared to be inert to cyclic stretch (not shown), both AP-1 and C/EBP abundance in the nucleus were transiently enhanced (maximum 1 h, not shown). Moreover, translocation of AP-1 as well as C/EBP was sensitive to both Y27632 and SB212190 (Fig. 4). According to super-shift analysis, primarily the β-isoform of C/EBP appeared to be stretch-sensitive.

To confirm the involvement of AP-1 and C/EBPβ activation in stretch-induced ET$_B$-R expression, 2 experimental approaches were chosen. First, SMC were pre-incubated for 4 h with dODN (10 µmol/L), mimicking the consensus binding sites of the candidate transcription factors. Both dODN concentration and pre-incubation period were chosen according to previous experiments with the cultured SMC, demonstrating maximum neutralization of the transcription factors under these conditions (cf. Ref. 10). As shown in Fig. 5, dODN blockade of AP-1 and C/EBP, but not ATF, significantly attenuated stretch-induced expression of the ET$_B$-R gene. Consecutive incubation of the SMC with both the AP-1 and C/EBP dODN, on the other hand, did not result in a further inhibition as compared to the effect of the C/EBP dODN alone (n=4, not shown).

As a second method to verify the involvement of AP-1 and C/EBP in stretch-induced expression of the ET$_B$-R gene, ROBERTA was employed. The principle of this method, described in detail in the methods section, is outlined in Fig. 6. Incubation of nuclei isolated from stretched SMC with a specific anti-C/EBPβ antibody attenuated ET$_B$-R gene expression in response to cyclic stretch, whereas the c-Jun antibody (AP-1) did not reveal a significant effect (Fig. 7A). The inhibitory effect of the antibody directed against C/EBPβ appeared to be specific, for neither an antibody directed against a transcription factor not functional in ET$_B$-R expression (STAT-1) nor an antibody
directed against an unrelated protein (fibronectin) revealed an effect on stretch-induced 
de novo synthesis of ET$_B$-R mRNA (Fig 7B). As a matter of principle, however, the anti-
STAT-1 antibody was clearly active for it efficiently attenuated cytokine-induced de novo
mRNA synthesis of the STAT-1 dependent gene CD40 (approximately 50% inhibition)
in nuclei isolated from cultured SMC under static conditions (not shown).
Discussion

Background

Vascular remodeling per se is an adaptive process enabling the vessel wall to accommodate an increased hemodynamic load. If exaggerated, however, this process becomes pathologic such as, e.g. in manifest hypertension, ultimately leading to fixation of peripheral resistance and eventually occlusion of the blood vessel. There is increasing evidence that the endothelin system is crucially involved in the onset of deformation-induced vascular remodeling (1,3), and that this effect is mediated primarily via the ET\textsubscript{B}-R rather than the ET\textsubscript{A}-R expressed on SMC.

Endothelin receptor antagonism

Although there are several ET\textsubscript{A}-R-specific and mixed antagonists available, these substances for several reasons do not provide an attractive means for challenging ET\textsubscript{1}-induced vascular remodeling. Most importantly, in conditions of enhanced blood pressure, ET\textsubscript{A}-R antagonism may have an adverse effect by weakening the ET\textsubscript{A}-R-mediated vasoconstrictive capacity of the SMC that is normally relieving the hemodynamic load of the vessel wall to some extent, thereby promoting deformation-induced ET\textsubscript{B}-R expression. Activation of the ET\textsubscript{B}-R, in turn, induces or supports the remodeling process (4,18). Therefore, in the absence of a specific non-toxic ET\textsubscript{B}-R antagonist, understanding stretch-induced ET\textsubscript{B}-R expression at the molecular level might lead to the definition of a novel target for interfering with the onset of an exaggerated remodeling process.
Interplay of the 2 signal transduction kinases involved in stretch-induced ET\(_B\)-R expression

Using inhibitors against kinases potentially involved in mechanotransduction, ROCK and p38-MAPK were characterized to contribute to stretch-induced ET\(_B\)-R expression while protein kinase C or the classical MAP kinase pathway were not. This finding was unprecedented for inhibition of protein kinase C, which often integrates multiple extracellular signals, was without effect whereas ROCK located up-stream of protein kinase C (19) and p38 MAPK, one of the best characterized down-stream kinases of protein kinase C (20), both constituted part of the signal transduction pathway mediating stretch-induced ET\(_B\)-R expression. Moreover, the inhibitors of these 2 kinases were similarly effective but when combined did not even produce an additive effect. The most plausible explanation for these results is that both kinases are part of the same signal transduction pathway. For example, ROCK may directly or indirectly activate p38-MAPK, bypassing protein kinase C. Indeed, such a direct signal transduction pathway has already been documented in monocytes/macrophages (21) and therefore may represent a fast and effective way to transmit mechanical deformation signals to the nucleus of vascular SMC.

If this hypothesis holds true, p38-MAPK, besides its well documented ability to phosphorylate c-Jun, hence activate AP-1, must be able to activate C/EBP, too, and this has in fact been documented in other cell types (15, 22).

Transcriptional regulation of the ET\(_B\)-R gene

The aforementioned characterization of ROCK and p38-MAPK and analysis of the ET\(_B\)-R gene promoter suggested 3 candidate transcription factors for stretch-induced ET\(_B\)-R expression, i.e. AP-1, ATF and C/EBP (or NF-IL-6). According to gel shift analysis, both AP-1 and C/EBP indeed translocated to the nucleus of the cultured SMC exposure to
cyclic stretch. Moreover, both kinase inhibitors attenuated the nuclear translocation of the 2 transcription factors to a similar degree, providing further evidence for the notion that ROCK acts through p38-MAPK and not via alternative pathways such as ERK1/2 and protein kinase C activation which also mediate AP-1 translocation to the nucleus (19, 23).

Nuclear translocation of ATF, on the other hand, was not detectable in the stretched SMC, even though phosphorylation, hence activation of this transcription factor has been reported in SMC exposed to cyclic stretch (24). As a consequence, this finding per se did not exclude ATF from being involved in deformation-induced ET_{B}-R expression, especially because proteins of the CREB/ATF family of transcription factors are not necessarily regulated by nuclear translocation.

Therefore, the role of all 3 transcription factors in deformation-induced expression of the ET_{B}-R in SMC was investigated further by using the dODN technique. Both, the AP-1 and C/EBP family dODN significantly inhibited ET_{B}-R expression in response to cyclic stretch, whereas the consensus dODN for the ATF/CREB family of transcription factors was without effect. Combined treatment of the SMC with the AP-1 and C/EBP dODN, however, did not produce a greater inhibitory effect. The most likely reason for this finding is that both dODN must compete for a common uptake mechanism in the cultured SMC (M. Hecker, unpublished observation). Nonetheless, one can safely conclude from the dODN data that both AP-1 and C/EBP, but not ATF, are involved in stretch-induced up-regulation of ET_{B}-R expression in vascular SMC.

**ROBERTA**

At least in the case of C/EBP, this conclusion was supported by ROBERTA, a direct method for monitoring transcription factor activity in the nucleus. This method, which was devised on the basis of a previously published nuclear run-on assay (9), seems to
be a valuable tool, complimenting other methods such as reporter gene analysis (25) or the dODN technique (26) for functional analysis of gene expression.

ROBERTA is an anti-body hence specific tool for demonstrating the involvement of a single transcription factor in the expression of the gene of interest. The advantage of this approach is that the genes and transcription factors remain in their intact functional environment, i.e. the nuclear chromatin. Perhaps the only factor limiting ROBERTA is the binding capacity (and specificity) of the antibody for the chosen transcription factor. This obviously was a problem with AP-1 that consists of different heterodimeric proteins. For factors belonging to the C/EBP family of transcription factors, on the other hand, gel supershift analysis and ROBERTA clearly suggested a role for C/EBPβ rather than another C/EBP family member in deformation-induced ET₃₉ expression. The specificity of this novel technique can be inferred from the fact that the inhibitory effect of the anti-C/EBPβ antibody was not mimicked by 2 different control antibodies directed against an unrelated transcription factor (STAT-1) or just another protein (fibronectin).

Conclusions

Taken together, the aforementioned findings demonstrate that stretch-induced expression of the ET₃₉-R in cultured vascular SMC is mediated at least in part by 2 different mechanosensitive transcription factors, AP-1 and C/EBPβ. It remains to be determined whether deformation-induced activation of these transcription factors fully accounts for the observed increase in ET₃₉-R expression or whether other truly mechanosensitive transcription factors also play a role therein. At present, C/EBPβ appears to be the most feasible target for limiting deformation-induced ET₃₉-R expression and thus an exaggerated remodeling of the vessel wall in response to an increased pressure load.
Acknowledgments

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References

Figure Legends

Figure 1: Increase in steady state levels of (A) ET$_{B}$-R mRNA and (B) protein in SMC upon exposure to cyclic stretch for 6 h (15% elongation, 0.5 Hz). (A) ET$_{B}$-R mRNA abundance as determined by RT-PCR analysis with elongation factor-2 as an internal standard (n=6). (B) ET$_{B}$-R protein content determined by Western blot analyses with β-actin as an internal standard (n=4). *P<0.05 vs. non-pressurized control, i.e. 100%. The insets depict a typical result for each analytical method.
Figure 2: (A) Analysis of ET$_B$-R mRNA stability. Cultured SMC were pre-incubated for 1 h with 1 µM actinomycin D (Act D) before exposure to cyclic stretch (15% elongation, 0.5 Hz, 6 h). ET$_B$-R mRNA abundance was analyzed by RT-PCR 1 h after termination of the stretch protocol. (B) Nuclear run-on analysis of ET$_B$-R gene expression. Nuclei isolated from SMC exposed to cyclic stretch (15% elongation, 0.5 Hz, 3 h) are compared to nuclei isolated from SMC incubated under static conditions. The negative control (0 min) is derived from the same amount of nuclei from the same batch of SMC lysed immediately. Both figures depict a representative result of a series of 3 identical experiments with different batches of SMC.
Figure 3: Deformation-induced expression of (A) ET$_B$-R mRNA and (B) protein in SMC depends on the activity of ROCK and p38 MAPK. Following 1 h pre-incubation with the specific kinase inhibitors Y27632 (1 µM, ROCK) or SB212190 (10 µM, p38-MAPK) alone or in combination, the cells were exposed to cyclic stretch for 6 h (15% elongation, 0.5 Hz). *P<0.05 vs. control. i.e. 100%. The insets depict a typical result for each analytical method.
Figure 4: Gel shift analysis plus supershift analysis of the nuclear translocation of (A) AP-1 and (B) C/EBP (β and δ isoform) in the cultured SMC exposed to cyclic stretch for 1 h (15% elongation, 0.5 Hz). Pre-incubation for 1 h with SB212190 (10 μM) or Y27632 (1 μM) resulted in marked decrease in deformation-induced nuclear translocation of the transcription factors. Shown is a representative result of 3 identical experiments with different batches of SMC. Note the low amount of AP-1 supershifted with the anti-c-Jun antibody.
Figure 5: Statistical analysis of dODN-mediated inhibition of stretch-induced ET$_B$-R expression in SMC. The cultured cells were pre-incubated with the respective dODN (10 µM) for 4 h and then exposed to cyclic stretch for 6 h (15% elongation, 0.5 Hz; n=3-8). *P<0.05 vs. non-pressurized control, i.e. 100%.
Figure 6: Principle of ROBERTA. Addition of antibodies directed against the transcription factor (TF) of interest to a nuclear run-on assay prevents binding of the transcription factor to its genomic target sequence(s) and hence expression of genes that are induced by this factor.
Figure 7: Stretch-induced ET$_B$-R expression in SMC as analyzed by ROBERTA. Nuclei from SMC exposed to cyclic stretch (15% elongation, 0.5 Hz, 3 h) were isolated and incubated with the appropriate antibodies (20-50 µg/mL) for 30 min. Thereafter, nucleotides and the ATP-regenerating system were added and the nuclei either lysed immediately (negative control; 0 min) or incubated for 30 min at 30°C. (A) Analysis of the role of C/EBPβ. (B) Negative control experiments. Upper panel: Lack of effect of the anti-STAT-1α antibody on stretch-induced ET$_B$-R mRNA expression compared to that of the anti-C/EBPβ antibody. Lower panel: Lack of effect of an anti-fibronectin (FN) antibody.
A

**ETB-R mRNA**

[Bar graph showing the expression of ETB-R mRNA (% of static control) under different conditions.]

B

**ETB-R protein**

[Bar graph showing the expression of ETB-R protein (% of static control) under different conditions.]
Fig. 2 Cattaruzza et al.
Fig. 3 Cattaruzza et al.
Fig. 4 Cattaruzza et al.

**A**

- c-Jun-supershift
- AP-1

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**B**

- C/EBPβ supershift
- C/EBPβ
- C/EBPδ

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Fig. 5 Cattaruzza et al.

The figure illustrates the effect of stretch and the addition of AP-1-dODN and C/EBP-dODN on ETB-R mRNA expression, measured as a percentage of the stretched control. The bars indicate the relative mRNA levels, with asterisks denoting statistical significance.
Fig. 6 Cattaruzza et al.
**Fig. 7 Cattaruzza et al.**

### A

30 min 0 min

- ETB-R
- GAPDH

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### B

- ETB-R
- GAPDH

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