THE EFFECT OF 1,25-DIHYDROXY VITAMIN D3 ON AT1 CELL RECEPTOR EXPRESSION IN HUMAN ILIAC ARTERY ENDOTHELIAL CELLS (HIAEC)

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Abstract

The renin-angiotensin system (RAS) plays an important part in homeostasis through angiotensin II's role as a vasoconstrictor. Defects in the renin angiotensin system can lead to hypertension, heart attack, and stroke (Weir, 1999). Studies have shown an inverse relationship between Vitamin D_3 and blood pressure (Li et al., 2002). While studies have been performed on vitamin D's effect on renin expression, little is known about vitamin D's effect on the angiotensin II high-affinity type 1 (AT_1) receptor. The hypothesis for this study was that human iliac artery endothelial cells (HIAEC) supplemented with 1,25-dihydroxy vitamin D_3 will express less AT_1 receptor protein expression than the control group. After exposing the cell to 1,25-dihydroxy vitamin D_3, RNA was isolated and RT-PCR was completed using AT_1 primers. Gel electrophoresis allowed for quantification of expression by measuring the density of bands. Two way ANOVA was used to determine significance, and it was determined that the presence of 1,25-dihydroxy vitamin D_3 had no significant effect on either AT_1 or B-actin expression in HIAE cells. The hypothesis can neither be rejected nor accepted however because the control group did not express AT_1, and therefore it cannot be determined whether AT_1 expression would have been reduced.

Background

An important participant in maintaining homeostasis is the renin-angiotensin system (RAS) which acts on blood pressure, electrolyte concentrations, and extracellular volume levels (Kong and Li, 2003). The system is activated when the kidney releases renin into the blood in response to physiological stimuli. Renin then converts angiotensinogen, released from the liver, by cleaving it into the 10 amino acid polypeptide angiotensin I (ANG I) (Li, 2003). The angiotensin-converting-enzyme (ACE) then in turn cleaves ANG I into angiotensin II (ANG II), which acts on its cell membrane receptors in the kidney, heart, and brain to increase vasoconstriction, to cause cell proliferation, and to stimulate the sympathetic nervous system (Li et al., 2002). The biological effects of ANG II are mostly caused by binding to its high-affinity type 1 receptor (AT_1), which plays a role in vasoconstriction, cell proliferation, and antinatriuresis (Weir, 1999 and Kong and Li, 2003). Both AT_1 and AT_2 receptors are present on myocytes, fibroblasts, endothelial cells, and vascular smooth muscle (Li et al., 1999a). Renin can also be regulated by the feedback inhibition of ANG II (Kong and Li, 2003). Irregular RAS activity is related to hypertension, heart attack, and stroke; therefore, understanding methods to regulate RAS is an important objective in treating these conditions (Li et al., 2002).

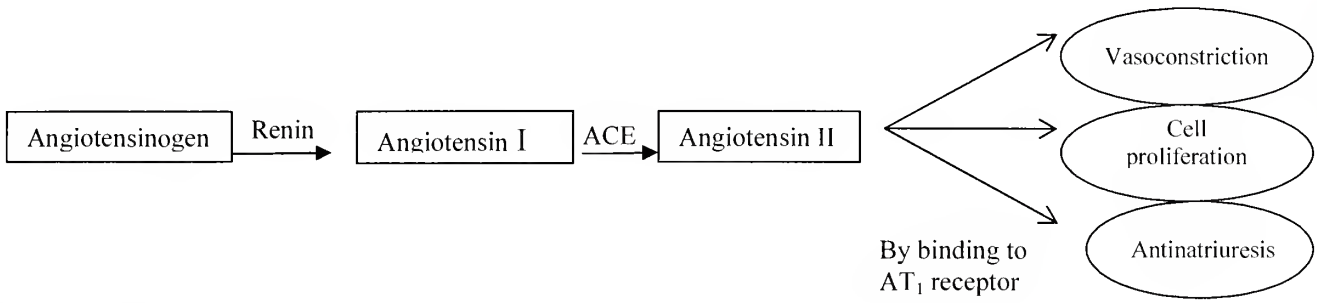

Fig 1. The Renin-Angiotensin System

In an attempt to control the RAS system, three classes of pharmaceuticals have been developed: β blockers, ACE inhibitors, and angiotensin receptor blockers (Weir, 1999). Each class has corresponding complications. AT_1 receptor blockers leave the other angiotensin receptors unaffected and could lead to adverse effects in long-term use (Contreras et al., 2003), while ACE inhibitors can be bypassed by alternative pathways (Weber, 1997). Because of the difficulties associated with the pharmaceuticals used to treat irregularities in the RAS system, other treatments are also being pursued. In recent clinical studies, an inverse relationship between the Vitamin D concentration in plasma and blood pressure readings was established and has led to further investigation of the use of Vitamin D in treatment for hypertension (Li et al., 2002).

The majority of people obtain Vitamin D through solar UV-B radiation as a primary source (Holick, 2006). Accumulating Vitamin D through dietary means is rare because few dietary sources include significant amounts of Vitamin D (Holick, 2006). The few that do include oily fish, egg yolks, fortified milk, and fish oils (Holick, 2006). Vitamin D consists of two forms: Vitamin D_2, which is formed from the irradiation of ergosterol in plants, and Vitamin D_3, which is produced in the skin after sunlight exposure (Holick, 2006). Vitamin D_3 has been the primary focus in research since studies revealed that an increase in UV irradiation resulted in a decrease in blood pressure (Dakshinamurti and Dakshinamurti, 2001). There is also an increase

in the frequency of hypertension with increased distance from the equator because the amount of circulating Vitamin D_3 fluctuates based on geographic location (Li et al., 2004).

In the body, exposure to UV radiation converts the chemical 7- dehydrocholesterol into Vitamin D_3 which is then transformed into 25-hydroxy vitamin D_3 in the liver (Thibodeau and Patton, 2007). Finally, the 25-hydroxy vitamin D_3 is then transformed in the kidney to produce the active form of Vitamin D, 1,25-dihydroxy vitamin D_3 (Thibodeau and Patton, 2007). Previously, it was thought Vitamin D's role in the body was limited to calcium homeostasis, but because its receptors are found extensively throughout various types of tissue, Vitamin D is believed to have other roles including ones in the immune system (Li, 2003).

Li's 2002 study provided evidence that 1,25-dihydroxy vitamin D_3 functioned as an endocrine suppressor to maintain the homeostasis of renin production thus lowering blood pressure. Kong's 2003 study suggested that this repression is independent of the ANG II feedback inhibition pathway. Further knowledge about Vitamin D's effect on the RAS system can aid in treatment of conditions such as hypertension and alleviate the problems associated with the current treatments. While most reports have been concerned with vitamin D on renin expression, little is known about Vitamin D's effect on AT_1 receptor expression. By studying the role of 1,25-dihydroxy vitamin D_3 *in vitro* on the receptor expression, its role as a negative regulator will be better understood. The hypothesis was that the human iliac artery endothelial cells grown with 1,25-dihydroxy vitamin D_3 will have decreased AT_1 receptor expression compared to the control, indicating that vitamin D also affects expression of the receptor along with renin activity. Results suggesting this interaction would help explain the observed inverse relationship between blood pressure and vitamin D.

Materials and Methods

Cells. Human iliac artery endothelial cells (HIAEC) were ordered from the American Type Culture Collection (Catalog Number CRL-2608), plated in 10mL sterile flasks, and incubated at 37°C in 95% air and 5% CO_2 (Li et al., 1999b). The cells were plated with 10 mL of F-12K modified medium (American Type Culture Collection Catalog No.30-2004) with 10% fetal bovine serum added, 0.1 µL of a 0.03 mg/mL solution of endothelial growth serum, and 0.01 µL of a 0.1 mg/mL solution of heparin. The cells were grown to 80% confluency then split into two 10 mL flasks. Third generation HIAEC were then split into seven 10mm dishes. The media was replenished every second day.

Vitamin D treatment. One 10mm dish served as the control and therefore contained only growth medium, heparin, and EGS. In another study, vitamin D was effective in enhancing osteogenesis at the concentrations of 1 nM, 10 nM, and 100 nM, and therefore those were the concentrations added to the growth media in the experimental groups (Malladi et al., 2006). Concentrations of 1 nM, 10 nM, and 100 nM of 1α,25-dihydroxy vitamin D_3 (Sigma D1530) were added in addition to the growth medium in 2 dishes of cells per treatment. One dish per treatment was treated for one hour, and the second dish was treated for four hours. Three trials were completed. All dishes were collected at the same time using trypsin, and stored using Qiagen's RNA Protect Cell Reagent in -20°C.

RNA isolation and spectrophotometry. RNA was isolated by using the Qiagen RNeasy Plus Mini Kit (Catalog No. 74134). The spectrophotometer was used to inspect the quality of RNA (260/280 nm) and determine the amount of each sample needed to run RT-PCR (Table 1).

RT-PCR. Using the Invitrogen SuperScript II RT First-Strand Synthesis system for RT-PCR (Cat No. 12371-019 Lot No. 1397500), reverse transcription was completed within a PTC-

100 Programmable Thermal Controller thermocycler (MJ Research Inc) following the directions provided using 4 μL of total RNA. The kit also contained the instructions to complete positive and negative control reactions with 1 μL of HeLa RNA. Polymerase chain reactions were completed with both B-actin and AT_1 primers as the B-actin expression was used to standardize AT_1 expression and ensure that procedures had been properly completed. Next, the Invitrogen kit contained Taq DNA polymerase recombinant (Cat No. 10342-053 Lot No. 1368151) and all the components needed to run the polymerase chain reaction which amplified the AT_1 and B-actin strands within the thermocycler. PCR with AT_1 primers included 40 cycles of 94°C for 40 seconds, 55°C for 1 minute, and 72°C for 1 minute, and when running PCR with B-actin specific primers, PCR consisted of 40 cycles at 95°C for 1 minute, 50°C for 1 minute, and 72°C for 1 minute (Li et al., 1999b).

Gel electrophoresis. Samples were then loaded into 0.7% agarose gels, along with a positive and negative control provided by the Invitrogen kit and Invitrogen's 1 kb ladder, and an electrical current (110 volts) was passed through the gel to separate the DNA based on size. The agarose gels contained ethidium bromide which would bind to all present DNA and would reveal bands when inspected under ultraviolet light (Figure 2). AT_1 expression was expected to be visualized at 532 bp and B-actin at 201 bp (Li et al., 1999b). The positive control was evident at 353 bp. Expression was quantified by measuring band densities (Gao et al., 2006). A polaroid picture was then taken of each gel and scanned into a computer for analysis. The densities of the bands were measured using Adobe Photoshop CS3 extended version 10.0. The images were inverted, and the integrated densities were measured of the band and the background gel. Integrated density is defined by Adobe as the sum of the values of the pixels in the selected area, or the product of area and mean gray value. The integrated densities of the bands were divided

by the integrated density of a random area of equal size of the same gel so that the densities could be compared.

Statistical Analysis. The independent variable in this experiment is concentration of 1,25-dihydroxy vitamin D_3, and the dependent variable is AT_1 protein expression level. After accounting for background density, the ratio of AT_1 density to B-actin was compared between samples. Mean ratios of for concentrations of 1 nM, 10 nM, and 100 nM of vitamin D were determined by compiling band density ratios of all trials (Table 2). These mean ratios were then compared against the mean ratios from other treatments of vitamin D to observe any differences with a standard deviation of 0.172 (Graph 1). The ratios of AT_1 to B-actin were run through a two-way ANOVA test where a value of $p < 0.05$ was considered to be statistically significant to determine if 1, 25 dihydroxy vitamin D_3 had any effect on AT_1 expression (Table 2). A two-way ANOVA test was run with B-actin densities also to test for any effect (Table 3).

Results

Table 1 lists the 260/280 nm ratio for all of the samples. This was used to determine how much of the sample was needed to run RT-PCR correctly, and the 260/280 nm ratio was used to determine the quality of the RNA with 260/280nm of 2.00 indicating the best quality RNA. Figure 2 contains the scanned gels that were used to determine the density of the bands of expression. AT_1 expression would have been evident at 532 bp, while B-actin was obvious at 353 bp. By looking at Figure 3, it can be determined that there is not much variation in the mean ratios of AT1 to B-actin between test groups, and therefore the presence of vitamin D had little effect. When the data was run through two-way ANOVA, there was a significance of 0.479 for AT_1 expression (using AT_1 to B-actin ratios) and 0.683 for B-actin expression. Because neither

significance is less than 0.05, 1,25 dihydroxy vitamin D_3 made no significant difference on the expression of either AT_1 or B-actin.

Table 1. Spectrophotometer readings for RNA quality

Sample	RNA concentration (ng/microlitre)	260/280 nm
1 nM 1 hr	10.30	1.57
1 nM 4 hr	10.00	1.44
10 nM 1 hr	9.30	1.43
10 nM 4 hr	13.70	1.97
100 nM 1 hr	8.10	1.28
100 nM 4 hr	11.60	1.56
0 nM	8.40	1.47
1 nM 1 hr	7.10	1.48
1 nM 4 hr	8.60	1.68
10 nM 1 hr	40.60	1.33
10 nM 4 hr	7.70	1.36
100 nM 1 hr	8.90	1.53
100 nM 4 hr	17.70	1.48
0 nM	10.00	1.59
1 nM 1 hr	5.30	1.68
1 nM 4 hr	7.80	1.39
10 nM 1 hr	12.00	1.37
10 nM 4 hr	6.70	1.94
100 nM 1 hr	7.20	1.45
100 nM 1 hr	10.00	1.26
0 nM	10.90	1.40

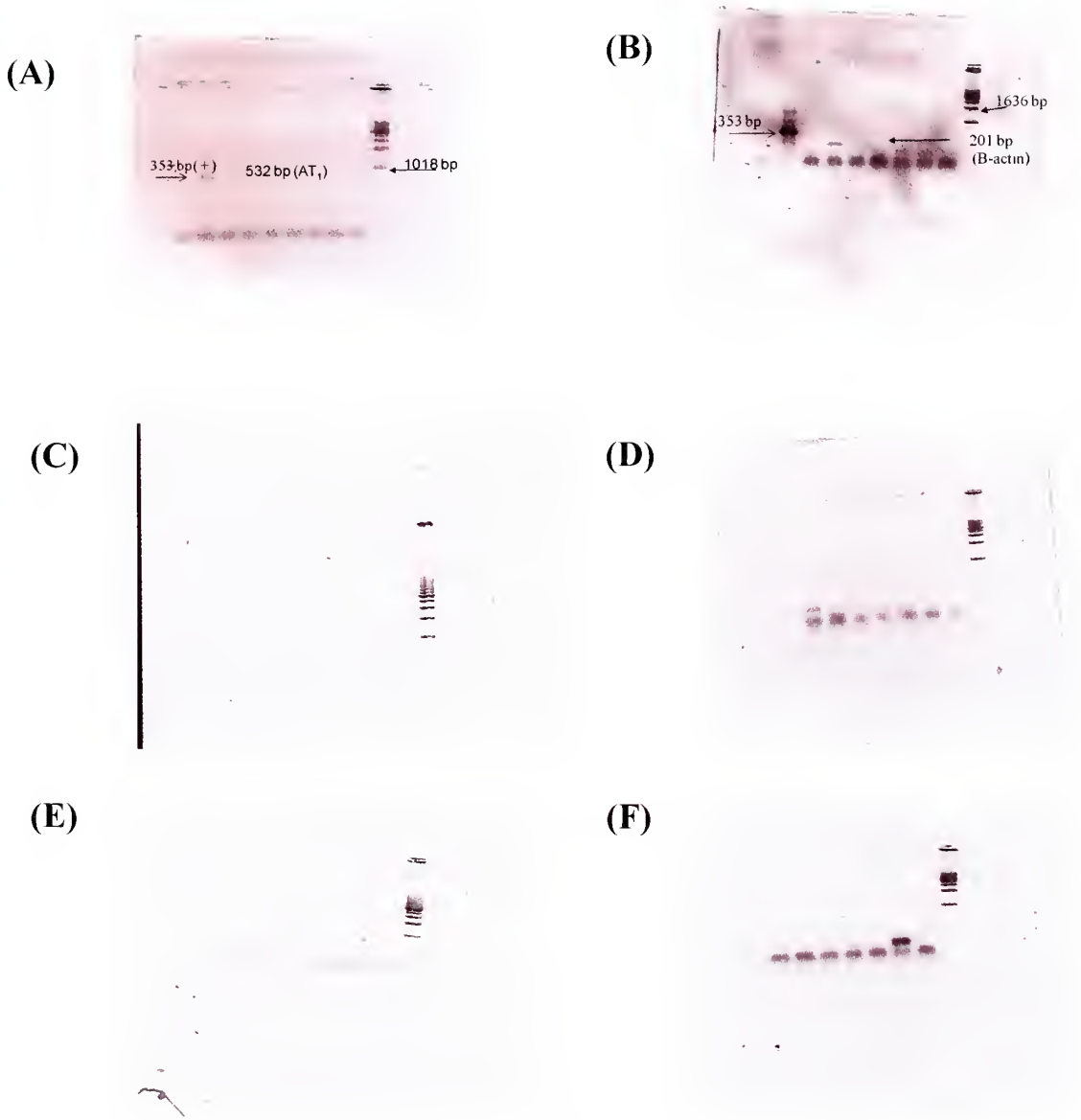

Fig 2. Gel electrophoresis results of samples run on agarose gels. **(A), (C), (E)** gels were samples that had AT_1 expression amplified. **(B) (D) (F)** gels were amplified with B-actin primers.

Table 2. Integrated densities of B-actin and AT_1 bands of inverted gels for all treatment samples and average ratios of AT_1 to B-actin among treatment groups.

AT_1 gel	--	+
353 bp	41131	17791

Background density	73827	73827
control density	0.56	0.24
B-actin gel		
353 bp	15223	4139
background density	17047	17047
control density	0.89	0.24

Trial 1	100 nM 4 hr	10 nM 4 hr	1 nM 4 hr	100nM 1 hr	10 nM 1 hr	1 nM 1 hr	0 nM
AT_1 bar	23687	31005	45486	46011	40479	48649	56066
background density	73827	73827	73827	73827	73827	73827	73827
AT_1 density	0.32	0.42	0.62	0.62	0.55	0.66	0.76
B-actin bar	12041	12812	10898	7521	7441	6551	8747
background density	17047	17047	17047	17047	17047	17047	17047
B-actin density	0.71	0.75	0.64	0.44	0.44	0.38	0.51
Ratio of AT_1 to B-actin density	0.45	0.56	0.96	1.41	1.26	1.71	1.48

Trial 2	100 nM 4 hr	10 nM 4 hr	1 nM 4 hr	100nM 1 hr	10 nM 1 hr	1 nM 1 hr	0 nM
AT_1 bar	28898	28580	28445	28560	28508	28141	28384
background density	28737	28737	28737	28737	28737	28737	28737
AT_1 density	1.01	0.99	0.99	0.99	0.99	0.98	0.99
B-actin bar	19572	23052	24029	23258	21314	23883	26349

background density	26506	26506	26506	26506	26506	26506	26506
B-actin density	0.74	0.87	0.91	0.88	0.80	0.90	0.99
Ratio of AT_1 to B-actin density	1.36	1.14	1.09	1.13	1.23	1.09	0.99

Trial 3	**100 nM 4 hr**	**10 nM 4 hr**	**1 nM 4 hr**	**100nM 1 hr**	**10 nM 1 hr**	**1nM 1 hr**	**0 nM**
AT_1 bar	24794	24197	24325	24196	24259	22749	24199
background density	24513	24513	24513	24513	24513	24513	24513
AT_1 density	1.01	0.99	0.99	0.99	0.99	0.93	0.99
B-actin bar	36591	36582	35915	36240	34461	14861	33409
background density	38653	38653	38653	38653	38653	38653	38653
B-actin density	0.95	0.95	0.93	0.94	0.89	0.38	0.86
Ratio of AT_1 to B-actin density	1.07	1.04	1.07	1.05	1.11	2.41	1.14

	100 nM 4 hr	**10 nM 4 hr**	**1 nM 4 hr**	**100nM 1 hr**	**10 nM 1 hr**	**1nM 1 hr**	**0 nM**
Average Ratio of AT_1 to B-actin density	0.96	0.92	1.04	1.20	1.20	1.74	1.21

Figure 3. Inverted average ratios of AT_1 to B-actin density among treatments

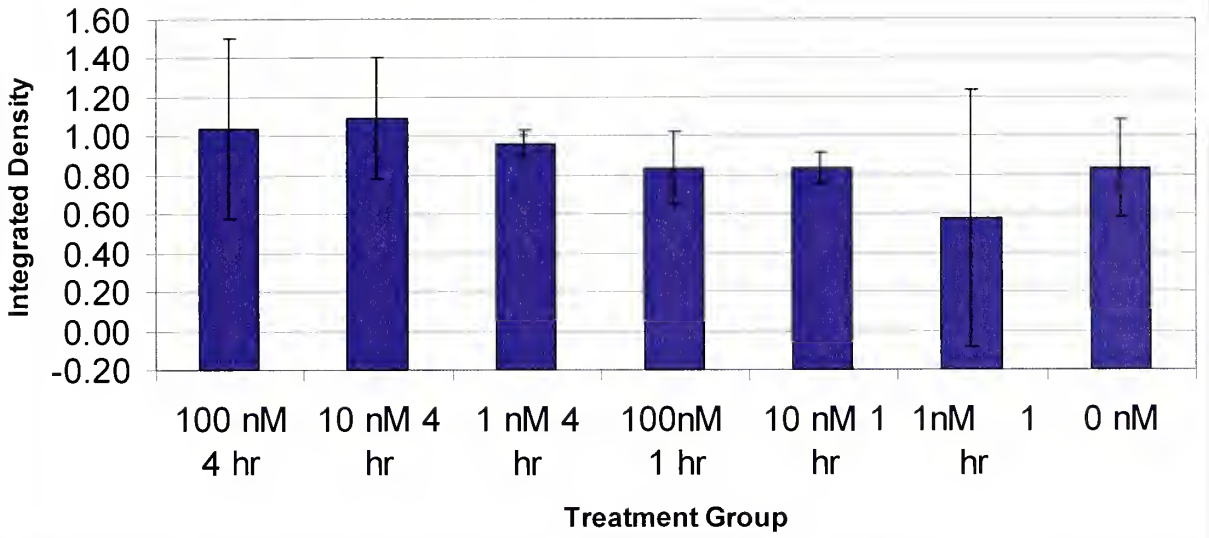

Table 3. Two-way ANOVA Test of Between-Subjects Effects on AT_1 expression as measured by ratio of AT_1 to B-actin					
Dependent Variable: VAR00003					
Source	Type III Sum of Squares	df	Mean Square	F	Sig.
VAR00001 * VAR00002	0.191	2	0.095	0.776	0.479

Table 4. Two Way ANOVA test of Between-Subjects Effects on B-actin band density					
Dependent Variable: VAR00003					
Source	Type III Sum of Squares	df	Mean Square	F	Sig.
VAR00001 * VAR00002	0.038	2	0.019	0.392	0.683

Discussion

After running the integrated density data through the two-way ANOVA test, the hypothesis is to be rejected because there is no significant difference after the treatment with 1,25-dihydroxy vitamin D_3. The test was used to measure effects on either B-actin or AT_1

expression, but there was no significant difference in either. It can be assumed that procedure was properly carried out because of the controls present throughout the experiment. Each trial contained one dish of control cells to ensure the proper treatment of the cells, and there were no indications of problems during incubation. There were some difficulties during treatments such as condensation on some flasks and second trial cells being stressed due to a malfunction in the delivery of CO_2. These incidents however did not seem to affect the data in that cells with condensation did not differ from other samples, and the data of the second trial does not conflict with the data of the first or third trials. The spectrophotometer was used to confirm the presence of RNA before proceeding onto RT-PCR. During RT-PCR, the positive control did appear as a strong band in the agarose gels. While the results of this experiment support the idea that 1,25-dihydroxy vitamin D_3 does not affect AT_1 expression, the control also did not express AT_1. Previous research on the vitamin D- RAS relationship has been studied on lab rats which may have made a difference. The cell line used in this experiment is human which may have led to different results. Also perhaps in an organism AT_1 is required and would be expressed by arterial cells as opposed to *in vitro* cells where the production of AT_1 would be energetically unfavorable. To further support the findings of this experiment, it would be necessary to test the effect of 1,25-dihydroxy vitamin D_3 on a cell line or organism where AT_1 is normally expressed in the control group. AT_1 receptor presence can be confirmed by the use of radiation binding where the cells are exposed to radioactive ANG II. If the cells retain radiation after washing, the cells used the AT_1 receptor to take in ANG II. If the AT_1 receptor is not present, cells can be encouraged to take up the gene for the receptor by use of a vector. In endothelial cells of cardiac tissue, the AT_1 receptor activation mediates major functional responses to ANG II, and the AT_1 receptor expression is higher (Li et al., 1999a). Therefore, working with different cells lines such

as human coronary artery endothelial cells may reveal more. In this experiment, we were unable to work with HCAE cells because of budget limitations, but there was no reason to believe that working with HIAE cells would produce different results. Oxidized low density lipoprotein can be used with human coronary artery endothelial cells to increase the expression of the AT_1 receptor (Li et al., 2000). Therefore if all test groups were pretreated with ox-LDL before exposure to 1,25 dihydroxy vitamin D_3 there may be a measurable effect in AT_1 expression. In a different study, rat pulmonary microvascular endothelial cells increased AT_1 expression with lipopolysaccharide (LPS) treatment (Zhang and Sun, 2006). Pretreatment with angiotensin II in rat pulmonary microvascular endothelial cells resulted in reduced AT_1 expression (Zhang and Sun, 2006). Growth hormone has also been shown to increase the expression of the AT_1 receptor in astrocytes (Wyse and Sernia, 1997). It is also possible that the angiotensin II type 2 receptor may have inhibited the expression of AT_1 by being an antagonist to the AT_1 receptor (AbdAlla et al., 2001). Therefore it may be necessary to treat cells with an AT_2 antagonist such as PD123,319 before AT_1 expression can be measured (Ford et al., 1996). Another line of research that could be followed would be to test the effects of 1,25-dihydroxy vitamin D_3 on other components of the RAS system. Most research has been focused on the effect on renin, and the results of this experiment support no effect on AT_1 expression, but the other steps of the RAS system should not be ignored and further research should be pursued.

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