H295R-cells as a model system for detection of toxic effects on the adrenal aldosterone synthesis pathways

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ABSTRACT

The adrenal gland is exposed to high concentrations of circulating xenobiotics due to its high rate of blood flow and it may also accumulate lipid soluble chemicals in its lipid rich tissue. These substances can affect the aldosterone synthesis in the glomerulosa cells by activation or suppression of the steroidogenic enzymes' gene expression, hence resulting in effects on blood pressure since this is the main action of aldosterone.

The purpose of this project was to set up a model system for detection of xenobiotic effects on aldosterone synthesis due to changes in gene expression of involved enzymes and transporter proteins. The human adrenocarcionoma cell line H295R was subjected to angiotensin II (ang II) and potassium acetate (KAc), in order to establish which modulator that most efficiently differentiates the cells into glomerulosa like aldosterone secreting cells. Further the cells were tested for differences in differentiation due to various time intervals and for Ultroser SF (USF) supplementation in the medium. Leads effect on the involved genes in aldosterone formation was investigated, since lead is a known activator of aldosterone secretion.

The results indicated that ang II was the best modulator for differentiating the cells, KAc had cytotoxic effects at higher concentrations. The results also indicated that USF supplementation in the medium had a rising effect on basal gene transcription levels of the steroidogenic enzymes, some genes differed as much as 70-fold in expression levels between the two medium types. USF- medium was therefore chosen for exposure experiments, since the chemical effect hence became clearer.

The lead exposures indicated that this substance did not affect the gene expression level of the investigated genes, except for small effects in medium without USF and ang II. Some other differences in gene expression were noted between the control and the samples, but they were very small even if they were statistically significant.

The model will need more testing with other substances and the aldosterone level during chemical exposure will need to be determined. We have so far established that ang II is an efficient stimulator of glomerulosa like differentiation and that USF- medium is favorable in the experiments.

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ABBREVIATIONS

12-LO	12-lipoxygenase
3β-HSD	3β-hydroxysteroid dehydrogenase
ACTH	adrenocorticotropic hormone
Ang II	angiotensin II
AT_1R	angiotensin type I receptor
ATF	activating transcription factor
CRAC	calcium relese activated channels
CRE	cAMP response element, same as Ad1
CREB	CRE-binding protein
CYP11A1	side-chain cleavage cytochrome P450, $P450_{SCC}$
CYP11B1	11β-hydroxylase
CYP11B2	aldosterone synthase
CYP17A1	17α-hydroxylase
CYP21A2	21-hydroxylase
DAG	sn-1,2-diacylglycerol
ER	endoplasmatic reticulum
IP_3	inositol 1,4,5-triphosphate
KAc	potassium acetate
MEK	mitogen-activated protein kinases kinase
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
QRT-PCR	quantitative real time polymerase chain reaction
SF-1	steroidogenic factor-1
StAR	steroidogenic acute regulatory protein
USF	Ultroser SF (serum replacement)

1. INTRODUCTION

It has been shown that there is often variable species responsiveness to chemical toxicity. Laboratory animals are usually used in order to assess toxic effects of xenobiotic chemicals in humans (Klaassen, 2001). Due to the interspecies difference it is of interest to establish a model more suitable for humans than using laboratory animals.

In this project the human adrenocarcinoma cell line H295R, was used to set up a model system for testing the gene expression effects of different substances i.e. drugs, pesticides and other xenobiotics on aldosterone production in the human adrenal gland.

1.1 The adrenal gland

The adrenal gland is a relatively small organ located above the upper pole of each kidney. It has a weight of 4-10 g (Boron and Boulpaep, 2003; Nussey and Whitehead, 2001; Berne et al., 1998) and one of the body's highest rates of blood flow per gram of tissue (Berne et al., 1998). With this high blood flow rate there most likely will be some toxic impact on these organs.

The adrenal cells are rich in lipid storages primary used for steroidogenesis. Due to this high lipid concentration lipophilic compounds might accumulate in this tissue, potentially causing a toxic effect on the steroid synthesis in the adrenal gland. Another way of toxically affecting the adrenal cells is through the metabolism of xenobiotic chemicals by the cytochrome P450 enzymes, generating reactive compounds (Klaassen, 2001).

1.1.1 Structure

An outer cortex and an inner medulla, derived from embryonic mesoderm and neural crest cells, respectively, build up the adrenal glands. The medulla is responsible for producing adrenaline and noradrenaline, also called catecholamines. These important stress hormones differ from the steroid hormones, derived from the cortex, in structure and function (Boron and Boulpaep, 2003). The cortex in turn is subdivided into three layers, which together make up 80-90% (Berne et al, 1998; Nussey and Whitehead, 2001) of the adrenal gland. Zona glomerulosa is the outermost layer of the cortex located in the direct vicinity of the capsule, which surrounds the adrenal gland. The following layer is the zona fasciculata, representing the wide midcortex. The cells of this layer are columnar in shape, forming long cords (*fig 1*) and contain a high number of vacuoles. Zona reticularis represents the innermost layer of the

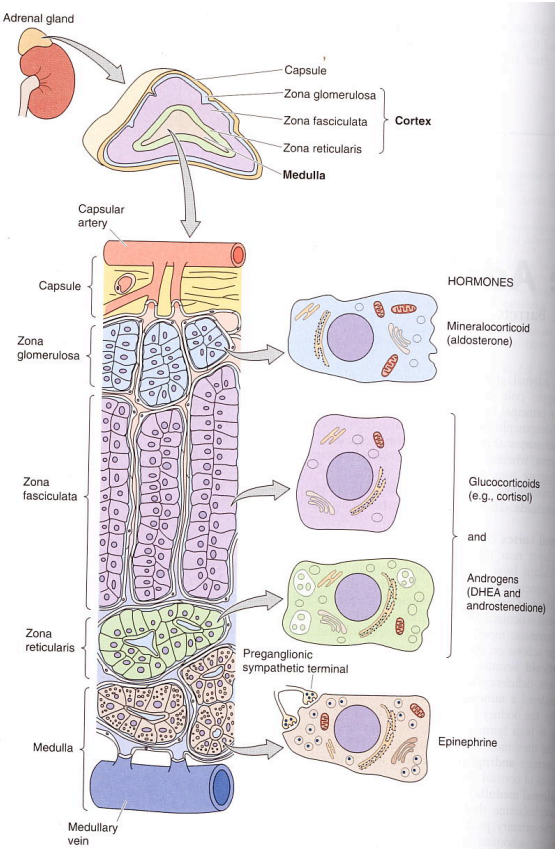

Fig 1. Location and internal structure of the adrenal gland (Boron and Boulpaep, 2003).

cortex and contains a smaller number of lipid droplets than the zona fasciculata. These cells are instead of long cords oriented in a network fashion (Berne et al., 1998).

Even if cholesterol is the starting point for all of the adrenal cortex steroids these are not equally synthesised all over the cortex. Glucocorticoids and the androgens are produced in the fasciculata and reticularis layers, with the main glucocorticoid production located in zona fasciculata (Berne et al., 1998). Due to lack of expression of the enzyme 17α-hydroxylase (CYP17A1) in the zona glomerulosa, no cortisol or androgen formation takes place in this layer, only production of the mineralocorticoid aldosterone (Nussey and Whitehead, 2001). The zona fasciculata and zona reticularis on the other hand lack the enzyme 18-hydroxysteroid dehydrogenase (CYP11B2), which is needed for aldosterone production (Klaassen, 2001).

1.1.2 Function of the adrenocortical hormones

The glucocorticoids are named after their initial discovery in glucose regulation (Boron and Boulpaep, 2003). Cortisol and corticosterone belong to this group and are regarded as critical to life, due to their effect on the metabolism of carbohydrates and protein. In humans cortisol is considered to be the dominant glucocorticoid, although if this pathway should be blocked corticosterone may step in its place upholding necessary activity (Berne et al., 1998).

Cortisol is responsible for increased glucose concentrations in plasma. Some other functions that this steroid possesses are immunosuppressive and anti-inflammatory activity, modulation of the CNS and to serve as an influent of the metabolism of fat, calcium and bone (Berne et al., 1998; Boron and Boulpaep, 2003).

As the name of the mineralcorticoid implies these steroids are involved in the mineral regulation of the body. The most prominent of the mineralcorticoids is aldosterone, responsible for re-absorption of sodium mainly in the kidneys, but also in colon, salivary glands and sweat glands (Berne et al., 1998; Boron and Boulpaep, 2003).

Androgens are the precursors to the sex steroids and they are mainly synthesised in the zona reticularis. The final conversion to testosterone and estradiol takes place in the peripheral tissues (Berne et al., 1998).

1.2 Aldosterone production

1.2.1 Angiotensin II activation

Synthesis of aldosterone is influenced mainly by the renin-angiotensin system, which releases renin. This is a response to reduced perfusion pressure in the kidney or decreased Na^+ delivery to the macula densa, which is a group of cells belonging to the juxtaglomerular apparatus in the kidney, responsible for salt regulation. Due to these signals renin is released into the bloodstream, cleaving the precursor angiotensinogen, produced by the liver, to angiotensin I (Nussey and Whitehead, 2001; Berne and Levy, 1998). This peptide is cleaved into angiotensin II by the angiotensin-converting enzyme, located in the endothelial cells of the lungs or kidney (Nussey and Whitehead, 2001).

1.2.2 Angiotensin II and ACTH induced signal transduction

Angiotensin II (ang II) interacts with the glomerulosa cells in the adrenal gland by the AT_1-receptor, responsible for activation of phospholipase C (PLC) (*fig 2*). Due to this activation insitol 1,4,5-triphosphate (IP_3) and sn-1,2-diacylglycerol (DAG) are formed. IP_3 influences the intracellular calcium concentration by release from calcium stores and opening voltage-dependent (T- and L-types) and calcium release activated (CRAC) channels (Capponi, 2004; Foster, 2004). DAG on the other hand activates the protein kinase C (PKC) pathway (Foster, 2004). Calcium channels may also be opened by increases in K^+ concentrations, which depolarize the cell membrane and activate voltage-dependent calcium channels without the need of internal Ca^{2+} release (Foster, 2004; Boron and Boulpaep, 2003). Since both ang II and potassium act by raising the calcium concentration they have a synergistic effect on the glomerulosa cells (Boron and Boulpaep, 2003).

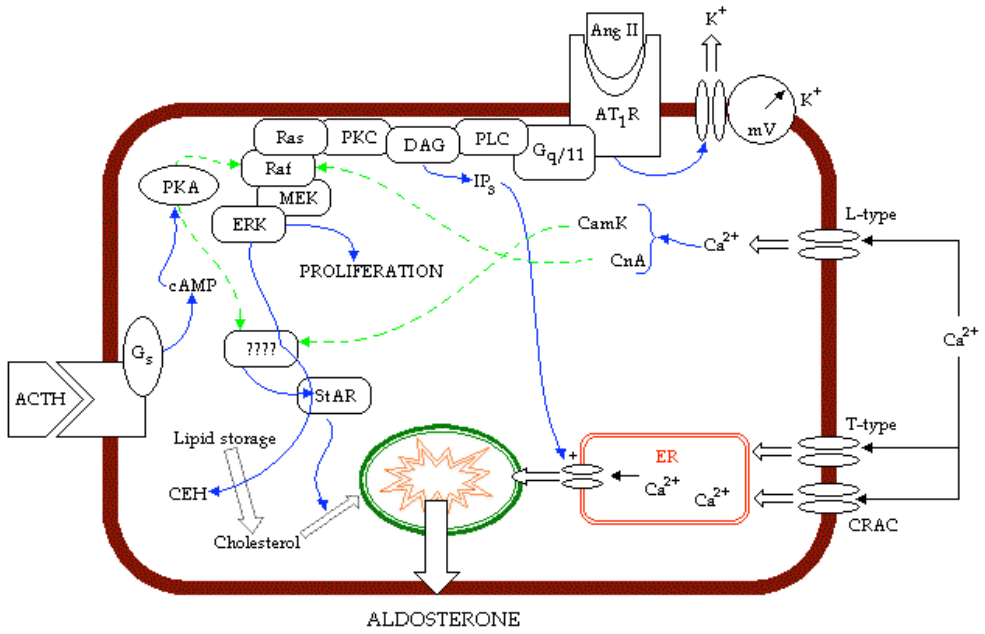

Fig 2. Different pathways in the induction leading to aldosterone formation, based on information from Foster, 2004. ERK – a mitogen-activated protein kinase, MEK – mitogen-activated protein kinases kinase. Dotted arrows indicate possible signaling pathways and regulators.

Adrenocorticotropin hormone (ACTH) also stimulates aldosterone synthesis, although with less effect compared to ang II and K^+. ACTH acts through another signaling pathway and binds a different G-protein-coupled receptor on the glomerulosa cell surface, thereby activating the formation of cAMP. cAMP in turn activates cAMP-dependent protein kinase A (PKA) (Foster, 2004; Boron and Boulpaep, 2003). PKA phosphorylates a number of proteins in the cytosol, which finally leads to Ca^{2+}-influx across the plasma membrane (Boron and Boulpaep, 2003). Forskolin, which is an adenylate cyclase activator, has been used to mimic ACTH in cellular experiments with steroidogenic induction (Romero et al., 2004).

The end result of the signal transduction pathways is an induction of aldosterone secretion through for example phosphorylation of the steroidogenic acute regulatory (StAR) protein (Foster, 2004) and above all, through increased expression of steroidogenic genes.

1.2.3 Gene regulation of genes involved in aldosterone formation

Transcription of the genes involved in aldosterone formation is regulated based on the signaling pathways activated by ang II, potassium or ACTH described above. These modulators influence the gene expression levels differently based on which cell type that is influenced. For example *CYP11B1* has a higher expression level in mainly fasciculata but also to some extent in the reticularis cells and is regulated by ACTH. *CYP11B2* on the other hand

is highly expressed in the glomerulosa cells and is induced by ang II or potassium (Lisurek and Bernhardt, 2004; Basset et al., 2004).

ACTH conducts its activation through cAMP signaling (Foster, 2004) and it has been shown that treatment with cAMP analogues preferentially enhances *CYP11B1* mRNA expression compared to *CYP11B2*. Even if it has been established that ACTH may cause an acute increase in aldosterone production it has also been recognized that it acts as an inhibitor on *CYP11B2* expression after chronic exposure (Basset et al., 2004). It is not clear how this chronic exposure inhibit *CYP11B2* expression, but it has been shown that cAMP has a negative effect on the expression of AT_1R in glomerulosa cells. In contrast to the ACTH pathway, ang II and potassium act through a calcium intracellular signal, conducted by calcium binding proteins (Basset et al., 2004). The end result of the different signaling pathways described here is the transcription of target genes, based on activation of gene specific transcription factors.

Many of the binding sites in the promoter region are common among the CYPs involved in the steroid synthesis. Ad1, a cAMP response element (CRE), in humans and Ad4 are necessary for cAMP-induced and basal transcription, respectively, in the case of *CYP11B1* (Basset et al., 2004). Ad4 is known to bind the steroidogenic factor-1 (SF-1) (Basset et al., 2004), which only seems to be present in the cells responsible for steroid synthesis (Omura and Morohashi, 1995). This factor regulates all genes except *CYP11B2*.

CYP11B2 is, in many cases, similar to the other enzymes involved in the steroid synthesis when it comes to the promoter region, with an Ad4 and a CRE site. But compared to *CYP11B1* there are some sites that seem to be specific for this gene, Ad5 and a *cis*-element named NRBRE-1. These are able to bind two members of the NGFIB family of orphan nuclear receptors, NGFIB and NURRI. It has been suggested that these proteins play an important role in the regulation of *CYP11B2*. In a test with a reporter construct NGFIB together with NURRI activate the construct whereas SF-1 on its own did not. This indicates a different regulation of *CYP11B2* compared to other genes involved in the steroid biosynthesis including *StAR*, *CYP11A1*, *CYP11B1* and *CYP17A1*, which are all positively regulated by SF-1 (Basset et al., 2004).

1.2.4 Reaction pathways from cholesterol to aldosterone

Cholesterol, which is the starting molecule for all steroid production of the adrenal cortex, is synthesised in the cytoplasm and stored in oil droplets within the cell (Omura and Morohashi, 1995). The esterfied cholesterol must be transferred from the outer to the inner mitochondrial

membrane by StAR (Lumbers, 1999). The transport of cholesterol is the main rate-limiting step in all steroid hormone biosynthesis. Cleavage of cholesterol takes place in the internal mitochondrial matrix by the cholesterol side-chain cleavage cytochrome P450 ($P450_{SCC}$ or CYP11A1) (Foster, 2004; Rainey et al., 2004; Harvey and Everett, 2003). A schematic picture of the reaction pathways in steroid biosynthesis is presented in *fig 3*.

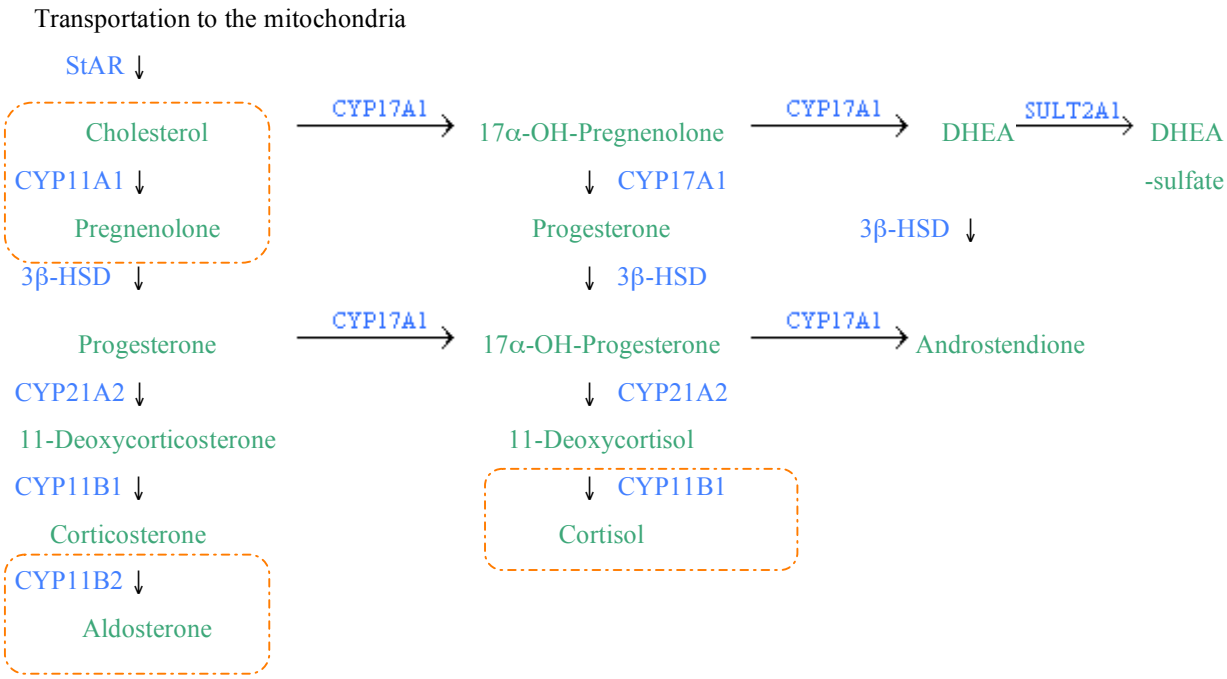

Fig 3. Steroid biosynthesis in the adrenal cortex. Squared intermediates and enzymes are located in the mitochondrial membrane, non-marked are placed in the endoplasmatic reticulum (ER). Androstendione is transported from the cortex and transformed into either testosterone or estradiol in the gonads (Omura and Morohashi, 1995; Lisurek and Bernhardt, 2004; Rainey et al. 2004; Xu et al., 2005).

The next step in aldosterone synthesis is the conversion of pregnenolone to progesterone. The enzyme responsible for progesterone formation is not a cytochrome P450 protein but 3β-HSD (3β hydroxysteroid dehydrogenase type II, HSD3B2). 21-hydroxylase also known as CYP21A2 hydroxylates progesterone to 11-desoxycorticosterone, which is transported back to the mitochondria where it is hydroxylated by CYP11B1 (11β-hydroxylases) to corticosterone. CYP11B2 (aldosterone synthase) converts the product from CYP11B1 into aldosterone (Lumbers, 1999; Rainey et al. 2004).

1.3 Aldosterone action

Aldosterone secreted by the adrenal gland has its main effect on the electrolyte transport across epithelial cells in mainly the kidney but also in salivary glands and colon. This has the effect of increasing the sodium and decreasing the potassium concentrations in the body and hence also saving the water from being excreted (White, 2003).

It has been noted that the mineralocorticoid receptor is expressed in the human heart. This indicates an effect of aldosterone directly on the heart and not only through the pressure effect that this mineralocorticoid possesses. The possible effect of aldosterone on the heart depends on which cell types that are affected and in which area of the heart (White, 2003). Since synthesis of aldosterone mainly takes place in the adrenal glands, but also in the heart, some interactions between these systems probably do exist. This means that higher levels of aldosterone production in the adrenal gland most likely affect the heart even if this is not the primary target (Hymes et al., 2004).

1.4 Toxicity connected to the adrenal gland

Aldosterone levels in the body can be raised through a number of different ways, not only by direct influence on the gene expression of involved enzymes and proteins in the steroid synthesis.

Some substances and compounds act through inhibition on the synthesized enzymes or other proteins in the steroid synthesis (Harvey and Johnson, 2002; Colby, 1981). Substances that possess this ability are represented in a number of groups, ranging from medical drugs to environmental toxins. These substances can act directly, or through their metabolite in order to inactivate the enzymes involved in steroidogenisis (Colby, 1981). For instance amino-glutethimide inhibits CYP11A1, etomidate inhibits CYP11B1 and ketoconazole inhibits CYP17A1 (Harvey and Johnson, 2002). But it is not all substances that act through enzyme interactions, some substances may probably affect the gene expression of the involved genes. Because of this it is important to determine what effect these substances actually may have on the level of gene expression.

1.4.1 Lead

Lead has been recognized as a toxic metal since ancient times when Hippocrates first described abdominal colic in miners. The toxicity of this metal has even been proposed to be the cause of the fall of the Roman Empire, since the Romans utilized lead for a number of

food connected tools. Lead toxicity is characterized with anemia, neuropathy, nephropathy, sterility and coma (Papanikolaou et al., 2005) as well as affecting the blood pressure. Toxic effects of lead also affect the fetus with increased stillbirths, neonatal deaths as well as decreased fertility rate in exposed women (Needleman, 2004).

Lead is a divalent cation and it has the ability to mimic and/or compete with calcium. The competition may lead to inhibition of calcium entry of cells and swelling and distortion of mitochondrial cristea. Most of its toxicity can be connected to its ability of binding sulfhydryl groups on proteins, thereby deforming enzymes and structural proteins (Needleman, 2004). This is clearly shown in the heme synthesis with lead inhibiting three of the important enzymes involved, which decreases the concentration of circulating hemoglobin in the body (Papanikolaou et al., 2005).

Rat experiments have shown that twelve weeks of lead exposure increases blood pressure. Adrenal cells from lead-treated rats differ from controls, showing increased aldosterone secretion. The biosynthesis and excretion of corticosterone though still remained at the same level as in non-treated rats, indicating a lead effect specific for the later steps of aldosterone formation. It was proposed that lead affects the 18-oxidation performed by CYP11B2 (Goodfriend et al., 1995).

1.5 The H295R cell line

The cell line called H295R originates from an adrenocortical carcinoma in a 48-year old black woman operated in the 1980s. The initial cell line from this carcinoma was named NCI-H295 and was shown to express all of the enzymes that participate in normal human adrenal steroidogenesis. The cells also respond to the same secondary messengers, activating the genes coding for these enzymes, in the same fashion as normal adrenal cells (Rainey et al., 2004).

There are a few substrains of the NCI-H295 cell line, derived by using different growth conditions in order to encourage substrate attachment and shorter cell cycle times. Compared to H295, which grow in suspension or in loosely attached patches, H295R and H295A grow more attached to the surface in a monolayer (Rainey et al., 2004).

1.6 Aim of project

The aim of this project was to set up a model system based on the human adrenocarcionoma cell line H295R and to optimize the experimental conditions. This system will make it possible to determine the effects various substances have on gene expression of the involved enzymes and transport proteins in aldosterone formation.

Aldosterone is produced in the glomerulosa cells of the adrenal gland. The H295R-cells were due to this be exposed to ang II and potassium acetate (KAc) in order to differentiate these cells towards glomerulosa like cells. In addition different time intervals for differentiation of the cells were tested in order to find the most appropriate one.

There may be cytotoxic effects on the cells at some concentrations of the tested substances. Therefore proliferation tests were performed to establish a non-cytotoxic concentration. It was also possible that Ultroser SF (serum replacement) supplementation in the medium had an effect on cell differentiation or gene expression response, therefore this was tested as well.

Lead has been recognized to activate aldosterone formation in the adrenal gland. This was investigated in this project by determine if there were any differences in gene expression levels of the involved genes.

In all cases gene expression levels of the key enzymes and transporter proteins involved in the mineralocorticoid synthesis was established by quantitative real time-PCR (QRT-PCR).

2. METHOD AND MATERIAL

2.1 Chemicals and cell culture

H295R cells can be commercially obtained from American Type Culture Collection (Rockville, MD, USA), in our case we have been given cells from Ingvar Brandt, Department of Physiology and Developmental biology, Uppsala university, Uppsala, Sweden. The cells were cultured in Gibco DMEM-F12 (1:1) medium (Invitrogen, Carlsbad, CA, USA) supplemented with 1% ITS PLUS culture supplement (BD Biosciences, Bedford, MA, USA), 1% 5000 U/ml penicillin and 5000 μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA) and 2% of the serum replacement Ultroser SF (USF) (Soprachem, France). Cells were grown in 37^{o}C with 5% CO_2 and was passaged every seventh day with Gibco Trypsin-EDTA (1x) (Invitrogen, Carlsbad, CA, USA). Human angiotensin II was obtained from SIGMA (Saint Louis, MO, USA), forskolin from SIGMA, potassium acetate from BDH (now Merck KGaA,

Darmstadt, Germany), lead (II) acetate from SIGMA-Aldrich (Saint Louis, MO, USA) and staurosporin from SIGMA (Saint Lousi, MO, USA).

2.2 Exposure and test of cell proliferation

2.5 ml of a medium-cell suspension with the concentration of 200 000 cells/ml were seeded in 6-well plates and 100 µl in a 96-well plate, all at 37°C in a humidified, 5% CO_2 atmosphere. Cells were grown in the incubator for three days, where after the medium was changed to medium containing the test substance Depending on exposure setup different modulators for differentiation towards glomerulosa like cells was used for different time points. The cells were tested with ang II and KAc with the most efficient inducer tested for optimal induction time. The inducer was also tested with and without USF supplementation, as well as how the cells responded to different concentrations of lead. The medium was after exposure withdrawn and stored in -20°C for future analysis and the remaining cells were harvested from the 6-well plates with lysis buffer according to manual from the NucleoSpin® RNA II Kit (Clontech, Mountain View, CA, USA). The harvested cells were stored in -70°C until the day of use.

The 96-well plate was used for proliferation/toxicity test. 20 µl of CellTiter 96® AQ_{ueous} One Solution Reagent (Promega corporation, Madison, WI, USA) was added to each well of cells at the same time as the 6-well plates were harvested after exposure. The CellTiter 96® is a MTS reduction test, wherein a tetrazolium compound is transformed to formazan by the cells. After incubation at the previous conditions for one hour each well was recorded for the absorbance at 490 nm utilizing Wallac Victor^2, 1420 Multilabel counter (PerkinElmer life and Analytical sciences, Inc., Wellesley, MA, USA). The absorbance at 490 nm is directly proportional to the number of living cells in the culture. Staurosporine at the concentration of 1µM was used as a positive control for toxicity in the 96-well plate.

2.3 RNA isolation and quantitation

Total RNA was isolated from the cells utilizing NucleoSpin® RNA II Kit (Clontech, Mountain View, CA, USA) following manufactures instructions. Basically the cells were lysed in the 6-well plates, if the RNA was not purified directly it was stored in -70°C until the day of use. 70% ethanol was added to the samples and the fluid was transferred to NucleoSpin® columns, where repeated washes and DNase I digestion took place. The RNA was eluted in nuclease-free water. Samples were stored at -70°C until use.

RiboGreen® RNA-Specific Quantitation Kit with DNase I (Invitrogen, Carlsbad, CA, USA) was used to determine the concentration of the isolated RNA samples. Manufactures instructions were followed during the procedure. Since the samples already had been treated with DNase this step was omitted and a volume of 40 µl sample were generated by mixing 1 µl RNA (diluted 10x in nuclease-free water) with 39 µl TE buffer (10 mM Tris-HCl, 1mM EDTA, ph 7,5). Each sample and RNA standard solution was later measured in duplicates. RiboGreen® reagent was added to each well and incubation took place at 37°C for 30 minutes. Measurement of the 96-well plate was performed in Wallacs Victor^2 at ~520 nm. The RNA concentrations were calculated based on the generated standard curve.

2.4 cDNA-synthesis and RT-PCR amplification

Reverse-iT™ 1st Strand Synthesis Kit (ABgene House, UK) was used according to protocol to synthesize cDNA from the purified total RNA. 1 µg RNA was mixed with random decamer primer (400 ng/µl), heated to 70°C to remove secondary structure and mixed with an solution of 5x First Strand Synthesis buffer, dNTP mix (0.2 nM final concentration), *Reverse*-iT™ RTase Blend and DTT (4 mM). Synthesis took place during 47°C-incubation for 50 minutes and the enzyme was deactivated by 75°C for 10 minutes. cDNA-samples were diluted to a final volume of 100 µl and stored at -20°C until the day of use.

ABgenes Absolute™ QPCR Mix (ABgene House, UK) was used and mixed according to manufacturers instructions. The protocol shortly has the following procedure: A mix of Absolute™ QPCR Mix, gene specific forward (0.4 µM) and reverse (0.4 µM) primers, hybridization probes (0.1 µM) and nuclease-free water is put together to a final volume of 20 µl. Template is added to this reaction mixture at a volume of 5 µl cDNA corresponding to <50ng RNA/reaction.

A Rotor-Gene™ 3000 (Corbett life science, Sidney, Australia) was used for QRT-PCR measurements. Amplification of cDNA was done by 15 minutes enzyme activation at 95°C, followed by 40 cycles of 15 seconds denaturation at 95°C and 1 minute of annealing/extension at 60°C. Primers used in the reactions were designed to anneal to the genes of the enzymes according to *table 1*. Detection of amplified products was done by gene specific Taqman probes, labeled: 5'-FAM and 3'-TAMRA. Two non-template controls were included in each run. The QRT-PCR program generated a standard curve based on five

known concentrations of the genes ranging from 10^2 – 10^6 copies, added in duplicate. This standard curve was used to calculate the copy number for each gene in the samples.

Table 1. Sequence information of human primers and probes utilized in the QRT-PCR.

Gene	Forward primer	Reverse primer	Probe
StAR	TTGCTTTATGGGCTCAAGAATG	GGAGACCCTCTGAGATTCTGCTT	CATGCGCTGGCAGTACATGTGCAC
CYP11A1	CTTCTTCGACCCGGAAAATTT	CCGGAAGTAGGTGATGTTCTTGT	CCCAACCCGATGGCTGAGCAA
CYP17A1	GCTGACTCTGGCGCACACT	CCATCCTTGAACAGGGCAAA	TCGCCAGCCTTCGATGCAGCT
HSD3B2	GCGGCTAATGGGTGGAATCTA	CCTCATTTATACTGGCAGAAAGGAAT	TGATACCTTGTACACTTGTGCGTTAAGACCCA
CYP21A2	TCCCAGCACTCAACCAACCT	CAGCTCAGAATTAAGCCTCAATCC	CTCCCTTCCTGACCCTCCGCTGC
CYP11B1	TCCCGAGGGCCTCTAGGA	GGGACAAGGTCAGCAAGATCTT	TGCTGCTTAGCCTGGCAAACCCTG
CYP11B2	TTGTTCAAGCAGCGAGTGTTG	GCATCCTCGGGACCTTCTC	TCCTCTGCTTCCTGAGCTGTCCCCT
SULT2A1	CCTCCAGCGGTGGCTACA	AATCGTCCGACATGATGATGAC	TTGAAACCCTCACACCACGCAGGA
β-actin	AGCCTCGCCTTTGCCGA	CTGGTGCCTGGGGCG	CCGCCGCCCGTCCACACCCGCC

The copy numbers of each gene for each exposure was calculated in Microsoft Excel 2003 to generate diagrams visualizing possible effects on gene expression. The levels of β-actin were gathered from each sample, if normalization of the expressed genes should be needed.

Statistical testing was performed by StatView 5.0.1 (SAS Institute, Cary, NC, USA) to determine if there were any significant effects on the cells compared to the control. Kruskal-Wallis and Mann-Whitney U tests were utilized in the statistical testing, which both are non-parametric tests.

3. RESULTS

3.1 Proliferation/toxicity tests

3.1.1 Proliferation effect on cells exposed to ang II or KAc

The result of the proliferation/toxicity assay after ang II and potassium exposure is shown in *fig 4*. Staurosporine was used as a positive control, inhibiting proliferation, the negative control in this case was water. KAc was shown to have a significant stimulatory effect on cell proliferation at the two lowest concentrations, 1 mM and 10 mM. The highest concentration of ang II (100nM) was also significantly different in cell proliferation compared to the control, with higher proliferation level. Staurosporine on the other hand had a negative impact on cell proliferation, as expected. In the case of the highest concentration of KAc there were

fewer cells in these wells compared to the wells of the other two concentrations. Even if there is no difference in proliferation, this does not rule out a cytotoxic effect on the cells.

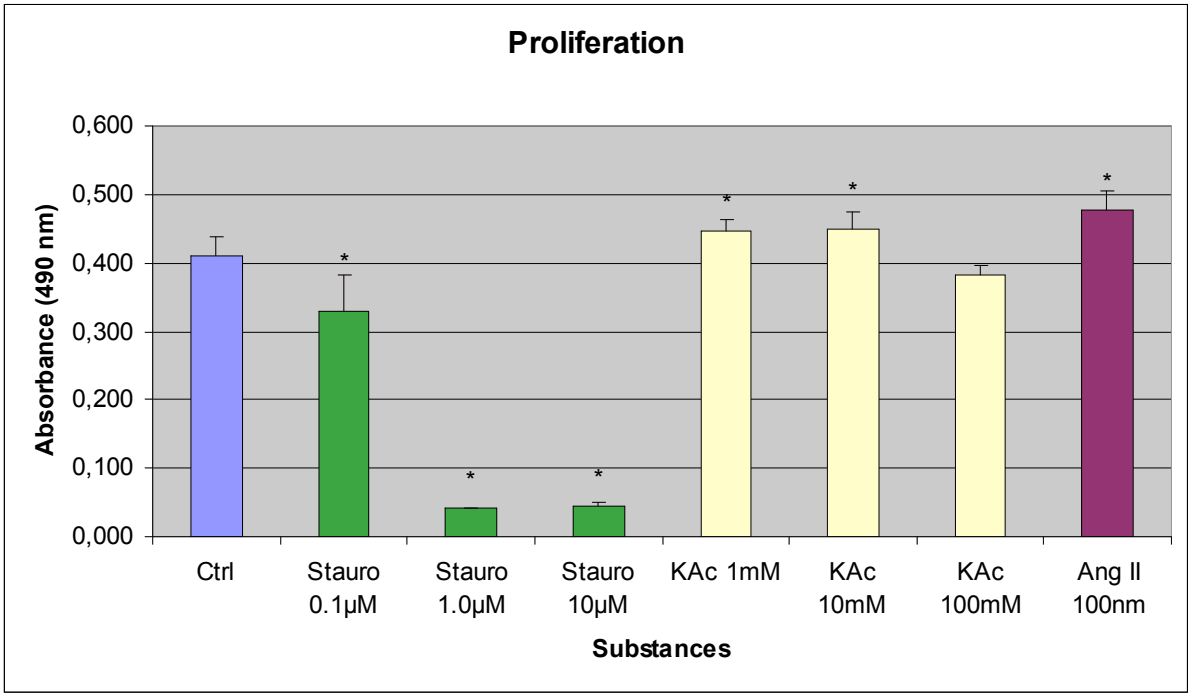

Fig 4. The result of the proliferation/toxicity assay after ang II and potassium exposure. Results expressed as mean ± SD; n = 5; $p < 0.05$ (marked with *). Statistical testing was performed with Mann Whitney U.

3.1.2 Test if USF addition has any effect on cell proliferation

This test was performed to determine if cell proliferation was influenced by USF supplementation in the medium. The result of the proliferation assay is presented in *fig 5*. When cells were exposed to ang II in USF supplemented (USF+) medium, small stimulatory effects on proliferation was noted. This was not the case in medium without USF (USF-) where the increasing concentrations of ang II had a negative effect on the cells, clearly notable as dead cells in the wells. This was not noted with the same concentrations in the exposure wells, where the cells seemed to proliferate just like the cells in USF+ medium. The decrease in proliferation in the absence of USF has however not been able to be reproduced (see discussion).

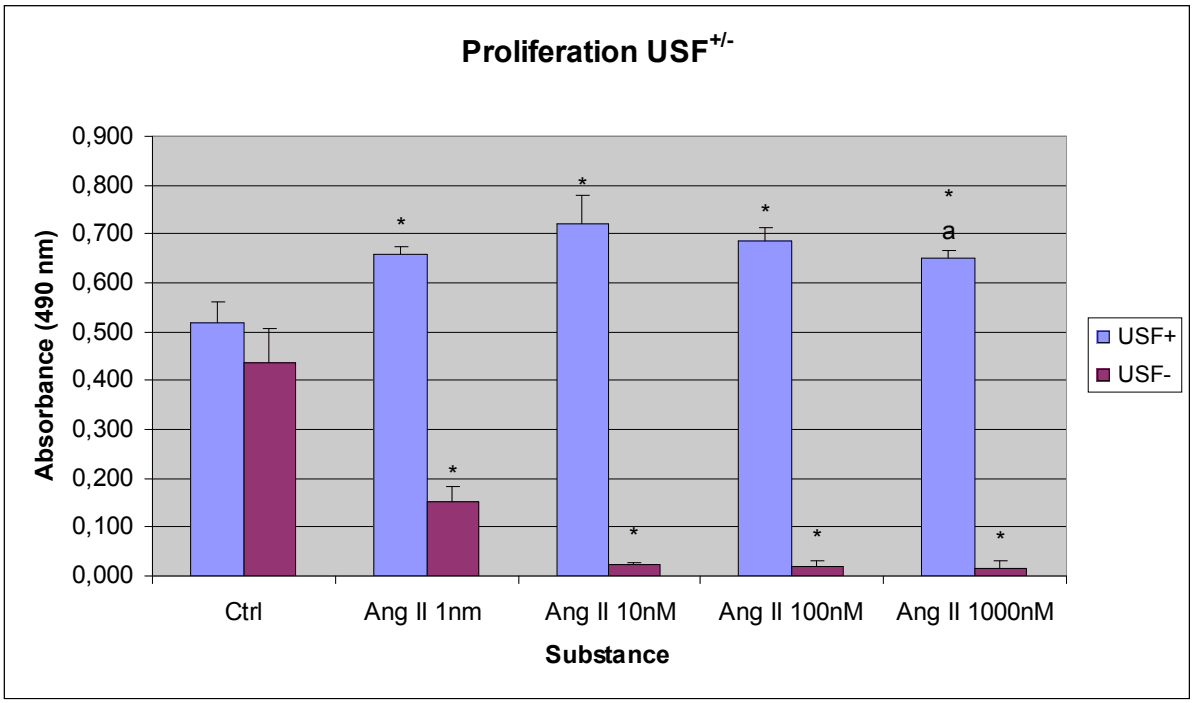

Fig 5. The proliferation/toxicity assay results with four different ang II concentrations in USF+/- medium. * indicate a p-value < 0.05 compared to the control. USF+, ang II 1000 nM is based on four absorbance values (indicated with "a") instead of five as the rest.

3.1.3 Test if lead exposure has any effect on cell proliferation in USF+ medium

In order to test the model system lead was chosen as test substance, since it has a known effect on aldosterone synthesis in the adrenal gland. The proliferation/toxicity effect of this exposure is shown in *fig 6*. Some effects on proliferation seem to be present in the lowest concentration of lead without ang II supplementation, and in the highest concentration of lead with ang II supplementation, according to statistical analysis. Both cases have a negative effect compared to the control, but these differences are not big.

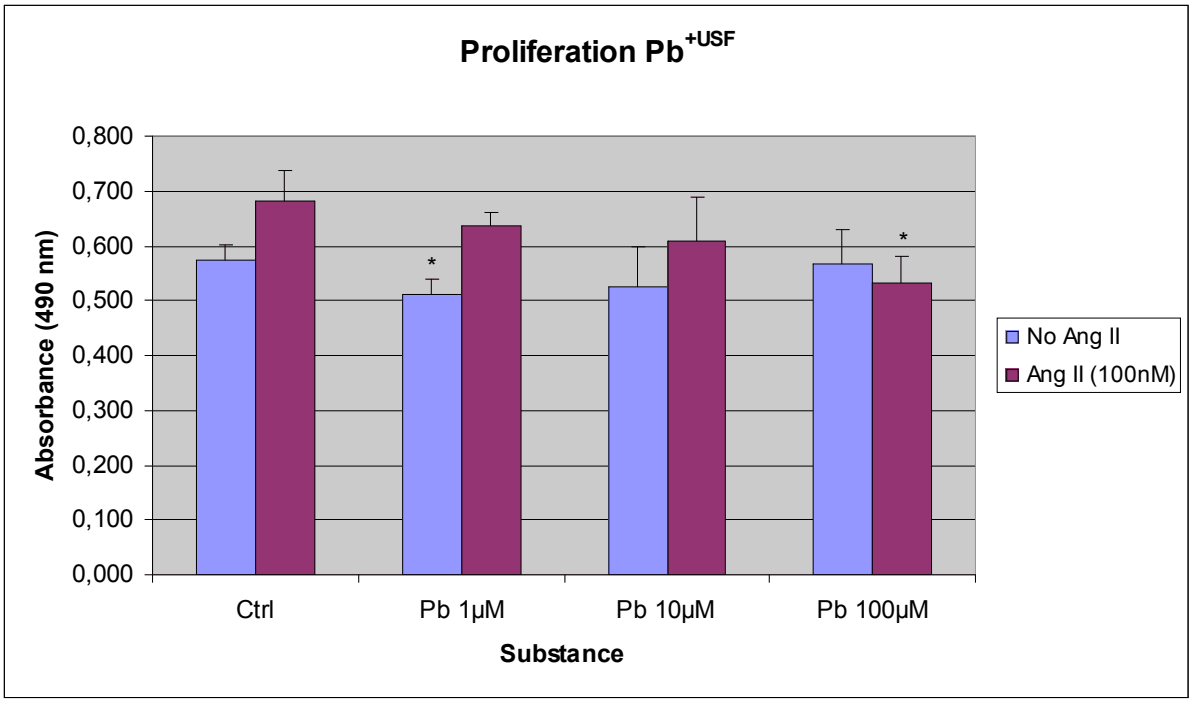

Fig 6. Proliferation/toxicity test of lead exposure to cells in USF+ medium. * indicate a p-value < 0.05 compared to the corresponding control. Each concentration and control is based on 5 measurements. Statistical analysis was performed with Mann Whitney U.

3.1.4 Effect of lead exposure to cell proliferation without USF supplementation

The proliferation/toxicity tests for lead exposure in medium without USF showed no significant toxic effects on the cells with ang II addition. Some differences in viability compared to cells in USF+ medium can be noted. In this case there is a higher level of viability for the cells treated without ang II compared to the same setup in USF+ medium. Since the previous proliferation assay for this particular comparison (*fig 5*) may be misleading it is hard to tell if the difference noted here is consistent or not.

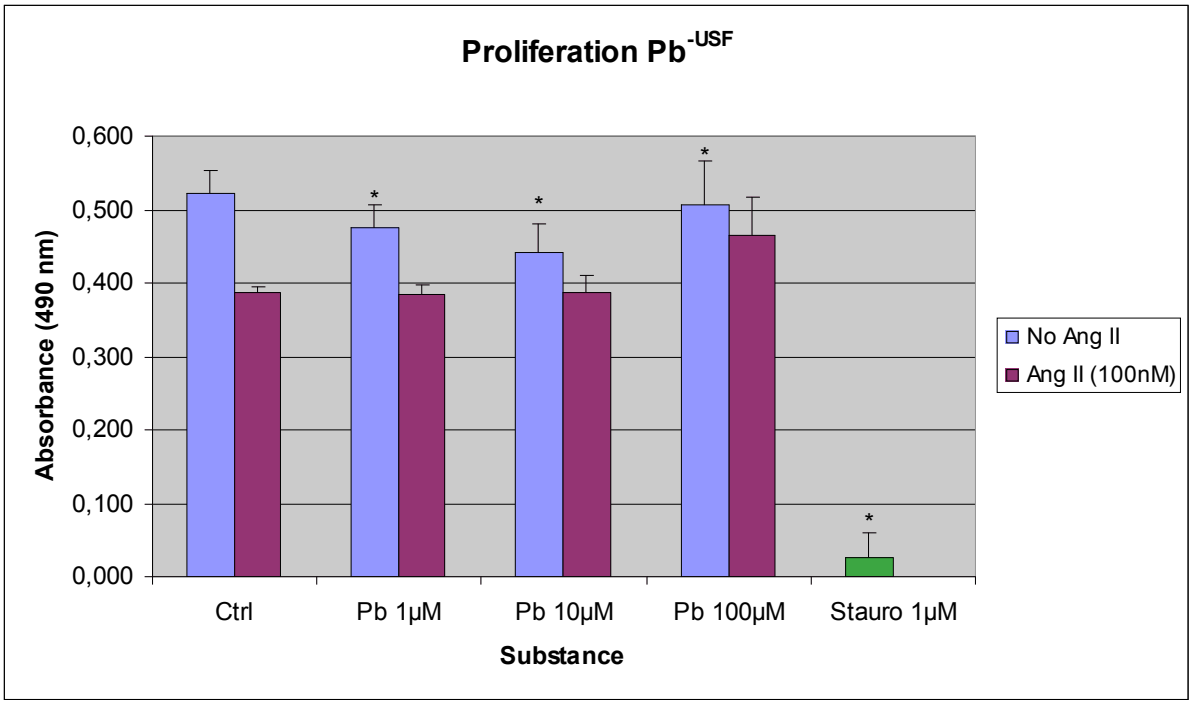

Fig 7. Proliferation/toxicity test of lead exposure in medium without USF. * indicate a p-value < 0.05 compared to the corresponding control and each test has been repeated five times. Analysis was performed with Mann Whitney U.

3.2 Quantitative RT-PCR analysis

3.2.1 Determination of optimal concentrations of modulator for gene induction

Ang II induction was most notable detectable by the level of *CYP11B2* expression. High expression levels of this gene were also noted with KAc treatment. This is expected since ang II and potassium is recognized as the inducers of aldosterone formation and hence activation of this specific gene. The expression levels for all genes declined at the highest concentration of KAc through out the experiment. This corresponded with the results from the proliferation test, indicating a small decrease in proliferation compared to the other concentrations of KAc. Ang II on the other hand showed increasing gene induction following higher concentrations for many of the involved genes, see *fig 8.*

Forskolin was used as a control and showed expected effects, with high induction of *CYP17A1* and *CYP11B1*, two important enzymes involved in cortisol formation.

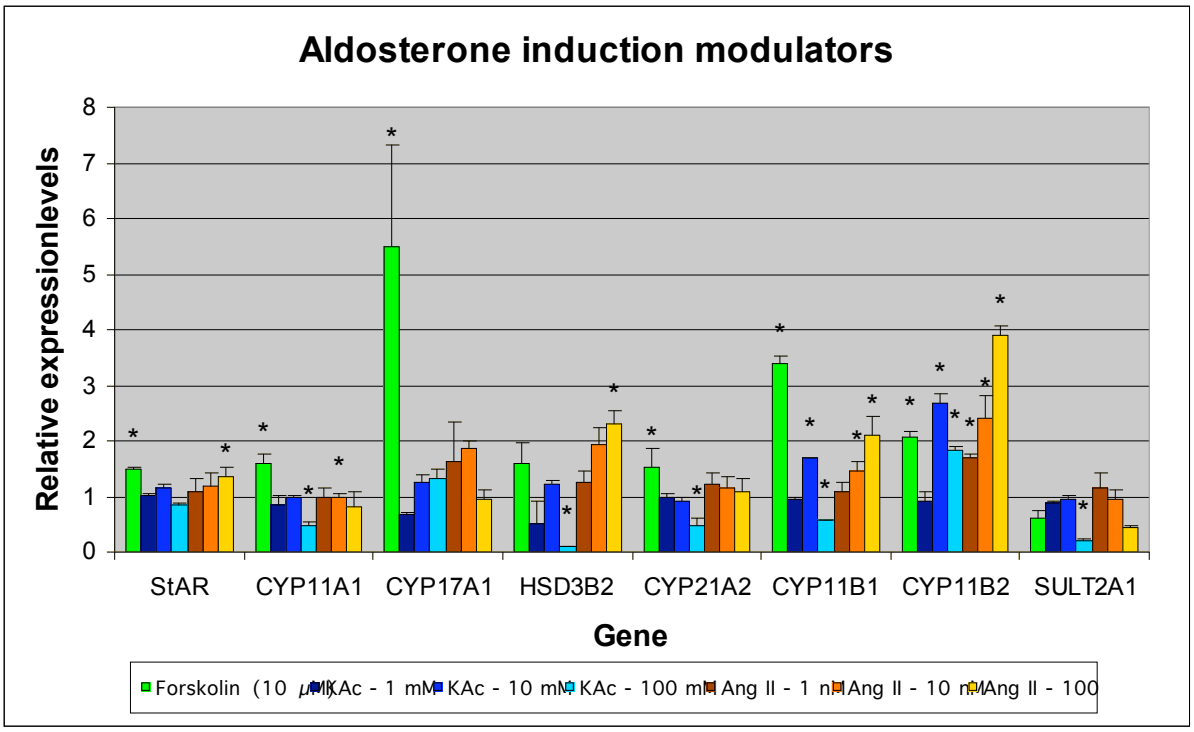

Fig 8. QRT-PCR result for the induction test with KAc and ang II in USF+ medium. The relative gene expression levels for forskolin, KAc and ang II induction expressed as mean ± SD; n=3; $p < 0.05$ (in comparison with the vehicle, indicated with *). The vehicle control is equal to one but not shown in the figure.

Since induction with ang II 100 nM did not show any toxic effects and gave the highest level of gene expression for genes like *CYP11B2* and *CYP11B1* this concentration was chosen for the following experiments.

3.2.2 Test of induction time with 100 nM of ang II

The cells were exposed to 100 nM ang II for 8 h, 24 h, 48 h and 72 h before harvest. This was done in order to determine if there was a time point more suitable for ang II induction. The results from the QRT-PCR on this experiment are shown in *fig 9*. No preferred time for ang II induction is indicated after this experiment. The only genes that were affected due to differences in time with ang II induction according to Kruskal-Wallis analysis were *HSD3B2* and *SULT2A1*. Differences between the time points for *HSD3B2* expression were only significant between 24 h and 48 h and between 48 h and 72 h for the ang II treatment. There were also a significant difference in gene expression of *HSD3B2* for the control between 8 h and 24 h. In the case of *SULT2A1* there were significant differences in gene expression levels between all time points, 8-24 h, 24-48 h and 48-72 h. There were also significant differences in gene expression levels for this gene between 24-48 h of the control. Kruskal Wallis

analysis was also performed for the controls and *CYP17A1*, *CYP21A2*, *CYP11B2* and *SULT2A1* were considered significant in this case.

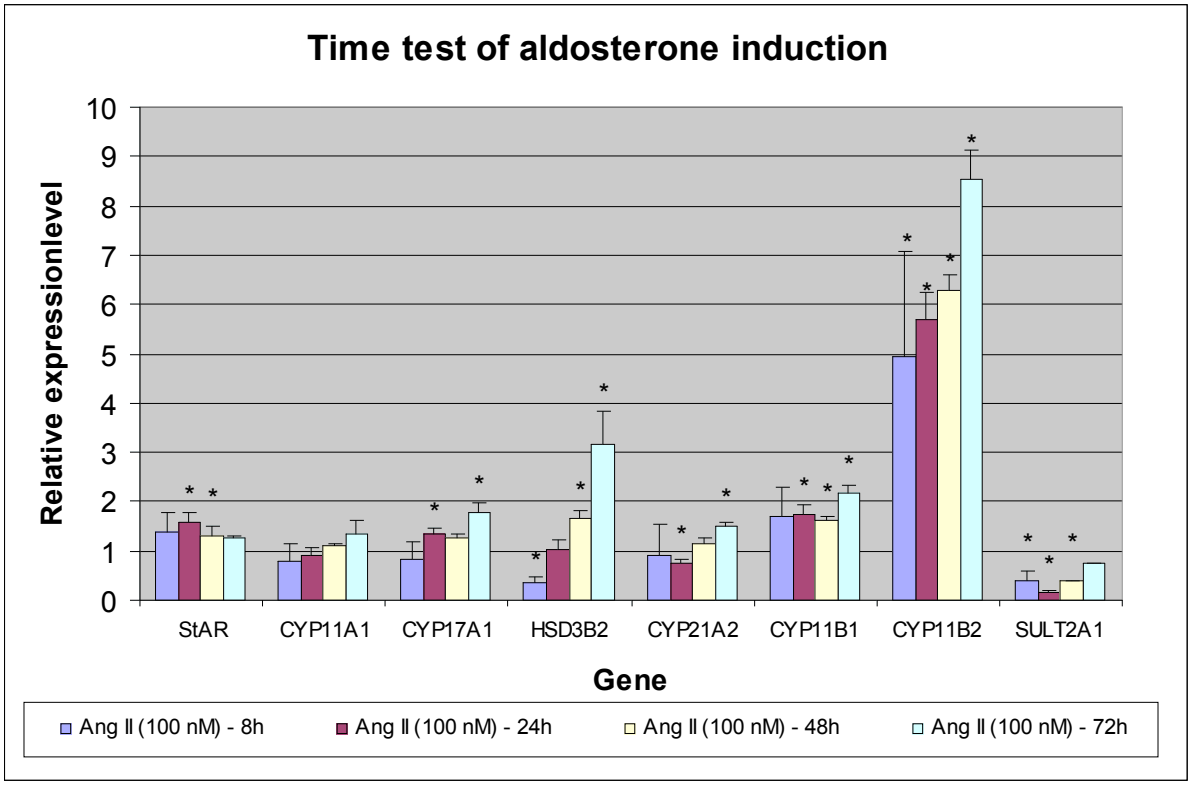

Fig 9. The QRT-PCR results for the time-induction test in USF+ medium shown in relative expression levels ± SD compared to the H_2O control (equal to one, but not present). The results are based on triplicates and the asterisks indicate $p < 0.05$ in comparison with the control, based on Mann Whittney U-analysis. No gene expression differences between the time points were noted with ang II induction based on Kruskal Wallis analysis, except for the expression levels of *HSD3B2* and *SULT2A1*.

3.2.4 Effect on gene expression based on USF supplementation in medium

The cells were exposed to ang II at the concentrations of 100 and 1000 nM with and without USF in the medium. This was done to determine if serum supplementation has an effect on gene expression levels. It was noted that there was a significant difference in gene expression due to USF supplementation, for all genes except *CYP11A1* for the control. In the comparison between the ang II samples in USF+ and USF- medium it was established that a significant difference in gene expression were present for all genes except *CYP11A1* in the 100 nM concentration and *CYP11A1* and *SULT2A1* in the 1000 nM concentration. *HSD3B2* was the gene that differed most in relative expression levels between the USF+ and USF- mediums, which is clearly visible in *fig 10*.

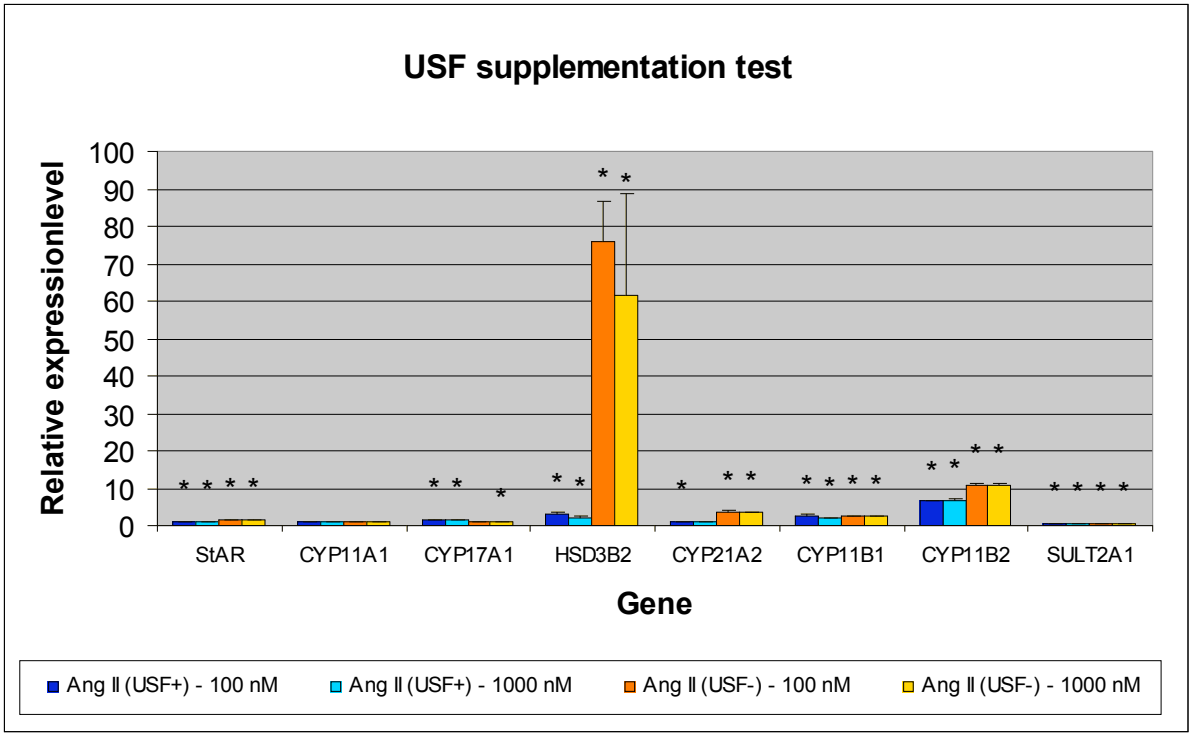

Fig 10. This is all of the data from the USF supplementation test based on triplicate values. The relative expression level for *HSD3B2* in USF- medium is extremely high. * indicates $p < 0.05$ (in comparison to the related control). There is a corresponding control without ang II for the USF+ as well as for the USF- exposure, both are set to 1.0 in this figure (although not present).

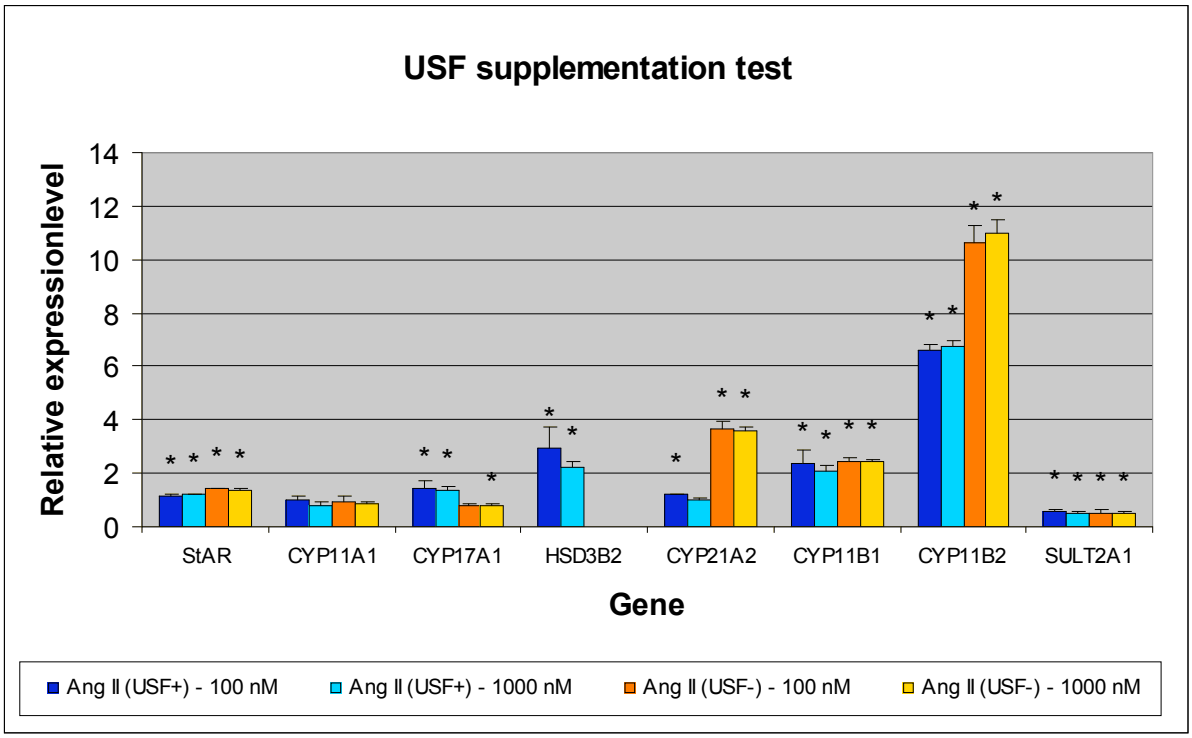

Fig 11. The same data as in *fig 10* but the values for *HSD3B2* in medium without USF has been excluded in order to visualize the expression levels of the other genes. * indicates $p < 0.05$ (compared

to the related control). There is a corresponding H_2O-control for the USF+ as well as for the USF- exposure, both set to 1.0 in this figure (although not present).

3.2.4 Test of lead exposure to cells grown in USF+ medium

Shown in *fig 12* are the results from the QRT-PCRs on the lead exposure in USF+ medium. Lead is known to increase aldosterone formation and we were hoping to find gene regulation effects based on this. The final conclusion is that no lead specific effects can be detected on the gene expression level except for a few genes, which have been marked in the figure. These effects may on the other hand be incidental due to a high number of comparisons.

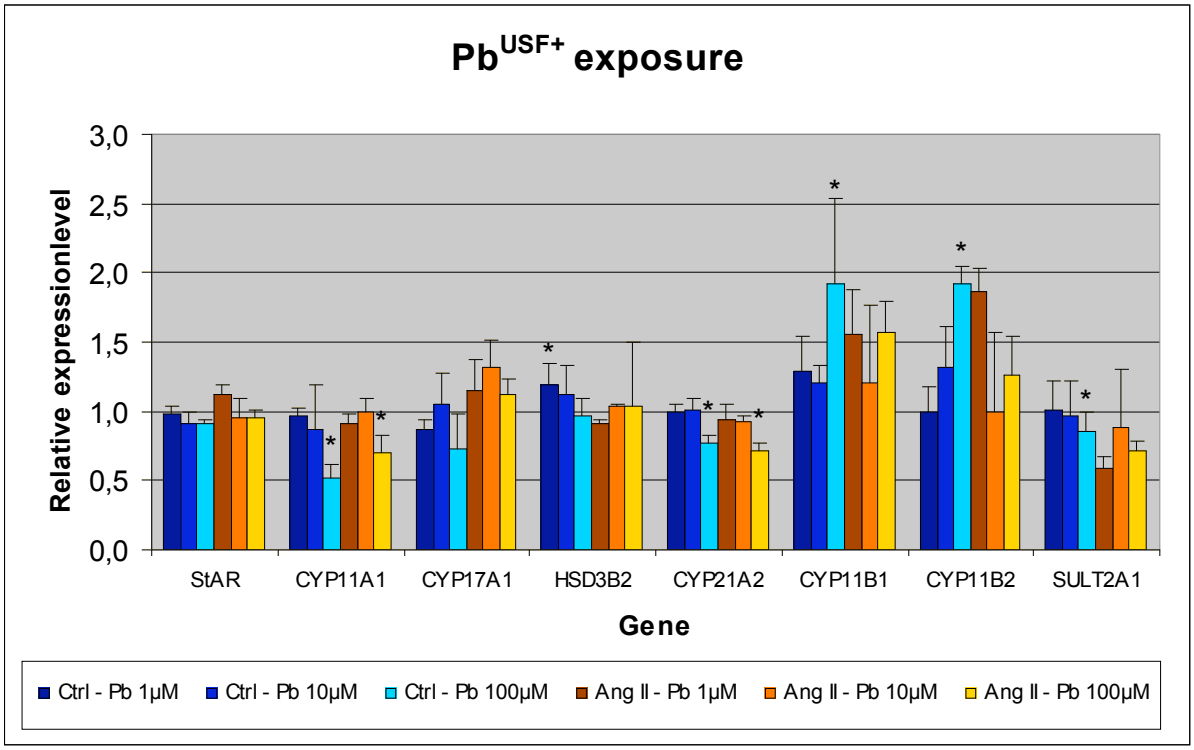

Fig 12. Relative gene expression levels for lead treatment in USF+ medium. Results are expressed as mean ± SD; n = 3; $p < 0.05$ (in comparison with the control, indicated with *). The H_2O-control as well as the ang II-control in this case are equal to 1.0 and have been excluded from the figure. Mann Whitney U was utilized for statistical analysis.

3.2.5 Test of lead exposure to cells grown without USF supplementation

The same setup was used in this test as the previous one with lead, except for no addition of USF to the medium. The outcome of this exposure did not turn out much different from the previous one except for some indications of lead effects in cells treated without ang II. The QRT-PCR results indicate that *StAR*, *CYP11A1* and *CYP21A2* are down regulated in a concentration dependent manner and that *CYP11B2* in fact is up regulated. These effects are

not present in the ang II-induced cells. Genes that are differently expressed in comparison to the corresponding control has been marked with an asterisk in *fig 13*.

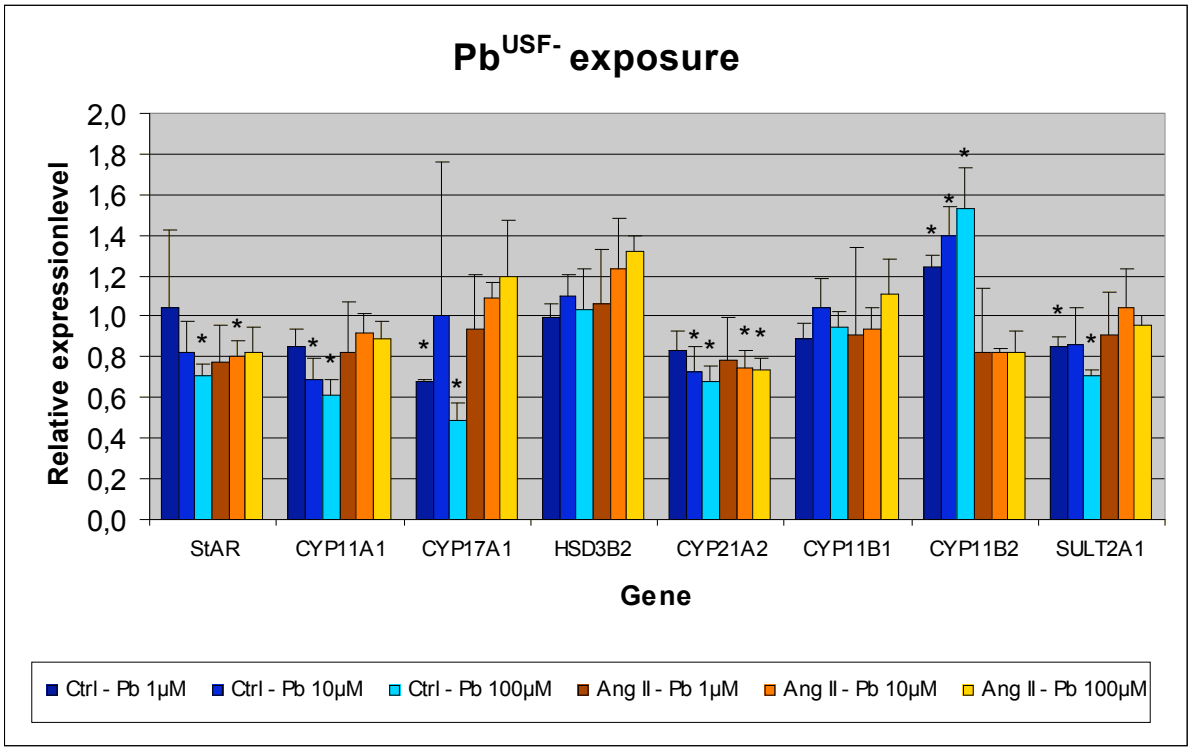

Fig 13. Lead induction without USF supplementation in relative gene expression levels. Results are expressed as mean ± SD; n = 3; $p < 0.05$ (in comparison with the control, indicated with *). There were one control with H_2O and one control with ang II, these have been left out in this figure but are both equal to 1.0. Statistical analysis was performed by Mann Whitney U.

4. Discussion

The purpose of this project was to utilize the H295R-adrenocarcinoma cell line in order to generate a system for determining the effects various substances have on the aldosterone production and gene expression of the involved enzymes and transport protein.

The cells were supposed to be differentiated into glomerulosa like cells using angiotensin II or potassium. The up- or down-regulation effects on the gene expression were determined utilizing quantitative real time PCR (QRT-PCR). Aldosterone secretion will later be determined using radioimmunoassay and therefore medium from the exposures has been stored for future analysis.

4.1 Modulators of differentiation towards aldosterone producing cells

Induction with angiotensin II (ang II) and potassium is considered to be a way of differentiating H295R cells into glomerulosa like cells. It is expected to find genes that are involved in the aldosterone synthesis are up-regulated to a higher extent than genes associated with for instance cortisol production, which is usually activated in the fasciculata cells. The first test that was performed was done in order to determine if ang II or potassium is the best inducer in this case. As can be seen in *fig 4* there seem to be toxic effects on the cells at the concentration of 100 mM KAc, since the proliferation decreases in this case. The cells were notably fewer at this concentration prior to harvest and as can be seen in the diagram from the QRT-PCRs, the gene expression for the involved genes are also lower. Since the same amount of RNA was used for each sample in the cDNA synthesis the explanation for the lower expression levels of these genes cannot be a smaller amount of total RNA.

Forskolin is more associated with cortisol induction, since it mimics ACTH activation, and should in this case provide a lower expression level for *CYP11B2*-mRNA than ang II. As can be seen in *fig 8* there is a twice as high induction of *CYP11B2* expression with ang II compared to forskolin, indicating a differentiation difference between the two modulators. Another proof of the difference in gene induction between forskolin and ang II is the raised levels of *CYP11B1* and *CYP17A1* expressions (clearly notable in *fig 8*) with forskolin induction, these genes are utilized in cortisol formation. 100 nM ang II was chosen for differentiation of the H295R-cells into glomerulosa-like cells and for induction of aldosterone production. Ang II was chosen over KAc due to higher levels of gene expression and the fact that high concentrations of KAc seemed to be cytotoxic.

4.2 Determining angiotensin II induction times

By inducing the cells for different time intervals it was possible to determine an appropriate time for ang II induction before exposure experiments took place. As can be seen in *fig 9*, there do not seem to be any differences in gene expression if the ang II induction continues for 8 hours or 72. This was confirmed with Kruskal Wallis analysis, where it was indicated that time had less effect on the gene expression with ang II compared to without. *HSD3B2* and *SULT2A1* where the only genes that were significantly different in gene expression over time due to ang II-induction. Except for these genes there seem to be no preferred time in this case, therefore ang II induction was set to 24 hours due to convenience.

4.3 Ultroser SF supplementation or not during exposure

Ultroser SF (USF) is a substitute for human serum. In serum there are a number of known as well as unknown substances. Most cells are considered to need serum addition for efficient growth. In this case we wanted to determine if a serum addition made any difference to the ang II differentiation towards glomerulosa like cells or not. The comparisons between USF supplemented cells and non-supplemented are shown in *fig 5*, *10* and *11*. As can be seen in *fig 5* there seem to be significant toxic effects of ang II addition in the concentrations of 10 and 100 nM in USF- medium. This phenomenon is not reproducible and could not be noted in the 6-well plates exposed at the same time. The total yield of RNA from the 6 wells plates without USF was generally the same as for the control and those with an addition of USF.

As can be seen in the diagrams from the QRT-PCRs (*fig 10* and *11*) there indeed is a difference between ang II induction in USF+ (lower gene expression levels) and USF- medium (higher gene expression levels for some genes). These USF-coupled differences are noted in proliferation as well as in gene expression levels and have been shown to be significant. The explanation for this is not clear and these differences will require more investigation.

The basal level of *HSD3B2* and *CYP21A2* are notably lower in the non-USF-supplemented cells, therefore the fold induction of these genes after ang II induction seem to be higher compared to the levels in the USF supplemented cells (data not presented). The expression of *SULT2A1* is extremely high in the cells grown without USF. Together with the results from *CYP21A2* and *HSD3B2* expression it is interesting to note that the cells grown in USF- seem to mimic the adrenal steroid synthesis in fetuses. Rehman et al. (2003) showed that the transcription level of *HSD3B2* was 127-fold higher in the adult adrenal compared to the fetal, with the levels in the fetal barely detectable. The transcription levels of *SULT2A1* were on the other hand 13-fold higher in the fetal adrenal compared to the adult. The reason for this is that the adrenal cells in the fetus have another role than they do in the adult, with hormone synthesis focusing on androgens during development and gluco- and mineralocorticoids in the adult (Rehman et al., 2003), which explains the different expression pattern. The results noted here do not indicate that serum depletion differentiated the H295R into fetal-like cells, to draw that assumption we need to do more investigations.

Statistical analysis indicates a significant difference in gene expression levels for a number of genes. *StAR*, *HSD3B2*, *CYP11B1*, *CYP11B2* and *SULT2A1* for all concentrations of ang II with and without USF supplementation are all significantly different from the corresponding

control. *StAR*, *HSD3B2*, *CYP11B1*, *CYP11B2* in these cases have a higher level of gene expression and *SULT2A1* has a lower expression level.

Even *CYP17A1* and *CYP21A2* are significantly different in gene expression levels due to USF supplementation, except for one concentration of ang II in each case. The levels for *CYP17A1* are lower for one ang II concentration in USF- and higher for both concentrations in USF+. *CYP21A2* has a significantly higher level of gene expression in USF-, but have a level close to the control in USF+ except for one ang II concentration that is significantly higher.

The only gene that does not seem to be affected by USF supplementation is *CYP11A1*, which also do not differ from the expression levels of the control. It would be interesting to see if the increased gene expression levels of *CYP21A2* and *CYP11B2* in USF- medium actually has had an effect on aldosterone secretion compared to cells treated with USF+ medium.

4.4 Lead exposure

The proliferation/toxicity tests did not indicate any lead toxicity that had to be taken into consideration, neither in USF+ nor in USF- medium (*fig 6* and *7*). The concentrations of lead determined to significantly have an effect on proliferation have not been excluded since the differences in proliferation is very small, even if they are statistically significant. 78% viability compared to the control was the lowest value calculated for the lead exposure test.

Lead exposure in USF+ medium did not provide any specific effects on gene expression. As can be seen in *fig 12* there seem to be some genes that are statistically significant, but these can also be incidental results due to a high number of comparisons. Practically all genes are expressed at the same level as its corresponding control in the exposure with USF+. The only genes that differ from this are *CYP11B1* and *CYP11B2*, which have higher expression levels. If this raised level of expression is due to the lead treatment or the effect of ang II is hard to tell.

In the case of lead exposure with USF- medium we have different patterns compared to the USF+. There do not seem to be any effects on gene expression when ang II treated cells are exposed to lead (*fig 13*), but this is not the case for the cells exposed only to lead. *StAR*, *CYP11A1* and *CYP21A2* seem to be down regulated in a concentration dependent fashion, *CYP11B2* on the other hand seem to be up regulated.

Lead exposure has been reported to induce aldosterone formation by a number of researchers, for example by Goodfriend et al. This also seems to be the case for H295R-cells grown in medium without USF and without ang II induction, based on gene expression levels. It has not been confirmed but the down regulation of the mentioned genes might be compensated for by the up regulation of *CYP11B2*. If this is the case aldosterone formation should not divert too much from the control in a test where the concentration of aldosterone secretion can determined. Aldosterone concentration determination has not been performed in this project. We will need to perform more lead exposures in order to determine if there is any biological significance in the differences noted here.

5. CONCLUSIONS

In this project the human H295R cell line was utilized as a model system for detection of effects on gene expression on steroid synthesis in the adrenal gland. This method has been optimized for angiotensin II induction in order to differentiate the cells towards glomerulosa like cells. The setup has been tested with lead, which is a known inducer of aldosterone formation.

The model will need more testing, in order to clarify that it fulfills the criteria for a workable model system in determination of steroidogenic gene expression effects. The different effects on steroid gene induction due to serum supplementation or not, will need some further investigation.

This model will need to be complimented by an assay that measures the concentration of secreted steroid products. This has to be done in order to establish if changes in gene expression can be correlated to changes in hormone secretion or not.

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