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Effect of Thyroid Hormones on AT1R MRNA Levels in HEK 293 Cells: Implications for Early-Onset Hypertension in Children Born of Preeclamptic Women

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Effect of Thyroid Hormones on AT1R mRNA Levels in HEK 293 cells: Implications for Early-Onset Hypertension in Children Born of Preeclamptic Women

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Effect of Thyroid Hormones on AT1R mRNA Levels in HEK293 cells: Implications for Early-Onset Hypertension on Children Born of Preeclamptic Women

Mandy Eddins

Abstract:

This experiment tested the effect of thyroid hormones, T_3 or T_4, on angiotensin II type 1 receptor mRNA levels in human embryonic kidney cells (HEK). Maternal preeclampsia is a prediction of higher ambulatory blood pressure (ABP) in the exposed offspring. The mother's blood pressure affects the blood pressure of the fetus since the placenta mediates blood flow. Thyroid hormone can increase production of angiotensinogen, which is a part of the renin-angiotensin system (RAS). RAS regulates blood pressure. Hypothesis: HEK 293 cells treated with T_3 and T_4 will have more angiotensin II type I receptors than those not treated with T_3 and T_4. Those HEK 293 cells treated with thyroid hormones along with angiotensin I will have even more angiotensin II type I receptors than those just treated with thyroid hormones. The HEK293 cells were treated with T_3+Ang I, T_3, Ang I, T_4+Ang I, T_4, and PBS (control). After the treated cells were collected of all three trials RNA was isolated and quantified. After RNA quantification was performed using a spectrophotometer, RT-PCR and a gel electrophoresis were completed. A one-way ANOVA and LSD Post-HOC test were run using SPSS software for statistical analysis. Angiotensin I led to an increase in mRNA levels of AT1R, which causes vasoconstriction and therefore higher blood pressure. This increase in AT1R mRNA levels is attenuated when T_3 is added.

Background:

Preeclampsia is the development of high blood pressure and proteinuria in pregnant women after 20 weeks gestation (Cnossen 2006). Preeclampsia occurs in 5% to 7% of all pregnancies (Tenhola 2003). It's also the leading cause of premature birth worldwide. Some risks associated with preeclampsia are maternal diabetes, persistent high blood pressure, kidney disease, stroke, autoimmune disorders, fetal death, fetal growth retardation, small for gestational age (SGA) size, and thrombophilias (Cnossen 2006, Walker 2000). The possible causes of preeclampsia include: poor diet, high body fat percentage and insufficient blood flow to the uterus (Cnossen 2006). Atypical implantation of fertilized egg and excessive placental tissue are also possible causes of preeclampsia (Cnossen 2006). The only way to treat preeclampsia is to remove the placenta, which cannot be done until delivery of the fetus. Prematurity often occurs with preeclampsia because the doctor may decide delivering the baby early is best for the mother. These are several possible causes for preeclampsia although the exact etiology remains unknown. An important point of interest is that mothers and fetuses affected by preeclampsia are more likely to have cardiovascular disease later in life.

Maternal preeclampsia is a prediction of higher ambulatory blood pressure (ABP) in the exposed offspring. The mother's blood pressure affects the blood pressure of the fetus since the placenta mediates blood flow. The amount of blood the fetus gets depends on the amount of blood that enters the placenta. Blood pressure is the force per area that the blood exerts on the walls of the blood vessels. Systolic blood pressure is pressure on

the arteries when the ventricles of the heart are contracting. Diastolic blood pressure is the measure of the force exerted on the blood vessels when the heart is at rest. As early as the age of six, maternal preeclamptic children had higher diastolic blood pressures than control subjects born to normotensive mothers (Palti 1989). Twelve-year-old children born to preeclamptic mothers (PRE) have significantly increased 24-hour systolic and diastolic ambulatory blood pressures than children of non-preeclamptic mothers (non-PRE) (Tenhola 2006). PRE children had significantly higher systolic and diastolic blood pressure values than non-PRE children, even after adjusting the values by current weight and height (Tenhola 2003). The child's gender is also a factor is elevated ABP in PRE children. PRE males at age 12 were shown to have a higher 24 hour systolic ABP than PRE females of the same age (Tenhola 2006). In addition, 17-year-old females with maternal preeclampsia had higher blood pressures than their coed controls (Seidman 1991). Menstruation also occurred at an earlier age in the preeclamptic group than in the normotensive group (Vatten 2003). Systolic blood pressure was significantly higher in the preeclamptic group than in the non-preeclamptic group (Vatten 2003). There was no significant difference in diastolic blood pressure between the two groups (Vatten 2003). There is a reduced risk of breast cancer in female offspring of preeclamptic pregnancies (Vatten 2003).

The body's nervous system and hormones determine its blood pressure. The autonomic nervous system regulates the rate of heart contractions. The vagus nerve controls the parasympathetic nervous system that lowers the heart rate. Both the cervical and upper ganglia regulate the sympathetic nervous system, which increases the heart rate. The adrenal medulla exerts hormonal control via epinephrine secretion to raise the

heart rate. PRE children have slightly higher mean plasma epinephrine concentrations than in non-PRE children (Tenhola 2003). SGA PRE children had the highest mean cortisol concentration, total cholesterol, LDL cholesterol, triglycerides, and insulin levels than AGA (average gestational age) PRE children and non-PRE children (Tenhola 2003). High systolic 24-hour ABP is associated with high plasma epinephrine concentrations and being born SGA in the PRE children (Tenhola 2006).

Thyroid Hormones' Relation to Preeclampsia

Thyroid Stimulating Hormone (TSH) is synthesized in the anterior pituitary gland. TSH is a tropic hormone, meaning it stimulates other endocrine glands to release hormones. Specifically, TSH stimulates the thyroid gland to absorb iodine and then synthesize and release thyroid hormones including thyroxine and triiodothyronine.

Thyroxine (T_4) and Triiodothyronine (T_3) are two thyroid hormones that are required for growth and neurological development in children. T_3 is the active form of thyroid hormone and T_4 is the form stored in the thyroid gland for use if T_3 levels are exhausted. However the T_4 must be converted to T_3 before becoming active. T_3 and T_4 increase the rate of cellular respiration, protein and fatty acid synthesis, and degradation in many tissues. Both T_3 and T_4 have vasodilatory actions and T_3 may maintain vascular tone (Gumieniak 2005). Variations in thyroid hormone concentrations define vasodilation and hypertension (Gumieniak 2005). TSH is positively correlated with systolic and diastolic blood pressure levels (Fommei 2002). High levels of T_4 and T_3 inhibit TRH and TSH, which causes plasma levels to return to normal (Figure 1).

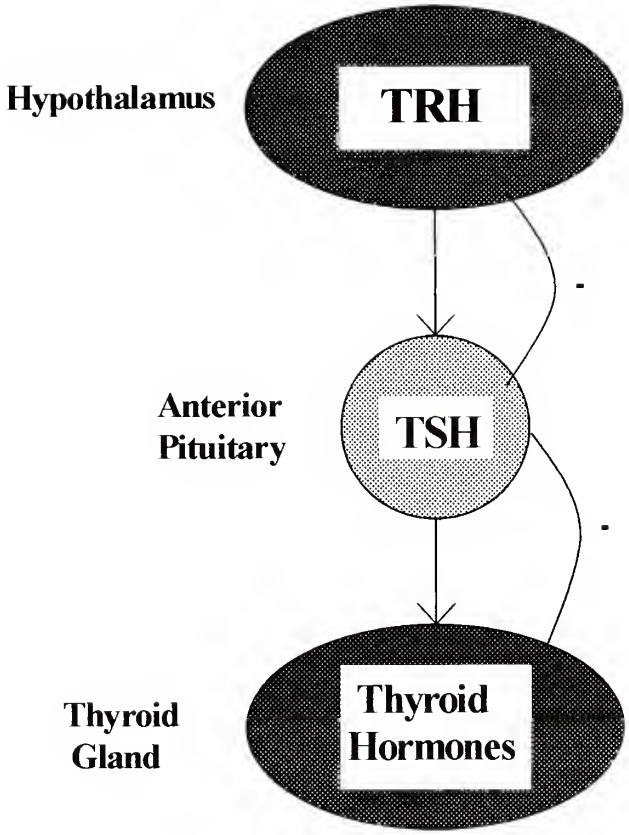

Figure 1.Thyroid Hormone Interactions derived from "Control of Thyroid Hormone Synthesis and Secretion" (Google Images 2007).

Thyroid Stimulating Hormone (TSH) levels are elevated in preeclamptic women compared to normotensive women (Kumar 2005, Basbug 1999). Maternal thyroid hormones are able to cross the placenta (Thyroid Australia LTD. 2001). Fetuses rely on their mother's hormones for the first 12 weeks of gestation because they are unable to produce their own (Thyroid Australia LTD. 2001). The thyroid hormones are needed for neurological development (Thyroid Australia LTD. 2001). Even after the fetus' thyroid gland begins functioning, the mother's thyroid hormones still cross the placenta (Thyroid Australia LTD. 2001). Birth weights of babies born to preeclamptic mothers were negatively correlated with TSH levels (Basbug 1999). TSH is a strong association factor

for the development of preeclampsia (Kumar 2005). The association between thyroid hormone levels and hypertension will be explained in the following section.

Hypothyroidism is linked with elevated systemic vascular resistance and hypertension (Gumieniak 2005). Hypothalamic-pituitary-thyroid pathway genes may be involved in blood pressure regulation (Gumieniak 2005). Spontaneously hypertensive rats (SHR) have high-normal TSH levels compared with normal rats. A significant proportion of hypertensives have high-normal TSH concentrations. Normotensive subjects who had a family history of hypertension had considerably higher serum TSH levels than those subjects who did not have a hypertensive family history; even after age, gender, race and free T_4 index were adjusted (Gumieniak 2005). Other metabolic systems also have an effect on blood pressure.

Thyroid hormone can increase production of angiotensinogen, which is a part of the renin-angiotensin system (RAS) (Saladin 2004). RAS regulates long-term blood pressure. RAS is activated when blood volume decreases or when there is a drop in blood pressure (Saladin 2004). Renin is an enzyme released from juxtaglomerular cells in the kidneys (Saladin 2004). Angiotensinogen is a peptide that is cleaved by renin to convert it to angiotensin I. Angiotensin converting enzyme (ACE) converts angiotensin I to angiotensin II. Angiotensin II causes blood pressure to rise by constricting blood vessels (Figure 2) (Saladin 2004). Renin levels are high in fetuses and angiotensin II levels are relatively low (Schutz 1996). From this information we know that increased blood pressure is positively correlated with the increase in thyroid hormone production, and that fetuses have a decreased angiotensin II production.

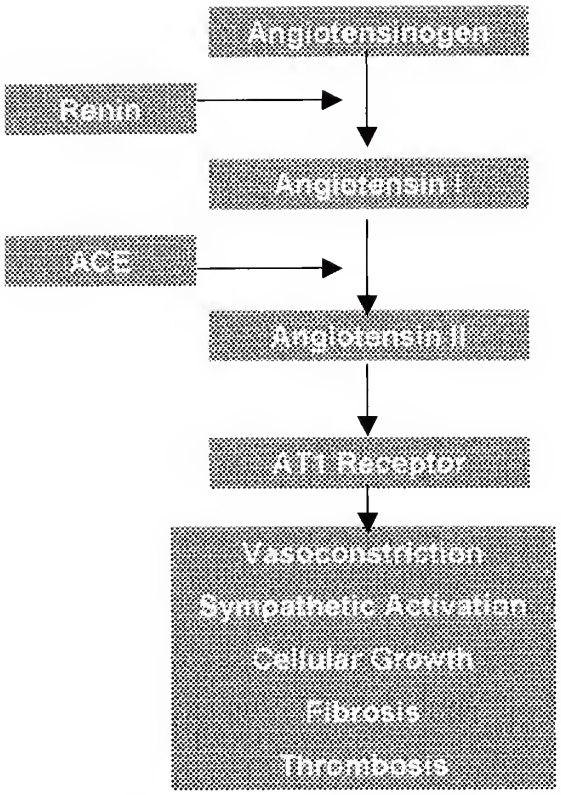

Figure 2. Derived from Renin Angiotensin-Aldosterone System (Chiaventone 2007)

This experiment tested the effect of thyroid hormones on angiotensin II type 1 receptor mRNA levels in human embryonic kidney cells (HEK). I was able to determine if T_3 and T_4 stimulated angiotensin II activity in cells. HEK cells were the best cells to use in this experiment because the kidney regulates blood pressure, and they are extracted from the source of interest: embryos. Knowing how thyroid hormones effects angiotensin I levels in HEK cells helped me determine if elevated T_3 and T_4 is the cause of early onset hypertension in children born of preeclamptic mothers. Hypothesis: HEK 293 cells treated with T_3 and T_4 will have more angiotensin II type I receptors than those not treated with T_3 and T_4. Those HEK 293 cells treated with thyroid hormones along

with angiotensin I will have even more angiotensin II type I receptors than those just treated with thyroid hormones.

Methods:

***Cell Cultures*:**

Human Embryonic Kidney (HEK) cells from cell line 293 were used in this experiment.

Dulbecco's Modified Eagle's Medium was used to grow the HEK 293 cells. Medium contained 2mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L of sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate, 90%; heat inactivated fetal bovine serum (FBS), 10% and 1% streptomycin/ampicillin antibiotics (ATCC 2007). Cells were maintained in an incubator at 37°C and 5% CO_2 levels (ATCC 2007). All cell culture procedures were performed in a laminar flow hood. Media was changed every 2 to 3 days (ATCC 2007).

Cell Culture Maintenance

HEK-293 cells were grown in 10 ml T-75 flasks. Once confluent, 5 ml of trypsin was added. Trypsin caused the cells to come off the bottom of the flask. Collected cells were added to 15 ml conical tubes containing 10 ml of media. This mixture was centrifuged at 20 rpm for 3 minutes. All supernatant liquid was removed and the cells were resuspended in 3 ml of media. 2 ml of cells from each passage were stored in liquid nitrogen. Then 31 ml of media was added to the remained 1 ml of cells in the 15 ml conical vial. 10 ml was added to each of the two new T-75 flasks and 12 ml was added to the six-well plate (2 ml per well).

Six-well plates containing HEK 293 cells plus the necessary hormones were used for this experiment (Figure 3). Each well plate was treated as follows once it is 75-90% confluent. Well I was treated with 100µl of 2 µM angiotensin I and 100µl of 43 nM T_3 (Papathanasiou 2007). Well II was treated with 100µl of 43 nM T_3 and 100µl of PBS (Papathanasiou 2007). Well III was treated with 100µl of 2 µM angiotensin I (Papathanasiou 2007). Well IV was treated with 100µl of 2.26 µM T_4 and 100µl of 2 µM angiotensin I (Papathanasiou 2007). Well V was treated with 100µl of 2.26 µM $T_{4\ and}$ 100µl of PBS (Papathanasiou 2007). Well VI served as a control and was treated with 200µl phosphate buffered saline (PBS). Angiotensin I was added to ensure that angiotensin II is produced. The concentrations of thyroid hormones and angiotensin I are based on the average ranges in humans. Each treatment lasted for 24 hours. Three trials were completed.

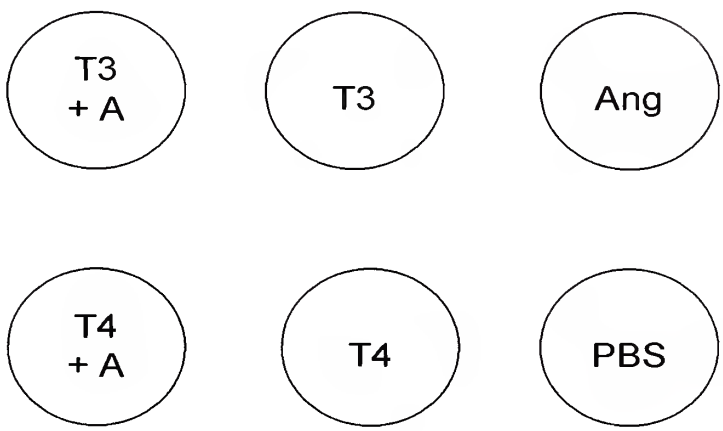

Figure 3. Six Well Plate

RNA isolation from cultured cells:

Isolation of RNA was performed as described in Qiagen RNA miniprep kit (Figure 4) (Qiagen 2006). Cells were grown in T-75 flasks then trypsinized and collected

as a cell pellet (Qiagen 2006). Adding Buffer RLT lysed the cells (Qiagen 2006). The RNA was bound to the spin column (Qiagen 2006). The column was washed two times and then the RNA was eluted with RNase free-water (Qiagen 2006).

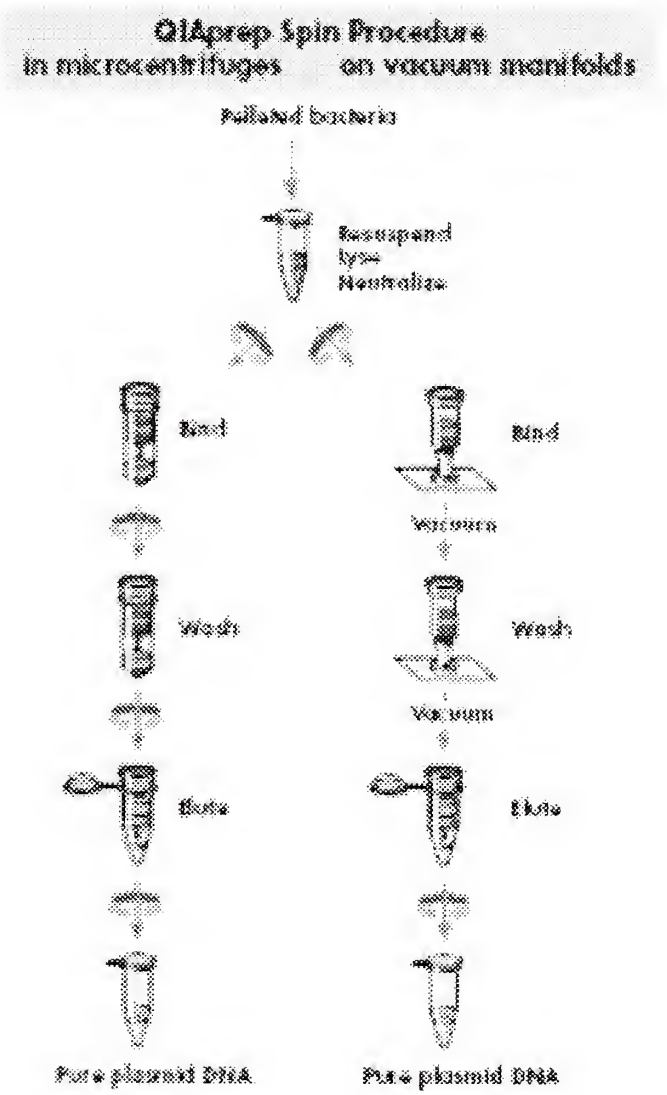

Figure 4. QIAprep Miniprep Handbook (Qiagen 2006)

RNA quantification:

A spectrophotometer will be used to measure the absorbance values of RNA at 260nm and 280nm. 260nm is when the RNA can be detected and 280nm is measured to determine purity. The expected $absorbance_{260/280}$ ratio values are 1.8-2.0.

Reverese Transcriptase- PCR

I will run reverse transcription-polymerase chain reaction analysis on the RNA fragment using SuperScript™ III First-Strand Synthesis System for RT-PCR by Invitrogen. A cDNA Synthesis Mix will be prepared according to Invitrogen. Then 10 µl of cDNA Synthesis Mix will be added to each RNA mixture (Invitrogen 2003). The PCR was run at 94°C (denature proteins) for 30 seconds, 56°C for one minute, and 72°C (amplification) for one minute. Forty-five cycles were run.

Gel Electrophoresis

Gel electrophoresis will be run on the RNA. The gel that was used for the electrophoresis will be 1% agarose gel with Ethidium bromide added. There was a lane for each of the six treatments from all three trials, a lane for the ladder, and a positive and negative control lane on the gel. The experimental (AT1R) gel will show the messenger RNA levels of Angiotensin II type I receptor. A primer pair for AT2-receptor with the genetic sequence: sense 5′-TTCCCTTCCATGTTCTGACC-3′, antisense 5′-AAACACACTGCGGAGCTTCT-3′ will detect Angiotensin II type I receptor (Tone 2007). Angiotensin II Type I receptor consists of 532 base pairs (Li 1999). Beta-actin messenger RNA levels will be used for the control gel. The forward primer pair for β-actin is TCGAATTCTGGAGAAGAGCTATGAGCTGCCG and the reverse primer pair is TCGGTACCGTGCCACCAGACAGCACTGTGTTG (Invitrogen 2007). The size of β-actin is 201 base pairs (Li 1999). The gels were run at 120V. The experimental gel ran

for 2.5 hours and the β-actin gel ran for 4 hours and 45 minutes. Normally the gels would only need to be run for about 1.5 hours, but the machine kept shorting out and resetting the voltage at 20V instead of 120V. The films for the gels were scanned into Adobe Photoshop so that the band densities between the experimental gel and the control gel could be compared. The bands were compared to a positive and negative control.

Statistical analysis:

A one-way ANOVA was performed to determine if the different treatments of angiotensin I and thyroid hormones had different affects on the HEK293 cells. An LSD Post-HOC test was also performed to determine where the significant differences lie. The ratios of band densities between the Angiotensin II type I receptor gel and the β-actin gel were compared for each of the five different treatments. An average ratio for each treatment was taken from the three trials. A standard deviation was used to measure the possibility of error in each trial.

Results:

Angiotensin I (Ang I) increased mRNA levels of Angiotensin II type 1 receptors (AT1R). AT1R mRNA levels in HEK293 cells treated with Ang I were significantly ($p<0.05$) higher than all other treatments except T_3 (Figures 6 & 7).

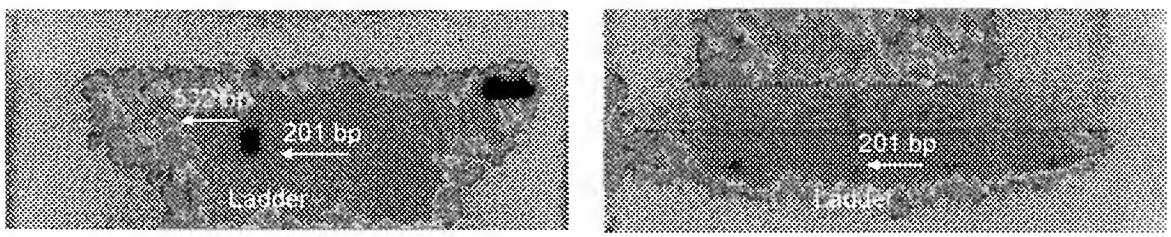

Figure 5. AT1R gel (left) and Beta-actin gel (right). Lanes from L-R in both images- **Trial 1** T_3+Ang I, T_3, Ang I, T_4+Ang I, T_4, control; **Trial 2** T_3+Ang I, T_3, Ang I, **LADDER**, T_4+Ang I, T_4, control; **Trial 3** T_3+Ang I, T_3, Ang I, T_4+Ang I, T_4, control; + **control, - control**

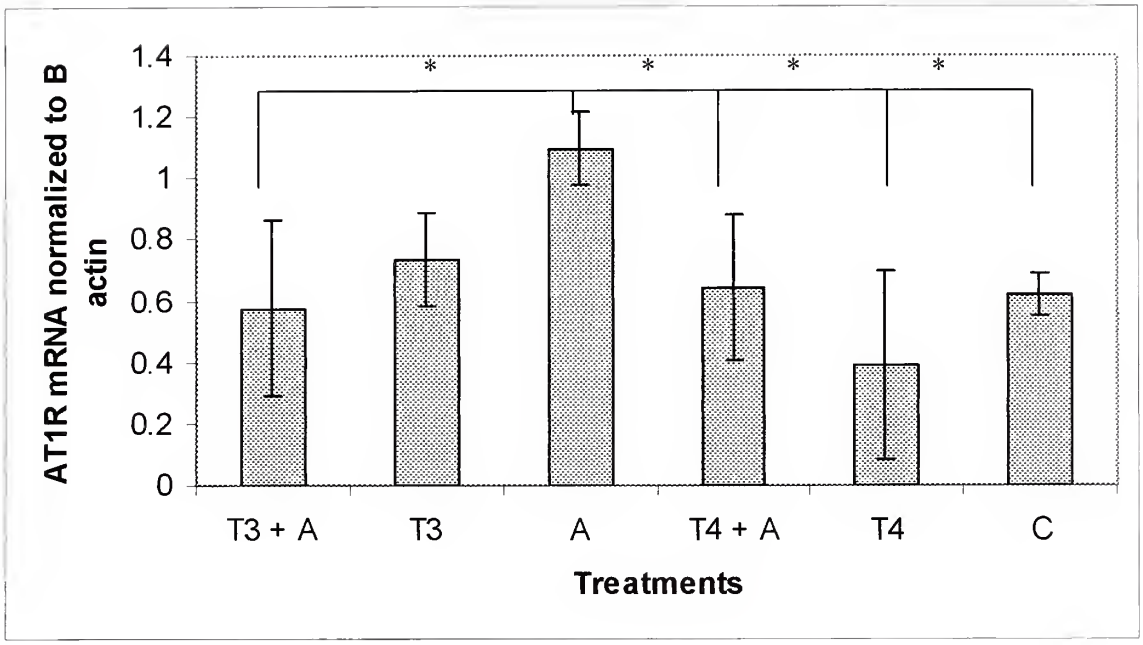

Figure 6. Mean AT1R mRNA levels normalized to B-actin of three trials.

Multiple Comparisons

Dependent Variable: Normalized AT1R mRNA
LSD

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Upper Bound	Lower Bound
1.00	2.00	-.16090	.175662	.378	-.54363	.22184
	3.00	-.51966(*)	.175662	.012	-.90239	-.13692
	4.00	-.06619	.175662	.713	-.44892	.31655
	5.00	.18778	.175662	.306	-.19496	.57051
	6.00	-.04397	.175662	.807	-.42670	.33877
2.00	1.00	.16090	.175662	.378	-.22184	.54363
	3.00	-.35876	.175662	.064	-.74149	.02397
	4.00	.09471	.175662	.600	-.28802	.47745
	5.00	.34868	.175662	.070	-.03406	.73141
	6.00	.11693	.175662	.518	-.26580	.49966
3.00	1.00	.51966(*)	.175662	.012	.13692	.90239
	2.00	.35876	.175662	.064	-.02397	.74149
	4.00	.45347(*)	.175662	.024	.07074	.83621
	5.00	.70744(*)	.175662	.002	.32470	1.09017
	6.00	.47569(*)	.175662	.019	.09296	.85842
4.00	1.00	.06619	.175662	.713	-.31655	.44892
	2.00	-.09471	.175662	.600	-.47745	.28802
	3.00	-.45347(*)	.175662	.024	-.83621	-.07074
	5.00	.25396	.175662	.174	-.12877	.63670
	6.00	.02222	.175662	.901	-.36052	.40495
5.00	1.00	-.18778	.175662	.306	-.57051	.19496
	2.00	-.34868	.175662	.070	-.73141	.03406
	3.00	-.70744(*)	.175662	.002	-1.09017	-.32470

	4.00	-.25396	.175662	.174	-.63670	.12877
	6.00	-.23175	.175662	.212	-.61448	.15099
6.00	1.00	.04397	.175662	.807	-.33877	.42670
	2.00	-.11693	.175662	.518	-.49966	.26580
	3.00	-.47569(*)	.175662	.019	-.85842	-.09296
	4.00	-.02222	.175662	.901	-.40495	.36052
	5.00	.23175	.175662	.212	-.15099	.61448

Based on observed means.

- The mean difference is significant at the .05 level.

Figure 7. LSD Post-HOC table from one-way ANOVA. Treatment 1= T3+Ang I, Treatment 2= T3, Treatment 3= Ang I, Treatment 4= T4+ Ang I, Treatment 5= T4, Treatment 6= Control (PBS)

Discussion:

Given these results both of my hypotheses must be rejected. HEK293 cells treated with thyroid hormones did not have significantly higher levels of AT1R than those not treated with thyroid hormones. This could be because thyroid hormones increase metabolism and therefore cause more ATP to be made. The increase in ATP production may have distracted the cells from making more AT1R. HEK293 cells treated with both thyroid hormones and angiotensin I also did not have more AT1R than those only treated with thyroid hormones. In fact, cells treated with both T_3 and Ang I had lower mRNA levels of AT1R than those treated with just T_3. It seemed that T_3 had an inhibiting effect on Ang I activity. Cells treated with T_4 and Ang I did have higher levels of AT1R mRNA than cells treated with only T_4. This could be because T_4 is the inactive thyroid hormone and therefore did not inhibit Ang I activity.

Angiotensin I led to an increase in mRNA levels of AT1R, which causes vasoconstriction and therefore increased blood pressure. This increase in AT1R is attenuated when T_3 is added.

Increased TSH leads to an increase in thyroid hormone production which leads to an increase in angiotensinogen. Women with preeclampsia are known to have increased

TSH levels. Renin levels are known to be high in fetuses, so renin would cleave the angiotensinogen into Ang I (Schutz 1996). Since fetuses begin producing their own hormones at 12 weeks gestation (Thyroid Australia LTD. 2001), the HEK293 cells should have angiotensin-converting enzyme needed to convert the Ang I into angiotensin II.

Further studies need to be conducted on this topic. An arterial (endothelial) cell line could be used in place of HEK293 cells to see if the results are similar to what I found in this experiment. Adding the thyroid hormone treatments a day before adding the angiotensin I treatment could determine if T_3 actually does have an inhibiting effect on Ang I. One other research idea would be to measure the mRNA levels of angiotensin II type 2 receptor. This receptor causes vasodilation instead of vasoconstriction, so the results may be opposite of what was found in this study.

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