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Differential phosphorylation of calreticulin affects AT1 receptor mRNA stability in VSMC

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ABSTRACT

The AT1 receptor plays a pivotal role for the pathogenesis of hypertension and atherosclerosis. AT1 receptor expression is regulated posttranscriptionally via destabilization of the AT1 receptor mRNA by mRNA binding proteins. Recently, we identified calreticulin as a novel binding protein within the 3′untranslated region of the AT1 receptor mRNA.

Calreticulin phosphorylation is essential for binding of the AT1 receptor mRNA. In crosslink experiments, we identified src kinase as the key enzyme for calreticulin phosphorylation. Overexpression of src sense DNA resulted in vascular smooth muscle cells (VSMC) in destabilization, overexpression of src antisense resulted in stabilisation of the AT1 receptor mRNA. Furthermore, phosphorylation/dephosphorylation sites of calreticulin and their impact on the AT1 receptor mRNA stability were investigated. VSMC were stimulated with AngII before tyrosine phosphorylation as well as serine phosphorylation of calreticulin were analysed via immunoprecipitation. Stimulation of VSMC with AngII resulted in enhanced tyrosine and reduced serine phosphorylation. Both effects are essential for AT1 mRNA stability as assessed by use of pharmacological inhibitors of serine dephosphorylation (cantharidin/ocadaic acid) or tyrosine phosphorylation (tyrphostin/orthovanadat).

These findings imply an important role of serine dephosphorylation and tyrosine phosphorylation on calreticulin mediated AT1 receptor mRNA stability.

The renin–angiotensin system (RAS) is important for the regulation of blood pressure, water- and sodium-retention, free radical release and cell growth. Dysregulation of the RAS contributes to common vascular diseases like hypertension and atherosclerosis [1]. Most of the biological effects of the RAS such as vasoconstriction, reactive oxygen species release and smooth muscle cell proliferation are mediated via the AT1 receptor. AT1 receptor expression is altered by angiotensin II(AngII), growth factors and lipoproteins [2].

The AT1 receptor is regulated at the posttranscriptional level via AngII induced destabilization of the AT1 receptor mRNA [3]. In prior publications we identified an important site for protein binding and destabilization within the 3′untranslated region of the AT1 receptor mRNA [4]. This region is located between bases 2175 and 2195. Coincubation of polyribosomal eluates containing binding proteins and AT1 mRNA transcripts including the region 2175–2195 resulted in rapid degradation of the AT1receptor mRNA.

Via MALDI-analysis we identified the protein calreticulin as an important binding protein within this region. Calreticulin rapidly promotes AT1 receptor mRNA decoy. This in turn leads to decreased AT1 receptor expression [5]. Calreticulin is a polypeptide consisting of 400 aminoacids with a molecular weight of 46 kDa [6]. The calreticulin-gene is located on chromosomes 19 and 8 in the human genome [7]. Calreticulin expression has been reported in various tissues and cellular compartments for instance in the cytoplasm, the endoplasmatic reticulum, the nucleus and on the cellular membrane [8,9].

Primarily, calreticulin has been identified as a calcium binding protein [6]. It contributes to the maintenance of intracellular calcium homeostasis and regulates calcium handling proteins. Additionally, it has been reported as a receptor for nuclear export [10]. Recently, calreticulin has been identified as an mRNA binding protein as well [11].

Of note, phosphorylated calreticulin only can attach to the AT1 mRNA. Calreticulin phosphorylation is induced by AngII stimulation [5]. So far it was not known which regions within the calreticulin structure undergo phosphorylation and which kinases or phosphatases mediate this process. In the present paper, we investigated potential phosphorylation mechanisms and phosphorylation sites of the calreticulin structure.

Materials and methods

Cell culture. Confluent vascular smooth muscle cells (VSMC) of rat origin were used. Cells were prepared as published previously [12]. Rat-VSMC were isolated from rat thoracic aorta (male Sprague–Dawley, 6 to 10 weeks old, Charles River GmbH, Sulzfeld, Germany). Cell culture media and materials were obtained from Gibco.

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Stimulation with serine/tyrosine phosphorylation/dephosphorylation inhibitors. VSMC were grown to confluency and afterwards stimulated with the substances listed below: cantharidin (2 μmol/l), ocadaic acid (0.06 nmol/l), tyrphostin (5 μmol/l), and orthovanadat (1 mmol/l). Incubation times varied between 2 and 4 h as indicated in the figures. All drugs were obtained from Sigma–Aldrich.

Stimulation with phorbol 12 myristate 13-acetate (PMA) and Forskolin. VSMC were grown to confluency and transfected via electroporation as described earlier [5] with either pEGFP as control vector or pEGFP containing the AT1 receptor 3′UTR. After 24 h the cells were incubated with phorbol 12 myristate 13-acetate (PMA, 10 μM) or Forskolin (25 μM) for 4 h. The cells were then harvested and the total mRNA eluted. The amount of AT1 receptor 3′UTR mRNA was analysed by real-time PCR. Forskolin and PMA were obtained from Gibco.

In vitro phosphorylation of calreticulin. In vitro phosphorylation of calreticulin was performed according to the manufactureŕs protocol using the β-insulin receptor kinase/Src kinase/JNK kinase kit supplied by stratagene.

mRNA isolation, northern analysis, and polymerase chain reaction. Cells were lyzed with 1 ml RNA-clean (AGS), scraped, and processed according to the manufactureŕs protocol. Northern blots were prehybridized for 2 h at 42 °C with random-primed, [^{32}P]-dCTP-labeled rat AT1 receptor cDNA probe. The rat AT1 receptor cDNA probe was a 479-bp fragment generated from an AT_1 receptor cDNA template by polymerase chain reaction. The same primer pair was used as described in the PCR-section. Isolated total RNA (2 μg) was reverse transcribed using random primers. The single stranded cDNA was amplified by PCR using Taq DNA-polymerase (Boehringer). Twenty-eight cycles were performed as described below: 30 s 94 °C, 45 s 55 °C, and 45 s 72 °C. The sequence for the AT1 receptor sense and antisense primers were: 5′-ACCCTCTACAGCATCATCTTTGTGGTGGGA-3′ and 5′-GGG AGCGTCGAATTCCGAGACTCATAATGA-3′. The same samples were used for GAPDH cDNA amplification to confirm that equal amounts of RNA were reverse transcribed. PCR amplification resulted in a 452-bp fragment of the AT1 receptor mRNA.

Cell transfections and calreticulin/SRCsense-antisense constructs. Cells were grown to confluency and then transfected using electroporation as published previously [5]. Full length calreticulin and src sense were obtained after PCR from isolated total RNA and cloning in the pcDNA3 vector.

UV mRNA protein crosslink assays. Recombinant calreticulin was mixed on ice with 4–10 pmol of [^{32}P]-dUTP-labeled RNA transcripts and UVmRNA protein crosslink experiments were performed as described earlier [5].

Real-time PCR. For quantitative PCR, a reverse transcription was performed (primer for AT1 receptor 5′-GAGGTAAACATACATTGCC-3′, GAPDH 5′-TGTTATGGGG TCTGGGATGGA-3′). Following 1:1000 dilution, the PCR was performed according to the manufacturer's instruction using the Sybr-Green Mastermix Kit (Applied Biosystems) and the Abi Prism 7700 Sequence Detector. PCR primer: AT1 receptor 5′-TTCAGCCAGTGTTTTAGA-3′ (sense) and 5′-GAGGTAAACATACATTGCC-3′ (antisense). GAPDH PCR served as control.

Statistical analysis. Values are expressed as means ± standard error of the mean and were compared between groups by using ANOVA. The Student–Newman–Keuls post hoc test was performed when significance is indicated. For comparisons between two groups the Students *t* test was used.

Results

Calreticulin binds and destabilizes the 3′UTR of the AT1 receptor mRNA

VSMC were cotransfected with pEGFP containing the 3′UTR of the AT1 receptor and pcDNA3 containing either calreticulin sense or antisense. Afterwards the amount of AT1 receptor 3′UTR mRNA was analysed via real-time PCR. Overexpression of calreticulin sense led to significantly reduced levels of AT1 receptor 3′UTR mRNA indicating binding and destabilization by calreticulin. Overexpression of calreticulin antisense significantly increased the number of AT1 receptor 3′UTR mRNA copies (Fig. 1A).

Destabilization of the AT1 receptor 3′UTR mRNA depends on protein phosphorylation and is induced via the Phosphokinase C (PKC)/ERK1/2 pathway

VSMC were transfected with pEGFP containing no insert or the AT1 receptor 3′UTR. Twenty-four hours later the cells were stimulated for up to 4 h with phorbol 12 myristate 13-acetate (PMA) or forskolin (Fig. 1B). Incubation with PMA as well as Forskolin

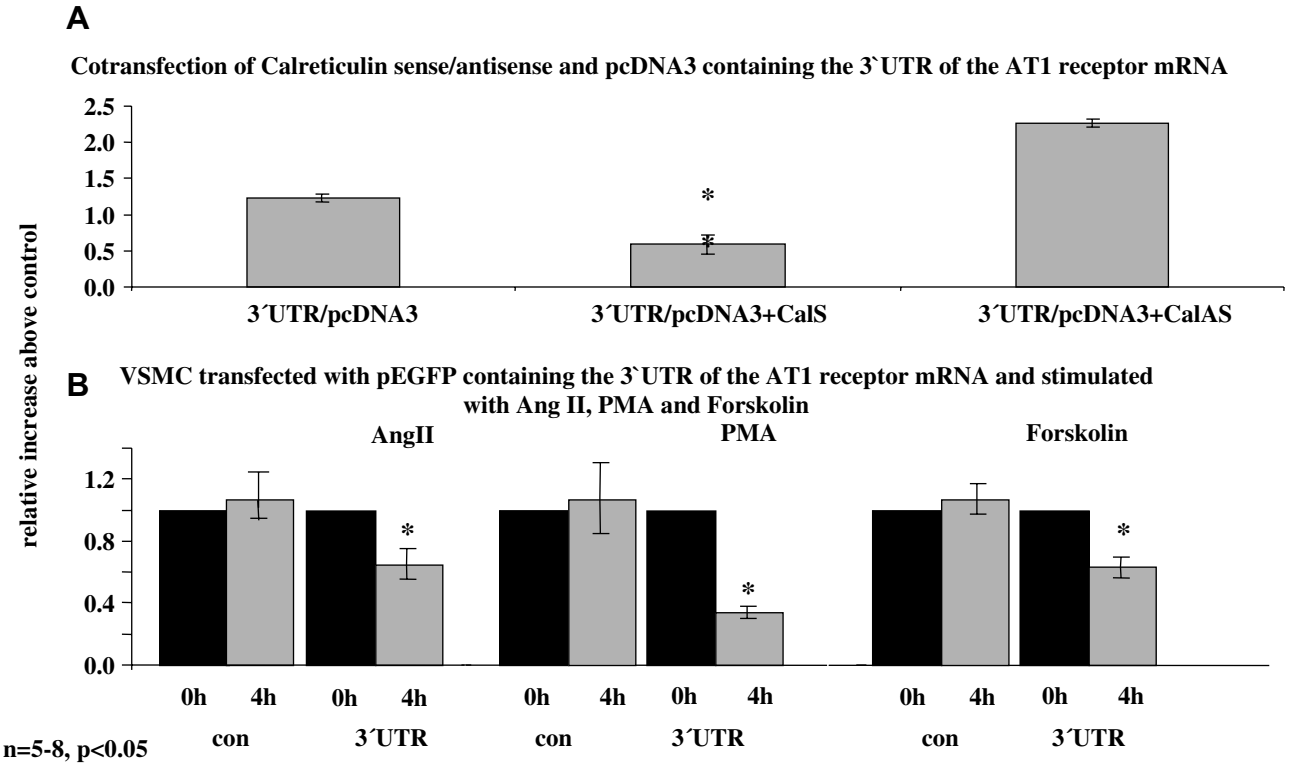

Fig. 1. (A) VSMC were cotransfected with pEGFP containing the AT1 receptor 3′UTR and pcDNA3 containing calreticulin sense or antisense. Overexpression of calreticulin sense resulted in decreased AT1 receptor 3'UTR stability, overexpression of calreticulin sense in increased AT1 receptor 3'UTR stability suggesting an important role of calreticulin in AT1 receptor regulation. $n = 5–8$, $p < 0.05$. (B) VSMC were transfected with pEGFP or pEGFP containing the AT1 receptor 3'UTR. Afterwards the cells were stimulated with AngII (10^{-6} mol/l), phorbol 12 myristate 13-acetate (10 mM) or forskolin (25 mM). Induction of the phosphokinase C/ERK1/2 pathway as well as elevated levels of cAMP led to destabilization of the AT1 receptor 3'UTR. These findings imply an important role for protein phosphorylation in respect to AT1 receptor mRNA regulation. $n = 5–8$, $p < 0.05$. ns = non-stimulated cells, AngII = angiotensin II, PMA = phorbol 12 myristate 13-acetate, CalS = calreticulin sense, CalAS = calreticulin antisense. $n = 5–8$, $p < 0.05$.