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TITLE:

AT_1 receptor Gαq protein-independent signalling transcriptionally activates only a few genes directly, but robustly potentiates gene regulation from the β-2Adrenergic receptor

Gitte L. Christensen[1,2] Steen Knudsen[4], Mikael Schneider[5], Mark Aplin[1,6], Steen Gammeltoft[2], Søren P. Sheikh[5] and Jakob L. Hansen [$,1, 3]

[1]Laboratory for Molecular Cardiology, Danish National Research Foundation Centre for Cardiac Arrhythmia, Department of Biomedical Sciences, University of Copenhagen, Blegdamsvej 3b, DK-2200 Copenhagen, Denmark, [2]Department of Clinical Biochemistry, Research Park Glostrup, Glostrup Hospital, Denmark, [3]Copenhagen University Hospital, Rigshospitalet, Juliane Mariesvej 20, section 9302, DK-2100 Copenhagen, Denmark, [4]Medical Prognostic Institute, Hørsholm, Denmark, [5]Department for Clinical Biochemistry & Pharmacology, Odense University Hospital, University of Southern Denmark, Denmark, [6]The Victor Chang Cardiac Research Institute, Darlinghurst, Sydney, Australia.

$ Corresponding author: Jakob Lerche Hansen, Laboratory for Molecular Cardiology, The Danish National Research Foundation Centre for Cardiac Arrhythmia, Department of Biomedical Sciences, University of Copenhagen, Blegdamsvej 3b, DK-2200 Copenhagen, Denmark. E-mail:jlhansen@sund.ku.dk, Phone: +45 28757645 Fax: +45 35327610

Keywords: Angiotensin II type 1 receptor, Gene expression, Microarray, Biased agonist, 7TMR, transcription factor

Abbreviations:

Angiotensin II type 1 receptor (AT_1R)
Angiotensin II (Ang II)
[Sar1, Ile4, Ile8] Ang II (SII Ang II)
Isoproterenol (Iso)
Forskolin (Forsk)
Seven-transmembrane receptor (7TMR)
Transcription element listening system based on the Jasper database (Telis)
Transcription factor binding site (TFBS)

Abstract :

The Angiotensin II type 1 receptor (AT_1R) is known to signal through heterotrimeric G proteins, and Gαq protein-independent signalling has only recently gained appreciation for profound impact on a diverse range of biological functions. β-Arrestins, among other central mediators of Gαq protein-independent signalling from the AT_1R interact with transcriptional regulators and promote phosphorylation of nuclear proteins. However, the relative contribution of Gαq protein-independent signalling in AT_1R mediated transcriptional regulation remains elusive. We here present a comprehensive comparative analysis of Gαq protein-dependent and –independent regulation of AT_1R mediated gene expression. We found Angiotensin II to regulate 212 genes, whereas Gαq-independent signalling obtained with the biased agonist, SII Angiotensin II only regulated few genes. Interestingly, SII Angiotensin II, like Ang II vastly potentiated β-2-adrenergic receptor-stimulated gene expression. These novel findings indicate that the Gαq protein-independent signalling mainly modifies the transcriptional response governed by other signalling pathways, while direct induction of gene expression by the AT_1R is dependent on classical Gαq protein activation.

1.Introduction:

The Angiotensin II type 1 receptor (AT_1R) regulates a wide range of biological functions including vasoconstriction and fluid homeostasis and is involved in the patho-physiology of cardiovascular diseases [1,2]. The AT_1R has been thoroughly investigated with respect to activation mechanisms and signal transduction, focusing mainly on heterotrimeric Gαq protein activation and consequential downstream signalling. In a Gαq protein-independent manner, the AT_1R recruits β-arrestins, which associate with clatrin resulting in receptor internalization and termination of G protein signalling. The understanding of β-arrestins is expanding from receptor-trafficking proteins to scaffolding proteins which bring signalling proteins in close proximity to initiate a second wave of signalling. In addition to β-arrestins, AT_1R signalling which is independent of Gαq may involve direct activation of tyrosine kinases and possibly minor contributions from alternate trimeric G proteins, such as G12/13, Gi and Go [3-5]. Due to the availability of excellent tools such as mutants and selective agonists, the AT_1R has been used as a model seven-transmembrane receptor (7TMR) to study Gαq protein-dependent versus -independent signal transduction. Terms such as "functional selectivity" and "biased agonism" have been used to describe the phenomenon that individual pathways may be dissected at the level of receptor activation [6]. Accumulating evidence suggest Gαq protein-independent signalling is involved in cellular processes such as proliferation, protection against apoptosis, protein synthesis and migration [7-11]. This is important from a pharmacological perspective, since these adaptive and protective cellular responses may be favourable for patients with cardiovascular disease. Thus, selective activation of Gαq protein-independent effects in conjunction with inhibition of Gαq protein-dependent signals may prove pharmacologically superior to current therapeutic strategies that involve complete blockade of all AT_1R pathways [2]. Mechanistically however, it remains unresolved if Gαq protein-independent signalling induces transcription directly or through crosstalk with other signalling pathways.

Several studies indicate that β-arrestins and Gαq protein-independent signalling may somehow play an active role in regulation of transcription. Recently, it was reported that the biased agonist SII Ang II regulated gene expression by Gαq protein-independent signalling in rat aortic vascular smooth muscle cells [12] and adrenocortical cells [13]. Furthermore, a great number of transcriptional regulators have been shown to associate with β-arrestins [14] and we have recently revealed substantial phosphorylation of nuclear proteins to be involved in the Gαq protein-independent signalling from the AT_1R, including a great number of transcriptional regulators [15]. One study has thus far addressed this pertinent question, albeit considering only 100 genes, and curiously concluded that Gαq protein-independent signalling does not result in regulation of gene expression in HEK293 cells [16].

In light of the pharmacological importance of G protein-independent signalling, we undertook a comprehensive analysis of gene expression by a global comparison of the gene regulatory potential of the full agonist Ang II versus the biased agonist SII Ang II, which does not activate Gαq protein signalling while still activating Gαq protein-independent signalling. We also extended this comparison to evaluate the importance of Gαq-dependent and -independent AT_1R signalling on modulation of the β2 adrenergic receptor (β2-AR)-stimulated and cAMP-dependent transcriptional response.

2. Results

To determine a suitable time point for studying maximum inducible deviation in gene expression compared to baseline, HEK293 cells stably expressing the AT_1R were stimulated with Ang II for various times between 1 and 48 hours. The gene regulation profile for 41 transcripts previously reported to be sensitive to AT_1R activation was analyzed [17-20]. As depicted in figure 1, gene regulation was most pronounced at 3 hours, but was also considerable at subsequent time points up to 24 hours. Based on this observation, we decided to perform our global analysis (in triplicate) with ligand stimulation for 3 and 24 hours, since this would enable us to detect genes that are regulated by both early and late signalling events. In the final analysis, we re-used the samples with 3 and 24 hours treatment from the time course experiment so the final analysis included samples from four independent experiments for each hormone and time point, and six independent samples of un-stimulated cells.

To identify regulated genes, we examined each gene for differences in expression levels between experimental groups by t-tests assuming equal variance. To adjust for multiple testing, we applied a significance cut-off which allows a maximum of one false positive. Furthermore, only genes regulated more than 2 fold were considered as regulated. This analysis revealed 212 transcripts that were regulated after 3 hours of Ang II treatment, and 133 transcripts regulated after 24 hours. A heatmap showing the time-resolved expression of the 212 Ang II regulated genes is depicted in Figure 2A and the complete list of regulated genes are reported in supplementary file A.1. Using the same parameters for the analysis, we only found a single gene (*RGS2*) that was regulated by SII Ang II stimulation. Furthermore, we performed a principal component analysis. This analysis showed that the overall changes in gene expression induced by Ang II treatment for 3 and 24 hours were substantially different from untreated cells and from each other as well (Figure 2B). The analysis illustrated that the regulation profile induced by SII Ang II was clearly distinct from the untreated cells, although being closer related to untreated cells than to Ang II stimulated samples.

We find it reasonable to assume that genes potentially induced by SII Ang II represent a subset of the genes that are also regulated by Ang II, since SII Ang II stimulation of the AT_1R entails Gαq-

independent signal transduction, prominently as a partial agonist for β-arrestin recruitment [21-23]. Furthermore, we would also expect the actual degree of regulation for each gene to be lower than that induced by Ang II, since SII Ang II is only a partial agonist. We wanted to exploit this background knowledge of the applied agonists to increase our statistical power and identify more genes regulated by SII Ang II. To do so, we narrowed our analysis to focus only on SII Ang II regulated genes within the group of genes regulated by Ang II. We applied t-tests assuming equal variance for each gene and to adjust for multiple testing we applied a significance cut-off allowing a maximum of one false positive. With this strategy, we identified 19 genes which were regulated by SII Ang II after 3 hours treatment and 23 genes after 24 hours, which represents a total of 25 genes (Reported in supplementary table A.2.). As indicated, these genes were all activated by Ang II and therefore we included all significant genes for further analysis, despite low fold changes induced by SII Ang II. However, only 6 of these genes were regulated more than 2 fold. As was the case for Ang II treatment, a time-course was also performed with SII Ang II stimulation, which allowed us to observe the time-dependent regulation of the identified regulated genes. The time-resolved regulation of the 25 SII Ang II regulated genes is shown in a heatmap in figure 2C. When looking at the time-course of gene regulation, there is a tendency for later and sustained regulation by SII Ang II. The Ang II regulated genes tend to be normalized to basal levels after 48 hours (Figure 2A), whereas many of the 25 SII Ang II regulated genes are still up regulated at this point (Figure 2C). We also observe that 9% (19 of 212) of the genes regulated by Ang II at 3 hours were concordantly regulated by SII Ang, whereas that proportion was 17% (23 of 133) after 24 hours treatment (The 25 most statistically significantly regulated genes upon 3 hours Ang II and SII Ang II treatment are listed in Table 1 and 2).

The 5 most regulated genes upon SII Ang II stimulation were re-evaluated by Quantitative PCR (Q-PCR) (Figure 3). These were the common alpha chain of the glycoprotein hormones (*CGA*), Cysteine-rich angiogenic inducer 61 (*CYR61*), the transcriptional activator *LBH* (human homologue to the mouse limb-bud and heart), regulator of G-protein signalling 2 (*RGS2*) and Dual specificity protein phosphatase 6 (*DUSP6* –also known as MAP kinase phosphatase 3). All 5 genes were statistically significantly regulated with SII Ang II, although with lower fold changes than observed after Ang II treatment. The correlation between fold changes determined by microarray and Q-PCR was very good (R=0.82, Pearson´s correlation), in the correlation analysis we left out genes that were regulated more than 30 fold (Figure 3G). Considering the strong correlation between the microarray and Q-PCR analysis together with the conservative cut-off of 1 false positive, we believe that the genes we have identified to be regulated by SII Ang II are reliable.

PTGS2 has previously been reported to be regulated by SII Ang II [13]. In our microarray analysis, *PTGS2* did not meet the strict borders for significance, but since fold changes were high for SII Ang II

treatment and to validate the previous observation, we included it for further evaluation by Q-PCR. Indeed we confirm the previous finding that *PTGS2* can be regulated in a Gαq protein-independent manner (Figure 3f).

It has been questioned whether Gαq protein-independent or β-arrestin dependent signalling has a positive role in transcriptional regulation from the AT_1R. To look further into the mechanisms behind the observed gene regulation, we used the Transcription element listening system (TELIS) based on the Jasper database [24]. TELIS is designed to identify over-represented transcription factor binding sites (TFBS) in the promoters of the regulated genes. The results of the TELIS analysis are depicted in Table 3. The most significantly over-represented TFBS among the genes regulated by Ang II treatment was that of the Serum Response Element (SRE), which was interestingly not among over-represented TFBS after SII Ang II stimulation. cAMP response element (CRE) which was the second most over-represented TFBS for Ang II-regulated genes was also the most over-represented in the SII Ang II promoter set. The regulation of SRF and CREB mediated transcription was further investigated with SRE and CRE coupled firefly luciferase reporter constructs. Treatment with 100 nM Ang II resulted in 40 fold increased luciferase activity for the SRE-reporter and a 5 fold increase for the CRE-reporter (Figure 4A and B). 30 µM SII Ang II only increased SRE mediated luciferase activity by 1.2 fold and CRE actity 1.4 fold (Figure 4A and B). Findings of direct association between β-arrestins and a number of transcription factors and our observation that SII Ang II induces substantial phosphorylation of transcriptional regulators [15] are in apparent contrast to the finding that SII Ang II treatment does not lead to substantial gene regulation. The inability of SII Ang II to induce a transcriptional response alone could indicate that if Gαq protein-independent signalling has a role in gene regulation it is in cooperation with Gαq protein-dependent signals. β-arrestins which are recruited to the receptor upon SII Ang II treatment have previously been shown to be able to integrate signals from different receptors and facilitate cross talk [25]. We therefore extended our study to test whether SII Ang II, though unable to significantly regulate SRF and CREB mediated transcription alone, might have modulatory effect on SRF and CREB mediated gene expression induced by isoproterenol, an agonist for the β-2-adrenergic receptor (β2-AR). Isoproterenol was not able to activate SRF and SII Ang II only induced 1.2 fold regulation of this transcriptional activator. In concert the two agonists did not significantly increase SRF mediated transcription (Figure 4A). The clear SRF induction achieved with full agonist stimulation of the AT_1R was also not potentiated by co-stimulation of the β2-AR. Interestingly, SII Ang II immensely potentiated isoproterenol stimulated transcription of genes containing CRE cis-elements (Figure 4B), similarly Ang II and isoproterenol co-stimulation had synergistic effect on CREB induced transcription. The ability of Gαq protein-independent signalling to increase isoproterenol induced CREB transcriptional activation could result from AT_1R scavenging of β-arrestins, thus reducing desensitising capacity and resulting augmented

signalling from the β2-AR. To ensure that the observed synergy between the receptor systems was in fact a result of direct signalling cross-talk and not caused by competition for β-arrestin, we overexpressed β-arrestins. Overexpression of β-arrestins decreased the response to both Ang II, SII Ang II and isoproterenol (data not shown). The most obvious explanation for this observation is that β -arrestin causes the receptors to internalize. To circumvent this problem, we took advantage of the fact that the cAMP-PKA-CREB pathway can be activated directly by forskolin. The forskolin induced response should not be affected by scavenging of β-arrestin as this direct activation of adenylyl cyclase is independent of receptor activation. Forskolin was a potent activator of CREB dependent transcription and both SII Ang II and Ang II potentiated this effect (Figure 4C), to support actual crosstalk between Gαq protein-independent AT_1 signalling and the cAMP-CREB pathway.

3.Discussion

With this analysis of the gene expression of Ang II mediated Gαq protein-dependent and –independent signalling, we have covered a time span of 48 hours treatment and examined the expression of more than 20,000 transcripts. In supplement to previous studies, this comprehensive approach offers an overview of Ang II mediated gene regulation and identifies a minor group of genes that are specifically regulated by Gαq protein-independent signal transduction. Our data demonstrate that the major part of Ang II-mediated gene regulation relies on Gαq protein-dependent mechanisms. Nevertheless, 9% (19 of 212) of the Ang II regulated genes were also regulated with lower fold changes by SII Ang II after 3 hours treatment. Interestingly, this fraction doubled at 24 hours treatment where 17% (23 of 133) of the Ang II regulated genes were regulated by SII Ang II. This tendency of later and sustained regulation by SII Ang II is also visible on the heat maps in figure 3. Ang II regulated gene expression tend to be normalized to basal levels after 48 hours (Figure 3A) whereas many of the 25 SII Ang II regulated genes are still up regulated after 48 hours (Figure 3C).

Many transcription factor genes including *FOS, EGR1-4* and *Nr4A1-3* were strongly regulated by Ang II. These have previously been reported to be Ang II regulated in adrenocortical cells [13,26,27], proving the validity of our system. The five most regulated genes after SII Ang II treatment were validated by Q-PCR and the correlation to the fold changes revealed by the microarray analysis was remarkably good (Figure 3). Of the six genes validated by Q-PCR, we believe that *CYR61, LBH, RGS2, DUSP6* and *PTGS2* are truly regulated by both G protein-dependent and -independent mechanisms. In contrast, *CGA* showing up to 250 fold induction in response to Ang II and only 2 fold increase with SII Ang II can reasonably be assumed regulated exclusively by Gαq protein-dependent mechanisms.

Intriguingly, we found that increased RGS2 expression is supported by AT_1R Gαq-independent signalling. Pathological cardiac hypertrophy resulting from Ang II stimulation relies primarily on Gαq protein-dependent signalling by the AT_1R [28]. As a "regulator of G protein signalling", RGS2 acts to attenuate Gαq signalling and thus shift the signalling towards Gαq protein-independent mechanisms. In line with this, AT_1R mediated cardiac hypertrophy is counteracted by RGS2 [29]. Our finding of *RGS2* upregulation in response to SII Ang II indicates that a biased agonist like SII Ang II may prevent cardiac hypertrophy by increasing expression of RGS2 and simultaneously inhibit the Gαq-dependent signalling.

We also find *DUSP6* transcriptional induction to be among the highest in response to Gαq-independent signalling from the AT_1R. It is known that Gαq protein-dependent hypertrophic growth is mediated by ERK1/2 phosphorylation [8]. ERK1/2 is specifically dephosphorylated by DUSP6, and *DUSP6-/-* mice show higher basal activity of ERK1/2 and associated cardiac hypertrophy [30]. In pathological situations with increased circulating Ang II levels, DUSP6 up-regulation might therefore be important in prevention of a constant ERK1/2 drive.

We likewise observe SII Ang II induced expression of LBH. LBH is a transcriptional activator known to increase AP1 and SRF mediated transcription [31]. To our knowledge LBH has not been reported to be regulated by Ang II before. A large part of the LBH regulation by Ang II might be caused by Gαq protein-independent signal transduction as the fold changes observed with the biased and partial agonist, SII Ang II, are around 50% of those achieved with Ang II.

It is possible that the degree of induction we report for the SII Ang II regulated genes may not image the maximal capacity of Gαq protein-independent gene regulation for each of the reported genes. In our analysis, we stimulated cells with a concentration of 1.87 μM SII Ang II. In a recent study looking at PTGS2 gene regulation, it was found that 5μM SII Ang II resulted in a minor regulation of PTGS2, while the response was substantially increased by using 50 μM. Although 50 μM is a very high hormone concentration that may be physiologically irrelevant, several studies have shown that SII Ang II is only a partial agonist on β-arrestin-dependent signalling [22,23]. Furthermore, it is also likely that our global approach and conservative data-analysis strategy does not allow us to identify all genes that are in fact regulated by Gαq protein-independent mechanisms. Although equal kinetics cannot be assumed, the applied concentration of SII Ang II was chosen to achieve a receptor-occupancy comparable to that obtained with Ang II stimulation at 10nM, and Ang II has 187 times higher binding affinity for the AT_1R than SII Ang II [21].

Gαq protein-independent signalling represents a more active component in regulation of transcription, than previously appreciated, which is reflected by the ability of SII Ang II to induce and positively

modulate gene expression despite lack of Gαq/11 activation. However, the impact of SII Ang II on gene expression is modest compared to the large numbers of transcription factors associated with β-arrestins [14], and the observation that SII Ang II conveys widespread phosphorylation to transcription factors and other nuclear proteins [15]. We therefore hypothesized that Gαq protein-independent signalling may have a modulatory effect on transcriptional regulation induced by other signal transduction pathways, including G protein-dependent ones. To test this, we investigated if gene regulation induced by another 7TMR, the β-adrenergic receptor (β2-AR) could be modulated by activation of Gαq protein-independent signalling induced by SII Ang II. This was in fact the case as SII Ang II potentiated isoproterenol-induced gene expression from a CRE-luciferase containing promoter more than 4 fold (Figure 4B). This shows that gene regulation by a G protein-dependent signal (here Gαs) can be profoundly influenced by Gαq-independent signalling and possibly β-arrestin-dependent scaffolding. The β2-AR primarily signals through Gαs and generation of cAMP, which eventually leads to CREB mediated transcriptional activation. Furthermore, both Ang II and SII Ang II equally potentiated transcriptional activity induced by direct stimulation of the cAMP-CREB pathway with forskolin. This indicates that the observed synergistic effects actually results from direct signalling events from the AT_1R and is not just a result of β-arrestin scavenging resulting in increased β2-AR signalling. Our findings support a role for Gαq-independent mechanisms in the signalling cross-talk that underlie synergistic regulation of CREB- mediated transcription. Ang II has previously been shown to increase cAMP accumulation upon β2-AR stimulation by a Gαq protein-dependent mechanism [32]. β-arrestin scaffolding may be involved in the regulation of gene expression as SII Ang II stimulates formation of β-arrestin-scaffolded signalling complexes. The sub-cellular distribution of β-arrestins, with β-arrestin 1 in both the nucleus and cytosol, and β-arrestin 2 shuttling between the two compartments [33,34], β-arrestin binding to various nuclear proteins [14], and our recent finding of SII Ang II induced phosphorylation of nuclear substrates [15], all strongly implicate β-arrestin-dependent signalling in regulation of gene expression. Mechanistically, β-arrestin 1 has been reported to facilitate histone deacetylation and CREB mediated transcription by the δ-opioid receptor, by way of scaffolding the histone acetyl transferase (HAT), p300 and the transcription factor, CREB to promoters of p27 and c-fos [35]. We speculate that the mechanism behind the synergy of isoproterenol and SII Ang II on CRE-driven transcription could be formation of arrestin-CREB-p300 complexes, regulating subcellular localization of CREB and p300 and thereby facilitate histone deacetylation and CREB dependent transcription. These results indicate a role for Gαq protein-independent signalling as a regulator of G protein-mediated transcription and warrant further studies on the interplay between G protein-dependent and –independent signalling in the regulation of transcription.

In conclusion, this study shows that the majority of the AT_1R regulated gene expression is induced by Gαq protein-dependent mechanisms, but the study also shows that Gαq protein-independent signalling modulates gene expression. We report 25 genes that are likely regulated by Gαq protein-independent signalling alone and demonstrate that Gαq protein-independent signalling influences the magnitude of gene expression in 7TMR systems and can increase the gene expression induced by a different signalling pathway, as here exemplified by the β2-AR. Our findings solidify mounting reports of long term cellular phenotypes associated with Gαq protein-independent signalling from the AT_1R, and warrant in-depth analysis of the mechanistic links.

4. Materials and Methods

AT_1R stable transfected HEK293N (AT_1R-HEK) cells were a generous gift from Dr. Robert J. Lefkowitz, Duke University Medical Center, Durham, NC [10]. Cells were maintained in DMEM (Lonza) supplemented with 10% FCS (Gibco), 50U/ ml Penicillin and 50U/ ml streptomycin (Lonza), 2mM glutamine (Sigma) and 300 ug/ml Zeocin (Invitrogen). AT_1R-HEK cells were starved overnight in serum free DMEM and stimulated with 10 nM Angiotensin (Sigma) or 1.87 uM SII Angiotensin II (Cleveland Clinic). Treated cells were trypsinized, centrifuged and resuspended in RLT buffer. RNA was purified with RNAeasy columns (Qiagen). For time course studies, one experiment was performed at each of the indicated time points. Vehicle treated cells were harvested at 0, 6 and 24 hours after onset of the experiment. There was no difference in expression of the known Ang II regulated genes between these basal samples. For statistical analysis of Ang II and SII Ang II regulated genes we performed three new independent experiments of cells treated with Ang II or SII Ang II for 3 or 24 hours.

The quality and quantity of the extracted total RNAs were assessed by agarose gel electrophoresis, spectrophotometric ultraviolet (UV) absorbance at 260/280 nm, and Agilent Bioanalyzer analysis (Agilent Technologies, Inc., Santa Clara, CA). Gene expression analysis was performed on Affymetrix GeneChip Human Genome U133A 2.0 Array (Affymetrix, Inc., Santa Clara, CA). Labelling, hybridization, washing and image scanning were performed according to the Affymetrix standard protocol: Two µg of total RNA was used to synthesize double-stranded cDNA with the Superscript Choice system (Invitrogen) using an oligo(dT) primer containing a T7 RNA polymerase promoter (GenSet). The cDNA was used as the template for an *in vitro* transcription reaction to synthesise biotin-labeled antisense cRNA (IVT labelling Kit, Affymetrix Inc.). After fragmentation at 94°C for 35 min in fragmentation buffer (40 mMTris, 30 mM magnesium acetate, 10 mM potassium acetate), the labeled cRNA was hybridised for 16 h to Affymetrix U133A 2.0 arrays (Affymetrix Inc.), that contain 22,277 probe sets. The arrays were washed and stained with phycoerythrin-streptavidin

(SAPE) using the Affymetrix Fluidics Station 450, and the arrays were scanned in the AffymetrixGeneArray 3000 scanner to produce raw data image files.

Raw data was processed using Robust Multichip Analysis (RMA) normalisation [36] in order to get an intensity value for each gene and to calculate fold changes. In order to identify differentially expressed genes, we applied logit normalization [37] and MBEI estimation [38]. A t-test with equal variance was conducted for each gene and a significance cut-off of max. 1 false positive due to multiple testing and a min. fold change of 2 was applied due to the low number of replicates. Heatmaps were generated using the Heatplus library of Bioconductor. Principal Components Analysis was performed on Ang II regulated genes using the prcomp package of R with variables scaled to unit variance.

SYBR green based real-time Q-PCR was used to detect and quantify mRNA of interest according to manufacturer's protocol on a 7900HT (Applied Biosystems). Primers were designed to be exon-junction spanning with the following sequences:
CGA-forward: 5´-aggagcgccatggattactac-3´, CGA-reverse: 5´-agaagaatgggttttcctgtagc-3´,
CYR1-forward: 5´-ggctggaatgcaacttcg-3´,CYR1-reverse: 5´-cccgttttggtagattctgg-3´,
LBH-forward: 5´-tcatgtctatatatttccccattcac-3´, LBH-reverse: 5´-agatctggtaggaaaggccatc-3´,
RGS2-forward: 5´-caaaagctgtcctcaaaagcaag-3´, RGS2-reverse: 5´-cttcttgtatattctgggcaatcag-3´,
DUSP6-forward: 5´-atcactggagccaaaacctg-3´, DUSP6-reverse: 5´-taggcatcgttcatcgacag-3´,
PTGS2-forward: 5´-tgtatgtatgagtgtgggatttgac-3´, PTGS2-reverse: 5´-agtatgtagtgcactgtgtttggag-3´,
GAPDH-forward: 5´-ggtgtgaaccatgagaagtatgac-3´, GAPDH-reverse: 5´-gagtccttccacgataccaaag-3´,
β-actin-forward: 5´-gacaagacctgtacgccaacaca-3´ β-actin-reverse: 5´-gctcaggaggagcaatgatcttga-3´.
Specificity of primers was validated by sequencing of amplicons. Q-PCR values for mRNA analyses were normalized against two experimentally verified stably expressed mRNAs (GAPDH and β-actin) by use of qBasePlus software as previously described [39].

CRE and SRF dependent transcription was measured using the Pathdetect cis-reporting constructs CRE-luc, SRE-luc and pCIS-CK (negative control plasmid) (Stratagene). AT_1R-HEK293N cells were transfected with reporter plasmid 48 hours before the assay. 18 hours after transfection cells were split into 96 well plates. After O/N starvation, the cells were treated with the respective hormones for 3 hours, lysed in 30 µl luciferase lysisbuffer (1% triton x- 100, 40mM tricine pH 7.8, 50mM NaCl, 2mM EDTA, 1mM $MgSO_4$, 5mM DTT) for 15 min at room temperature. 10 µl lysate was transferred to a white 96 well-plate followed by addition of 100 µl luciferase reagent (40mM tricine pH 7.8, 0.5mM ATP, 10mM $MgSO_4$, 0.5mM EDTA, 10mM DTT, 0.5mM coenzymeA, 0.5mM D-luciferin (Promega)). Luciferase

activity was measured in a Tecan infinite M200 reader using an integration time of 5 seconds. Relative Luminescence Units (RLU) for the negative control plasmid (pCIS-ck) were subtracted from the experimental RLUs.

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7. Figure legends

Figure 1: Time course of Ang II gene regulation

To determine the optimal time point to study AT_1R induced gene expression, AT_1R-HEK cells were treated with 10nM Ang II for the indicated times spanning 1-48 hours. RNA was isolated and translated into cDNA, hybridized to affymetrix HGU 133A microarrays. Time resolved expression of 41 transcripts, previously reported to be regulated by Ang II stimulation is shown as affy-fold change compared to mean of untreated cells (3 samples, 0h, 6h and 24h), on the affyfold scale +2 means twofold up-regulation whereas -2 means twofold down-regulation. The maximum fold change for each of the transcripts is listed to the right.

Figure 2:

A: Time resolved heatmap of Ang II regulated genes

A heatmap showing the time resolved expression of the 212 genes which were significantly regulated after 3 hours of Ang II treatment. The expression of the same 212 genes with SII Ang II treatment is shown to the right. Basal reflects a mean of 3 untreated samples (0h, 6h and 24h). Green color represents upregulation, red color represents downregulation. A (Ang II), S (SII Ang II).

B: Principal component analysis

A principal component analysis of the 212 Ang II regulated genes showing the variation of the samples treated with Ang II and SII Ang II for 3 or 24 hours compared to untreated samples. 6 control

samples were used (4 harvested at the beginning of the experiment and one after 6h and 24h treatment with vehicle).

C: Time resolved heatmap of SII Ang II regulated genes

A heatmap showing the time resolved regulation of the 25 genes found to be significantly regulated by SII Ang II.

Figure 3: Validation of SII Ang II regulated genes with Q-PCR

A-E: Expression values determined by microarray versus Q-PCR.

The 5 most regulated genes upon SII Ang II treatment identified with microarray (plus *PTGS2*) were validated with Q-PCR. Fold changes from microarray and Q-PCR are shown for each of the 6 genes, doted lines represent 1. Statistically significant difference from untreated cells was tested with a paired student´s t-test. All expression values were significantly different from untreated samples (Except *CYR61*, SII Ang II 24h, array), $p < 0.05$, $n=3$, error bars represent S.E.M.

F: Correlation of fold change values from microarray versus Q-PCR analysis.

Correlation between array and Q-PCR measurements was evaluated using Pearson´s correlation coefficient, R=0.82.

Figure 4: SII Ang II potentiates CRE mediated transcription

AT_1R-HEK cells were transfected with SRE-luc or CRE-luc plasmids or a non cis-element-luc plasmid. Following 3 hours stimulation with isoproterenol (Iso), SII Ang II (SII), Ang II (Ang), forskolin (Forsk) or a combination, cells were lysed and luciferase activity was measured by addition of D- luciferin. Fold changes in comparison to untreated cells are shown (Background activity as reflected by counts in the cis-luc samples was subtracted).

A: SRE driven gene expression is induced by Ang II, but not by SII Ang II, and Isoproterenol does not affect this pathway.

B: Ang II and SII Ang II potentiate Isoproterenol mediated induction of CREB dependent transcription.

C: Ang II and SII Ang II potentiate forskolin mediated induction of CREB dependent transcription.

Note the logarithmic scale. Statistical significance of differences between groups was tested with a paired student´s t-test. * $p < 0.05$, $n=3-4$, *error bars reflect S.E.M.*

Table 1: Ang II regulated genes

The 25 genes most significantly regulated by Ang II treatment and their fold change values at 3 and 24 hours treatment with Ang II (A) and SII Ang II (S).

Table 2: SII Ang II regulated genes

The 25 genes most significantly regulated by SII Ang II treatment and their fold change values after 3 and 24 hours treatment with Ang II (A) and SII Ang II (S).

Table 3

A: Transcription factors regulated by Ang II.

B: Transcription factors regulated by SII Ang II.

Transcription factor binding sites over-represented in the promoters of the regulated genes. Results of analysesusing the Transcription element listening system based on the Jasper database (Telis: http://www.telis.ucla.edu)[24]

8. Appendices

A.1.: Ang II regulated genes

A.2.: SII Ang II regulated genes

A.3.: All expression values

A.4.: Expression values from time courses

A.5.: Expression values from replicates

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Table 1 Ang II regulated genes

Affy. No.	GENE	A3	A24	S3	S24
204637_at	*CGA*	7.9	128	1.1	2.5
202768_at	*FOSB*	89.5	2.0	1.5	1.3
206115_at	*EGR3*	69.3	1.9	1.6	1.6
211506_s_at	*IL8*	56.9	3.5	0.9	1.1
202340_x_at	*NR4A1*	54.4	10.3	1.3	1.3
210090_at	*ARC*	48.4	1.9	1.2	1.0
204748_at	*PTGS2*	46.6	9.5	2.2	3.9
205249_at	*EGR2*	41.7	1.2	1.2	1.3
207768_at	*EGR4*	40.2	1.0	0.9	1.0
209189_at	*FOS*	38.1	2.8	2.2	2.6
209959_at	*NR4A3*	36.8	3.6	1.0	1.1
206291_at	*NTS*	6.0	36.3	1.0	1.3
36711_at	*MAFF*	32.2	8.3	1.5	1.4
204614_at	*SERPINB2*	31.1	13.5	1.0	1.1
204621_s_at	*NR4A2*	27.1	7.0	1.7	1.8
201693_s_at	*EGR1*	24.4	1.8	1.6	1.8
204622_x_at	*NR4A2*	21.0	7.2	1.3	1.7
204222_s_at	*GLIPR1*	19.0	5.4	1.0	1.3
209101_at	*CTGF*	17.9	9.3	2.9	3.4
205239_at	*AREG*	17.5	13.1	1.0	1.1
204472_at	*GEM*	17.2	5.1	1.2	1.8
210764_s_at	*CYR61*	16.4	6.3	2.8	2.6
209278_s_at	*TFPI2*	11.1	15.2	1.2	1.0
202241_at	*TRIB1*	14.6	1.6	1.2	1.5
208078_s_at	*SNF1LK*	14.0	1.7	1.0	1.2

Table 2 SII Ang II regulated genes

Affy. No.	GENE	A3	A24	S3	S24
208891_at	***DUSP6***	7.1	7.1	2.9	2.9
210764_s_at	***CYR61***	16.4	6.3	2.8	2.6
221011_s_at	***LBH***	6.0	3.4	2.0	2.8
204637_at	***CGA***	7.9	128	1.1	2.5
202388_at	***RGS2***	4.0	5.5	1.6	2.5
203349_s_at	*ETV5*	7.8	5.5	2.0	2.1
207060_at	*EN2*	9.7	5.0	1.4	1.9
206442_at	*SEMG1*	2.1	8.5	1.3	1.9
204472_at	*GEM*	17.2	5.1	1.2	1.8
216017_s_at	*NAB2*	6.3	1.8	1.7	1.3
216598_s_at	*CCL2*	2.9	12.3	1.5	1.7
204622_x_at	*NR4A2*	21.0	7.2	1.3	1.7
208881_x_at	*IDI1*	2.5	3.0	1.5	1.2
207332_s_at	*TFRC*	2.2	4.3	1.2	1.5
208370_s_at	*DSCR1*	3.1	3.4	1.4	1.5
203499_at	*EPHA2*	6.6	3.6	1.5	1.9
201325_s_at	*EMP1*	7.1	3.7	1.4	1.4
216977_x_at	*SNRPA1*	1.3	2.2	1.1	1.3
202340_x_at	*NR4A1*	54.4	10.3	1.3	1.3
219098_at	*MYBBP1A*	1.3	2.0	1.0	1.2
219397_at	*FLJ13448*	2.3	1.5	1.2	1.1
205647_at	*RAD52*	0.5	0.6	0.8	0.9
208682_s_at	*MAGED2*	0.9	0.4	1.0	0.8
202447_at	*DECR1*	0.7	0.3	0.9	0.8
208791_at	*CLU*	1.6	2.5	1.0	0.7

Table 3

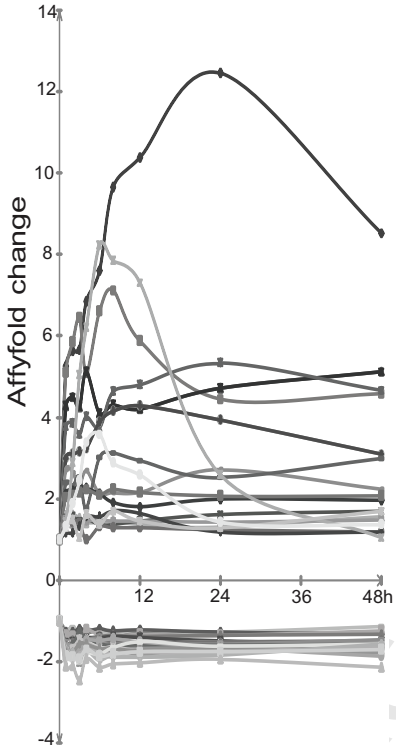
A: Transcription factors regulated by Ang II

	TF	p-val	n=147	%	% background
1	SRF	2.50E-05	5	3.4	0.23
2	CREB	9.00E-05	40	27	15
3	bZIP910	3.00E-04	26	17	8.5
4	HLF	1.50E-02	4	2.7	0.6

B: Transcription factors regulated by SII Ang II

	TF	p-val	n=25	%	% background
1	CREB	6.00E-04	8	32	15
2	Myf	2.00E-03	7	28	8.6
3	TEF-1	2.40E-03	4	16	3.8
4	FREAC-4	4.20E-03	7	28	12

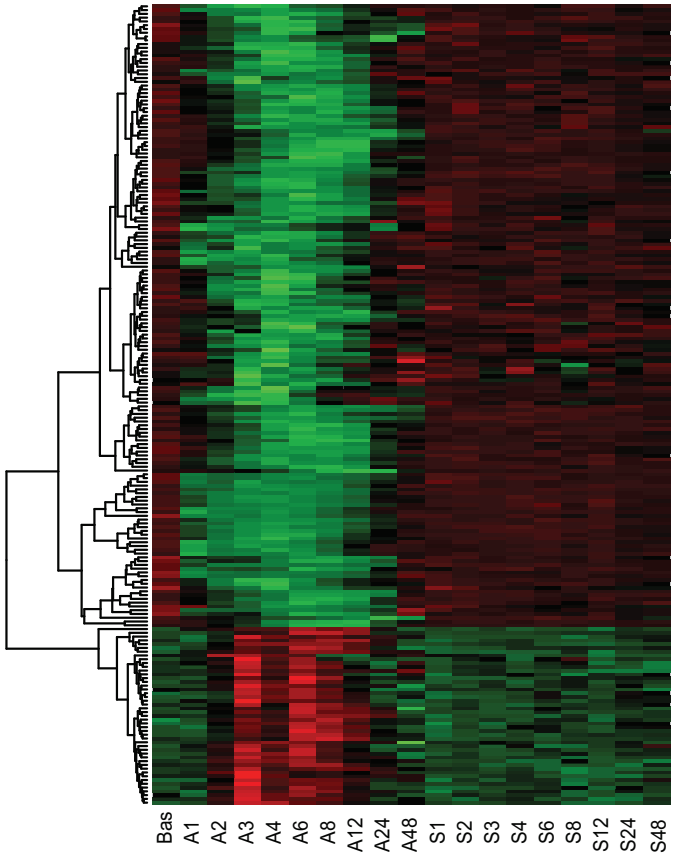
FIGURE 1



GENE NAME	MAX AFFY FOLD	GENE NAME	MAX AFFY FOLD
PRSS25	12.4	FGF2	-2.4
FOSL1	8.2	MEOX2	-2.0
SCNN1B	7.1	JAG1	-2.0
PTGFR	5.3	EPO	-2.0
P2RY2	5.1	TIMP1	-1.9
E2F3	5.1	PRPS1	-1.9
CDKN1A	4.3	MYLIP	-1.9
GSTM1	3.7	MMP2	-1.8
ETS2	3.6	IL1B	-1.8
S100A10	3.1	EPHX1	-1.8
NOS1	2.7	NCF1	-1.8
SOD3	2.3	LDHA	-1.7
PTGIS	2.2	EDNRA	-1.7
IRF1	1.7	PDGFRA	-1.7
DDT	1.7	SERPINE1	-1.7
ARVCF	1.7	FOXA1	-1.6
C20orf67	1.6	ID3	-1.6
P2RY1	1.5	AQP9	-1.5
SPP1	1.4	CASP9	-1.5
		THRB	-1.4
		CCNF	-1.3
		E2F1	-1.3

FIGURE 2

A



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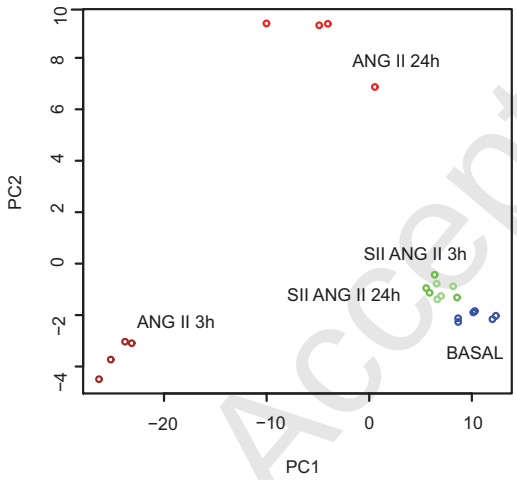

C

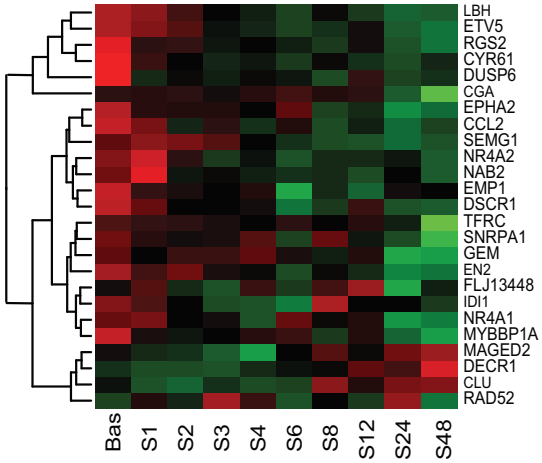

FIGURE 3

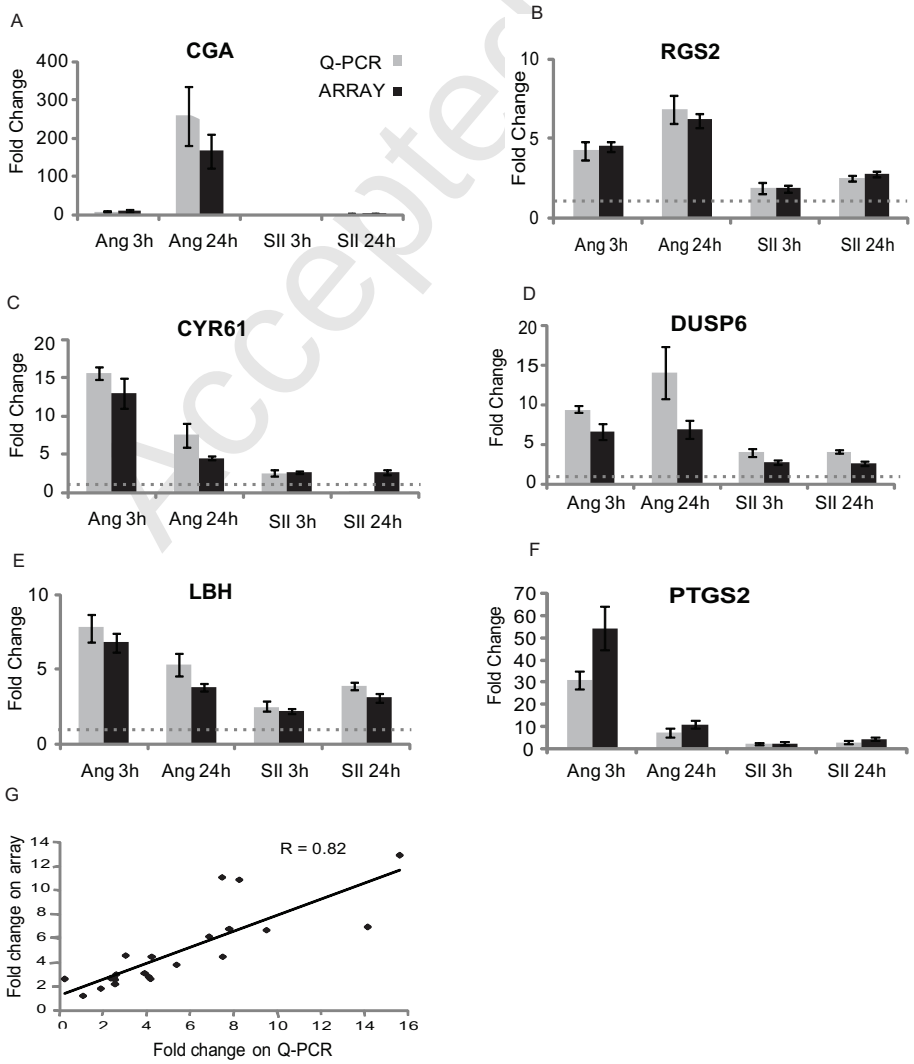

FIGURE 4