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Abstract

Normal abundant dietary sugars such as fructose and sucrose can contribute to hypertension and other health issues. To avoid these health complications, many individuals use artificial sweeteners. An equivalent intake of some artificial sweeteners also can lead to hypertension. However, Stevia, a sweetener that is isolated from a Paraguayan plant, was shown in relevant literature to decrease blood pressure in both rat specimens and humans. The general purpose of this research project was to study the effect of Stevia, saccharin, and sucrose on the expression of two key components of the renin-angiotensin-aldosterone system (RAAS): prorenin receptor (PRR) and angiotensin II type 1 receptor (AT1). Increased expression of renin and angiotensin can lead to vasoconstriction and systemic hypertension. Their effects are mediated by their binding to PRR and AT1. Therefore, decreases in the expression of these receptor proteins can result in lowered blood pressure.

Rats were fed diets supplemented with sucrose, saccharin, or Stevia over a six-week period and the kidneys were obtained. qPCR designs were developed to measure the relative amounts of PRR receptor and AT1 receptor. The methods had efficiencies greater than 97% and gave reproducible results. Then the developed methods were used to measure the expression of AT1 and PRR in the different rat kidney samples. A general increase in the relative expression of both AT1 and PRR in the sucrose diet was observed. However, this increase and all other differences in the test groups were not significantly different than the control group. These results suggest no differences in AT1 or PRR expression for different sweetener diets.

1. Introduction

Sugar is a large part of the American diet, something that pervades many of our processed foods and beverages. Many have linked the excess consumption of sugar to health problems, most notably obesity, hypertension, and diabetes. As a result, many individuals have turned to the use of other sweeteners in order to consume the foods and beverages they like with less health detriment. Nevertheless, recent research has shown a correlation between artificially sweetened beverage consumption and a variety of health problems such as weight gain and cardiovascular disease.[1] Unfortunately, our population still has significant health problems. High blood pressure is a serious issue, because more than 30% of adults in the United States have hypertension.[2]

A major hormone system that regulates blood pressure and blood volume is the renin-angiotensin-aldosterone system, located primarily in the kidneys. This system involves the hormone aldosterone and its major two proteins, renin and angiotensin. These proteins bind cell receptors in order to cause a local vasoconstrictive effect, among other functions. Two of these cell receptor proteins are prorenin receptor (PRR), which binds both prorenin and renin, and angiotensin II type 1 receptor (AT1), which is one of a group of receptors that are

bound by angiotensin II. Figure 1 shows a few of the different interactions of angiotensin II and renin, including those with AT1 and PRR, and their effects on the body. Binding to these receptors causes local vasoconstriction that can cause hypertension. This idea is supported by Contreras et al. (2002) who found that an increase in AT1 expression correlates with hypertension.[3]

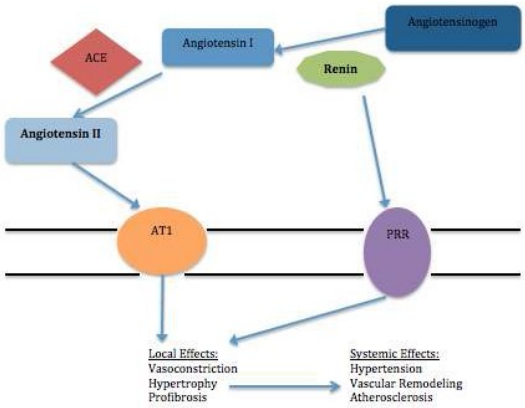

Figure 1. Simplified Diagram of Renin-Angiotensin System

For sweeteners to have an effect on hypertension, it seems possible that they could change the expression of both AT1 and PRR. Iyer et al. (1996) observed that AT1 expression and blood pressure increased in hyperinsulinemic rats fed with fructose.[4] An increase in AT1 expression was also observed in normal rats with high sucrose intakes[5], and high fructose intakes.[6] Though theorized to have a similar effect as angiotensin II type 1 receptor, the role of prorenin receptor is less clear, making it a good target for experimentation.

Stevia is a newer, promising sugar substitute that is derived from a Paraguayan plant. The effects of Stevia on AT1 have not been studied. However, Melis (1997) showed in a lengthy study that an injected Stevia extract decreased blood pressure in rats.[7] This study was supported by further evidence that demonstrated a correlation between Stevia intake and hypotension (low blood pressure) in hypertensive humans patients.[8]

This objective of this study was to measure the relative expression of AT1 and PRR mRNA in the kidneys of rats fed different sweetener diets. These diets were supplemented with sucrose, saccharin, and Stevia and were compared to a standard diet group. By measuring the relative expression of AT1 and PRR between sample groups, associations can be made about the effect of sweeteners on the renin-angiotensin system and the entire body. Based on past research, an increase in mRNA expression for both sucrose and saccharine was expected. A corresponding decrease in the mRNA expression of the Stevia group was also anticipated.

There are three specific objectives for this research. First, to isolate RNA samples from rat kidneys with consistent purity. Second, to design and test the efficiency of qPCR primers and probes to measure the expression of prorenin receptor (PRR), angiotensin II type 1 receptor (AT1), and GAPDH (endogenous control). Lastly, to measure the expression of AT1 and PRR relative to GAPDH in the control and experimental rat kidneys.

2. Materials and Methods

2.1 Rat Diets and Kidney Collection

Male Wystar-Kyoto rats around the same age were separated into different dieting groups for six weeks. The control rat group was feed a standard diet of liquid osmolite. Experimental groups were fed the liquid osmolite supplemented with sucrose, saccharin, and Stevia. Another group of rats was given a mixed dieting schedule, beginning with three weeks of only liquid osmolite followed by a week of the osmolite supplemented by glucose for a week and then Stevia for two weeks.

For each of the primary groups, kidneys were collected from four rats after they were

sacrificed. Kidneys were also collected from rats in the mixed diet but only two were tested. After the kidneys were collected, they were immediately frozen with liquid nitrogen, and stored at -80°C for further experimentation.

2.2 Tissue Homogenization & RNA Isolation

Approximately 25 milligrams of Rat kidney tissue was ground using a pre-chilled mortar and pestle and liquid nitrogen. Samples were homogenized using QIAshredders (Qiagen). After homogenization, RNA was isolated using the mirVana miRNA Isolation Kit (Ambion) and phenol-chloroform extraction. Short RNAs were separated from the long RNAs for use in a different analysis. The concentrations and A_{260}/A_{280} readings were determined using a Nanodrop Lite Spectrophotometer (Thermo Scientific). Then samples were stored at -20°C.

2.3 Reverse Transcription (RT)

RNA samples were thawed and reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). This was done so that all samples had a final RNA concentration of 50ng/uL. A negative control sample using RNase free water also underwent the entire reverse transcription process. Once samples were prepared, they were added to a reaction plate and placed in a thermal cycler (Applied Biosystems) and run for 10 min at 25°C, 2 hr at 37°C, and 5 min at 85°C. Afterward, the reaction plate was stored at -25°C.

2.4 Quantitative Polymerase Chain Reaction (qPCR)

Primers and probes for GAPDH, AT1, and PRR were developed using Primer Express 3.0 program (Applied Biosystems), and are summarized in Table 1. These were designed to ensure that one of the primers or the probe crossed the exon junction. qPCR samples were prepared using TaqMan Gene Expression Master Mix. Each biological sample was run in quadruplicates, and the negative control samples for each target were run in triplicates.

Table 1. Primers and Probes for GAPDH, PRR , and AT1

	Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	Angiotensin II Receptor type 1 (AT1)	Prorenin Receptor (PRR)
Forward	5' ACGGGAA ACCCATCAC CAT 3'	5' GCTGGCAT TTTGTCTGG ATAAAT 3'	5’ TCGCTCC CCCTCAAT TCTCT 3’
Reverse	5' CCAGCATC ACCCCATT TGA 3'	5' GGGTTGAGT TGGTCTCAGA CACT 3'	5’ CAGCACTTG CAGTTCAGAA AGAAA 3’
Probe	5' TTCCAGGAG CGAGATC 3'	5' AGTGAT CACCAGG TCAAGT 3'	5’ GGAATAA TGAAGTTGA CCTGC 3’

GAPDH served as the endogenous control in order to calculate relative quantities. Samples were prepared in a 96-well reaction plate so that the final concentration of cDNA in each 20uL reaction was 0.4ng/uL. The 96-well plate was run using a STEPONE PLUSthermal cycler (Applied Biosystems). Resulting data was analyzed using the RQ = $2^{(-\Delta\Delta C_T)}$ method. Biological replicates were grouped in order to determine that average relative quantity for each diet group and its standard deviation. To test the significant differences between diet sample groups, a student’s t-test was used.

3. Results and Discussion

3.1 RNA Isolation

RNA was isolated with consistent purity between 1.97 and 2.04 A_{260}/A_{280}. Concentrations of long RNA samples varied between 110 ng/uL and 800 ng/uL. The control diet samples were labeled E1-E4, and give a good representation of the range in both A_{260}/A_{280} and concentration. E1 had a concentration of 592.7 ng/uL and a purity of 2.01. E2 had a concentration of 111.9 ng/uL and a purity of 1.97. E3 had a concentration of 596.7 ng/uL and a purity of 2.02. Figure 2 demonstrates the separation between long and short RNA samples using the

mirtVAna procedure. The high quality samples were then ready for experimentation.

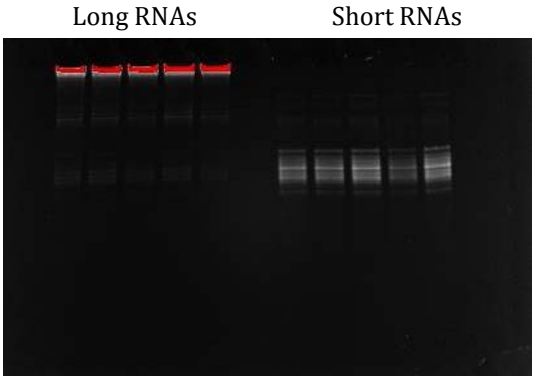

Figure 2. 15% denaturing gel demonstrating the separation of short and long RNAs in the mirVana procedure

3.2 Efficiencies of RT-qPCR Methods

An efficient system is able to double the cDNA each cycle or increase the sample tenfold over 3.32 cycles ($2^{3.32}$=10). A high efficiency, between 90-100 %, means that testing using the same sample can be reproducible. In order to determine that the methods were efficient for the mRNA of interest, a standard curve was developed by running qPCR with a range of RNA amounts for the same control kidney sample. 0.1 ng, 1 ng, 10 ng, and 50 ng of RNA from a control kidney were run for AT1, PRR, and GAPDH targets in quadruplicate. All systems were determined to be efficient, with 97% efficiency for AT1, and 98% efficiency for both PRR and GAPDH.

3.3 Relative Expression of AT1 and PRR mRNA

Using the total RNA sample and the developed methods, the relative expression of different sweetener diets was determined. These results are summarized in Figures 3 and 4 for AT1 and PRR respectively. Compared to the standard diet (1.0±0.2), the sucrose (1.2±0.2) and saccharine (1.2±0.3) diets appeared to show an increase in expression, though neither was significantly different once their standard error was compared with the control error. Neither the Stevia diet (0.9±0.2) nor the mixed diet (1.00±0.04) appeared to have any differences in the expression of AT1, and were not significantly different from the control or other samples.

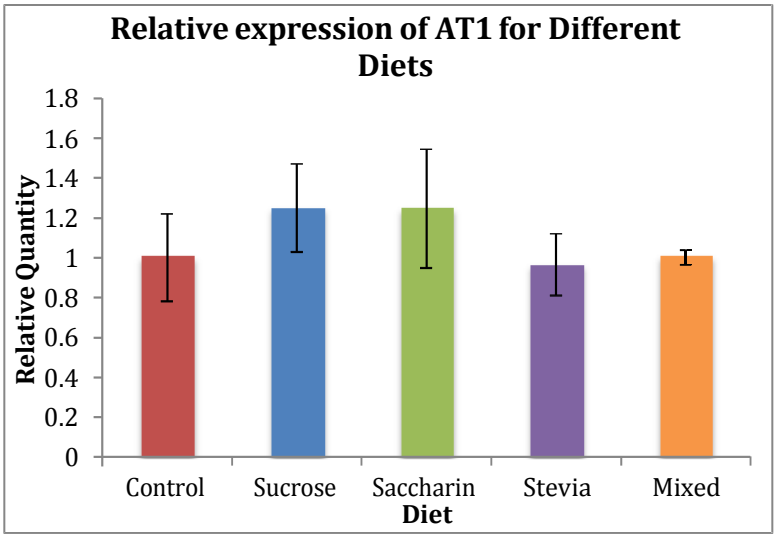

Figure 3. Relative AT1 mRNA quantity for each diet group

For prorenin receptor, only the sucrose diet kidneys (1.17±0.05) seem to show an increased relative expression compared to the control diet (1.0±0.3). The relative expression of PRR for saccharine was 1.0±0.1, similar to relative expression for the Stevia diet (1.0±0.1) and the mixed diet (1.0±0.1). Unfortunately, none of these mRNA levels were significantly different for PRR due to their overlapping standard deviations.

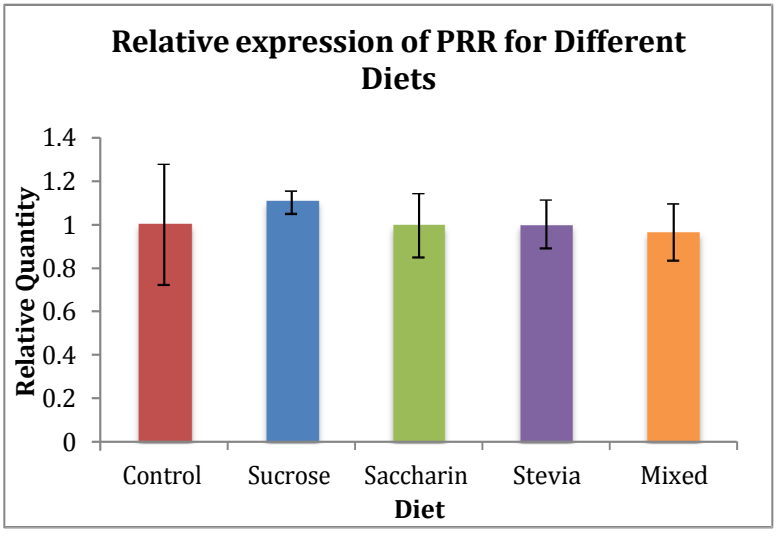

Figure 4. Relative PRR mRNA quantity for each diet group

4. Discussion

RNA was isolated from rat kidneys with consistent purity. This was important so that potential future experimentation will be run with long RNA samples that correctly represent actual mRNA expression and can show reproducible results. Concentrations of the RNA samples varied, which was most likely due to the extraction and elution steps for the mirVana procedure. The samples with higher concentrations of mRNA were eluted with warm nuclease free water and allowed to sit before centrifugation.

The developed methods for qPCR were determined to be very efficient. Repeated practice with the procedure and careful experimentation probably contributed to the high efficiencies. Nevertheless, any subsequent testing using these primers and probe systems should demonstrate reproducible results, even if the RNA samples are from very different studies.

Despite a general increase in expression of both AT1 and PRR for sucrose, a large standard deviation in each group meant that none of the diets showed any significant difference from the control diet for either target. Whenever different samples are pooled into test groups, standard error will occur. However, a few specific factors contributed to the error in these experiments. The main reason for the large standard deviations was due to the differences among the biological replicates. Within the same diet test group, rats likely had different caloric intakes. If one rat eats much more than the rest of a certain sweetener diet, this data point can contribute a lot of error with respect to their relative mRNA expression. Comparing the mRNA expression for these different targets was limited, since only four kidneys were isolated from each test group. With a larger sample size for each diet, results might show significant differences. Nevertheless, the greatest difference between the control and sucrose diet was 1.2 fold relative increase of angiotensin II type 1 receptor, or a 20% increase in expression. Whether or not this would cause a substantial effect on blood pressure or other functioning in the body is unknown.

From what was expected based on literature review, the sucrose did not demonstrate an increase in expression for AT1 like previously suggested. Though no molecular data was determined for the differences in expression for saccharine and Stevia diets, the expectation was that saccharine would increase the expression and Stevia would decrease expression for both mRNA targets. Based on the results in this research, this did not occur.

If the sweetener diets should have altered expression as theorized, multiple explanations can account for these findings. A different experimental design using much higher concentrations of each sweetener as part of the diet might have clearly suggested an increase in expression of sucrose, for example. Nevertheless, this study was more realistic as a representation of the general population. Within individuals who drink diet beverages for example, one person might rarely drink these beverages and another drink multiple each day. The differences in protein expression between these two individuals might be very different and specific expression differences would be hard to determine, similar to the results for this experiment.

Another possible way these results do not support past research is that the mRNA expression between these targets does not change significantly, but differences in protein expression are tightly regulated. In this way, a dietary change effects the amount of proteins in the cell, whereas the change in mRNA expressions remains relatively constitutive. One explanation for this would be inhibition of transcription by miRNAs, another possible avenue of future research. Western blot experiments would elucidate potential protein expression differences and complement the mRNA findings in this experiment.

Unfortunately, this research does not garner any insightful information about positive or negative effects of sweeteners on the expression of AT1 and PRR. Further research might lead to more information about this relationship. More data will only help us understand the effect of dietary sweeteners in our bodies, and hopefully change food consumption habits that lead to both hypertension and more serious diseases that plague our society today.

5. Acknowledgements

I would like to thank Dr. Theresa Salerno for her excellent mentorship of this project. Rat kidneys were graciously donated by Dr. Mary Hadley, Dr. Penny Knoblich, and Dr. David Bisonnette. The RNA isolation help of Natalie Young and the kidney collection by Dr. James Rife is greatly acknowledged. I would also like to thank the Department of Chemistry and Geology, the Undergraduate Research Center, and the Honors Program for their grants and support.

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Student Biography

Jacob Ball, from Hudson, Wisconsin, graduated Summa Cum Laude from Minnesota State University Mankato with a B.S. in Biochemistry and a B.S in Biology: Human Biology in May 2014. He minored in Spanish by studying abroad in Cuenca, Ecuador and also graduated from the Minnesota State Mankato Honors Program. As an undergraduate, he was a four time All-Conference athlete in Cross Country and Track & Field. He also was named Academic All-American in 2012 and 2013. He presented this research at the National Conference of Undergraduate Research in Lexington, Kentucky, and at the Undergraduate Research Symposium in Mankato, Minnesota. In August, he will attend medical school at the University of Wisconsin, Madison School of Medicine and Public Health and aspires to become a family practice physician in Northwest Wisconsin or Southwest Minnesota.

Mentor Biography

Dr. Theresa Salerno is an associate professor in the Department of Chemistry and Geology at Minnesota State University, Mankato. She instructs courses in Biochemistry, Principles of Biochemistry, Biochemical Techniques, and Chemistry of Life Processes. Outside of MSU, she has helped youth find a passion for science by setting up fun scientific activities at the Science and Nature Conference and volunteering at the Southern Minnesota Regional Science and Engineering Fair. Dr. Salerno is an excellent faculty mentor for many students majoring in Biochemistry who have gone on to research careers, and graduate or professional schools. Due to her dedication to these students, it is no surprise that she received the College of Science, Engineering, and Technologies Award for Excellence in Advising for the 2002-2003 academic year. Dr. Salerno also has mentored more than 30 research projects that were presented at the Undergraduate Research Symposium in the past 15 years. Frequently, she mentors more than three students a year and works well past her office hours to ensure that students are successful. Dr. Salerno's investment in undergraduate research has helped her students develop their experimentation skills, realize their research goals, and be prepared for their future careers.