Interplay between EGR1 and SP1 is critical for 13-cis retinoic acid-mediated transcriptional repression of angiotensin type 1A receptor

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Abstract
Recently, we have demonstrated that 13-cis retinoic acid (13cRA) downregulates rat angiotensin type 1A receptor (Agtr1a) gene transcription through a MAP kinase (ERK1/2)-dependent mechanism in rat liver epithelial and aortic smooth muscle cells. However, the exact mechanism remained unknown. In this study, we determined the signaling intermediates activated by ERK1/2 involved in 13cRA-mediated Agtr1a downregulation. Rat Agtr1a chloramphenicol acetyltransferase (CAT) promoter construct containing a sequence -2541 and -1836 bp upstream of the start site demonstrated reduced CAT activity; this region possesses a specificity protein 1 (SP1) consensus sequence (5'-TGGGGCGGGG-3'). Mobility shift analysis using untreated nuclear extracts in the presence of mithramycin A suggests that the trans-acting factor binding to this cis-acting element is SP1. 13cRA significantly reduced specific binding without any change in SP1 protein expression. Studies showed that 13cRA treatment maximally phosphorylates ERK1/2 within 5–10 min, which translocates to the nucleus, activating early growth response protein 1 (Egr1) mRNA expression at 20 min followed by de novo protein synthesis, leading to an EGR1/SP1 interaction. siRNA silencing of Egr1 restored Agtr1a mRNA and protein expression in 13cRA-treated cells, and Sp1 silencing results in complete loss of Agtr1a expression. Our study suggests that 13cRA-mediated activation of ERK1/2, through EGR1, is capable of disrupting SP1, the requisite trans-activator for Agtr1a expression, providing a novel paradigm in Agtr1a gene transcription.

Key Words
- Gene transcription
- Gene regulation
- Renin–Angiotensin system
- Retinoic acid
- Signal transduction

Introduction
Angiotensin II (AngII) is a crucial hormone in fluid volume control and vasoconstriction through its stimulation primarily of the angiotensin type 1 receptor (AGTR1; Burnier 2001, Atlas 2007). As a result, it has become a key factor in the realm of hypertension research. Moreover, a greater understanding of AngII stimulation of AGTR1 effects as a growth factor, stimulator of angiogenesis, and oxidative stressor has expanded the interest in AGTR1 research beyond hypertension (Braunwald 2008, Pan et al. 2010, Abadir 2011). AGTR1 is a ubiquitously expressed protein with significant effects in renal, cardiovascular, hepatic, and neural physiology. However, AGTR1 protein
expression is tissue and cell specific primarily due to differential transcription of the Agtr1 gene (Hannan et al. 2004, Tower et al. 2010, Braga 2011). Changes in Agtr1 transcription also occur under pathophysiological conditions, such as damaged cardiac tissue after myocardial infarction (Sun & Weber 1994), atrial tissue and the rostral ventrolateral medulla in chronic heart failure (Kaprielian et al. 1997, Gao et al. 2008), and in cardiac, renal, and neural tissue in cases of spontaneous hypertension (Esler 1993, Raizada et al. 1993, Lenkey et al. 1997). The coordination of Agtr1 gene regulation is complex. Research has shown that the basal transcription of Agtr1 largely relies on the activity of the transcription factor specificity protein 1 (Sp1; Zhao et al. 2001). Moreover, upregulation of AGTR1 in the vascular wall is involved in the induction of oxidative stress and enhancement of endothelial dysfunction and plaque instability (Nickenig & Harrison 2002). The cellular stress resultant from generation of reactive oxygen species (ROS) induces greater binding activity of activator protein 1 (Wu et al. 2005), leading to a cyclic pattern in which Agtr1 becomes deregulated, first upregulated by oxidant stress then contributing to a further ROS burden in the vasculature. Our own studies have shown that hormonal signaling has diverse effects on Agtr1 transcription. Growth factors, such as GH, insulin, platelet-derived growth factor, and epidermal growth factor, have a direct upregulatory effect on Agtr1 transcription. Alternatively, Agtr1 (angiotensin type 1A receptor (Agtr1a) – in studies of rodent species) transcription may be downregulated by treatment with tannic acid (Yesudas et al. 2012), the estrogen metabolite 2-methoxyestradiol (Koganti et al. 2012), or high glucose (Thomas & Thekkumkara 2004), a study in which we isolated a novel glucose response element, though characterization of cis-acting elements for the other agents remains undetermined. As this receptor is regulated in multiple ways, addressing the mechanism of gene transcription is essential to develop better strategies to control the effects of AngII.

Retinoic acids, metabolites of preformed vitamin A, exert their function through the binding of nuclear receptors (RAR and RXR) and forming complexes with conserved sequences on gene promoters termed retinoic acid response elements (Mark et al. 2006). 13-Cis retinoic acid (13cRA) is a synthetic form that functions similar to the other isoforms, but its exact mechanism of action is unclear. It may spontaneously isomerize to all-trans-retinoic acid or possess a novel signaling pathway of its own (Kim et al. 1994, Ganceviciene & Zouboulis 2007). However, 13cRA has relatively weak transactivation activity for RAR/RXR compared with the endogenously formed isoforms all-trans and 9-cis retinoic acids (Mangelsdorf et al. 1994). Although 13cRA has multiple applications as a dermatological treatment (Landthaler et al. 1980) and as an effective therapeutic for numerous myelodysplastic and proliferative conditions (Piattella et al. 1999, Zhang & Duvic 2003, Siitonen et al. 2007), the cellular mechanisms and molecular targets are not well understood. Studies have shown that 13cRA can act as a potent inhibitor of many of the retinoid and hydroxysteroid-mediated pathways (Gamble et al. 1999). This may indicate how 13cRA mediates its antagonistic effects on sebum secretion and skin growth factors. However, many of these studies cannot provide compelling evidence of a distinct cellular mechanism. Interestingly, it has been proposed that 13cRA may have non-nuclear targets, based upon differential activity from all-trans and 9-cis retinoic acids (Blaner 2001).

In a recently published study, we have demonstrated that in both rat liver epithelial and aortic smooth muscle cells, 13cRA is capable of downregulating AT1R by inhibiting Agtr1a mRNA transcription (Snyder & Thekkumkara 2012). Additionally, the study revealed that 13cRA-induced Agtr1a downregulation is dependent on the activation of MAPK p42/p44 (ERK1/2). Importantly, this study suggested that the response in AT1R expression was effective in cells of hepatic origin, in which it has been shown that AT1R signaling blockade or interference provides substantial attenuation of inflammatory processes leading to fibrosis and steatosis (Ratziu & Zelber-Sagi 2009). Angiotensin converting enzyme inhibition (ACEI) and angiotensin receptor blocker (ARB) use can significantly lower fibrosis index scores (Kim et al. 2008, Yoshiji et al. 2009). Thus, the findings of our preceding study were valuable in demonstrating a method of downregulating Agtr1a expression in hepatic and cardiac tissue, possibly providing an alternate approach to traditional ARB and ACEI pharmacology. However, the downstream mechanism(s) involved in 13cRA induction of ERK1/2 leading to Agtr1a downregulation remained undetermined, as the classical retinoic acid receptors (RAR/RXR) were not involved. The purpose of this study was to identify the downstream effectors responsible for the inhibition of Agtr1a transcription to further elucidate the mechanism of ERK1/2-mediated Agtr1a repression.

Materials and methods

Materials

Continuously passaged rat liver epithelial cells (WB cells) were kindly provided by Dr H Shelton Earp, University of
North Carolina at Chapel Hill (Chapel Hill, NC, USA). Richter’s improved minimal essential medium was obtained from Cellgro-Mediatech, Inc. (Manassas, VA, USA). Fetal bovine serum (FBS) was from Equitech-Bio, Inc. (Kerrville, TX, USA). Oligonucleotide primers and EMSA nucleotide duplex probes were obtained from Integrated DNA Technologies, Inc. (Coralville, IA, USA). PCR master mix was from Roche. Losartan was provided by Merck Sharp and Dohme Research Laboratories (Rahway, NJ, USA). 13cRA, insulin, mithramycin A, and gentamicin were from Sigma. DNA/RNA extraction reagents were from Ambion/ABI (Austin, TX, USA). [3H]AngII was from Perkin-Elmer (Waltham, MA, USA). PD98059 was from Calbiochem (La Jolla, CA, USA). SP1 antibody (SC-59) was from Santa Cruz Biotechnology. PD98059 was from Calbiochem (La Jolla, CA, USA). SP1 antibody (SC-59) was from Santa Cruz Biotechnology. Early growth response protein 1 (EGR1) (cat no. 4153), phospho-ERK1/2 (cat no. 4370), and total ERK1/2 (cat no. 4695) antibodies were from Cell Signaling (Danvers, MA, USA). siRNA Silencer Select pre-validated oligonucleotides, Opti-MEM, and transfection reagents (Lipofectamine LTX and RNAiMAX) were from Life Technologies. Electrophoresis regents were from Bio-Rad, and all other chemicals and molecular biology grade agents were purchased from Fisher Scientific (Fairlawn, NJ, USA).

**Construction of expression plasmids**

All DNA manipulations were carried out according to previously published works (Wyse et al. 2000). Three thousand three hundred and seventy-two base pairs of the 5′ promoter sequence of the rat Agtr1a, (ref# NC005116.2) were custom-cloned into pUC57 carrier vector and sequence confirmed by GenScript (Piscataway, NJ, USA). Using the genomic clone as a template for PCR and oligonucleotides corresponding with the published sequence of the rat Agtr1a promoter, fragments of varying length were amplified. The oligonucleotide corresponding exon 1 (+23 to +45 bp) of the rat Agtr1a (5′-ACA-GATCTTTCTCCAGCGGGACA-3′) was used as the antisense primer for each reaction. The varying sense primers (rP1, 5′-GCTTATCTGTCTCTCCCTTATCCC-3′; rP2, 5′-CCCTTCTGCTAGCCTTCTTCTCT-3′; rP3, 5′-GGGTACCGCGAAGAGCCA-3′; rP4, 5′-AAGGCTAGCCGACTACAC-3′; rP5, 5′-GGTTGAGCTAGCATAATGAGGGGAC-3′; rP6, 5′-AAATCTGAGGCTTAAGGTACG-3′; and rP7, 5′-GATCGAGGCTCCGTACCTCCGCAAGCGA-3′) yielded fragments of 310, 652, 1266, 1836, 2541, 2824, and 3321 bp respectively. Each fragment was double digested with BglII and to the presence of an Nhsl digestion site downstream to the 5′ terminus of the rP7 fragment. Fragments rP1–rP7 used primers that had incorporated restriction sites for 5′ selective Nhsl/KpnI digestion and 3′ selective BgllI digestion. Double-digested fragments were unidirectionally ligated into the multiple cloning site of the pCAT-Basic vector (Promega) digested with the same enzymes. The authenticity of clones was confirmed by automated bidirectional dideoxy sequencing. Mutated rP7X promoter construct was assembled using similar techniques, with additional modification by selective PCR amplification of sequences flanking the upstream SP1 response element (SP1RE), and subsequent BamHI digestion of modified primer sequences followed by ligation to exclude said response element.

**Cell culture and transfection of reporter gene constructs**

The WB cells were maintained in Richter’s improved MEM supplemented with 10% FBS, 10 mM glucose, 17.8 mM HEPES, 5.4 μg/ml insulin, and 44.6 μg/ml gentamicin (complete medium) at 37°C in 5% CO2 with 100% humidity. For the studies, cells were grown to 70–80% confluence and the medium was exchanged with treatment medium (Richter’s improved MEM supplemented with 5% FBS, 5 mM glucose, 17.8 mM HEPES, 5.4 μg/ml insulin, and 44.6 μg/ml gentamicin). After 24 h, cells were exposed to treatment medium containing 13cRA for indicated concentrations and times. For transient transfection of DNA constructs, WB cells were seeded in six-well plates and grown to 70–80% confluence in complete medium. Cells were transiently transfected with 3 μg reporter plasmids and cotransfected with 1 μg pSV-β-galactosidase expression construct (to act as an internal control for transfection efficiency) using Lipofectamine LTX and PLUS reagent according to manufacturer’s instructions (Invitrogen) and grown for 24 h in complete medium. Following 24-h growth in complete medium, treatment medium and 13cRA were added consistent with all other studies.

**Chloramphenicol acetyltransferase assay**

The chloramphenicol acetyltransferase (CAT) assays were performed according to the manufacturer’s instructions (Invitrogen FASTCAT Protocol). Briefly, cells were rinsed with PBS three times and harvested in the same buffer. Cells were then centrifuged and the resultant pellet was resuspended in 100 μl 0.25 M Tris–HCl, pH 7.8. Cellular
extracts were prepared by freeze–thaw cycling from the −80 °C freezer to the 37 °C water bath for four times. The cells were then centrifuged (17,530 × g at 4 °C) for 10 min. Thirty microliters of the supernatant were removed and β-galactosidase activity was measured using a colorimetric assay according to the previously published method (Wyse et al. 2000). The remaining supernatant was heated at 70 °C for 10 min to inactivate endogenous acetylases and centrifuged further to remove cell debris. The assay for CAT activity was performed as described in the FAST-CAT protocol. The green fluorescence was visualized under the VERSA-Doc System at 504 nm absorbance and 510 nm emission. The acetylated chloramphenicol was then measured by fluorescent densitometry and normalized to arbitrary β-galactosidase activity/mg protein.

Electrophoretic mobility shift assay

Nuclear extracts were prepared using NE-PER nuclear protein extraction kit (Thermo-Scientific, Rockford, IL, USA). Protein concentration was determined using Bio-Rad protein assay reagent based on the Bradford method (Bradford 1976), and extracts used immediately after preparation. Mobility shift assays were performed as described previously (Wyse et al. 2000). Briefly, a double-stranded Sp1 consensus sequence with a 5′ overhang was custom synthesized as duplex DNA by Integrated DNA Technologies (sense primer, 5′-CCCCGCCCCGCCCCCA-3′; antisense primer, 5′-TGGGGCGGGG-GCGG-3′). The double-stranded SPIRE was labeled by filling the overhang with DNA polymerase Klenow in the presence of [α-32P]dGTP. The labeled probe was purified using a Sephadex G-25 column. Ten micrometers of nuclear extracts were preincubated with 2 μg poly(dIdC) in a total volume of 20 μl binding buffer comprising 0.1 M Tris–HCl, 50% glycerol, 0.2 M KCl, 0.5 M EDTA, and 1.0 M dithiothreitol (DTT) at 22 °C for 20 min. Where appropriate, the reaction mixture was supplemented with either proteinase K, 10 μM mithramycin, or varying concentrations (0- to 200-fold excess) of double-stranded, unlabeled Sp1RE. The labeled probe (300,000 c.p.m.) was added and the reaction mixture was further incubated for 30 min at 22 °C. Reactions were separated on a 6% native polyacrylamide gel, dried, and exposed to Kodak XR-film at −70 °C with intensifying screens.

Angiotensin receptor binding studies

Receptor binding studies were performed in triplicate on WB cells as described previously (Snyder & Thekkumkara 2012). Briefly, cells were washed twice with PBS and incubated with [3H]AngII (20 pM) at 22 °C in a binding buffer containing 50 mM Tris–HCl (pH 7.5), 120 mM NaCl, 4 mM KCl, 5 mM MgCl2, 1 mM CaCl2, 10 μg/ml bacitracin, 2 mg/ml dextrose, and 2.5 mg/ml BSA. After 60-min of incubation, cells were washed with ice-cold PBS three times to remove free radioactivity. Cells were then lysed with 0.1% Triton X-100 in PBS. The cells were scraped and transferred to counting vials and radioactivity was determined using a Beckman liquid scintillation counter. Specific [3H]AngII binding was defined as that portion of the total binding displaced by 1 μM unlabeled AngII. At equilibrium, specific binding was more than 95% of the total binding. Protein concentrations were determined using Bio-Rad protein assay system based on the Bradford method (Bradford 1976).

Reverse transcriptase/dual-PCR and real-time PCR

Cells were grown to 70–80% confluency and treated with agents for indicated times. Total RNA was isolated using guanidinium thiocyanate–phenol–chloroform method as described previously (Snyder & Thekkumkara 2012). Total RNA (5 μg) was processed for cDNA template conversion using MLV-RT. The reaction without reverse transcriptase served as control for DNA contamination. For real-time quantitative analysis of Egr1 mRNA, two parallel PCRs each containing 5 μg total mRNA and either Egr1 (Egr1 sense – 5′-GTGGCTCTCCCATCCTA-3′; Egr1 antisense – 5′-CAG AGGAAGACATG-3′), Agtr1a (Agtr1a sense – 5′-TGATTACGTGGG CGTCATCCA-3′; Agtr1a antisense – 5′-TTTCTCTGAGAGGTGAGGG-3′), or β-actin-specific primers (β-actin sense – 5′-CGGAAACACGGTATCCTGCTGCC-3′; β-actin antisense – 5′-AACCACACTGGTTGGCATCTG-3′) were performed using 2X SYBR Green Master Mix (Applied Biosystems). Following the reaction, threshold cycles (Ct) were calculated for each sample for Egr1 and β-actin mRNA reactions, and quantitative concentrations were calculated using ΔΔCt calculations described by Perkin-Elmer Applied Biosystems.

Western blot analysis

Cells were lysed by sonification in lysis buffer containing 50 mM HEPES, 1% Triton X-100, 50 mM NaCl, 50 mM NaF, 10 mM sodium pyrophosphate, 5 mM EDTA, and one tablet per 10 ml Mini-Complete Protease Inhibitor (Roche). Equal amounts of protein (25 μg/lane) were resolved by SDS-PAGE and transferred onto a nitrocellulose filter (Millipore, Billerica, MA, USA). The filters were blocked with Tris-buffered saline (TBS) containing 5%
were precleared by a 1-h incubation with 50% PAGS. After washing three times with TBS containing 0.5% Tween 20, bound primary antibody was detected with anti-rabbit HRP-conjugated goat-IgG (1:5000 dilution, Santa Cruz). Immunoreactive proteins were visualized using the chemiluminescent substrate kit from Pierce Biomedical (Thermo Scientific Pierce, Rockford, IL, USA).

**Immunofluorescent microscopy**

Cells were seeded and grown in four-chamber microscope slides (Bio-Tek). After indicated treatment times, the cells were washed once with cold PBS and fixed for 20 min in 4% paraformaldehyde. Cell nuclei were further perforated by incubation in 100% pure methanol at −20 °C for 10 min. The cells were then blocked in 5% goat serum for 1 h and incubated overnight at 4 °C in rabbit primary antibody (1:1000 dilution, Cell Signaling Technologies) directed against rat EGR1, SP1, or phospho-ERK1/2 proteins. Cells were washed five times with cold PBS and incubated at room temperature for 2 h with FITC-conjugated secondary goat anti-rabbit antibody (1:3000 dilution, Santa Cruz). Cells were washed an additional five times and stained with 10 nM 4′,6-diamidino-2-phenylindole (DAPI) for 48 h. Image capture was performed at 22 °C using an Olympus IX-81 microscope equipped with an Olympus U-CMAD3 camera under a PlanApo 60x/1.4 oil immersion objective. Images were analyzed using Slidebook software.

**Co-immunoprecipitation**

Nuclear extracts were prepared as mentioned earlier from 100 mm plates of 90–100% confluent culture plates using the NE-PER nuclear extraction kit (Thermo Scientific). Protein content was determined by Bio-Rad DC protein assay system, and equivalent portions of 150 μg were aliquoted for each reaction group to 25 μl nuclear extract buffer, and the sample was further diluted to 150 μl in reaction buffer (150 mM NaCl, 10 mM HEPES, pH 7.5, 0.2% Nonidet P-40, 5 mM sodium fluoride, 5 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 10 mg/l aprotinin, 10 mg/l leupeptin, and 1 mM phenylmethylsulfonyl fluoride). Protein A/G Sepharose (PAGS) (Cell Signaling) was washed with reaction buffer and samples were precleared by a 1-h incubation with 50% PAGS. After centrifugation, the precleared lysate was suspended with 4 μg capture antibody (either anti-EGR1 (Cell Signaling) or anti-SP1 (Santa Cruz)) and incubated with gentle rocking for 90 min at 4 °C. Fifty microliters of washed 50% PAGS were added to the reaction and incubated for a further 90 min at 4 °C with gentle rocking. The PAGS was separated by brief centrifugation and washed with 500 μl cold wash buffer (150 mM NaCl, 10 mM HEPES, pH 7.5, and 0.2% Nonidet P-40) three times. A final wash with 500 μl 50 mM HEPES, pH 7.5, was performed, and the supernatant was removed as completely as possible from the PAGS beads. The proteins/antibody complexes bound to sepharose were eluted by addition of 25 μl reducing 2X SDS sample buffer (120 mM Tris–HCl, pH 6.8, 3.3% SDS, 10% glycerol, 40 μg/ml bromophenol blue, and 200 mM DTT) and incubation at 100 °C in a tightly capped tube for 5 min. The PAGS was pelleted by centrifugation (14 000 g 5 min, RT) and 20 μl of the supernatant was transferred to a clean tube. The sample was then loaded into a SDS–PAGE and analyzed by western blot, directed against EGR1 or SP1, as dictated by the capture antibody employed before.

**siRNA transfection**

Cells were plated in 100 mm plates and grown to 60% confluence. Cells remained in growth media. In order to ensure effective gene knockdown, we employed a combination of three separate 21 mer Silencer Select validated siRNA oligos at a concentration of 50 pmol/10 ml transfection media for each knockdown experiment. Provided successful transfection, each oligo is guaranteed to specifically knockdown target gene expression at least 80%. Transfection mixtures were prepared as follows for the 100 mm plates: 50 pmol of each interfering RNA suspensions combined (a total of 150 pmol for the three respective siRNA molecules provided (Egr1 KO oligos – Life Technologies Cat # s127689, s127690, s127691; Sp1 KO oligos – Life Technologies Cat # s128430, s128431, and s128429)). These siRNAs were diluted in Opti-MEM serum-free media (150 pmol/ml). In an additional tube, 30 μl Lipofectamine RNAiMAX was diluted in 1 ml Opti-MEM. The diluted lipofectamine was added to the RNA containing Opti-MEM solution. The 2 ml reaction mixture was incubated for 20 min at 22 °C. At the end of this incubation, the lipofectamine/RNA complex was added dropwise to the 100 mM plates containing 10 ml growth media. The plates were allowed to grow an additional 24 h, when the growth media were replaced with treatment media, and either Agtr1a, Egr1, or Sp1 expression was assessed by previously mentioned methods.
Statistical analysis

Sequence analyses and alignments were performed using MacVector 12.0 sequence analysis software. Results are presented as mean ± S.E.M. and the value of $P < 0.05$ was considered statistically significant. Statistical analysis included column statistics to test for normal distribution and parametric $t$-test or one-way ANOVA with post hoc Bonferroni analysis as appropriate. Values are normalized to milligrams of protein determined by Bio-Rad DC protein assay system based on the Bradford method (Bradford 1976). Data were analyzed using the GraphPad Prism$^®$ software (La Jolla, CA, USA).

Results

Agtr1a promoter activity

In our previous study, we have shown that exposure to 13cRA reduced the expression of rat Agtr1a protein via transcriptional downregulation (Snyder & Thekkumkara 2012). To further determine whether there was promoter activity of Agtr1a and, if so, whether downregulation is involved with specific cis-acting elements, we investigated the involvement of the −3276 to +45-bp promoter sequence in the regulation of the rat Agtr1a gene transcription. Functional analysis of the rat Agtr1a gene transcriptional regulatory unit was performed using a reporter gene assay, which relies on the linkage of putative regulatory sequences to a reporter Cat gene, whose transcription is detected after transfection into WB cells. To determine specific regions responsible for 13cRA-mediated Agtr1a downregulation, serial deletions were constructed and placed upstream of the Cat reporter gene (Fig. 1A). These serially deleted promoter fragments containing reporter gene constructs were transiently transfected into cells and were then treated with 25 μM 13cRA for 24 h. Each group was compared parallel to a control group transfected with the same fragment construct but not treated with 13cRA, thereby accounting for any variable activity not due to the 13cRA treatment, as the gene may be affected by any number of different factors when the promoter is truncated unrelated to treatment (Thomas & Thekkumkara 2004; Fig. 1B). Activity relative to control was not significantly different in groups pCAT-rP1 (−310 bp – $P > 0.05$) through pCAT-rP4 (−1836 bp – $P > 0.05$). However, the CAT activity (and thus the Cat gene expression) was significantly suppressed in retinoic acid-treated cells expressing pCAT-rP5 (−2541 bp, 61.1 ± 5.641% reduced activity, $P < 0.001$) through pCAT-rP7 (−3321 bp, 62.26 ± 5.748% reduced activity, $P < 0.001$), indicating the presence of a cis-acting element between −2541 and −1836 bp responsible for inhibition. Although this sequence is considerable in length, consensus binding sites to characteristic response elements are rare in this region. Sequence analysis revealed a single true match for an SP1-putative response element (5′-TGGGGCCGGGGCGGGG-3′) consensus site within this upstream region, which led to further investigation of SP1’s involvement in 13cRA-induced downregulation of Agtr1a.

Determination of the role of putative Sp1 binding site in 13cRA-mediated downregulation of AT1aR

To determine whether the distal SP1RE is sensitive to 13cRA exposure, we performed a gel shift assay with nuclear extracts of control and 13cRA-exposed WB cells using $[^{32}P]$-labeled SP1RE as a probe (Fig. 2A). The result

Figure 1

CAT activity of rat Agtr1a reporter gene constructs in 25 μM 13cRA. CAT activity for serially deleted promoter fragments in PCAT vector was determined. (A) Schematic representation of the pCAT reporter expression vector containing serially deleted 5′ promoter region of the rat Agtr1a gene. (B) Solid bars represent the CAT activity of control plasmids, the value set automatically at 100% activity relative to β-galactosidase co-transfection control, patterned bars represent the comparative CAT activity in 25 μM 13cRA-treated cells. Data are expressed as mean ± S.E.M., ***$P < 0.001$, $n = 3$. 

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showed a distinct mobility shift of the labeled probe (indicated by arrow) with control nuclear extracts (Fig. 2A, lane 2), which was significantly reduced after pretreatment with 13cRA (Fig. 2A, lane 3). Incubation of the extracts with proteinase K eliminated the signal, indicating the formation of a protein–DNA complex (Fig. 2A, lanes 4 and 5). The protein–DNA binding was specific for nuclear factor(s) as evidenced by observed lack of mobility shift with the cytosolic extract (Fig. 2A, lane 6). To determine the specificity of this reaction, nuclear extract was incubated with labeled probe and increasing concentrations (0–200×) of unlabeled SP1RE probe that progressively inhibited the appearance of labeled DNA–protein complex, demonstrating the specificity of the nuclear protein(s) binding to SP1RE (Fig. 2B). To further demonstrate the identity of the protein as SP1, nuclear extracts were prepared after pretreatment with mithramycin A, a nuclear factor inhibitor when used in conjunction with the consensus sequence allowed positive identification of SP1 as the binding protein of this element (Fig. 2C). When the nuclear extracts from mithramycin A-treated cells were added to the probe, the binding of the protein to the labeled DNA was significantly reduced (85.31 ± 4.44%, **P < 0.01, n = 3) (Fig. 2C, lane 3). However, western blot analysis revealed that SP1 expression remained unaffected by the treatment with 13cRA in these cells (Fig. 2D). Additionally, we performed radio-ligand binding studies to show the effect of mithramycin A, and thus SP1 selective inhibition, on the expression of AGTR1A protein (Fig. 2E). The results demonstrate that after treatment with 20 μM mithramycin A significantly reduced [3H]AngII binding (reduction of 52.7 ± 9.91% compared with untreated control, P = 0.006, n = 3). Moreover, this reduction was not only similar to that resulting from 25 μM 13cRA treatment (P = 0.6345, n = 3), but combinatorial treatment of both mithramycin A and 13cRA resulted in no additive or synergistic effect (reduction of 57.9 ± 9.14% compared to untreated control, P = 0.0032; when compared to 13cRA-treated group, P = 0.6905; when compared to mithramycin A-treated group, P = 0.4166, n = 3), suggesting that SP1 inhibition by 13cRA was not enhanced by further addition of mithramycin A. Therefore, we propose that 13cRA may induce a trans-acting factor to interact with SP1, thereby inhibiting SP1’s ability to interact with the Agtr1a promoter. However, in order to validate the distal SP1 consensus sequence, a mutant promoter construct lacking the putative response element (5′-TGTAAGGCGGCGG-GCCGGG-3′) was created and designated as promoter construct ‘rP7X’ (Fig. 2F). CAT assay results indicate that
following 13cRA treatment, there was a significant reduction in acetyltransferase activity in rP7 non-mutated constructs as shown in Fig. 1B (reduction of 48.1 ± 4.37% compared with untreated control, $P=0.0027$, $n=3$) (Fig. 2G). However, after similar treatment with 25 μM 13cRA, rP7X-mutated constructs showed no significant reduction of CAT activity (increased activity of 3.82 ± 4.28% compared with untreated rP7X control, $P=0.2629$, $n=3$), indicating that loss of the putative SP1RE eliminates 13cRA’s ability to alter Agtr1a promoter activity.

13cRA-mediated de novo synthesis of Egr1

From previous studies, we confirmed that phosphorylation of ERK1/2 was essential for downregulation of the AGTR1A protein. In this study, when cells were exposed for different time periods, as indicated, we observed ERK1/2 was maximally phosphorylated within 5 min, followed by progressive dephosphorylation for the remainder of the 60-min test period (Fig. 3A). Immunofluorescent microscopy studies revealed that phosphorylated ERK1/2 was primarily resident in the nucleus (Fig. 3B). Upon further investigation, we observed increased Egr1 expression in 13cRA-exposed cells. We performed qPCR analysis using total RNA isolated from cells treated with 13cRA at different time points. qPCR results revealed that Egr1 mRNA was upregulated 403 ± 57.8% ($P<0.001$, $n=3$) at 20 min, which indicated that 13cRA mediates increased Egr1 gene transcription (Fig. 4A). A 4-h time course western blot analysis directed against EGR1 showed a significant increase in EGR1 expression in 13cRA-treated cells, reaching maximum response at 40–60 min followed by a rapid decrease in expression for the remainder of the time points (Fig. 4B). EGR1 is a resident nuclear protein and early response gene, but in order to validate the western blot data, we conducted a 120-min time course immunofluorescent microscopy study. The results of this study demonstrate that EGR1 protein is upregulated in 13cRA-treated cells, consistent with the western blot data, and the protein is localized primarily to the nucleus (Fig. 4C). In order to determine whether EGR1 upregulation requires de novo
synthesis, we performed a study in which cells were pretreated with the protein synthesis inhibitor cycloheximide. This analysis revealed that no EGR1 protein upregulation occurred if cells were pretreated with cycloheximide, and thus, the EGR1 upregulation was due to de novo protein synthesis (Fig. 5A). EGR1 upregulation was rapid and significant; however, we had not yet proven that this upregulation was due to ERK1/2 phosphorylation. Western blot analysis in which cells were pretreated with the MEK inhibitor PD98059 revealed that if MEK and subsequent phosphorylated ERK1/2 were inhibited, no increase in EGR1 production occurred (Fig. 5B). After correlation with ERK1/2 activation, the next objective in our study was to establish EGR1 (Fig. 5B). After correlation with ERK1/2 activation, the next objective in our study was to establish EGR1 synthesis is dependent on ERK1/2 transcriptional activation and de novo synthesis. (A) Western blot analysis of EGR1 after 1-h treatment with 25 μM 13cRA, with or without the MEK inhibitor (20 μM) PD98059. (B) Western blot analysis of EGR1 after 1-h treatment with 25 μM 13cRA, with or without the translational inhibitor cycloheximide (Chx). (C) Western blot analysis of nuclear extracts treated with and without 13cRA, probed with anti-SP1 primary antibody after immunoprecipitation with anti-EGR1. (D) Western blot analysis of nuclear extracts treated with and without 13cRA, probed with anti-ERK1 primary antibody after immunoprecipitation with anti-SP1. (E and F) Corresponding reprobe controls with appropriate capture antibody to confirm efficient immunoprecipitation of target proteins.

Figure 5
EGR1 synthesis is dependent on ERK1/2 transcriptional activation and de novo synthesis. (A) Western blot analysis of EGR1 after 1-h treatment with 25 μM 13cRA, with or without the MEK inhibitor (20 μM) PD98059. (B) Western blot analysis of EGR1 after 1-h treatment with 25 μM 13cRA, with or without the translational inhibitor cycloheximide (Chx). (C) Western blot analysis of nuclear extracts treated with and without 13cRA, probed with anti-SP1 primary antibody after immunoprecipitation with anti-EGR1. (D) Western blot analysis of nuclear extracts treated with and without 13cRA, probed with anti-ERK1 primary antibody after immunoprecipitation with anti-SP1. (E and F) Corresponding reprobe controls with appropriate capture antibody to confirm efficient immunoprecipitation of target proteins.

this study demonstrate that there is significant interaction between EGR1 and SP1 in 13cRA-treated cells (Fig. 5C and D). In EGR1 western blot analysis, some interaction is observed in untreated cells; however, this interaction is 518 ± 67% stronger in 13cRA-treated cells. The membranes were reprobed using the respective capture antibody to confirm that the target-precipitated protein was effectively transferred following protein electrophoresis (Fig. 5E and F). Collectively, these experiments suggest that there is a protein–protein interaction occurring between EGR1 and SP1.

Sp1 dictates basal transcription of AT1R
In order to determine Sp1’s control of Agtr1a expression, we selectively targeted Sp1 with siRNA oligonucleotides to silence gene expression. In order to ascertain the ability for the siRNA to silence Sp1 expression, western blot analysis was performed. Western blot analysis using specific antibodies targeting Sp1 show significant knockdown of Sp1 expression in cells transfected with Sp1-specific siRNA compared with both scrambled negative control siRNA and untransfected cells (Sp1 knockout (KO) control and Sp1 KO treated with 13cRA mean difference from control 90.80±2.963 and 92.54±2.502% respectively, P<0.0001, n=3) (Fig. 6A and B). Consistent with our promoter analysis, AT1aR mRNA expression studies using dual-PCR amplification indicate that upon knockdown of Sp1 expression, AT1aR mRNA expression downregulates both in untreated control cells and in cells exposed to 25 μM 13cRA for 24 h (Sp1 KO control and Sp1 KO treated with 13cRA mean difference from control 84.53±3.248 and 84.92±3.113% respectively, P<0.0001, n=3) (Fig. 6C). Furthermore, 3HAngII binding studies indicate that receptor density on the plasma membrane is reduced (88.5±3.352%, P<0.0001, n=9) in cells transfected with Sp1-specific siRNA (Fig. 6D). AngII binding among the untreated control and 13cRA-exposed cells was not significantly different (P>0.05) among Sp1 knockdown groups. From the siRNA studies, we concluded that Sp1 is a key regulator in the basal transcription of Agtr1a.

Establishment of EGR1 as a critical mediator of 13cRA-induced Agtr1a downregulation
Studies up to this point established that 13cRA-mediated downregulation of Agtr1a involves activation of ERK1/2; additionally, investigations demonstrated that Sp1 binding is affected on distal response elements, ERK1/2 activates the transcription and production of EGR1,
EGR1 and SP1 interact in a protein–protein complex detectable by co-immunoprecipitation, and SP1 is essential for the basal transcription of the Agtr1a gene. However, the studies did not demonstrate that EGR1 is involved directly with the downregulation of Agtr1a. In order to determine whether EGR1 is integral in ERK1/2-mediated Agtr1a downregulation, we performed siRNA knockdown of Egr1, similar to our method used for Sp1 knockdown, to determine EGR1’s effect on Agtr1a mRNA transcription and [3H]AngII binding in the presence of 13cRA. Western blot analysis indicates that EGR1 expression was significantly reduced compared with both untreated and scrambled siRNA when transfected with Egr1–specific siRNA, and the siRNA-transfected cells showed no significant increase in Egr1 expression when exposed to 25 μM 13cRA for 24 h (Fig. 7A). Immunofluorescent microscopy targeting EGR1 indicated that expression of the nuclear resident EGR1 is significantly reduced in siRNA-transfected cells, with no detectable increase in expression upon exposure with 13cRA (Fig. 7B). Next, we examined the effect of Egr1 siRNA transfection on 13cRA-mediated Agtr1a mRNA expression. Agtr1a mRNA expression studies using dual-PCR amplification specific to β-actin and Agtr1a mRNA display no significant reduction in siRNA-transfected cells when treated with 13cRA (mean difference in 13cRA-treated Egr1 knockdown and Egr1 knockdown control 2.663±8.842%, P=0.7783, n=3) (Fig. 7C). Under similar conditions, we determined whether receptor

![Figure 6](image-url)

**Figure 6**
Knockdown studies reveal that Sp1 expression is essential for Agtr1a basal transcription. (A) Western blot displaying sufficient Sp1 silencing by siRNA targeted for Sp1. Gel separation shows doublet of protein bands characteristic with polyclonal antibody SC59-X. (B) Densitometric analysis of Sp1 bands from western blot is shown in panel A. Displayed is a knockdown by Sp1 siRNA of 90–95% relative to control. 13cRA has no effect on level of expression of Sp1. (C) Agtr1a mRNA expression eliminated after Sp1 knockdown (−out). mRNA transcription is significantly reduced after 13cRA treatment. (D) Radiolabeled ligand binding assay showing elimination of binding of AngII to membrane receptors after Sp1 knockout. All data are expressed as mean±s.e.m. ‘−’Ctrl and ‘−’13cRA indicate cells that are transfected with scrambled negative control siRNA. All 13cRA results shown are treated with 13cRA for 20 h.

![Figure 7](image-url)

**Figure 7**
siRNA silencing of Egr1 reverses 13cRA-mediated downregulation of Agtr1a. (A) Western blot analysis of cells treated with or without 13cRA and transfected with scrambled negative control siRNA oligonucleotides or Egr1-targeted siRNA oligonucleotides. (B) Immunofluorescent microscopy using anti-EGR1 to confirm effective silencing of 13cRA-induced Egr1. EGR1 antibody labeled with FITC (green) and nuclei are stained with DAPI (blue). (C) Agtr1a mRNA expression restored after Egr1 knockdown (−out) (D). Radiolabeled ligand binding assay showing restoration of binding of AngII to membrane receptors after Egr1 knockdown. All data are expressed as mean±s.e.m. ‘−’Ctrl and ‘−’13cRA indicate cells that are transfected with scrambled negative control siRNA. EGR1 imaging indicates 13cRA treatment for 60 min while AngII binding and mRNA studies reflect 20-h 13cRA treatment. (E) Accompanying mobility shift in selective gene knockout studies included. Samples were analyzed on 6% non-denaturing polyacrylamide gels and visualized by autoradiography. The position of the protein–DNA complex is indicated by arrow. Labeled probe in the absence of nuclear extract (lane 1), in the presence of untreated nuclear extract (lane 2), in the presence of 13cRA-exposed nuclear extract (lane 3), nuclear extracts of both untreated and treated with 13cRA after siRNA-mediated silencing of Sp1 (lanes 4 and 5 respectively), and nuclear extracts of both untreated and treated with 13cRA after siRNA-mediated silencing of Egr1 (lanes 6 and 7 respectively).
binding studies correlated with the detected restoration of mRNA expression. [3H]AngII binding displayed a complete restoration in AngII binding in Egr1 siRNA transfected and 25 µM exposed cells (mean difference in 13cRA-treated Egr1 knockdown and Egr1 knockdown control 9.055 ± 7.224%, P = 0.2281, n = 6) (Fig. 7D). Lastly, we performed additional EMSA analysis to correlate previous findings in mobility shift with the effect of selective knockdown of SP1 and EGR1 proteins on characteristic mobility shift patterns. Protein/DNA interaction was almost entirely eliminated after Sp1 knockdown in both untreated and 13cRA-treated nuclear extracts (Fig. 7E, lanes 4 and 5 respectively), whereas robust protein/DNA interaction occurred in Egr1 knockout nuclear extracts, regardless of 13cRA treatment (Fig. 7E, lanes 6 and 7). Our results suggest that EGR1 is essential for 13cRA-mediated downregulation of Agtr1a, and we propose that its mechanism involves the disruption of SP1 to initiate basal transcription of Agtr1a mRNA by direct protein/protein interaction.

Discussion

In our previous studies, we have shown that phosphorylation of ERK1/2 (MAPK p42/p44) results in the downregulation of Agtr1a mRNA expression (Snyder & Thekkumkara 2012). However, signal transduction following the phosphorylation of ERK1/2 was not investigated. Therefore, we extended this study to elucidate the downstream regulators responsible for Agtr1a gene transcription. We observed that SP1 binds to a distal cis-acting element on the Agtr1a promoter for basal transcription, and upon 13cRA treatment, EGR1, a trans-activator, binds to SP1 through protein–protein interaction, thereby downregulating Agtr1a expression. The observed effect requires ERK1/2 phosphorylation, which is unrelated to RAR/RXR activation, as retinoid receptor blockade has no effect on 13cRA-mediated downregulation of Agtr1a (Snyder & Thekkumkara 2012). SP1, along with SP3, has been identified as an essential transcription factor for expression of human Agtr1 in H295-R cells (Zhao et al. 2001) and expression of rat Agtr1a may be enhanced by SP1 overexpression (Sugawara et al. 2001). Our study indicates that SP1 is a constitutively active transcription factor. There is no current evidence of the involvement of distal SP1 elements in the expression of human AGTR1, particularly because most promoter studies examine much shorter promoter sequences. Therefore, it may be that any sequestration of SP1 by EGR1 in humans disrupts transcription by a similar mechanism, requiring additional studies; however, human studies are difficult, particularly because of the limited availability of cell lines with reliable expression of AGTR1. However, to our knowledge, this is the first instance in which disruption of a distal SP1 binding element has resulted in a down-regulatory effect on Agtr1a expression, suggesting that 13cRA’s effect is unique and is indicative of an entirely novel mechanism.

After determining the time course of early ERK1/2 phosphorylation, we had yet to establish the link between ERK1/2 and SP1 binding disruption. The potential candidate was EGR1. Egr1 expression has been shown to be upregulated upon ERK1/2 activation (Hoffmann et al. 2008). In addition, EGR1 has been associated with disruption of Sp1 binding ability with gene promoters, including response elements found in the AT1aR promoter (Hsu et al. 2009). Upon observing Cxcr3 KO mice have suppressed expression of EGR1 and increased expression of AGTR1A protein, the authors suggested a possible involvement of EGR1 and SP1 in the expression of AGTR1A protein. These conclusions were based upon a previous study showing that SP1 activity plays a major role in human Agtr1 gene transcription (Elton & Martin 2007). EGR1, also known as zif268, NGFI-A, Krox24, and TIS8, is a zinc-finger transcription factor belonging to the same nuclear factor family as SP1 and SP3 (Lim et al. 1998). However, EGR1 is distinct in its binding characteristics and is particularly active at 5′-GGGGGGGCGG-3′ moieties within GC-rich regions (Silverman & Collins 1999). In fact, mutations of this characteristic element have shown that affinity for GC elements is lost if a single internal guanine residue or the 3′ cytosine is exchanged for an alternate base (Zhang et al. 2007). Often, EGR1 elements are integrated within regions sharing the particularly GC-rich motifs specific for SP1 and SP3 binding 5′-GGGGGGGCGG-3′ (Khachigian et al. 1995, Zhang et al. 2007). EGR1, like many transcription factors, has either activation or repression function depending upon whether co-activators or co-repressors are recruited. EGR1 can interact directly with CREB-binding protein and p300, very similar to SP1, in order to activate gene transcription by the co-activators’ inherent histone acetyltransferase activity. As far as EGR1’s relevance in the current study, the distal SP1 binding element used in our mobility shift does not possess a putative EGR1 binding sequence. Sequence analysis revealed no characteristic EGR1 binding elements located within the 3300 bp investigated in the rat Agtr1a promoter. Therefore, EGR1 binding may not be necessarily important as a direct trans-acting factor binding to cis-acting elements on the Agtr1a

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promoter, but rather an indirect protein interference factor inhibiting binding of SP1 by a protein–protein interaction as shown in this study.

EGR1, as its name suggests, is an immediate response gene, though some sources characterize it as an intermediate response protein (Zhou et al. 2010) putting it in the same time frame for activation of other kinase-dependent effector transcription factors like c-fos and c-jun. Its steady-state expression is relatively low, but upon activation by MAPKs, it is rapidly induced (Hoffmann et al. 2008). In hypoxia-reoxygenation experiments, Zhou et al. (2010) found that Egr1 expression was increased upon hypoxic conditions followed by reoxygenation. This was accomplished by activation of cellular stress kinases (p38, p42/p44, JNK); the subsequent activation of EGR1 led to transactivation of downstream targets such as TGF-β, ICAM-1, tissue factor, and PDGF (Khachigian et al. 1995, Silverman & Collins 1999, Okada et al. 2001, Khachigian 2006) that lead to vascular hyperpermeability, coagulation, and inflammation. Therefore, EGR1 production is detrimental to the outcome of hypoxia-reoxygenation. However, in the findings of this study coupled with what is known about exposure to 13cRA, EGR1 is not itself an inflammatory mediator, and the transient upregulation seen in these studies had no damaging effects in these cells. EGR1 activation in the past has been accomplished by chronic cell stress leading to multiple MAPK activations and subsequent activation of negative pathways, but the data suggest that 13cRA is more selective as it is not associated with the activation of p38 or JNK (Jameel et al. 2009), and specific activation of ERK1/2 may give different results. The direct interaction between EGR1 and SP1 has been recognized in previous studies. Jain et al. (1996) have suggested that EGR1 and SP1 can directly bind one another and may limit their respective binding to gene promoters (Srivastava et al. 1998), but this interaction is dictated by EGR1 phosphorylation by casein kinase II. When phosphorylated by casein kinase II, EGR1 has lower binding affinity than SP1; interestingly, casein kinase II has also been associated with the inactivation of SP1 by phosphorylation (Harris et al. 2008). If EGR1 first binds to SP1, then dissociates by upregulation of casein kinase II, there could be indirect inactivation of SP1 by the same mechanism, which requires further investigation.

As we have shown in this study, EGR1 upregulation is an ERK1/2-dependent process. It has also been demonstrated that 17β-estradiol levels are associated with improved vascular condition (Kublikiene et al. 2008, Kitamura et al. 2009). EGR1 is activated in MCF-7 cells upon treatment with 17β-estradiol (Chen et al. 2004). Moreover, this activation is through a non-genomic or ‘extra-nuclear’ pathway. The extra-nuclear effect could have a strong parallel with the current study as we have shown that 13cRA mediates its effects independent of retinoic acid nuclear receptors (Snyder & Thekkumkara 2012). In further support of the estrogen theory, the aforementioned study by Srivastava et al. (1998) controlled phosphorylation of EGR1 and subsequent binding to SP1 by estrogen replacement in ovariectomized animals. Estrogen replacement reduced the activity of casein kinase II and thus increased the formation of interactions between EGR1 and SP1. Especially, with regard to our own research (Koganti et al. 2012), which has shown a down-regulatory effect by estrogen metabolites through extra-nuclear mechanisms, this comes as a link between disparate phenomena connected by ERK1/2. With regard to direct ERK1/2 activation by 13cRA, it may bind a number of cytosolic binding proteins, such as CRABP-II or FABP5, leading to divergent effects through distinct signal transduction pathways (Schug et al. 2008) resulting in tissue-specific effects. These suggested upstream mechanisms of ERK1/2 phosphorylation in this model require further investigation. In summary, our results show for the first time that the mechanism of 13cRA-mediated Agtr1a downregulation is reliant on both ERK1/2 phosphorylation and EGR1 synthesis ultimately leading to the disruption in binding of SP1 to the Agtr1a promoter.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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