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Sarnoff Fellowship: Essay

Cardiovascular disease has remained one of the leading causes of death globally, accounting for a combined 15.2 million deaths in 2016 per the World Health Organization.[1] Hypertension is a major risk factor for heart disease, with a global prevalence in adults of 1.13 billion.[1] The prevalence of hypertension continues to remain high despite global efforts focused on prevention and modification of disease through behavioral risk factors such as obesity, high salt intake, and physical inactivity.[1] These statistics beg the following question: Should we be focusing efforts on early intervention in utero to prevent future risk of cardiovascular disease? A few decades ago, Barker and Hales advanced the hypothesis of "altered programming," which states that chronic diseases in adulthood may be the consequences of altered programming, when a stimulus or insult at a critical time in early life has permanent effects on structure, physiology, and metabolism.[2] Intrauterine growth restriction (IUGR) is a prenatal finding that is confirmed by low fetal growth rate due to maternal or fetal risk factors which cause placental insufficiency.[2] This hypothesis has been tested in subsequent studies which have shown that intrauterine growth restricted fetuses are prone to the development of insulin resistance in the postnatal life where fetal adaptation to save glucose as a response to intrauterine malnutrition manifests as insulin resistance.[3]

Classically, insulin functions to promote glucose metabolism. Studies have depicted that insulin additionally acts to increase blood flow to skeletal muscle beds, suggesting that insulin resistance dampens molecular pathways that lead to arterial vasodilation.[4] This supports the notion that vascular actions of insulin can be coupled to glucose metabolism in order to regulate blood flow in intrauterine growth restricted fetuses (Fig 1). The direct association between insulin resistance and the development of hypertension, however, are not clearly understood.[5] I am motivated to study the effects of insulin resistance on the vascular endothelium of intrauterine growth restricted fetuses and to attempt to reverse harmful insulin-mediated changes in the fetal vascular endothelium.

Binding of insulin to its receptor, a member of a large family of tyrosine kinases, initiates its biologic actions. Subsequent insulin-mediated increases in blood flow are dependent on endothelial derived nitric oxide (NO). NO is synthesized in the vasculature by endothelial nitric oxide synthase (eNOS) which acts as a catalyst to produce NO in the vascular endothelium by hydroxylation of L-arginine to N-hydroxy-L-arginine, followed by oxidation of this intermediate to NO and L-citrulline.[5] Endothelial-derived NO then diffuses into adjacent vascular smooth muscle where it activates guanylate cyclase, leading to increased levels of cyclic guanosine monophosphate (cGMP) and vasodilation.[4,5] A clearly defined mechanism linking receptor kinase signaling to activation of eNOS in endothelial cells has not been fully elucidated.[5] *In vitro* studies conducted by Montagnani et al. have provided novel insight into specific insulin pathways that may activate eNOS, by measuring production of NO in response to insulin in human umbilical vein cells (HUVEC) using an NO specific electrode.[5] Three pathways described by these studies include the insulin receptor tyrosine kinase pathway, the phosphatidylinositol 3-kinase (PI3K) pathway, and the Akt serine-threonine kinase pathway downstream of PI3K.[5] Montagnani et al. additionally showed that preincubation of the HUVECs with an insulin receptor tyrosine kinase inhibitor completely blocked production of NO in response to insulin.[5] Additionally, insulin stimulated production of NO was blocked when cells were incubated with an inhibitor of PI3K or overexpressed with a kinase-deficient mutant of Akt.[5]

Researchers have proposed treatment with NO mediators for improving endothelial function and mitigating long-term outcomes in intrauterine growth restricted fetuses. *In vitro* and *in vivo* studies have suggested type 5 phosphodiesterase (PDE-5) inhibitors, specifically sildenafil citrate, as promising agents to increase vasodilation due to increased cGMP as a result of PDE-5 inhibition.[6] Sharp et. al conducted a multicenter randomized, placebo-controlled, double blind trial of 135 pregnant women with early-onset fetal growth restriction where sildenafil citrate was compared to placebo. Sidenafil citrate showed no beneficial effects in comparison to placebo on uteroplacental and fetal circulation upon measurement of blood flow, as depicted by the pulsatility index of the umbilical artery, middle cerebral artey, ductus venous, and uterine artery on Doppler ultrasound, whereby a high index reflects blood flow resistance.[6] Notably, cGMP is a second messenger of NO, therefore, targeting NO-dependent vasodilation by working upstream of cGMP, early in the NO pathway at the level of eNOS activation can possibly lead to desired vasodilatory effects on uteroplacental and fetal circulation.

There is substantial evidence regarding the vasodilatory effects of NO on the vascular endothelium. However, there are still gaps in the literature regarding the mechanism of action of insulin signaling along the NO pathway and the effects of increased insulin sensitivity on the vascular homeostasis of the growth restricted fetus. I hypothesize that increasing insulin sensitivity through modulation of the Akt serine-threonine kinase and eNOS signaling pathways *in utero* can prevent hypertension and adverse cardiovascular outcomes in postnatal life. I plan to test this hypothesis by 1) determining if treatment with insulin sensitizers leads to vasodilatory effects in the vascular endothelium of insulin-resistant growth restricted fetuses using *in vitro* cell culture models, *in vivo* IUGR pregnant mice models, and pregnant human subjects with IUGR as noted on antenatal ultrasound and 2) studying the insulin-mediated effects on vascular endothelium in insulin-resistant growth restricted fetuses.

Specific Aim 1: To Determine Whether Induction of Insulin Sensitivity in Insulin-resistant Cultured Endothelial Cells Alters NO Production *in vitro*

The thiazolidinedione (TZD) pharmacologic agents are indicated for the treatment of diabetes mellitus, as they work to decrease insulin resistance and cardiovascular risk.[7] TZDs activate the nuclear peroxisome proliferator-activated receptor gamma (PPARy) which increases transcription of insulin sensitive genes and improves action of insulin.[7] Growing evidence has suggested that TZDs possibly work to improve endothelial function through PPARy dependent phosphorylation of the Akt serine-threonine kinase at serine ^{437} and eNOS at serine^{1177} and threonine^{495}, which would aid in the promotion of vasodilatory effects along the NO pathway.[8,9] I propose the use of TZDs to increase insulin sensitivity in insulin-resistant cultured endothelial cells. In order to induce insulin resistance in endothelial cell lines, I propose the use of tunicamycin or palmitic acid. Tunicamycin and palmitic acid impair insulin signaling through increased endoplasmic reticulum (ER) stress. Both agents suppress insulin-induced Akt serine-threonine kinase phosphorylation, which leads to suppressive action on the JNK pathway.[10,11] The agents described will allow for modulation of insulin-resistant and insulin-sensitive states through the Akt serine-threonine kinase pathway.

I propose the use of human umbilical vein endothelial cells (HUVECs) as previously described.[5] The HUVEC model involves culturing cells in media which contains 10% fetal bovine serum (FBS).[5] Insulin resistance can be induced by applying 5 μg/ml tunicamycin for 4 hours.[13] Insulin sensitivity can be induced with 10 uM of pioglitazone (PIO), a pharmacological agent of the TZD class, as previously described.[12] Insulin signaling in the cells can be stimulated by applying 10 nM insulin for 10 minutes and measuring glucose uptake before and after treatment with PIO, which would inform whether cells are in an insulin resistant state.[13] Additional cell lines can be treated with N(gamma)-nitro-L-arginine methyl ester (L-NAME), which is an inhibitor of eNOS.[13] The presence or absence of L-NAME will inform whether insulin mediated effects are directly related to eNOS activity. Thereby, the following 6 groups can be generated: insulin-resistant cells, which will act as the control (IR), non-IR cells, IR cells/PIO, IR cells/PIO/L-NAME, non-IR cells/PIO, non-IR cells/PIO/L-NAME.

Intracellular NO can be estimated by exposing the cells to 10 μmol/L of the fluorescent dye 4-amino-5-methylamino-2',7'-difluorofluorescein (DAF-FM) and subsequently measuring fluorescence on a microplate reader.[13] Western blot analysis can be used to quantify NO activity by extraction of total protein from HUVECs, followed by washing and harvesting of protein in a lysis buffer and centrifugation. The protein sample can then be separated by electrophoresis and probed against total eNOS, eNOS phosphorylated at serine^{1177} and threonine^{495}, total Akt, and Akt phosphorylated at serine^{437}.[13] Proteins can then be quantified by densitometry. Taqman-based qPCR techniques can also be used to quantify NO activity, where primers and probes can be designed for complementary DNA of eNOS and the Akt serine-threonine kinase based on available sequence data.[13] I predict that both techniques will result in PIO treated IR cells exhibiting increased levels of eNOS and Akt serine-threonine kinase activity in comparison to control cells and non-IR cells. These findings would also further support existing knowledge that insulin resistance has anti-vasodilatory effects on the vascular endothelium, where I expect control cells to exhibit decreased eNOS and Akt serine-threonine kinase activity. In the event that these findings are not observed as I predicted, I propose conducting successive rounds of the experiment with an alternate PPAR-y agonist or inducing insulin resistance with palmitic acid, as the absence of findings can be due to nonspecific targeting effects, inappropriate titration of agent, or ineffectiveness of the pharmacologic agent. Elevated eNOS and Akt activity level after suppressing insulin-induced serine-threonine kinase phosphorylation with tunicamycin and subsequently treating cells with pioglitazone to increase phosphorylation at Akt serine^{437} would constitute insulin sensitizers as a potential treatment to target vascular changes in the insulin-resistant growth restricted fetus.

Specific Aim 2: To Determine Whether eNOS Expression Varies According to Insulin Sensitivity in Growth Restricted Pups *in vivo*

A second approach to testing the effects of insulin sensitizers on the fetal vasculature can be achieved *in vivo*. I propose feeding one group of pregnant female rats a 20% protein diet (non-IUGR) and a second group an 8% protein diet (IUGR) starting on day 1 of pregnancy until birth of their pups, as previously described in a C57BL/6J mouse model of maternal-diet induced IUGR.[14] Both the non-IUGR and IUGR pregnant dams will be randomized to a control group or treatment group on gestational day (GD) 14.5. The control group (control) will consist of non-IUGR and IUGR pregnant dams who will continue their respective 20% protein or 8% protein diet. The treatment group (PPAR) will have a PPAR-y agonist added to their diet in the form of 0.0125% pioglitazone (PIO) as previously described in Lane et al.[15] This approach generates the following 4 study groups for comparison: control/non-IUGR, PPAR/non-IUGR, control/IUGR, PPAR/IUGR.

Following delivery, thoracic aortas of non-IUGR and IUGR pups will be fixed, embedded in paraffin**,** and sectioned. Sections can then be incubated with antibodies directed against eNOS and visualized using confocal

microscopy at a wavelength of 488 nm in order to visualize fluorescein.[16] eNOS expression can be quantified by extracting protein and total RNA from thoracic aortas of non-IUGR and IUGR pups and performing western blot analysis or real-time PCR, as described above.[13] I predict that the IUGR pups treated with the PPAR-y agonist diet will have elevated or similar levels of eNOS expression compared to non-IUGR pups from control and treatment groups.

In the event that this finding is not observed, insulin resistant versus insulin sensitive states can be induced by *in vivo* gene deletion. A previous experimental model of IUGR, showed that impaired activation of IRS-2 precedes the onset of insulin resistance.[17] This secondary study can be designed to mate male wild type mice with female wild type and IRS-$2^{-/-}$ mice. Pregnant female wild type and IRS-$2^{-/-}$ mice can be separated into a control group and a treatment group with a PPAR-y agonist. We can then use methods discussed above to assess if growth restricted fetuses of IRS-$2^{-/-}$ knockout mothers treated with insulin sensitizers have increased eNOS activity. I expect that IRS-$2^{-/-}$ knockout mothers treated with insulin sensitizers will have increased eNOS activity compared to the control group and comparable eNOS activity to the wild type group.

With these two methods of modeling IUGR *in vivo,* targeting insulin sensitivity during gestation can serve as a potential treatment option to prevent vascular changes in growth restricted fetuses.

Specific Aim 3: Translating Insulin Sensitizers to Prevention of Future Cardiovascular Disease in Growth Restricted Fetuses

Pregnant women noted to have a decreased fundal height are at risk of fetal growth restriction. On ultrasound, fetal growth restriction is defined as an abdominal circumference less than the third percentile, estimated fetal weight at less than the third percentile, and no end-diastolic flow in the umbilical artery as noted by the pulsatility index.[18] I propose recruiting a cohort of pregnant women with fetal growth restriction noted on the routine 20th week ultrasound and randomizing the cohort to control and treatment groups, where the treatment group can receive treatment with a low dose of PPAR-y agonist, pioglitazone. Use of pioglitazone in pregnancy is considerably safe if benefits outweigh risk to the fetus and thus considerably safe to use in this study approach, as no teratogenic effects have been observed in previous mice studies.[19] At the start of the third trimester, notably the 28th week of pregnancy, the pulsatility index can be measured to assess changes in maternal and fetal vasculature. Activity levels of NO can be then quantified twelve to twenty-four hours after delivery by collection of infant serum and subsequent measurement of NO concentration. Existing methods for measuring NO in human biological samples are limited to final products of NO metabolism, nitrite (NO_2^-) and nitrate (NO_3^-), via Greiss's reaction. Briefly, samples are deproteinized and supernatants are extracted from serum and exposed to Greiss reagents and absorbance is measured by a microplate reader at 540 nm which reflects nitrite levels. [20] Alternatively, NO can be measured through mass spectrometry detection of S-nitrosothiols (RSNOs), where RSNOs represent downstream NO signaling after oxidation and S-nitrosation of NO.[20] I anticipate that infants of mothers treated with insulin sensitizers will have increased levels of serum NO metabolites which reflects the vasodilatory effects of insulin sensitizers on fetal vascular endothelium. Measurement of the pulsatility index and serum NO metabolites after treatment with pioglitazone can inform whether treatment with insulin sensitizers in the mid-late stages of IUGR pregnancies can have beneficial cardiovascular effects on the vascular homeostasis of the fetuses and aid in reversing the "altered programming" concept.

Conclusion:

While all of the approaches can serve to test the hypothesis, the *in vivo* model offers the best approach as it offers direct manipulation of maternal factors. The *in vitro* model does not allow for a true uterine environment such as the *in vivo* model and the human randomized control trial is limited by the inability to truly decipher the driver of the fetal insulin-resistant state, especially in the presence of multiple maternal risk factors. The *in vivo* approach can elucidate the following: 1) effects of insulin resistance on NO release in growth restricted fetuses 2) direct correlation between insulin signaling to fetal vascular homeostasis and 3) if increasing insulin sensitivity leads to vasodilatory effects in growth restricted fetuses. This approach would provide qualitative and quantitative data on the actions of insulin sensitizers on the vasculature of the growth restricted fetus and inform further translational research which can serve to modify current treatment strategies in this patient population.

As stated, hypertension remains a major risk factor for ischemic heart disease globally despite significant gains made by the medical community through pharmacological and lifestyle modification. Shifting focus to the *in utero* environment and offering therapies to target insulin mediated effects on the vasculature of growth restricted fetuses may possibly decrease the rates of hypertension in adult years for these patients. Growth restricted fetuses are a subset of patients considerably implicated in the "altered programming" phenomenon. Continuing to identify early indicators of "altered programming" in varied patient populations would advance the scientific community and clinical practice markedly and can serve as a model of approaching cardiovascular risks globally.

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