13-cis-Retinoic acid specific down-regulation of angiotensin type 1 receptor in rat liver epithelial and aortic smooth muscle cells

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Abstract
Transcriptional repression through cis- and trans-acting factors enabling an alternate approach to control angiotensin type 1 receptor (AT1 or AGTR1 as listed in the MGI database) expression has not been studied. In previous investigations, treatment with retinoic acid was found to be associated with enhanced insulin sensitivity. In our previous study, expression of AT1 was found to be inversely correlated with intracellular glucose concentrations. Therefore, we hypothesized that 13-cis-retinoic acid (13cRA), an antioxidant, enhances insulin-sensitive glucose-mediated down-regulation of the AT1. In this study, we used continuously passaged rat liver epithelial cells. Our study shows that cells exposed to 13cRA specifically down-regulated the AT1 protein in a dose- and time-dependent manner, independently of any change in receptor affinity. Down-regulation of the AT1 expression leads to reduced AngII-mediated intracellular calcium release, a hallmark of receptor-mediated intracellular signaling. Similarly with receptor down-regulation, we observed a significant reduction in AT1 mRNA; however, the AT1 down-regulation was independent of insulin-sensitive glucose uptake and retinoic acid receptor activation (RAR/RXR). Treatment with 13cRA resulted in phosphorylation of p42/p44 MAP kinases in these cells. Subsequent studies using MEK inhibitor PD98059 prevented 13cRA-mediated AT1 down-regulation and restored AngII-mediated intracellular calcium response. Furthermore, 13cRA-mediated inhibitory effects on AT1 were validated in primary rat aortic smooth muscle cells. In summary, our results demonstrate for the first time that 13cRA has a glucose- and RAR/RXR-independent mechanism for transcriptional inhibition of AT1, suggesting its therapeutic potential in systems in which AT1 expression is deregulated in insulin-sensitive and -insensitive tissues.

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Introduction
Angiotensin II (AngII) through the angiotensin type 1 receptor (AT1 or AGTR1 as listed in the MGI database) is a powerful vasoconstrictor and a key hormone controlling plasma volume and is thus highly interesting to the realm of hypertension research (Nickenig 2002, Wassmann & Nickenig 2006). However, AngII is proving itself more ubiquitous in its importance to homeostasis outside its well-characterized hypertensive effects. It is a potent mitogenic/angiogenic stimulator (Escobar et al. 2004, Numaguchi & Murohara 2009) and an inducer of growth factors relevant to hyperplasia and fibrosis development (Skulttetyova et al. 2007), and responsive tissues outside the cardiovascular and renal systems are almost innumerable (Leung 2004). In particular, AngII has shown to play a part in the development of liver fibrosis and steatosis, and if one blocks AT1 with selective competitive antagonists, the exacerbation of these conditions by AngII ceases (Sookoian et al. 2005, Kim et al. 2008). Therefore, intervention against AngII’s binding of AT1 is associated with not only a decrease in blood pressure but also a multisystemic effect. Vitamin A, also known as retinol (an alcohol) or retinal (an aldehyde), is an essential dietary nutrient important for supporting a healthy immune system, maintaining normal eyesight and red blood cell production, and facilitating growth and development through gene transcriptional control (Blomhoff 1994). Retinoic acid, a metabolite of preformed vitamin A, exerts its function by binding to nuclear receptors (RXR and RAR) and forming complexes with specific sequences on promoter regions of target genes called retinoic acid response elements (Mark et al. 2006). Therefore, activity of a gene is dictated by recruitment of enhancer or repressor proteins such as nuclear–coactivator complex or nuclear–corepressor complex respectively. These are of course examples in a much larger panoply of effector molecules that may be recruited by an activated and DNA-bound nuclear receptor (Rosenfeld & Glass 2001). The primary isomers of retinoic acid formed in vivo are all-trans-retinoic acid (atRA) and 9-cis-retinoic acid (9cRA); each
binds separate retinoic acid receptor types, thus acting upon a select subset of genes (Chambon 1996). The 13-cis-retinoic acid (13cRA) is a synthetic form that may function similar to the other isomers, although the exact mechanism of action is unclear; in other words, its activity may be due to spontaneous isomerization to atRA or a novel activity of its own (Kim et al. 1994, Levin 1995, Ganceviciene & Zouboulis 2007). 13cRA has been used in the past as a dermatological preparation applied orally or topically to influence skin growth factors in such a way as to lower sebum secretion (Landthaler et al. 1980). As a result, its marketability to treat acne has overcast some of its more recent applications. For instance, it can be used as a monotherapy or in combination for the treatment of cutaneous T-cell lymphoma (Zhang & Ducic 2003) and oral leukoplakia (Piattellia et al. 1999), and as a differentiating agent in myelodysplastic conditions (Siitonen et al. 2007). Though 13cRA has multiple applications and therapeutic targets, the cellular mechanisms and molecular targets are not understood.

Previously, we had reported that there is an inverse relationship between glucose levels and expression of the AT1 in proximal tubule epithelial cells (Thomas & Thekkumkara 2004). Several studies have shown that the AngII-induced cellular response (i.e. hyperplasia) may be blocked by retinoic acid treatment (Wu et al. 1996, Wang et al. 2002, Lu et al. 2003). Indeed, retinoic acid has been recognized to be associated with renin–angiotensin system signaling (Choudhary 2006, Wang et al. 2002, Lu et al. 2003). From the perspective of these studies, one of the two possibilities is represented: either the members of the multicomponent signal transduction cascade mediated by stimulation of the AT1 are being blocked specifically by retinoic acid (i.e. ERK1/2, p38, and STAT–JAK) or the receptor is being down-regulated specifically by retinoic acid (i.e. ERK1/2, p38, and MAP kinase activation). Down-regulation we observed in these cells was determined to be due to MAP kinase activation. Furthermore, the observed effect was consistently reproduced in primary rat aortic smooth muscle cells (RASMC) in culture. To our knowledge, this is the first study elucidating the effects of 13cRA on AT1 expression and function.

Materials and methods

Materials

Continuously passaged rat liver epithelial cells (WB cells) were provided by Dr H Shelton Earp, University of North Carolina at Chapel Hill (Chapel Hill, NC, USA). Primary RASMC were from Lonza (Walkersville, MD, 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 biotinated probes were obtained from Integrated DNA Technologies, Inc. (Coralville, IA, USA). PCR master mix was from Roche (Branchburg, NJ, USA). Losartan was provided by Merck Sharp and Dohme Research Laboratories (Rahway, NJ, USA). 13cRA, insulin, actinomycin D, and gentamicin were from Sigma (St. Louis, MO, USA). DNA/RNA extraction reagents were from Ambion/ABI (Austin, TX, USA). [3H]D-glucose was from Amersham. Selective RAR and RXR antagonists BMS453 and HX531 were from Tocris Biosciences (Ellisville, MO, USA). [3H]D-glucose was from Perkin–Elmer (Waltham, MA, USA). Fura-2 AM and PD98059 were from Calbiochem (La Jolla, CA, USA). AT1 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and phospho-MAP kinase antibody was from Cell Signaling (Danvers, MA, USA). Real-time PCR reagents and equipment were from Applied Biosystems (Carlsbad, CA, USA). Electrophoresis reagents were from Bio-Rad (Richmond, CA, USA), and all other chemicals and molecular biology grade agents were purchased from Fisher Scientific (Fairlawn, NJ, USA).

Cell culture

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 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 primary culture of
RASMC, the cells were maintained in Richter’s improved MEM supplemented with 20% FBS, 10 mM glucose, 17-8 mM HEPES, 5-4 μg/ml insulin, and 44-6 μg/ml gentamicin at 37 °C in 5% CO₂ with 100% humidity. For the studies, cells were grown to 70–80% confluency and the medium was exchanged with treatment medium (identical to the WB treatment medium) and exposed to indicated agents for indicated times. The cells used in this study were from passages 3 to 5.

**Angiotensin receptor binding studies**

Receptor binding studies were performed in triplicate on WB or primary RASMC before and after 13cRA exposure as described previously (Thakkumkara et al. 1995). Briefly, the 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 incubation, the 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 the Bio-Rad protein assay system based on the Bradford method (Bradford 1976). Data were analyzed and nonlinear regression curves were obtained using the computer software GraphPad Prism (la Jolla, CA, USA); Kᵰ and Bₘₐₓ were calculated as described previously (Swillens 1992).

**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% nonfat milk and incubated with monoclonal antibody against phospho-MAP kinase p42/p44, total MAP kinase p42/p44 (1:1000 dilution; Cell Signaling), or monoclonal antibody against actin (1:3000 dilution; Santa Cruz, USA). After washing three times with TBS containing 0.5% Tween-20, bound primary antibody was detected with antirabbit HRP-conjugated goat IgG (1:5000 dilution; Santa Cruz Biotechnologies, Santa Cruz, CA, USA). Immunoreactive proteins were visualized using the chemiluminescent substrate kit from Pierce Biomedical (Thermo-Scientific Pierce, Rockford, IL, USA).

**Reverse transcriptase: dual PCR, real-time qPCR, and Southern blot analysis**

Cells were grown to 70–80% confluency and treated with or without 25 μM 13cRA for 24 h. Total RNA was isolated using the guanidium thiocyanate–phenol–chloroform method as described previously (Thakkumkara & Linas 2003). Total RNA was quantified and 5 μg/condition was processed for cDNA template conversion using MLV-RT. The reaction without reverse transcriptase served as a control for DNA contamination. The cDNA was then amplified with a dual-PCR primer set for AT1 and β-actin mRNA (AT1 sense: 5’-TGATCCAGCTGGGGGTCACTCCA-3’, AT1 antisense: 5’-TTTCGCTAGACAGGGTTCATGGG-3’; β-actin sense: 5’-CGGAACCCGTCATTGC-3’, β-actin antisense: 5’-ACCCACACTGTGCCA TCTA-3’). The PCR was performed in a 50 μl sample volume subjected to 30 cycles and the amplicons were analyzed on a 2% agarose ethidium bromide gel. Bands were visualized under u.v. to assure appropriate band density among β-actin controls and transferred to a nylon membrane using Southern capillary transfer as described previously (Brown 2001). The amplicon was then hybridized with biotinylated internal probes specific for both AT1 (5’-CTGACATCGTGGACACTGCGAT-3’) and β-actin (5’-AGAGGAATTCGTTGGGTACATT-3’). The membranes were then washed and bands visualized using Chemiluminescent Nucleic Acid Detection Module according to the manufacturer’s instructions (Thermo-Scientific). The intensity of the bands was captured by the Bio-Rad VersaDoc and quantified using Quantity One software. For real-time quantitative analysis of AT1 mRNA, two parallel PCRs, each containing 5 μg total mRNA and either AT1- or β-actin-specific primers, were performed using 2X SYBR Green Master Mix (Applied Biosystems). Following the reaction, threshold cycles (Ct) were calculated for each sample for AT1 and β-actin mRNA reactions, and quantitative concentrations were calculated using ΔΔCt calculations described by Perkin–Elmer Applied Biosystems. For the mRNA stability studies, cells were preincubated with 1 μg/ml actinomycin D for 15 min and then treated with or without 13cRA for indicated times. Total RNA was isolated using the method as described above. The densitometric data were further analyzed by linear regression using GraphPad Prism software to determine the half-life of the AT1 mRNA.
[3H]Glucose uptake assay

Cells were grown to 70–80% confluence in 12-well plates. Growth media were exchanged for media supplemented with or without insulin and in the presence or absence of 13cRA, leaving a total of four treatment groups. At the end of 24 h, cells were washed twice with warm PBS followed by replacement with growth medium containing 1% FBS and no glucose. After 15 min, the media were exchanged for growth media containing 1% FBS and 5 mM glucose. To each well, 100 nCi [3H]glucose was added, and the cells were incubated for 20 min at 37 °C. The medium was aspirated, and the cells were washed three times with chilled PBS. The cells were then lysed with a lysis buffer containing 1% SDS and 200 mM NaOH. The lysates were collected and the radioactivity was determined in a liquid scintillation counter. Specific activity was correlated with picomoles of glucose uptake per milligram of protein per unit time.

Immunofluorescent microscopy

Cells were seeded and grown in chamber slides (Nalge-Nunc International, Rochester, NY, USA) and then exposed to 13cRA for 24 h. At the end of 24 h, cells were washed once with ice-cold PBS and fixed for 20 min in 4% paraformaldehyde. Cells were then blocked in 5% goat serum for 1 h and incubated in primary antibody (dilution 1:1000; Santa Cruz) directed against rat AT1 receptor (dilution 1:3000; Invitrogen). Washed cells and the radioactivity was determined in a liquid scintillation counter. Specific activity was correlated with picomoles of glucose uptake per milligram of protein per unit time.

Measurement of cytosolic free Ca\(^{2+}\) concentration

WB cells grown to 70–80% confluence in 35 mm optical bottom plates (MatTek #P35G-0-10-C, Ashland, MA, USA) and AngII-induced changes in intracellular calcium were monitored using the microspectrofluorometry method as described previously (Gryniewicz et al. 1985). Briefly, cells were loaded with 1 μM fura-2/AM in HBSS for 20 min. The cells were washed twice with HBSS and changes in intracellular Ca\(^{2+}\) were measured. The microscope’s emission wavelength was set at 510 nm and the excitation wavelengths at 340 and 380 nm. Image capture was performed at 22 °C using an Olympus IX-81 microscope equipped with an Olympus U-CMAD3 camera under a PlanApo 60×/1.40 oil immersion objective. Excitation was monitored by a high-speed wavelength-switching device, recorded with a CCD camera. Images were collected and analyzed using Slidebook image analysis software. Alternatively, we quantified the AngII-induced increase in intracellular calcium by the fluorescent imaging plate reader (FLIPR) method as described in Gryniewicz et al. (1985). In short, 70–80% confluent cells in six-well plates were loaded with 1 μM fura-2/AM in 1 ml Dulbecco’s PBS (DPBS) for 1 h at 37 °C. The cells were then washed once with warmed DPBS and twice with FLIPR buffer (145 mM NaCl, 2.5 mM KCl, 10 mM HEPES, 10 mM glucose, 1.2 mM MgCl\(_2\), and 1.5 mM CaCl\(_2\)). They were then analyzed in 900 μl FLIPR buffer on the BioTek SynergyMx fluorescent plate reader for baseline readings for 2 min, and AngII was added to a final concentration of 1 μM for an additional 4 min. At the end of each experiment, the maximum emissions for Ca\(^{2+}\)-bound fura-2 and free fura-2 were obtained by sequential addition of 20 μl of 15 mM digitonin and 20 μl of 300 mM EGTA. Changes in nanomolar concentrations of intracellular calcium were calculated using the formula:

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\Delta [\text{Ca}^{2+}] = \frac{K_d (F - F_{\text{min}})}{(F_{\text{max}} - F)} - \frac{K_d (F_x - F_{\text{min}})}{(F_{\text{max}} - F_x)}
\]

\(K_d\) in this equation is predetermined at 224 nM. F corresponds to the fluorescence reading after AngII exposure, while \(F_x\) corresponds to the fluorescence reading just before AngII application. \(F_{\text{min}}\) is the minimal fluorescence detected after addition of digitonin. \(F_{\text{max}}\) is the maximum fluorescence detected after addition of digitonin.

Statistical analysis

Results were presented as mean ± s.e.m. and the value of P<0.05 was considered statistically significant. Values were 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, and the binding constants were determined as described previously (Swillens 1992).

Results

13cRA reduces AT1 density

In this study, to determine the effects of 13cRA on AT1 expression, we used a continuously passaged rat liver
epithelial cell natively expressing the AT1. This cell line was originally isolated by Dr Grisham and coworkers in 1984 and is characterized as a normal adult rat liver epithelial diploid continuously passaged cell line (Tsao et al. 1984). This cell line has been extensively used by Dr Earp and coworkers, as well as other investigators, to delineate AngII-mediated AT1 cell signaling and function (Tsao et al. 1986, McCune & Earp 1989, Huckle et al. 1990, 1992, Earp et al. 1995, Bokkala & Joseph 1997, Li & Earp 1997, Li et al. 1997, 1998, Bokkala et al. 2001). In this study, we used these cells up to passage 26 at which point there were no changes to the cell’s phenotype (Huckle et al. 1990).

Receptor binding studies have shown that tritiated AngII in the presence of the AT1 antagonist losartan displaces 88-89 ± 3.849% (P<0.0001, n=9) of the specific binding determined by cold AngII 10⁻⁶ M. However, when the cells were treated with the AT2R-specific antagonist PD123319, there was no detectable displacement of AngII-specific binding (mean difference = -0.6520 ± 6.126%, P=0.9168, n=9), indicating that these cells predominantly express the AT1 (Fig. 1A). Upon completion of 24-h treatment with 25 µM 13cRA, there was a marked reduction in AT1 binding (72.56 ± 5.359%, P<0.0001, n=9). However, upon full blockade of the AT1 with losartan in the 13cRA-treated cells, the remainder of the binding was equivalent with the control losartan-treated group. Additionally, there was no observed effect on the AT2R blockade group, when treated with 13cRA (mean difference = 2.726 ± 5.359%, P=0.3647, n=9). This suggests that the observed reduction in AngII binding by 13cRA is specifically reducing the binding of AngII to the AT1 (Fig. 1A). To determine the specificity of this effect, we performed dose–response and time course studies. The dose–response study from 1 to 150 µM shows a dose-dependent inhibition (Fig. 1B). For our future studies, we chose 25 µM concentration for 24 h as it reliably showed 53.0 ± 6.5% (P<0.0001) down-regulation with no cytotoxicity (determined by Hoescht staining, data not shown). Furthermore, the study using 25 µM concentration shows a time-dependent effect up to 24 h (reduction of 43.49 ± 3.814%, P<0.0001 at 24 h; Fig. 1C). The above studies indicate that 13cRA is capable of reducing AngII binding specifically to the AT1 in a dose- and time-dependent manner.

However, the binding studies cannot fully rule out the change in receptor density because of the possibility that AngII binding to the receptor can be altered due to changes in the receptor affinity. Therefore, we performed competition binding studies to determine the affinity of the receptor in untreated cells and 13cRA-treated cells (Fig. 2). Calculated Kᵢ in untreated cells was 3.28 ± 0.66 nM. These receptors are in the high-affinity state, which is consistent with the

**Figure 1** (A) 13cRA inhibits AT1-specific binding in WB cells. Radioligand binding assay after 24-h treatment with 25 µM 13cRA (RA) and either losartan (Los) or PD123319 blockade. Cells were exposed to 13cRA (25 µM) for 24 h and [³H]AngII binding was measured in the presence or absence of AT1 blocker losartan or AT2R blocker PD123319 (n=9). (B) 13cRA-mediated AT1 inhibitory effect is dose dependent. Radioligand binding assay after variable concentrations (1–150 µM) of 13cRA treatment for 24 h. 13cRA demonstrates IC₅₀ at 25 µM (n=9). (C) 13cRA-mediated AT1 inhibitory effect is time dependent. Radioligand binding assay after exposure to 25 µM 13cRA for variable treatment times as indicated. 13cRA inhibits AT1 binding ≥45% within 8 h and remains inhibited for the remainder of time-points (n=9). Data are expressed as mean ± S.E.M. ***P<0.0001 vs untreated control.
maximum binding was reduced in 13cRA-treated cells exposed to normal medium and a 2.97 nM dissociation constant (K_d), suggesting that there is no apparent change in receptor affinity after 13cRA treatment. The 13cRA-treated cells’ K_d was 2.97 nM, while the untreated cells yielded a mobilized Ca^2+ concentration of 554 ± 56.2 nM in the untreated cells, while the 13cRA-treated cells yielded a mobilized Ca^2+ concentration of 159 ± 5.21 nM (a reduction of 71.24 ± 10.26%, P=0.0023; Fig. 4B). The observed reduction in ELISA readings was consistent with our findings in fluorescence imaging for individual cell response. We performed an additional study to confirm the variable responsiveness at increasing doses of AngII to confirm the validity of using 10^{-6} M AngII for eliciting a Ca^{2+} release (Fig. 4C). The results of the study indicated that the effect is dose dependent in cells in the presence or absence of 13cRA and was maximally stimulated by 10^{-7} M AngII, with an equivalent response at 10^{-6} M AngII (mean difference between control groups, 2571 ± 14.29% (P=0.8598, n=8), mean difference between treated groups, 1410 ± 9.32% (P=0.8819, n=3)). In summary, studies performed so far show a significant reduction in the expression of AT1 on the cell surface with no change in receptor affinity and a coinciding reduction in receptor-mediated cellular signaling.

13cRA down-regulates AT1 mRNA expression

We extended our studies to further investigate the expression of AT1 mRNA. We performed real-time qPCR calculating AT1 mRNA expression quantitatively to β-actin mRNA concentration. In Fig. 5A, the ethidium bromide-stained gel image shows specificity of the primers used and the purity of the RNA. The purpose of this image is to demonstrate successful PCR amplification with given primers with no DNA contamination. For results of the qPCR, see Fig. 5B. Our findings show that after exposure to 25 μM 13cRA for 24 h, there is a reduction of 54.37% ± 10.45% (P=0.0065, n=3) in the level of AT1 mRNA compared with untreated cells (Fig. 5B). To determine whether this reduction was due to transcriptional repression, we estimated 13cRA-mediated changes in AT1 mRNA half-life in the presence of a transcriptional inhibitor,
actinomycin D. Cells were pretreated with 5 μM actinomycin D for 20 min followed by exposure of 25 μM 13cRA in treated groups. mRNA was extracted at 2-h intervals, and dual-PCR was carried out to calculate AT1 mRNA relative to β-actin density. The half-lives were calculated to be 7.04 ± 0.179 h for untreated cells and 7.13 ± 0.054 h for treated cells, demonstrating no significant difference, and further supporting that the reduction in AT1 mRNA is mediated at the transcriptional level (Fig. 5C and D). Alternatively, we treated the cells with actinomycin D following a 16-h preincubation in 13cRA-treated groups. The data were consistent with our findings from the previous actinomycin D study (data not shown). These studies indicate that 13cRA causes a significant reduction in the expression of AT1 mRNA without affecting the rate of mRNA degradation, suggesting that there is 13cRA-mediated transcriptional repression leading to down-regulation of AT1 protein.

**13cRA down-regulates AT1 expression through MAP kinase p42/p44 activation**

In order to establish a mechanism for the transcriptional down-regulation of AT1 transcription by 13cRA, we performed studies in which we selectively inhibited retinoic acid receptors, RAR and RXR, with the specific antagonists BMS453 (250 μM) and HX531 (50 μM) respectively (Fig. 6). The results of these studies demonstrate that blockade of either receptor has no effect on 13cRA-mediated down-regulation of AngII binding, and thus AT1 expression (mean reduction in respective controls of 59–11 ± 6–450%, \( P<0.0001, n=3 \) without RAR/RXR antagonism, 83–01 ± 4–914% \( P<0.0001, n=3 \) with RAR antagonism, and 66–78 ± 5–890% \( P<0.0001, n=3 \) with RXR antagonism). Had 13cRA mediated its effects through these receptors, we would have observed restoration of AngII binding; therefore, an alternate mechanism was investigated. Previous studies have shown that retinoic acid is capable of activating MAP kinase p42/p44 involved in cellular differentiation (Yen et al. 1998). Therefore, we determined the role of 13cRA on MAP kinase p42/p44 and AT1 down-regulation. Twenty-four hour treatment with 25 μM 13cRA induced MAP kinase p42/p44 phosphorylation without a change in the total protein (Fig. 7A). This 13cRA-mediated phosphorylation was completely inhibited by MEK inhibitor PD98059. To determine the effect of MEK inhibition on the 13cRA-mediated down-regulation of mRNA expression and cell surface binding, studies were performed incorporating PD98059. Dual-PCR analysis shows the mRNA to be restored to control levels (mean difference increased 12.33 ± 4.372%, \( P=0.0765, n=3 \) when treated with both PD98059 and 13cRA (Fig. 7B and C). Under similar conditions, binding data showed that there was little to no effect by 13cRA on binding of \([3H]\)AngII when MEK signaling was inhibited in the presence of PD98059 (reduction in binding from PD control 12.7% ± 6.78, \( P=0.089; \) Fig. 7D). Similarly, FLIPR calcium imaging showed significant restoration of Ca\(^{2+}\) signaling by AngII stimulation when cotreated with PD98059 and 13cRA (PD group Ca\(^{2+}\) nanomolar mobilized 442 ± 42.6 vs PD + 13cRA group Ca\(^{2+}\) nanomolar mobilized 389 ± 62.0, \( P=0.5140; \) Fig. 8). Combining the results of receptor binding, mRNA, and calcium mobilization studied in the presence of PD98059, our study shows that 13cRA-mediated down-regulation is MAP kinase p42/p44 dependent.

**13cRA-mediated down-regulation is glucose and insulin independent**

The WB cells used in this study are insulin sensitive, and intake of glucose is largely regulated through the facilitative transporters. Our study shows that glucose uptake was significantly reduced (40.33 ± 9.772%, \( P<0.0001; \) Fig. 9A) in the absence of insulin compared with insulin-exposed cells. Glucose has been known to

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**Figure 3** Immunofluorescent study shows the AT1 down-regulated in cells exposed to 13cRA. Immunofluorescent staining using primary rabbit anti-AT1 IgG followed by secondary antirabbit IgG conjugated with Alexa Fluor 488. Nuclei stained with 4’,6-diamidino-2-phenylindole (DAPI). (Left) Native AT1 in WB cells with no 13cRA exposure. (Right) AT1 expression after treatment with 25 μM 13cRA for 24 h.
Figure 4  (A) Fluorescent imaging shows that 25 µM 13cRA (RA) exposure for 24 h significantly inhibited AngII-mediated increase in intracellular Ca²⁺, a G-protein-coupled response. Transient free Ca²⁺ was measured with the Ca²⁺ indicator fura 2-AM using fluorescent imaging microscopy before and after AngII stimulation (10⁻⁷ M), with or without 13cRA treatment. Ca²⁺-bound fura 2-AM emits green fluorescence. (B) Ca²⁺ FLIPR assay shows a significant reduction in fluorescent emission after 25 µM 13cRA exposure for 24 h. Representative tracing of AngII-mediated transient increase in intracellular Ca²⁺ (left) without and with (right) 13cRA treatment. The tracings are representative of three separate experiments, an overall reduction of 71.24 ± 10.26%. (C) Ca²⁺ FLIPR assay performed with logarithmic dose of AngII to determine dose–response of calcium release in both treated and untreated WB cells.
both activate MAP kinase p42/p44 signaling and down-regulate the receptor (Park et al. 2002, Thomas & Thekkumkara 2004, Wang et al. 2010). Therefore, we examined whether glucose uptake was enhanced by 13cRA treatment. Glucose uptake assay shows that there was no significant change in the quantity of glucose uptake by 13cRA-treated cells compared with untreated cells (mean difference $3.33 \pm 2.88\%$, $P=0.3828$, $n=3$; Fig. 9A). The response was unaffected by the withdrawal of insulin (mean difference $5.21 \pm 7.88\%$, $P=0.5135$, $n=3$), indicating that 13cRA-mediated down-regulation is insulin independent. Furthermore, a binding study was performed to demonstrate the effect of insulin deprivation on 13cRA’s down-regulatory function. While there was no effect among the 13cRA-treated groups (mean difference $1.49 \pm 8.81\%$, $P=0.8669$), there was a significant increase in binding of $25.8 \pm 5.18\%$ ($P=0.0008$) when control cells were deprived of insulin (Fig. 9B), which is consistent but inversely related to our previous studies showing that increases in intracellular glucose concentrations result in decreased expression of the AT1. These studies show that 13cRA can mediate the down-regulation of AT1 independently of insulin-induced glucose uptake.

### 13cRA-mediated effects on primary vascular smooth muscle cells

To validate the WB cellular model of AT1 down-regulation by 13cRA and thus create relevance to vasculature, we performed additional studies using primary RASMC (Fig. 10). The treatments were conducted using the same protocol established in the WB cell line, and the results show that 13cRA is capable of down-regulating AT1 in vascular smooth muscle cells in the rat. The study validated our previous findings that the down-regulation by 13cRA is AT1 specific and is mediated by MAP kinase p42/p44 activation. Upon treatment with 25 μM 13cRA, AngII binding in RASMC decreased by $58.58 \pm 5.87\%$ ($P<0.0001$, $n=3$). AT2R blockade by PD123319 had no effect in control cells (mean difference in control and PD123319-treated

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**Figure 5** 13cRA inhibits AT1 mRNA expression without changing mRNA stability. (A) Representative image of ethidium bromide gel of a dual-RT-PCR using AT1-/actin-specific primers to determine the specificity of the primers and purity of RNA preparation. Lanes 2 and 3 are PCRs lacking reverse transcriptase showing RNA purity. Two specific bands detected are at 289 and 206 bp for β-actin and AT1 mRNAs respectively. (B) AT1 expression was determined by real-time quantitative PCRs after normalization to β-actin and data are expressed as mean ± S.E.M., $n=3$. **$P<0.01$ compared with untreated control.** (C) Representative dual-PCR ethidium bromide gel of actinomycin D exposed cells that were treated with (lower) or without (upper) 25 μM 13cRA for indicated times. Bands were captured and quantified using Bio-Rad Quantity One software. AT1 bands are normalized to β-actin control. Bands detected are at 289 and 206 bp for β-actin and AT1 mRNAs respectively. (D) mRNA half-lives were calculated using linear regression analysis ($n=3$).
in intracellular signaling that is insulin and glucose independent, as well as unrelated to classical retinoic acid receptor activation. The observed down-regulation is further correlated with intracellular signaling, such as intracellular calcium release, showing that the reduction in receptor density significantly disrupts AngII’s ability to enact cellular response. The results specific to MAP kinase-dependent AT1 down-regulation were further confirmed in primary RASMC of vascular origin.

The blockade of AT1 stimulation has profound protective effects in pathologically affected tissues relative to AngII signaling (Baumann et al. 2010). In this particular study, preconditioning with AT1 blockers showed improved prognosis in spontaneous hypertensive heart failure rats; additionally, these rats showed reduced sensitivity and lower receptor expression in cardiac and aortic tissue. We may therefore suggest that reduced receptor expression as well as a reduction in the normal cellular response from either circulating or locally (tissue) derived AngII is beneficial in those at risk of overactive renin–angiotensin system consequences. For the liver, in particular, one may take account of AngII’s potent activity. Studies have shown that blockade of AT1 in liver hepatocytes can attenuate fibrosis and steatosis (Ratziu & Zelber-Sagi 2009). AngII is not the causative agent of these conditions, but it can influence the rapid progression to more irreversible states if left untreated by inducing oxidative stress in at-risk livers (Wei et al. 2008). In previous studies, ARBs and ACEIs have lowered the fibrosis index score of at-risk livers (Kim et al. 2008, Yoshiji et al. 2009). This was taken as evidence that one may slow the progression of steatosis and fibrosis by inhibiting the renin-angiotensin system of the liver or at least by preventing the AT1/AngII interactions by ARB or ACEI therapy. Therefore, the observation in this liver cell model represents a finding of significant clinical importance.

Retinoids have been associated with improved responses to cardiac cellular injury. Vitamin A and its subsequent metabolism into retinoic acid have been proposed as a potential means for the regression of cardiac hypertrophy (Maier 2008). The atRA treatment prevents medial thickening of intrarenal and intramyocardial arteries in the SHR model (Lu et al. 2003), a model known to have up-regulated AT1 expression. Also, atRA supplementation has been shown to have protective effects on hypertrophic cardiomyocytes (Wang et al. 2002). AngII treatment in these cells resulted in increased protein production, as well as expression of a number of genes encouraging a hypertrophic phenotype. The atRA treatment significantly attenuated the expression of the hypertrophic effects after AngII exposure. In a related study involving an in vivo model, retinoic acid significantly attenuated ventricular remodeling after myocardial infarction, a

Discussion

Numerous studies have shown that alterations in AT1 expression leading to altered regulation of downstream intracellular modulators dictate specific patterns in downstream effectors; however, the role of these intracellular modulators on AT1 transcription remains enigmatic (Bonde et al. 2010, Wakui et al. 2010, Ozawa et al. 2011). In this study, we examined the effect of 13cRA on the expression of AT1 in rat liver epithelial cells known to express the native protein. Our results show that 13cRA is capable of down-regulating AT1 cell surface expression by down-regulating its mRNA expression through an MAP kinase-mediated
process known to be enhanced by AngII stimulation (Paiva et al. 2005). It has been reported that MAP kinase phosphatases are up-regulated during atRA treatment (Palm-Leis et al. 2004). However, as with many of the recent analyses, MAP kinase activation as the characteristic marker for hypertrophic activation is contingent upon activation of AngII stimulation of the AT1. For example, a study showed that atRA treatment prevented cardiac remodeling in aortic-banded rats, a procedure that leads to increased AngII release (Choudhary et al. 2008a). This study indicated that if the rats are pretreated with atRA, MAP kinase p42/p44 activation is prevented after aortic constriction. The finding is distinct from our observation, in which AngII is not a significant stimulator of MAP kinase p42/p44. In other words, MAP kinase activation in our study is unrelated to AT1 activation by AngII and therefore may be considered as a distinct acute signaling mechanism. Our study clearly demonstrates that MAP kinase p42/p44 is essential to 13cRA-mediated down-regulation of AT1. This is contrary to much of the existing data involving AT1-induced MAP kinase activation. Furthermore, studies in the past have implicated retinoic acid-mediated AngII/AT1 response disruption by downstream signaling pathways. The atRA treatment has been shown to up-regulate superoxide dismutase (Choudhary et al. 2008a). This was strongly supported from a previous observation that atRA treatment prevented AngII-induced oxidative stress in cardiomyocytes (Choudhary et al. 2008b). Therefore, the conclusion was that reduction of the oxidative stress by retinoic acid nullified the AngII-mediated cellular damage and inhibited cellular apoptosis. The atRA has been shown to directly affect the transcription of the AT1 gene in vascular smooth muscle cells (Takeda et al. 2000), partially supporting our observation of the
transcriptional repression of AT1 by retinoic acids. However, the proposed mechanism in the current study and previous work involves different retinoids. Although the protein directly affecting the transcription of the AT1 was unknown from the Takeda et al. (2000) study, it was shown that RAR/RXR-dependent transcription of this unknown protein was critical for the down-regulatory effect by atRA. Interestingly, the researchers found that there was no detectable competition between RAR/RXR complexes and CBP, even though MAP kinase activation was shown to have an accelerative effect on the transcription of the AT1 gene due to CBP binding to the proximal region of the promoter in studies investigating PPARγ (Sugawara et al. 2003), suggesting a further level of complexity in the mechanism found in our study. However, the mechanism of action of 13cRA has been shown to be significantly different than either atRA or 9cRA, with potent inhibition of many of the retinoid and hydroxysteroid-mediated pathways (Gamble et al. 1999, Blaner 2001) and relatively weak transactivation compared with atRA and 9cRA (Mangelsdorf et al. 1994). In other words, 13cRA is not a potent activator of either RAR or RXR and should therefore probably be considered not as a comparable compound with other retinoic acids, but as a distinct compound with specific therapeutic applications. To further delineate our findings with the Takeda et al. (2000) study, we have shown that 13cRA-mediated down-regulation is related to an entirely unrelated mechanism, as RAR/RXR antagonism has no effect on 13cRA-mediated down-regulation. 13cRA studies are not exclusively devoted to cardiac tissues. Indeed, there is one study that indicates 13cRA may impede AngII-induced hypertension by a direct effect on kidney tissues (Morath et al. 2009). Upon infusion of AngII, blood pressure was shown to be markedly elevated. However, with simultaneous treatment with 13cRA at both high and low doses, the increase in blood pressure by AngII was abolished. Additionally, renal damage in subtotally nephrectomized rats was markedly attenuated with concomitant treatment with 13cRA. Between our observations in two different cell types, the lowering of blood pressure in the Morath model as well as down-regulation of stimulation by AngII in the renal cortex, and observed antihypertrophic effects of retinoic acid (Wu et al. 1996, Wang et al. 2002, Lu et al. 2003), we believe that the effect of 13cRA on AT1 expression is not tissue specific.

There are numerous benefits in the choice of 13cRA as a therapeutic agent. Other retinoids are shown to have increased toxicity at high doses, particularly if consumed as preformed vitamin A (Mulholland & Benford 2007). In fact, adverse effects may be observed with intake of retinol equivalents of only 1500 µg, which is less than twofold greater than the recommended...
daily allowance. Liver toxicity is also a concern, as the liver acts as a reservoir for fat-soluble vitamins such as retinol. In contrast, 13cRA, aside from its potent teratogenic effects, carries very little danger as an acutely toxic agent. In one case, an overdose of 900 mg resulted in only minor and temporary dermatological and corneal manifestations with no permanent damage (Aubin et al. 1995). In other words, 13cRA has been referred to as the ‘liver-sparing retinoid’. To add to its advantages, 13cRA does not induce cytochrome p450 enzymes (CYP1A 2C, 2E1, 3A, and 2D6) in the way that atRA does (Muindi et al. 2008), thus stabilizing its therapeutic window in chronic treatment. However, there are disadvantages in 13cRA application as a therapeutic agent. The principal concern for 13cRA therapy is its profound teratogenic effects (Ganceviciene & Zouboulis 2007). Even low concentrations of 13cRA can cause significant birth defects in all stages of development, in particular, the suppression of cardiac development in the first trimester. AngII stimulation is critical in the development of cardiac tissue in the developing embryo; we may propose the mechanism by which the cardio-suppressive effect is mediated through the down-regulation of AT1 in the embryonic tissue via 13cRA, leading to unresponsiveness in tissues to AngII. This proposed mechanism requires future studies for validation. In addition, delivery of retinoids is relatively inefficient as distribution is restricted by their high lipophilicity and limited absorption from oral administration. Consequently, the dose of a 13cRA regimen is relatively high (40–80 mg/day; Rigopoulos et al. 2010); there are promising innovations in drug delivery methods, such as cyclodextrins, to improve the plasma concentrations of smaller doses of 13cRA (Lin et al. 2007, Trichard et al. 2007).

In summary, our study provides compelling evidence of a down-regulatory role by 13cRA on the AT1; this role is apparently dependent on MAP kinase p42/p44 and dictates transcriptional repression. Further studies

![Figure 9](https://example.com/figure9.png)

**Figure 9** 13cRA treatment caused no significant increase in glucose uptake; insulin withdrawal had no effect on 13cRA-mediated down-regulation of AT1. (A) Cells were exposed to 25 μM 13cRA (RA) for 24 h with or without insulin (Ins), followed by 15 min [3H]-glucose uptake assay. Data were calculated and expressed as picomoles of labeled glucose uptake per milligram protein per 15 min. Untreated control with insulin compared with untreated control without insulin shows a reduction in glucose uptake of 40.33 ± 9.77%, ***P<0.0001. (B) Radioligand binding assay after 24-h exposure to 25 μM 13cRA in the presence or absence of insulin. Data are expressed as mean ± S.E.M. *P<0.05 and ***P<0.0001 compared with untreated control (n=3).

![Figure 10](https://example.com/figure10.png)

**Figure 10** 13cRA down-regulates AT1 in rat aortic smooth muscle cells (RASMC). 13cRA down-regulates [3H]AngII binding in a AT1-specific fashion. RASMC are not significant expressers of AT2R as indicated by PD123319 blockade (10 μM). MEK inhibition and subsequent MAP kinase p42/p44 inhibition with the MEK inhibitor PD98059 (20 nM) restores [3H]AngII binding to control levels in the presence of 13cRA. Radioligand binding assay was performed after 24-h exposure to 25 μM 13cRA (RA) with blockade of AT1/AT2R or MEK with the respective agents indicated. Data are expressed as mean ± S.E.M. ***P<0.0001 compared with control, n=9.
must be performed to elucidate the exact nature of this repressor mechanism. Sequence analysis shows a characteristic COUP-TFII response element in the AT1 promoter, which may show some importance with MAPK p42/p44 signaling (Aerbajina et al. 2009). However, insulin and glucose were not involved in 13cRA down-regulation of AT1 as we had originally hypothesized. Moreover, mechanisms leading to 13cRA-mediated activation of MAP kinase in our study also need further investigation. It is possible that binding of cytosolic-binding proteins, such as cellular retinoic acid binding protein II or fatty acid binding protein 5, has opposing effects through divergent signal transduction pathway activation (Schug et al. 2008), leading to differential signaling in a tissue-specific manner. Nevertheless, the results presented in this study favor the possible benefits of 13cRA as a suppressor of AT1 expression and function, resulting in potential multisystem protective effects.

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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