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Effect of Altered VEGFR2 Expression on Oxidative Stress in a Rodent Model of Preeclampsia Pathology

Madison Kaser

Abstract

Preeclampsia is a disease that results in pathologies in both the mother and the fetus that lead to oxidative stress. A decrease in vascular endothelial growth factor (VEGF) signaling may be one factor that contributes to this pathology. We hypothesize that by increasing vascular endothelial growth factor receptor 2 (VEGFR2) expression via administration of L-tyrosine phosphate (LTP) nanoparticles, the conditions of both the mother and fetus will be improved. Following treatment with the nanoparticles, we will employ a lucigenin chemiluminescence assay to determine levels of oxidative stress within the placenta. There are three groups that will be compared which are the control group and two experimental groups. The control group is the SHAM, one experimental group is the reduced uterine perfusion pressure (RUPP), a common model for preeclampsia, and the other experimental group is the RUPP treated with the nanoparticle. Once finished, we hypothesize that placental samples from VEGFR2-treated RUPP animals will display less oxidative stress than untreated RUPP animals, similar to that seen from SHAM controls. These studies will indicate whether RUPP pathology is associated with placental oxidative stress, and identify potential therapeutic roles for VEGFR2 nanoparticles.

Introduction

Preeclampsia is a hypertensive disease of pregnancy that is hypothesized to be the result of endothelial dysfunction (Roberts, 1998). This induces detrimental vascular changes such as vasospasm, increased vasoconstriction, and decreased vasodilation (Crews, 2000). Some commonly seen effects of the disease are edema, renal dysfunction, and fetal growth restriction (Lindheimer, 1993). The cardiovascular and renal changes associated with the disease result in decreased blood flow to the fetal placental unit. A positive feedback loop results in

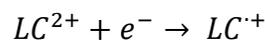
complications for both the mother and the fetus (Maynard, 2008). We hypothesize that a decrease in the placental perfusion somehow leads to a decrease in the vascular endothelial growth factor (VEGF) signaling. This growth factor is a vasodilator which uses vascular endothelial growth factor receptor 2 (VEGFR2) to activate endothelial nitric oxide synthase and plays a role in uterine remodeling and placental development (Facemire, 2009). Furthermore, it is vasodilator mechanisms which contribute to the pathology of preeclampsia. This includes hypertension, constrictive phenotype small arteries, and decreased uterine artery blood flow. For the fetus, it results in placental insufficiency, growth restriction, and fetal morbidity (Grummer, 2009). In addition to these negative effects on both the mother and fetus, oxidative stress can occur as a result of any of these deficiencies.

Currently, the only treatment for preeclampsia is to deliver the baby prematurely (Roberts, 2000). The goal is to find an alternative therapy to promote the safety of both the mother and fetus. One possibility is to examine the role of the vascular endothelial growth factor and the vascular endothelial growth factor receptor 2 (VEGF and VEGFR2) in uterine perfusion. We have hypothesized that increasing the expression of VEGFR2 can improve overall uterine perfusion in the pathology of preeclampsia.

The model we employed for preeclampsia pathology is the surgical Reduced Uterine Perfusion Pressure (RUPP) pregnant rat. This model is used because it mimics the utero-placental under-perfusion of preeclampsia. It has been shown that the RUPP model replicates common pathology associated with preeclampsia such as maternal hypertension, vascular dysfunction and fetal demise (Reho et al., 2011). Silver clips are placed around the abdominal aorta and the uterine-ovarian arteries after two full weeks of gestation. This surgical procedure decreases the uterine blood flow by 40% (Crews, 2000). Decreased blood flow causes the

decrease in uterine perfusion, and therefore deprives the fetus of sufficient blood. This cascade contributes to the maternal hypertension because her physiology must work in overdrive to provide the fetal-placental unit with sufficient blood, oxygen, and nutrients.

Another common characteristic of placental under-perfusion that is thought to occur is oxidative stress. Oxidative stress is a result of oxygen free radicals. One example of this is a superoxide anion. Increased oxygen radicals result in damage to lipids and DNA which leads to organ dysfunction (Sies, 1985). It has previously been shown that an increase in oxidative stress results in detrimental vascular behavior, seen as vasoconstriction, in RUPP models while investigating the effects of ascorbic acid, an antioxidant (Reho et al., 2011). In order to measure the levels of oxidative stress, we will employ a chemiluminescence assay that uses the compound lucigenin. In the presence of superoxide, this compound undergoes a one-electron reduction reaction, forming a cation radical which then reacts with superoxide to form an intermediate that decays by a light emitting process (Vásquez-Vivar, 1997). This signal can be read on a luminometer. The equation for this reaction is:



This research will specifically investigate the amount of oxidative stress in rats with increased VEGFR2 expression and those with unaltered VEGFR2 expression. The rats will be treated with L-tyrosine polyphosphate (LTP) nanoparticles, a nonviral gene therapy, designed to overexpress VEGFR2 and its co-receptor neuropilin-1 (NRP1) (Neufeld, 1999). The aim of this therapy is to specifically improve the utero-placental blood flow of both the maternal and fetal pathologies by selectively overexpressing VEGFR2 and NRP1, which increases NO (Ditto, 2006).

Materials and Methods

Preparation of HEPES buffer

HEPES buffer was used because it maintains conditions similar to physiological conditions in the body. We prepared 1 liter of 10X HEPES buffer by adding in order, 83g of NaCl, 3.50g of KCl, 1.60g of KH₂PO₄, 2.88g of MgSO₄*7H₂O, 3.68g of CaCl₂*2H₂O, and 23.83g of HEPES. Each of the solutes were added in the order listed after the previous one was dissolved. Deionized water was then added to the mixture until it equaled 1 liter. A stir bar was placed in the mixture to facilitate the process. The 10X HEPES buffer was then diluted to 1X to use for the homogenate and the lucigenin assay.

Ex: 50ml 10x HEPES buffer + 450ml deionized water = 1X HEPES

0.5 grams of glucose were added to the 1X HEPES. A stir bar was placed in the mixture and it was spun until the glucose was dissolved. The pH was taken and the mixture was titrated with 1 M NaOH until the pH was as close to 7.37 as possible without going above 7.40.

Animal Maintenance

The animals used in the experiment were housed in the vivarium at the University of Akron. Standard Operating Procedures from the IACUC were followed and other reference materials are available from the IACUC. The staff in the laboratory consisted of one animal medicine veterinarian and two technicians. The animal facility provided high quality of animal care, which included observing the animals daily for signs of illness. The animals were provided routine medical care. This included being observed daily by a technician and having their health status recorded twice weekly. Both the medical records and documentation of experimental use were kept and updated on each animal's cage card.

Preparation of Homogenate

Tissue samples from the placenta were taken and weighed. The goal was to obtain between 0.3-0.4 grams. To determine the amount of buffer needed for the homogenate the grams of tissue were divided by 0.1. Then the mass was subtracted from this number to determine how many milliliters of 1X HEPES to add. This was then converted to microliters.

Ex: tissue = 0.05g so: $0.05/10\% = 0.5\text{mL}$ $0.5-0.05 = .45\text{mL} = 450\text{uL}$

50 microliters of protease inhibitor cocktail were added to the placental tissue and 1X HEPES buffer. This was homogenized until no large chunks were present. The homogenate was placed in the centrifuge at 3 RPM for about 6 minutes. After this the red protein supernatant was collected in 100 microliter increments and each was placed in a bullet tube. All samples were stored at -80C.

Measurement of Protein Content in Placental Samples

A Bradford protein assay was used to determine the protein content of each placental sample. The standard curve consisted of six samples, each containing 600 microliters of Bradford Reagent. The first one contained no Bovine Serum Albumin (BSA) and 2400 microliters of deionized (DI) water. The second one contained 3.9 microliters of BSA and 2397 microliters of DI water. The third one contained 7.5 microliters of BSA and 2391 microliters of DI water. The fourth one contained 15 microliters of BSA and 2385 microliters of DI water. The fifth one contained 22.5 microliters of BSA and 2376 microliters of DI water. The sixth one included 30 microliters of BSA and 2370 microliters of DI water. For the placenta tissue, 4 samples were made. These each consisted of 200 microliters of the homogenate, 1800 microliters

of DI water and 600 microliters of Bradford Reagent in a test tube. The test tubes were all vortexed to make sure the contents were mixed and then let sit for 3 minutes. After all 10 samples were made, 6 standard and 4 homogenate, they were run in the spectrophotometer to record the absorbance at 595 nanometers. The standard curve was created using excel and the homogenate absorbances were compared to the curve to determine the protein content of the homogenate. It was necessary to determine the protein content of the placental samples because the amount of oxidative stress is relative to the amount of protein in the tissue. This is important because being able to compare levels of oxidative stress is the main goal of this specific research.

Superoxide Detection

A Lucigenin Chemiluminescence Assay was used for superoxide detection. The lucigenin solution was made at 100 micromolar/ liter. The NADPH solution was made at 10 millimolar/ liter. The tiron solution was made at 100 micromolar/ liter. The pH of the 1X HEPES buffer was measured and titrated with 1 M NaOH until about 7.37. The buffer was then preheated at 37C in a water bath for 10 minutes again and then the pH was measured again to make sure it was still at 7.37 and no higher than 7.40. The buffer that was not in use was kept in the water bath during the entire assay.

A luminometer from Turner Biosystems was used for this assay. The reactive light units (RLUs) were recorded every 5 seconds until 15 measurements were recorded. The average of each sample was taken and all numbers were recorded in excel. When the different assays were run for each tissue, the average RLU value for the baseline treatment was subtracted from each of the subsequent treatments. The 4 samples run consisted of a baseline, a superoxide baseline, NADPH stimulated superoxide production, and addition of tiron. The superoxide baseline

created a standard for the homogenate sample because it gave a measurement for any superoxides in the tissue before NADPH was used to stimulate it. The other baseline did not include any homogenate. Tiron was used to determine if the assay was superoxide specific because tiron reacts with superoxide anions (Greenstock, 1975). An internal standard was created using the same SHAM placental tissue to create a standard to compare the other assays to.

Baseline

400 microliters of 1X HEPES buffer and 0.6 microliters of lucigenin were mixed in a bullet tube and then incubated in the water bath at 37C for 3 minutes. The bullet tube was placed in the luminometer and the RLU's were recorded until 15 measurements were taken.

Superoxide baseline

100 microliters of homogenate, 400 microliters of 1X HEPES buffer and 0.6 microliters of lucigenin were mixed in a bullet tube covered in foil then incubated in the water bath for 3 minutes (still covered in foil in order to prevent light energy from activating the superoxides). The bullet tube was placed in the luminometer and RLU's were recorded until 15 measurements were taken.

NADPH Stimulated Superoxide Production

100 microliters of homogenate, 400 microliters of 1X HEPES buffer, 0.6 microliters of lucigenin and 10 microliters of NADPH were mixed in a bullet tube and

then incubated in the water bath at 37C for 3 minutes. The bullet tube was placed in the luminometer and the RLUs were recorded until 15 measurements were taken.

Tiron Protocol

100 microliters of homogenate, 400 microliters of 1X HEPES buffer, 0.6 microliters of lucigenin, and 50 microliters of Tiron were mixed in a bullet tube and then incubated in the water bath at 37C for 3 minutes. The bullet tube was placed in the luminometer and the RLUs were recorded until 15 measurements were taken.

Statistics

Data was normalized before doing statistical analysis. To normalize the data, the signal values will be divided by the protein concentration and subsequently compared with students T-test and ANOVA.

Results

Bradford Assay

Table 1. Standards Used in the Bradford Assay

BSA Std Curve 595 nm Abs Values

uL in Assay	ug in Assay	Abs
3	0	0
3	390	0.046
3	750	0.09
3	1500	0.148
3	2250	0.217
3	3000	0.261

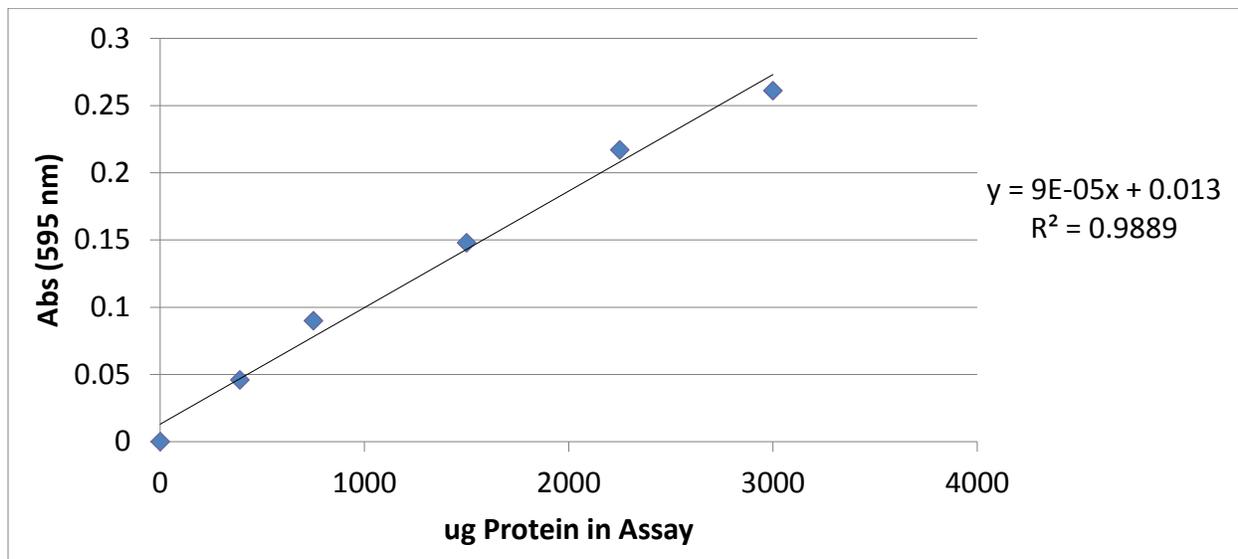


Figure 1: Standard curve for determination of protein concentrations in tissues. At a wavelength of 595 nm, the absorbance values were measured for known protein concentrations using BSA and Bradford reagent. The equation of the line determined from these values can be used to calculate the protein concentration (ug of protein) of unknown samples.

The standard curve in Figure 1 was used to determine the protein concentrations of the samples. The standard curve had to be corrected to start at an absorbance reading of 0 for a protein concentration of 0. Using the equation $y=mx+b$, protein concentrations for the homogenate samples were calculated. For example, both m and b are known from the standard, 0.00009 and 0.013 respectively, and y is the absorbance value obtained for the homogenate sample (the absorbance values for the samples are included in Table 2). Then the equation is solved for x , which is the protein concentration.

Ex: Sample 1

$$0.566 = (0.00009)x + 0.013$$

$$x = 6144.44 \text{ ug/mL}$$

However, because the homogenate was diluted when prepared this 1:10 dilution needs to be considered in the calculation.

$$6144.44 * 10 = 61444.4 \text{ ug/mL} = 61.444 \text{ ug/uL}$$

This calculation was done for the four homogenate samples and then the average was calculated to be used later to normalize data from the lucigenin assay. The calculations are listed below in Table 3. Because the standard curve had to be corrected, protein concentration calculations may be incorrect.

Table 2. Absorbance values for placental samples

Unknown Samples 595 nm Abs Values

uL in Assay	Sample 1	Sample 2	Sample 3	Sample 4
2600uL	0.566	0.524	0.562	0.565

Table 3. Calculated protein concentrations for placental samples

Unknown Sample Concentration (ug/uL)

uL in Assay	Sample 1	Sample 2	Sample 3	Sample 4
2600uL	61.444	56.778	61.000	61.333
AVERAGE=	60.139			

Lucigenin Assay

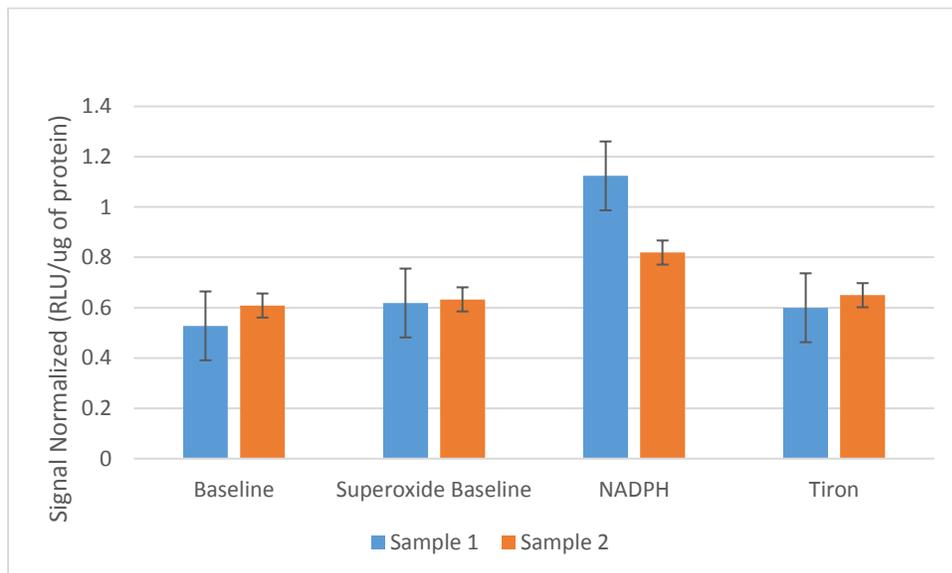


Figure 2: Measurement of superoxide levels in placental tissue from SHAM animals. Placenta was isolated from SHAM animals and X ug of protein was isolated and used to normalize superoxide measurements. Basal levels of superoxide were obtained using a Lucigenin Chemiluminescence Assay and compared to a positive control (NADPH addition). Reactive oxygen species was also measured after the addition of the superoxide scavenger tiron to confirm specificity (n=2 animals).

These two sets of data are for samples from the SHAM placental tissue. After recording 15 RLUs for the baseline, superoxide baseline, NADPH, and tiron an average was calculated for each. This was done for each of the SHAM samples. These averages were then normalized by dividing by the protein concentration of the samples so that the numbers can be compared. The baseline consisted of just lucigenin and buffer to create a baseline for the lucigenin. The superoxide baseline consisted of homogenate plus the lucigenin and buffer. This allows for a baseline of the superoxide in the homogenate before being stimulated to react with the lucigenin. One of the main sources of oxidative stress in vascular smooth muscle membranes is NADPH

oxidase (Sorescu, 2000). Therefore, more reactive oxygen species are created when NADPH is used as the substrate. The lucigenin then has more free radicals to react with and this results in the larger RLU measurements (Figure 2). Tiron is a direct $O_2^{\cdot-}$ scavenger and is used to confirm detection of radicals in this assay. In Figure 2, the RLUs for tiron are similar to the baselines indicating that tiron reacts with the superoxide anion and prevents the lucigenin from reacting with the superoxide (Münzel, 1995).

Discussion

At the time of writing, these experiments are still ongoing. As seen in the results section, data was only collected for the SHAM placental tissue. Throughout the rest of the experiment, data will also be recorded for the RUPP placental tissue and the RUPP placental tissue that received the nanoparticle. After this, the data of the three groups will be compared to see if it supports our hypothesis. The SHAM group was used as the control to create a baseline for oxidative stress levels in the rats. The RUPP group was the experimental group which exhibited pathologies similar to preeclampsia (Reho et al., 2011). Oxidative stress levels measured in this tissue would give insight into the damage that results from the preeclampsia pathology. Based on these two baselines for oxidative stress, the placental samples from VEGFR2 treated RUPP animals should exhibit normalized oxidative stress levels significantly different from the untreated RUPP animals but similar to the SHAM controls, in order for our data to support our hypothesis.

The RUPP rats treated with the LTP nanoparticle represent the group with increased VEGFR2 expression and the other group of RUPP rats have unaltered VEGFR2 expression (Neufeld, 1999). We hypothesize that there is a decrease in VEGF signaling due to the decrease

in placental perfusion. Because VEGF signaling results in things such as maternal hypertension, constrictive phenotype small arteries, placental insufficiency and fetal growth restrictions, it contributes to the pathology of preeclampsia (Li, 2007). The hypothesis is that this therapy with LTP nanoparticles will improve both the maternal and fetal pathologies resulting from preeclampsia.

My specific part in the experiment was to measure the oxidative stress levels of placental tissue samples each of the three groups: SHAM, RUPP, and RUPP treated with the nanoparticle. Oxidative stress results from the placental under-perfusion and any deficiencies caused by this. The decrease in uterine perfusion is due to decreased blood flow to the fetus. This then requires the mother to work in overdrive to provide the fetal-placental unit with sufficient blood, oxygen, and nutrients, resulting in maternal hypertension (Negi, 2011). This means that when comparing the SHAM to the RUPP, the levels of oxidative stress should be significantly lower because it does not exhibit the damage due to conditions of preeclampsia. Along with this, when comparing the RUPP to the RUPP treated with the nanoparticle, the levels of oxidative stress should be significantly higher because the overexpression of VEGFR2 we hypothesized would improve overall uterine perfusion and therefore decrease oxidative stress levels (Grummer, 2009).

The luminometer was used to measure the emission of light from the reaction of lucigenin and superoxide. The lucigenin yielded a chemiluminescence that is specific for extracellular superoxide anion (Vásquez-Vivar, 1997). This measurement gave us a signal which indicates the oxidative stress level. For each sample the signal was divided by the protein content so as to normalize each number so the different samples can be compared.

Although what is previously described is what was expected to happen, we encountered some difficulties that did not allow us to complete the experiment by this time. We were only

able to record measurements for oxidative stress for the SHAM placental tissue. Most of the data we collected gave us inaccurate readings so we could not create a solid baseline from the SHAM group. Without the baseline, it would have been impossible to compare the other two groups. We did numerous things to try to correct this issue but none seemed to be successful enough to move onto the RUPP groups knowing we had a solid baseline. Some things we did were remake the buffer, remake the lucigenin, and make the homogenate fresh every time we were recording measurements using the luminometer. One more possible solution would be to ask an expert to make sure the luminometer is functioning properly. For the future of this experiment, I hope that the rest of the data will be obtained so as to determine whether or not the hypothesis is supported. If it does support the hypothesis, it could result in an alternative treatment which can improve conditions and increase the safety of both the mother and fetus.

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