NIH SHORT-TERM RESEARCH TRAINING FOR MINORITY STUDENTS

SYMPOSIUM PROGRAM & ABSTRACTS
HEALTH PROFESSIONS, NURSING, PHARMACY (HPNP)
BUILDING ROOM G-316

UF UNIVERSITY of FLORIDA College of Medicine

UF UNIVERSITY of FLORIDA

July 24, 2009
9:00 A.M.

University of Florida College of Medicine
Office of Minority Affairs

NIH SHORT-TERM RESEARCH TRAINING FOR MINORITY STUDENTS

The program's success is due largely to the active participation of the faculty mentors listed below:

Christine Baylis, Ph.D.	Department of Physiology & Functional Genomics
David Bloom, Ph.D.	Department of Molecular Genetics & Microbiology
Arturo Cardounel, Ph.D.	Department of Physiology & Functional Genomics
Kirk P. Conrad, M.D.	Department of Physiology & Functional Genomics
Judy Muller-Delp, Ph.D.	Department of Physiology & Functional Genomics
Carrie Haskell-Luevano, Ph.D.	Department of Pharmacodynamics
Linda Hayward, Ph.D.	Department of Physiological Sciences
Mavis Agbandje-McKenna, Ph.D.	Department of Biochemistry & Molecular Biology
Lucia Notterpek, Ph.D.	Department of Neuroscience
S. Paul Oh, Ph.D.	Department of Physiology & Functional Genomics
Deborah Scheuer, Ph.D.	Department of Physiology & Functional Genomics

The Short-Term Research Training for Minority Students program is funded by a grant from the National Institutes of Health, National Heart, Lung and Blood Institute and supported by the University of Florida College of Medicine. The grant's principal investigator, Charles E. Wood, Ph.D., is the Professor and Chair for the Department of Physiology at the University of Florida.

STUDENTS	AFFILIATION
Erie, Christine	Cornell University
Flores, Viktor	University of Florida
Fraga, Kelli	University of Florida
Gonzalez, Rafael	University of Central Florida
Herrera, Diana	University of Houston
Johnson, Michael	University of Central Florida
Quintana, Ana	California State University, Monterey Bay
Khan-Thomas, Shehzad	Florida A&M University
Rangel, Alyssa	University of Notre Dame
Sterling, Myrline	Barry University

SYMPOSIUM PROGRAM

WELCOME 9:00-9:15

Charles E. Wood, Ph.D., Professor and Chair for the Department of Physiology

Michelle E. Jacobs, M.D., Assistant Dean, Office of Minority Affairs and Assistant Professor, Department of Psychiatry, College of Medicine

SESSION I

SESSION CHAIR:
Deborah Scheuer, Ph.D., Associate Professor, Department of Physiology and Functional Genomics

Diana Herrera 9:15-9:30
University of Houston
Project: **"Validation and Characterization of a Trophoblast Cell Lineage"**
Mentor: Kirk P. Conrad, M.D., Professor, Department of Physiology and Functional Genomics

Viktor Flores 9:30-9:45
University of Florida
Project: **"Synthesis of Peptides for the Melanocortin Receptors and Functional Characterization at the mMC3R"**
Mentor: Carrie Haskell-Luevano, Ph.D., Professor, Department of Pharmacodynamics

Christine Erie 9:45-10:00
Cornell University
Project: **"Immunohistochemical c-fos expression in the parabrachial nucleus and the afferent connections of areas related to acute stress in a SHR rat"**
Mentors: Linda Hayward, Ph.D., Associate Professor, Department of Physiological Sciences and Deborah Scheuer, Ph.D., Associate Professor, Department of Physiology and Functional Genomics

Michael Johnson 10:00-10:15
University of Central Florida
Project: **"Vascular Smooth Muscle Response to AMP-Activated Kinase"**
Mentor: Judy Muller-Delp, Ph.D., Associate Professor, Department of Physiology and Functional Genomics

Myrline Sterling 10:15-10:30
Barry University
Project: **"Exercise effects on renal endothelial enzymes: Impact of Ischemia/Reperfusion induced Acute Renal Failure in the Fischer 344 (F344) rat"**
Mentor: Chris Baylis, Ph.D., Professor, Department of Physiology and Functional Genomics

BREAK 10:30-11:00

SESSION II

SESSION CHAIR:
Arturo Cardounel, Ph.D., Assistant Professor, Department of Physiology & Functional Genomics

Kelli Fraga 11:00-11:15
University of Florida
Project: **"Determining whether peripheral myelin protein 22 is palmitoylated at cysteine 85"**
Mentor: Lucia Notterpek, Ph.D., Associate Professor and Chair, Department of Neuroscience

Rafael Gonzalez 11:15-11:30
University of Central Florida
Project: **"Role of Dihydrofolate Reducate in Diabetic Vasculopathy"**
Mentor: Arturo Cardounel, Ph.D., Assistant Professor, Department of Physiology & Functional Genomics

Ana Quintana 11:30-11:45
California State University, Monterey Bay
Project: "**ALK1 Conditional and Null Endothelial Cells Permanently Expressing Green Fluorescent Protein"**
Mentor: S. Paul Oh, Ph.D., Associate Professor, Department of Physiology and Functional Genomics

Shehzad Khan-Thomas 11:45-12:00
Florida A&M University
Project: **"Structural Identification of Receptor Binding Sites on Adeno-Associated Virus Serotype 6"**
Mentor: Mavis Agbandje-McKenna, Ph.D., Professor, Department of Biochemistry and Molecular Biology

Alyssa Rangel 12:00-12:15
University of Notre Dame
Project: **"Characterization of the Herpes Simplex Virus Type 1 Insulator Function by the Deletion of Two CTCF-binding Motifs in the Latency Associated Transcript Region"**
Mentor: David Bloom, Ph.D., Professor, Department of Molecular Genetics and Microbiology

LUNCH 12:15-1:15

PRESENTATION OF CERTIFICATES 1:15-1:30

STUDENT GROUP PICTURE 1:30-1:45

Valadation and Characterization of a Trophoblast Cell Lineage

Diana Herrera, University of Houston
Kirk P. Conrad, M.D., Department of Physiology and Functional Genomics,
University of Florida

Background: Preeclampsia is a disease that occurs during pregnancy with symptoms including hypertension and proteinuria. Although the etiology of the disease is unknown, maternal disease manifestations are due to endothelial dysfunction (1). Trophoblast cells are placental cells that, during normal pregnancy, invade the uterine wall and spiral arteries, ensuring unimpeded blood flow to the placenta (and fetus). These trophoblast cells have many endothelial cell-like properties and they transform the spiral arteries into low-resistance, high-flow channels that allow the fetus to be supplied with appropriate amount of oxygen and nutrients. In preeclampsia, trophoblast invasion is impaired which in turn hinders the transformation of the spiral arteries. The reduced blood flow causes hypoxia within the placenta and oxidative stress upon reperfusion. It is theorized that the hypoxia and oxidative stress cause the placenta to release anti-angiogenic and various other factors that affect the maternal endothelium, and therefore produce the known symptoms of preeclampsia (reviewed in 2).

Endothelin and the endothelin B receptor (ETB) are known to activate endothelial nitric oxide synthase (eNOS) to produce NO which is a vasodilator. In addition, this pathway contributes to the migration of endothelial cells (3). Various research supports the theory that vascular endothelial growth factor/ placental growth factor (VEGF/PlGF) and matrix metalloproteinases (MMP-2 and MMP-9) are upstream activators of this pathway that are activated through relaxin and its receptor, the leucine-rich repeat-containing G protein-coupled receptor (LGR7) (4). The focus of this research is to determine whether the genes within this pathway are expressed by trophoblast cells. By activating this pathway, relaxin may promote spiral artery vasodilation and trophoblast migration and invasion via production of NO.

Methods: Immortalized HTR-8/SVneo (first trimester trophoblast) cells propagated in culture were obtained from Queen's University Department of Anatomy and Cell Biology (Charles H. Graham, PhD). The cells were cultured in T-75 flasks to 90% confluency in RPMI-1640 with L-Glutamine Media with 5% FBS and a 1:1000 dilution of penicillin/streptomycin at 37°C and 5% CO_2. The cells were lysed using 2.5 mLs of Tri reagent and the extracted RNA was dissolved in 30µL of RNA secure resuspension solution. DNAse-Free protocol was performed to remove any contaminating DNA. The yield and purity of the RNA was determined by spectrophotometry and agarose gel electrophoresis, respectively. cDNA was then synthesized. RT-PCR was performed to determine the presence of β-actin (housekeeping gene), placental alkaline phosphatase (ALPP), aromatase enzyme, chorionic somatomammotropin hormone (CSH1), major histocompatibility complex (HLA-G), LGR7, VEGF, PlGF, MMP-2, MMP-9, ETB receptor, eNOS, sFlt-1, Flt-1, KDR and relaxins – H1, H2, and H3. All primers were designed using OligoPerfect. RT-PCR was performed with a 25µL sample volume constituting of: 14.25 µL of NFW, 5 µL of 5x Buffer, 1.5 µL of 25mM $MgCl_2$, 1 µL of dNTPs, 1 µL of Forward Primer, 1 µL of Reverse Primer, 0.25 µL of GoTaq DNA Polymerase and 1µL of cDNA. RT-PCR was performed for 20-40 cycles at 94°C for 30 seconds, 50-55°C for 1 minute and 72°C for 1 minute along with 94°C for 3 minutes and 72° for 10 minutes as initial denaturation and final elongation steps, respectively. The products were then run on a 1% agarose gel containing 0.5 µL of Ethidium Bromide in 0.5 x TBE Running Buffer. UV light was used to visualize the products and analyze results. Negative controls containing 1µL of NFW instead of 1µL of cDNA were run alongside each respective RT-PCR product.

Results: The RNA A_{260}/A_{280} ratios were between 1.8-1.9 documenting the purity of the samples, and the RNA gel electrophoresis showed strong 18 and 28s RNA bands. By RT-PCR, the HTR8/SVneos clearly expressed hALPP, HLA-G, and aromatase enzyme, but not hCSH1. The cells also express LGR7, VEGF, PlGF, MMP-2, MMP-9, ETB receptor, eNOS and H2. Bands were excised and sent for sequencing. All matched their respective gene sequences $\geq$ 97%. All negative control lanes were blank.

Conclusions: The expression of hALPP, HLA-G and the aromatase enzyme strongly supports the trophoblast lineage of the cells. hCSH1 may not have been expressed, because it is typically present in multinucleated cells which are few in culture (5). The identification of LGR7 within the cells indicates the potential for relaxin signaling. Finally, all of the components of the relaxin vasodilatory pathway are also expressed by the HTR-8/SVneo cells. In future studies, therefore, we will test whether relaxin can increase NO production in HTR-8/SVneo trophoblast-derived cells, promote their migration, and invasion into a Matrigel matrix.

Synthesis of Peptides for the Melanocortin Receptors and Funtional Characterization at the mMC3R

Viktor Flores, University of Florida
Prof. Carrie Haskell-Luevano, Ph. D, Department of Pharmacodynamics, University of Florida

Background

The melanocortin receptor system consists of five (MC1R-MC5R) G-protein coupled receptors (GPCRs) that regulate a myriad of different physiological functions [Haskell-Luevano, "Melanocortin Ligands", 2004]. Of these five receptors, the MC3R and MC4R are currently the most fascinating to researchers because of their role in regulating energy and weight homeostasis and controlling food intake. When the melanocortin receptors are stimulated by their agonist ligands, they activate the cyclic adenosine monophosphate (cAMP) signal transduction pathway, which generates a cascade of intracellular reactions that give a particular physiological function. The endogenous agonist of these melanocortin receptors are α-, ß-, and γ-melanocyte-stimulated hormones (α-, ß-, γ-MSH) and adrenocorticotropin hormone (ACTH), which are all derived from posttranslational processing of the proopicomelanocortin (POMC) gene transcript [Haskell-Luevano, "Progress in Development", 2004]. Unlike any other receptor in the GPCR, the melanocortin receptor system contains two endogenous antagonist ligands, the Agouti and Agouti-related protein (AGRP). It has been shown that when the Agouti or AGRP is overexpressed in the knockout mice without the POMC gene, it causes the mice to become overtly obese. Similarly, when α-MSH is administered through ICV to these mice, they experience substantial weight loss [Yaswen, 1999]. These results are very promising to researchers wanting to synthesize therapeutic drugs for obesity and other related diseases because with the development of potent and selective small molecules that can bind to the MC3R or MC4R similarly to an agonist; food intake can be controlled and stabilized.

In order to develop small molecules that can be used as therapeutic agents for treating obesity at the MC3R and MC4R, researchers must first study the ligand-receptor relationship that exist in the melanocortin system and identify the role and effect of each amino acid of the agonist in stimulating the receptors. All Melanocortin agonist have the core tetrapeptide sequence of His-Phe-Arg-Trp that has been attributed to melanocortin receptor binding and stimulation [Haskell-Luevano, Melanocortin Ligands, 2004]. However, it has been found that the stereochemical inversion of Phe to D-Phe increases the potency of the tetrapeptide. Studies of the endogenous agonist also identified a potent 13 amino acid peptide NDP-MSH that is similar in sequence to α-MSH but is more potent and biologically stable due to the substitution of Nle for Met at the four position and the substitution of D-Phe for Phe at the seven position. This peptide has been used extensively to characterize the melanocortin receptor and has been the lead compound in the generation of many Structure-activity relationship (SAR) studies [Haskell-Luevano, Melanocortin Ligands, 2004]. The objective of this study was to learn the technique for synthesizing the NDP-MSH and other peptides using Fmoc solid phase peptide synthesis (SPPS), and to purify and characterize the final product.

Method

To synthesize the NDP-MSH peptide (Ac-Ser-Tyr-Ser-Nle-His-DPhe-Arg-Trp-Gly-Lys-Pro-Val-NH_2) and a pentapeptide (Ac-His-DPhe-Arg-Gly-Trp-NH_2), I utilized Fmoc SPPS. Fmoc SPPS is a method in which a solid polymeric support, called the resin, is used to anchor a nascent peptide. This resin allows the nascent peptide to sequentially grow one amino acid at a time. The Fmoc is a temporary protecting group attached to the N-terminus of the amino acid which protects each intermediate peptide from making undesired residues. Rink Amide MBHA was

used for the synthesis of the NDP-MSH and the pentapeptide. To couple an amino acid into the growing chain, 20% Piperidine in DMF was used to deprotect the Fmoc from the reactive N-terminal of the amino acid. For each amino acid being coupled, excess of the sequential Amino acid was added to the resin-peptide with the coupling reagents DIEA and BOP in DMF for 2 hours under nitrogen gas. Cleavage of the resin from the peptide was performed with cleavage cocktail (95% TFA: 2.5% H_2O: 2.5% TIS). The cleavage product was then washed and purified by reverse phase HPLC and then lyophilized. The purified peptides were analytically characterized by reverse phase HPLC using water and methanol.

To learn the functional characterization of the synthesized peptide in the melanocortin receptors, NDP-MSH peptide synthesized by my co-worker was used and tested on human embryonic kidney cells (HEK 293) expressing mouse MC3R. These cells were transfected with (CRE)/ß-galactosidase cells for 24 hours. Forty-eight hours after transfection, the cells were stimulated with the peptide compound in solution with IBMX, 1% BSA in PBS, and DMEM. The cells were then lyse with a buffer and placed in a 96 well plate. ONPG was then added to the cell plate, which is a substrate for the ß-galactosidase, and ONPG turns to ONP, a yellow solution. ONP is produced in proportion to the amount of ß-galactosidase enzyme, which was produced in proportion to the amount of cAMP, which was formed in amounts proportional to the degree of stimulation of the receptor. The amount of the yellow solution was then measured with a spectrophotometer to assess the potency of the peptide at the MC3R.

Results

For the pentapeptide (Ac-His-DPhe-Arg-Gly-Trp-NH_2), 26.5mg of the pure compound was synthesized and characterized by RP-HPLC. The NDP-MSH was synthesized but due to time constraints, I was not able to purify the compound.

Conclusion

This study successfully showed me how to synthesize and purify peptides and how to characterize the functionality of an agonist ligand on the melanocortin receptors using CRE/ß-gal technique. Fmoc SPPS is a fundamental technique used by pharmacologist to synthesize peptides efficiently using a resin as polymeric support to extend an amino acid chain. In this study I synthesized a pentapeptide that will be used in future studies to characterize the effect of adding a Gly amino acid at the fourth amino acid of the core tetrapeptide His-DPhe-Arg-Trp. The NDP-MSH compound will also be used in future studies with a fluorophore attached at the end of the chain in order to better understand the ligand-receptor relationship of the melanocortin system. Characterizing the functionality of a peptide on the melanocortin receptor using CRE/ß-gal is what helps pharmacologist determine the selectivity and/or potency of an agonist ligand.

References

Haskell-Luevano, Carrie, and Jerry Holder. "Melanocortin Ligands: 30 Years of Structure-Activity Relationship (SAR) Studies." *Current Pharmaceutical Design* 24(2004): 325-356.

Haskell-Luevano, Carrie, et al. "Progress in the Development of Melanocortin Receptor Selective Ligands." *Current Pharmaceutical Design* 10(2004): 3443-3479.

Yaswen, Diehl, Brennan MB, Hochgeschwender U. "Obesity in Mouse Model of Pro-opiomelanocortin Deficiency Responds to Peripheral Melanocortin." *Nat Med* 5(1999): 1066-1070.

Immunohistochemical c-fos expression in the parabrachial nucleus and the afferent connections of areas related to acute stress in a SHR rat

Christine J. Erie, Cornell University

Linda F. Hayward, PhD., Department of Physiological Sciences, University of Florida
Deborah A. Scheuer, PhD, Department of Physiology and Functional Genomics, University of Florida

Background and significance: Cardiovascular disease is the number one cause of death in the world (WHO). In fact, stress is a risk factor for cardiovascular disease and heart attacks. Acute stress rapidly increases blood pressure and chronic stress leads to hypertension, a long term increase in blood pressure. The Spontaneously Hypertensive Rat (SHR) model expresses an exaggerated stress response and develops age-related hypertension. Additionally, the amygdala is known to play role in blood pressure regulation in response to acute stress (Saha 2005 & Gianaros 2008). Previous experiments in our lab have shown elevated activity in the amygdala of the SHR rat in response to stress. However, the neural pathways and mechanism of this exaggerated cardiovascular stress response are not well understood. The focus of this investigation was to determine if another brain region, the parabrachial nucleus (PBN) mediates amygdala activation during acute stress.

Methods: This investigation contained three treatment groups: air jet stress (AJS), noise control (NC) and no treatment (NT) group. Each treatment group contained a normotensive strain (Wistar) and SHR rats. The AJS group (n=7/strain) was subjected to 90 minutes of baseline blood pressure recording from a femoral catheter, 20 minutes of 20 psi AJS [random- jet stream location changing every 30-60 seconds]. The NC group (n=3-4/strain) was subjected to pressurized air noise for 3 hours. The NT group (n=5-6/strain) was not subjected to any stress stimulus. To determine if SHR had greater c-fos expression in response to stress in the PBN, the brain tissue was obtained 2 hours after the initiation of stress then processed for c-fos immunohistochemistry and counted blind to the rat strain and treatment group.. To detect if the PBN directly projects to the CeA, fluorogold (FG) retrograde tracer was injected into the central nucleus of amygdala (CeA) of the SHR 5-6 days before exposure to AJS (n=2) or the NC stimulus (n=1). The brains were the process for c-fos and fluorogold. The number of c-fos and fluorogold co-labeled neurons was counted while blinded to treatment group.

Results: Regardless of rat strain, the AJS group expressed more c-fos in all subnuclei of the parabrachial nucleus than the NC group and the NT group. There was not a significant difference in c-fos expression between NC SHR rat and NC Wistar rats. Also, there was not a difference in c-fos expression between NT SHR rats and NT Wistar rats. However, the AJS SHR rats expressed more c-fos in several of the subnuclei in the middle region of the parabrachial nucleus than the AJS Wistar rats ($P<0.05$). The fluorogold tracings were observed in the parabrachial nucleus, nucleus tractus solitarius (NTS), and the paraventricular nucleus (PVN).

Conclusions: These results show a relationship between acute stress and neural activation in the parabrachial nucleus of both the Wistar rat and the SHR rat. Like previous experiments which investigated amygdala activation, the SHR rat expressed more neural activation in the parabrachial nucleus than the Wistar rat. This suggests that the parabrachial nucleus plays a major role in regulating an SHR's response to stress stimuli. The fluorogold tracer injected into the amygdala traced to the PBN, PVN and NTS, indicating that these brain regions all project to the CeA. These results will help reveal the neurological pathway of the SHR exaggerated cardiovascular response and may lead to the specific treatments for cardiovascular disease in hypertensive individuals.

Vascular Smooth Muscle Response to AMP-Activated Kinase

Michael Johnson, University of Florida

Judy Muller- Delp, Ph.D., University of Florida, Department of Physiology and Functional Genomics

Background and significance:

AMP- Activated Kinase (AMPK) plays a major role in homeostasis. AMP: ATP ratios regulate the amount of AMPK present in the cell. AMPK can play a significant role via two pathways in the vascular smooth muscle. The protein affects the ability of the vascular smooth muscle to proliferate via the mTOR pathway (negative feedback) and it increases the contractibility of the vascular smooth muscle (positive feedback). The purpose of this study was to determine whether AMPK contributes to regulation of vascular homeostasis in the vascular smooth muscle of coronary resistance arteries, and in particular, to determine whether AMPK contributes to regulation of aging in the coronary vasculature.

Hypothesis

Age decreases AMPK in vascular smooth muscle of coronary resistance arteries, contributing to a transition from predominantly contractile phenotype to a more proliferative phenotype.

Methods:

Young (6 months) and old (24 months) male Fisher 344 rats were anesthetized with isofluorane and the hearts were removed. Coronary resistance arteries (100- 200 microns inner diameter) were isolated from the myocardium. In vitro videomicroscopy was used to study vasoconstrictor responses of pressurized arteries to the thromboxane analogue, U46619. Other coronary resistance arteries were isolated and prepared for immunohistochemical and immunoblot analysis.

Results:

Contractile responses to U46619 were significantly greater in arteries from young rats as compared to arteries from old rats. Measure of wall thickness and luminal diameter showed wall-to-lumen ratio increased in arteries from old rats. Data from the immunohistochemistry and western blots are still being acquired; however, preliminary results suggest that there is a trend of increased proliferation with age, as well as, the amount of AMPK present decreases with age.

Conclusions:

These data indicate that a transition from a more contractile phenotype to a more proliferative phenotype occurs in the vascular smooth muscle of coronary resistance arteries with age. Ongoing immunohistochemical analysis will determine if vascular smooth muscle cells of arteries from old rats are in a more proliferative state. Immunoblots will determine the amount of AMPK present in arteries from young and old rats. Further studies will provide more conclusive finding regarding the role played by AMPK in aging coronary vascular smooth muscle.

Exercise effects on renal endothelial enzymes: Impact of Ischemia/Reperfusion induced Acute Renal Failure in the Fischer 344 (F344) rat.

Myrline Sterling[1], Natasha Moningka[2], Mark Cunningham[2], Chris Baylis[2, 3].
[1]Barry University, Miami Shores, FL, [2]Department of Physiology and Functional Genomics, University of Florida, Gainesville, FL, [3]Department of Medicine, University of Florida, Gainesville, FL

Background and significance:

Exercise is beneficial to the cardiovascular system for many reasons. Some of the benefits are shear stress mediated and related to increase blood flow which stimulates endothelial nitric oxide (NO) production. NO is generated by the enzyme endothelial NO synthase (eNOS). It acts as a potent vasodilator and inhibits platelets aggregation and vascular smooth muscle proliferation. NO deficiency contributes to hypertension and increased risk of cardiovascular disease. Extracellular superoxide dismutase (EC SOD), an antioxidant located in the extracellular space, protects NO bioavailability. Both eNOS and EC SOD production are stimulated by increases in blood flow. While exercise increases blood flow to many organs such as skeletal muscle, heart, and lung, exercise ***decreases*** renal blood flow which by decreasing endothelial shear could decrease production of eNOS and EC SOD. Therefore, exercise could reduce renal NO bioavailability and increase oxidative stress in the kidney. In addition to impacting basal kidney function this could also make the kidney more vulnerable to acute injury. The most common cause of acute renal failure (ARF) is ischemia/reperfusion (I/R) injury and much of the damage is secondary to oxidative stress. Physical injury occurs as a result of intense renal vasoconstriction and renal hypoxia, leading to prolonged falls in glomerular filtration rate (GFR) and tubular damage. We will use an animal model to test the hypothesis that I/R induced ARF injury is exacerbated by chronic exercise. By measuring a number of determinants of the NO pathway, we hope to better understand the impact of exercise on the kidney.

Hypotheses:

1. Exercise will lead to falls in renal eNOS and EC SOD.
2. This will render the exercised kidney more vulnerable to I/R-ARF injury.

Materials and Methods:

Male F344 rats 10-12 wks old were randomly divided into a sedentary control (SED-Contra, SED-IR; n=6-12) and an exercise (EX-Contra, EX-IR; n=6-12) group. They were housed singly and allowed 24 hr access to food and water. EX rats were given access to individual wheels (Lafayette Instrument co., Lafayette, IN) for 12 wks. Odometers interfaced with a computer continuously counted and recorded the number of wheel rotations. Wheel running was completely voluntary and all rats increased their running over a 3-4 week period. 48 hours prior to I/R surgery, rats were given a low NOx diet and placed in metabolic cages for overnight urine collection. Rats were anesthetized and had their left renal pedicle completely occluded with a clamp (full sterile technique) for 35 min, during which their right (control or contralateral; contra) kidney was removed. Rats were then sutured, recovered and 24 hr later prepared for a terminal renal function study. Rats were anesthetized with Inactin (ip, 120 mg/kg) and placed on

heated table (body temp. maintained at 37±1°C). A tracheostomy was performed to provide an oxygen rich environment by exposing the tracheal tube to 95% O_2 and 5% CO_2 to stabilize blood pressure (BP). The left femoral artery and vein were catheterized for BP monitoring and arterial blood sample collection and infusion of 0.9 % NaCl containing inulin (for GFR measurement), respectively. Artificial plasma was also infused to maintain a euvolemic state. A non-occluding catheter was placed in the renal vein for measurement of renal inulin extraction and calculation of renal plasma flow (RPF). The bladder was catheterized for urine collection. After 60 min of stabilization period, 2 x 20 min urine collections and mid point blood samples were taken for inulin. Relative protein abundances of eNOS, EC SOD, Mn SOD, CuZn SOD, p22phox in renal cortical tissues were measured via Western blot. Bands were detected using enhanced chemiluminescent reagents and quantified by densitometry using the VersaDoc Imaging System and One Analysis Software (BioRad). The H_2O_2 assay was conducted according to the Amplex Red H_2O_2/Peroxidase Assay Kit (A22188) with the following modifications: Tissues were homogenized with 1x PBS (1:3 dilution) and H_2O_2 activity was confirmed using 2000 units of catalase. Urine protein was analyzed using the Bradford Protein assay and urine/tissue NOx (as a measure of NO production) was analyzed using the Greiss reaction. Plasma creatinine was measured by HPLC.

Results:

Running activity stabilized by week 4 and continued throughout the 12 wks (~3-4 km/day). In response to 12 wks of exercise the relative abundance of eNOS, EC SOD (and the other SOD isozymes CuZn and Mn), increased in the renal cortex from the control right kidney compared to the control right kidney of the SED rat. Oxidative stress markers of p22phox and H_2O_2 in the renal cortex also increased with exercise. Moreover, although not significant, the concentration of NOx (stable metabolites of NO; NOx = NO_2 + NO_3) in the kidney cortex had a tendency to also increase with exercise.

In response to I/R-induced ARF, renal function decreased as expected as seen by the reduction of GFR, RPF, and increased plasma creatinine (PCr). 12 wks of exercise training prior to I/R-induced ARF did not affect the functional response since the increase in plasma creatinine and falls in GFR and RPF were similar in EX vs. SED groups.

Conclusion:

Twelve weeks of voluntary exercise led to increases in renal eNOS and EC SOD. This is in contrast to our hypothesis since we predicted falls in eNOS and EC SOD in EX vs. SED, as a result the known exercise-induced reductions in RBF. Therefore, there is another mechanism by which renal eNOS and EC SOD are regulated during exercise. This was unexpected and very interesting and motivates further study on the mechanism of eNOS/EC SOD regulation in high flow parts of the circulation during exercise. Despite the enhanced renal eNOS and EC SOD abundance there was no significant protection afforded by exercise against the declines in renal function as a result of I/R-induced ARF.

Determining whether peripheral myelin protein 22 is palmitoylated at cysteine 85.

Kelli Fraga, University of Florida
Lucia Notterpek, Ph.D., Susie Zoltewicz, Ph.D.
University of Florida, Department of Neuroscience

Background:

Myelin proteins, such as peripheral myelin protein 22 (PMP22) must be present in the correct amount in the plasma membrane of Schwann cells to support neuronal activity. PMP22 is one myelin protein that leads to disease if mutated, over, or under expressed. Point mutations and duplications are associated with Charcot-Marie-Tooth disease, while deletions are associated with Hereditary Neuropathy with Liability to Pressure Palsies. Both of these diseases are characterized by overall muscle weakness with numbness, pain, and/or deformities in the extremities and can lead to muscular atrophy and paralysis. Yet to date it is largely unknown how correct amounts of PMP22 are trafficked to the plasma membrane and what post-translational modifications of the protein are involved. One mechanism that could mediate the membrane trafficking and insertion of PMP22, and other myelin proteins, is palmitoylation. S-palmitoylation is a reversible post-translational modification that results in the addition of a C16 saturated fatty acid chain to cytoplasmic cysteine residues. PMP 22 sequence was run though palmitoylation site prediction software (CSS-Palm) and cysteine 85 (C85) received the highest score of 0.95, suggesting that C85 may be palmitoylated.

Short Term Aim:

To determine whether PMP22 is palmitoylated at C85.

Long Term Aims:

1. To determine if palmitoylation is critical to the membrane localization of PMP22.
2. To assess and verify other potential sites of palmitoylation or lipidation within PMP22.

Methods:

Within the mouse PMP22, C85 was mutated to a serine (C85S) using PCR. The DNA was transformed into turbo competent E-coli cells and grown on amp plates over night. DNA was made from the colonies using mini preps. The DNA was quantified using a DNA spectrophotometer, cut with HindIII and XhoI enzymes, and run on 1% agarose gels to determine DNA size. The DNA was then sent out for sequencing to identify DNAs having desired point mutation. A palmitoylatable yellow fluorescent protein (Palm-YFP) was used as a positive control; this protein can be palmitoylated at two sites. The C85S mutant, wild-type, and Palm-YFP DNA were transfected into primary rat Schwann cells. Transfected cells were incubated for 48 hours. During the second 24 hours, 50 or 100 μM of the lipid 17-Octadecynoic acid (ODYA) added to the cell media. ODYA is taggable palmitate analog that can be used to detect palmitoylated proteins. ODYA was dissolved in dimethyl sulfoxide (DMSO). As a control, DMSO only was added to the media of duplicate plates. The cells were then lysed in detergent containing buffers, and the lysates were divided into soluble and insoluble fractions that were analyzed by SDS-PAGE followed by western blotting. In some cases the lysates were digested overnight with Endoglycosidase H (EndoH) which cuts off immature N-glycans added to proteins in the early secretory pathway. If the proteins are EndoH resistant then they have already passed though the endoplasmic reticulum and medial golgi, while EndoH sensitivity indicates an immature protein remaining in the early secretory pathway. Portions of insoluble lysates were reacted with biotin-azide in standard click chemistry conditions to label ODYA-containing proteins with the biotin reporter group. Then biotin-containing proteins were visualized on a western blot. The same blot was subsequently probed for Palm-YFP and PMP22, and the banding patterns compared to see if there were matches.

Results:

We were successful in making the desired point mutation to PMP22. After the proteins were transfected and cells lysed, there were many more proteins present in the soluble fraction than in the insoluble portion. PMP22 appeared primarily in the insoluble fraction, while Palm-YFP appeared in both soluble and insoluble fractions. In ODYA treated cells, there was an increase in the quantity of Palm-YFP in the insoluble fraction in comparison to the DMSO cells. In PMP22-transfected cells, the wildtype protein also showed an increase in quantity in insoluble extracts, and surprisingly the C85S mutant protein also increased, but not as much as the wildtype. The proportions of N-glycosylated isoforms of PMP22 differed between wildtype and C85S proteins. Preliminary experiments on biotin-labeled cells suggest that Palm-YFP and wildtype PMP22 are biotin labeled in ODYA-treated cells.

Conclusions:

These results suggest that PMP22 has a site of palmitoylation or lipidation at cysteine 85. The observation that the C85S mutant protein also increased in quantity in response to ODYA suggests that PMP22 could possibly have other sites of lipid attachment. The results showed sensitivity of wildtype and C85S PMP22 to EndoH, indicating that the transfected proteins have not proceeded beyond the early secretory pathway. Because of this, rat Schwann cells may not be the best cells to use for examining whether palmitoylation affects trafficking of PMP22 to the plasma membrane. The finding that wildtype and C85S PMP22 show different N-glycosylated isoforms in ODYA-treated extracts suggests that palmitoylation has an effect on N-glycosylation. These results provide novel insights into the post-translational modifications involved in the maturation of PMP22, a protein that is essential to the function of the myelin membrane.

ROLE OF DIHYDROFOLATE REDUCATE IN DIABETIC VASCULOPATHY

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Background: Diabetes has long been associated with increased oxidative stress and impaired vascular function. Nitric oxide synthase dysregulation and decreased NO bioavailability have been implicated as a central mechanism in vascular endothelial dysfunction observed in both type I and II diabetes. Several studies have demonstrated that while eNOS protein levels are increased in the diabetic state, NO bioavailability decreases and superoxide production increases. Among the proposed mechanisms that lead to decreased NO bioavailability and oxidative stress is eNOS "uncoupling" secondary to oxidation of the essential NOS cofactor Tetrahydrobipetrin (BH_4). BH_4 is highly redox sensitive and can be readily oxidized to its inactive form dihydrobiopterin (BH_2). Under normal physiological conditions the endothelium is able to recycle BH_2 back to BH_4 through the activity of the salvage pathway enzyme Dihydrofolate reductase (DHFR).

Hypothesis: It is our hypothesis that diabetes is associated with dysfunctional DHFR activity which results in accumulation of the catalytically incompetent NOS cofactor BH_2. As a consequence of BH_2 accumulation, NOS derived NO production is reduced while its oxidase activity is enhanced.

Results: To test this hypothesis, we examined the effects of diabetes on endothelial function, eNOS activity, DHFR expression and DHFR activity. Results demonstrated that diabetes was associated with a 35% reduction in endothelial dependent relaxation and a greater than 50% loss in both DHFR expression and activity. BH4 supplementation was unable to restore endothelial function in the intact vessel suggesting increased conversion to BH2 in the diabetic vasculature.

Conclusions: These observations have important implications for the role of oxidative stress and its effects on DHFR activity as it relates to the diabetic state.

Structural Identification of Receptor Binding Sites on Adeno-Associated Virus Serotype 6

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Background and significance:
Adeno-Associated Viruses (AAVs) are small (25nm), non-enveloped, ssDNA icosahedral viruses. AAV belongs to the Parvoviridae family, and are of the genus Dependovirus. As such, AAVs require a helper virus, either Adeno or Herpes simplex virus, for infection and replication. The genome of AAV has the length of 4.7 kbs, consisting of two Open Reading Frames (ORF), rep and cap. The cap ORF can produce three different Viral Proteins (VP1, VP2, and VP3) which are responsible for capsid assembly. Sixty copies of VP monomers are required to construct a capsid in the ratio of 1:1:10. Glycan arrays and biochemical studies have proved that sialic acid or heparin is required as primary receptors for AAV6 to enter the cells. Upon human infection, AAVs produce no severe immune effects, and thus they are not defined as pathogenic. AAV's lack of pathogenicity and its ability to transduce various human tissues makes it an ideal viral vector for gene therapy. There are twelve antigenic AAV serotypes, and over 100 variants are isolated from both human and nonhuman primates. Still, structural studies of AAVs are necessary to further enhance the understanding of the receptor binding, transduction, tropism, and specificity of the virus' different serotypes and variants. The goal is to produce crystals that can be used for the structural identification of the receptor binding sites on the AAV6 capsid structure.

Methods:
AAV6 cap gene was constructed in the Baculovirus expression system followed by the expression in SF9 cells. Next, purification of the AAV6 capsid was performed using sucrose gradient sedimentation. To determine the concentration of the AAV6 sample, a spectrometer was used to provide Optical Density (OD) readings. For quality assurance, SDS-PAGE was necessary to confirm the AAV6 sample was pure. To verify the presence of intact capsids, negative stained Electron Microcopy (EM) was used. Following confirmation of purified, intact capsids, structural determination and identification of receptor binding sites can be performed. For structural determination, crystal trays were created at a concentration of 10mg/ml VP with the drop ratio of <1:1> in each condition. These screening conditions are believed to be conducive to crystallization. If crystals form, the proceeding techniques for structural determination are x-ray crystallographic data collection, data processing, molecular replacement, and electron density mapping, and model building respectfully. To identify receptor binding sites, crystal trays are set up using AAV6 and Heparin complex in the drop ratio of <1:1:1>. Finally, the different map must be used to identify the binding sites.

Results:
SDS-PAGE and negative stained electron microscopy both initially showed signs of impurities. As a result, purification was revisited and a second sucrose gradient was performed. The second SDS-PAGE and Electron Microscopy showed significant improvement in the purification of the AAV6 sample. However, performing a second sucrose gradient lead to a reduced quantity of sample; 366μL less to be exact. Nevertheless, the crystal tray that was constructed from the sample showed promising signs of crystallization after a week.

Summary:
AAV6 capsids were successfully purified. The SDS Page Gel showed VP2 and VP3 bands. The EM Showed Intact Particles. A crystal tray was prepared from the purified sample. Crystallization can take anywhere from two weeks to several months to a year. No crystal has been formed yet that is sufficient for structural analysis.

Characterization of the Herpes Simplex Virus Type 1 Insulator Function by the Deletion of Two CTCF-binding Motifs in the Latency Associated Transcript Region

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Introduction

Herpes simplex virus type 1 (HSV-1) establishes lifelong latent infections in host sensory neurons during which lytic gene expression is repressed and the latency associated transcripts (LATs) are expressed. Two insulator elements, CTRL1 and CTRL2, containing reiterated copies of CTCF-binding motifs (CTCCC) flank the latently-expressed region of the genome containing the LAT promoter and 5' exon. CTCF insulators are thought to regulate gene expression with cell type specificity (Chen et al 2007). Therefore, regulation of HSV-1 gene expression *in vivo* may be due in part to the CTCF insulators. To begin the characterization of CTRL1 and CTRL2, single-step growth curves were performed by infecting mouse neuroblastoma cells (N2A cells) and rabbit skin cells (RS cells), representing neuronal and non-neuronal cells, respectively, with recombinant viruses containing CTCF-binding motif deletions in CTRL1, CTRL2, and both CTRL1 and CTRL2, with multiplicities of infection (m.o.i.) of 0.001 pfu and 5 pfu. This was done to attain qualitative characterization of the recombinant virus replication *in vitro*. Because CTRL2 is located between the inactive ICP0, an immediate early gene regulating the lytic cascade, and the actively transcribed LAT promoter (Amelio et al 2006), we hypothesized that CTRL2 mutants would increase replication in N2A cells and demonstrate unaltered replication in RS cells. Due to previous studies conducted on the recombinants by Feldman et al. (2007) *in vivo*, we predicted that replication in recombinant viruses with deletions in both CTRL1 and CTRL2 would be significantly reduced in the neuronal-like N2A cells.

Materials and Methods

Single-step and multi-step growth curves were performed using low-passage stock of HSV-1 strain 17*syn*$^+$ prepared from a master stock obtained from J. Stevens and previously constructed fosmid viruses: TFN 27-6, WT Fosmid; TFN 27-4, ΔB1; TFN 26, ΔB2; and TFN 27-1, ΔB1/B2 obtained from L. Feldman. TFN 27-4, ΔB1 (17ΔB1) contains a 170-bp deletion removing 27 CTCF-binding motifs in the CTRL1 insulator. TFN 26, ΔB2 (17ΔB2) contains a 132-bp deletion removing 9 CTCF-binding motifs in the CTRL 2 insulator. TFN 27-1, ΔB1/B2 (17ΔB1/B2) contains both deletions. The single-step growth curve (m.o.i. 5 pfu) was performed in triplicate using 35mm dishes by infection of RS cells obtained from E. Wagner and N2A cells obtained from ATCC and maintained in Eagle's minimal essential medium (Life Technologies) supplemented with 5% calf serum, 250μg of streptomycin/ml, and 292μg of L-glutamine/ml (Life Technologies) in a 37°C humidified incubator at a CO_2 concentration of 5%. Each virus type was harvested at 0, 4, 12, and 24 hours post-infection (hpi). The multi-step growth curve was similarly performed with an m.o.i. of 0.001 pfu with an additional harvesting at a 72 hpi A standard plaque essay using 24-well plates seeded with RS cells, using the aforementioned conditions, was used to quantify the virus (Thompson et al 1983).

Results

The plaque assay tested viruses: HSV-1 17*syn*$^+$ (wild-type), 17$^+$ fosmid (a version of wild-type reconstructed from the wild-type fosmids for use as a control for the fosmid constructed deletion viruses), and the fosmid-constructed deletion viruses 17ΔB1, 17ΔB2, and 17ΔB1/B2 for replication in RS cells and N2A cells at m.o.i. 0.001 pfu and m.o.i. 5 pfu. Any significant alteration in the replication of the 17ΔB1, 17ΔB2, and 17ΔB1/B2 recombinants was determined

by a comparison to their respective 17^+ fosmid. At both m.o.i. 0.001 pfu and m.o.i. 5 pfu, 17ΔB1 demonstrated a significant increase in replication in RS cells at 24 hpi and 72 hpi, respectively. 17ΔB2 recombinant replication was unaltered in N2A cells (m.o.i. 5 pfu) at 24 hpi and RS cells (m.o.i. 0.001 pfu) at 72 hpi, and increased in RS cells (m.o.i. 5 pfu) at 24 hpi.

With respect to N2A cells (m.o.i. 5 pfu), there was no significant difference in the replication of 17ΔB1, 17ΔB2, and 17ΔB1/B2 recombinants and the 17^+ fosmid replication at 24 hpi.

In both N2A cells (m.o.i. 5 pfu) and RS cells (m.o.i. 5 pfu), there was a significant difference between the replication profile of the 17*syn*$^+$ strain and that of the 17^+ fosmid. Titers have not yet been performed for the N2A cell (m.o.i. 0.001 pfu) infections.

Discussion

Unexpected results in HSV-1 recombinant replication in N2A cells and RS cells have led to a revision of our original hypothesis. Rather than increasing replication in N2A cells, the 17ΔB2 recombinant increased replication in the RS cells (m.o.i. 5 pfu) by 24 hpi. The 17ΔB1 mutant increased replication at both high and low multiplicities in RS cells. Also in contrast to our hypothesis, 17ΔB1/B2 replication was not attenuated in N2A cells (m.o.i. 5 pfu).

The 17ΔB2 recombinant replication was expected to increase in N2A cells because previous studies conducted by Amelio et al. (2006) and Chen et al. (2007) have demonstrated that the LAT intron containing the insulator element CTRL2, once binding CTCF, has an enhancer blocking function preventing the activation of nearby genes. There is also preliminary evidence that suggests that CTRL2 promotes LAT transcription by its association with euchromatic marks at the LAT promoter during latency (Feldman et al 2007). CTCF cluster deletions in the CTRL2 insulator should consequently result in increased viral replication if N2A cells behave with neuronal-like properties. This is supported by the observed increase in 17ΔB2 mutant virulence when compared to the virulence of 17^+ fosmid *in vivo* (Feldman et al 2007). In the current study, however, 17ΔB2 recombinant replication increased in RS cells (m.o.i. 5 pfu) only. It was also observed that neither 17ΔB1 nor 17ΔB1/B2 mutant replication varied significantly on N2A cells (m.o.i. 5 pfu). It is possible that these observations parallel observations made of infected sensory neurons, which immediately reactivate post-explant; it may be the case that the neuronal-like N2A cells used in the current experiment may be unable to appropriately utilize the insulator elements leading to viral replication in all cases.

The increase in 17ΔB1 recombinant replication in RS cells (m.o.i. 0.001 pfu and m.o.i. 5 pfu) and 17ΔB2 replication in RS cells (m.o.i. 5 pfu) when compared to their respective 17^+ fosmid was surprising. The function of the CTRL1 and CTRL2 insulators is typically tied to their binding the cellular protein CTCF, which may help to regulate gene expression during latency. A change in gene expression during the lytic phase, however, was not expected. As HSV-1 latency is known to occur only in sensory neurons, perhaps binding of CTCF to the CTRL1 and CTRL2 insulators does not occur in the same manner in non-neuronal cells (Spivack and Fraser 1987). The observation may be due to an association of the insulator elements with factors in non-neuronal cells that lead to the suppression of HSV-1 replication.

It is questionable whether the observations made for the recombinant viruses may be applied to the actual phenotypes of HSV-1 because this is an *in vitro* system. Also, there was a significant difference between the replication of the 17*syn*$^+$ strain and that of the 17^+ fosmid suggesting a problem with the parent fosmid strain. This may be the most significant reason for which replication was not attenuated for mutant 17ΔB1/B2 as expected. Experiments toward creating rescues of the deletion viruses are currently underway.