CHRONIC NOREPINEPHRINE EXPOSURE AND WHOLE BLOOD DETECTION IN FETAL SHEEP

BY

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STATEMENT BY AUTHOR

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Abstract

Placenta insufficiency results in decreased nutrient supply between the mother and fetus, which induces hypoxemia and hypoglycemia in the fetuses causing intrauterine growth restriction. The fetus increases circulating norepinephrine concentrations in response to this stress, which can cause adrenergic receptor desensitization. The aim of this study was to determine what adrenergic receptors were detectable in fetal whole blood mRNA and to determine whether tissue desensitization manifested in the form of low adrenergic receptor mRNA concentrations. Three of the nine adrenergic receptor subtypes (α2A, β1 and β2) were detectable in RNA extracted from fetal whole blood of control and placenta insufficient treatment groups. Of these three, adrenergic receptor α2A was expressed in the greatest concentration and was chosen for further study. In placenta insufficient fetuses adrenergic receptor α2A mRNA concentrations were 73% lower than the control group. We also measured adrenergic receptor α2A mRNA in fetuses without an adrenal medulla, which were not responsive to hypoxemia-induced elevation of norepinephrine. These placental insufficient fetuses were no different from the controls and therefore, the evidence supports the increase of norepinephrine, rather than the hypoxic conditions, as the cause of desensitization in whole blood samples.

Introduction

Overview

The ovine placental insufficient IUGR model is used to study the effects of IUGR conditions on developing fetuses because these fetuses share several similarities with human IUGR pregnancy. The similarities include impaired fetal and placenta vascularization, placenta-fetal nutrient transfer, blood flow, β-cell function, placenta proliferation, fetal weight and asymmetric fetal growth (Barry *et al.*; 2008).

Intrauterine growth restriction (IUGR) is caused by a variety of factors divided into three categories: maternal, fetal and placental in origin (Faraci *et al.*; 2011). One of these categories, placenta insufficiency, decreases placental transport of nutrients and oxygen from the mother to the fetus (Leo *et al.*; 2010). This condition causes low blood glucose and low blood oxygen concentrations in the fetus resulting in fetal stress. Fetal norepinephrine concentrations increase in response to fetal stress (Greenough *et al.*; 1990). During stressful states such as hypoxemia, high plasma norepinephrine concentrations can result for days or weeks, which have been shown to desensitize tissues. More importantly, adrenergic desensitization may have permanent effects on the body, which manifest as metabolic disorders like insulin resistance and type II diabetes (Leos *et al.*; 2010). It would be beneficial to have a detection method for clinical applications to detect whether small for gestational infants were stressed. This would allow us to develop treatment for affected infants. Because all infants undergo neonatal screening, the process for a simple biomarker in whole blood that is associated with fetal stress would be the most useful.

Scientific Objectives

The purpose of our study was (1) to determine which adrenergic receptors are expressed in fetal white blood cells in both control and placenta insufficient groups and (2) to determine declines in detectable adrenergic receptor concentrations in placenta insufficient fetuses.

Methods

mRNA Isolation and Preparation

Blood samples were collected from fetuses at 90% gestation using catheters surgically inserted into the femoral arteries. Four treatment groups were evaluated: control or intrauterine growth restricted (IUGR) fetuses that had either a SHAM or bilateral adrenal demedullation (DeMed) surgery. Animal preparations were performed as descried by Leos *et al.* 2010. Total mRNA was extracted from whole blood using TRI-Reagent RT Blood (Molecular Research Center) and cleaned up using the RNeasy Micro Kit (Qiagen, Valencia, CA). mRNA concentrations were measured using the NanoDrop ND-1000 Spectrometer (NanoDrop, Wilmington, DE). cDNA was synthesized using the extraction. mRNA (1ng mRNA in 11ul solution) was first prepared by adding 1 µl of oligo$(dT)_{20}$ and 1 µl of a 10 mM dNTP mix, incubating at 65ºC for 5 minutes then on ice for at least one minute. Then 7 ul of a solution containing 4 ul 5x First Stand Buffer, 1 ul 0.1 M dichlorodiphenyltrichloroethane (DDT), 1 ul RNaseOUT, and 1 ul SuperScript III RT (Invitrogen, Life Technologies, New York) was added to each reaction tube followed by a 5 minute incubation period at room temperature. Next, each reaction tube was incubated at 50ºC for 60 minutes and then 70ºC for 15 minutes.

Receptor Detection

PCR products of adrenergic receptors α1 (A, B, D), α2 (A, B, C), and β (1, 2, 3) were created using whole blood cDNA. 1 ul cDNA was added to a solution containing 17.8 ul distilled water, 0.75 ul 25 mM $MgCl_2$, 0.5 ul dNTP mix, 0.5 ul Forward Primer, 0.5 ul Reverse Primer (Forward and Revers primers match those described in Leos *et al.*;2010), 1.25 ul Dimethyl sulfoxide (DMSO), and *Taq* DNA Polymerase (Qiagen). The following cycle was used for PCR amplification: (1) 96ºC for 3 minutes, (2) 96ºC for 45 seconds, (3) 60ºC for 1 minute, (4) 72ºC 1 minutes, (5) Repeat steps 2 through 3, 35 times, (6) 72ºC 10 minutes, (7) 4ºC for ∞.

DNA gel electrophoresis was performed to separate and visualize PCR products of ovine adrenergic receptors in both control and IUGR groups using a 1% TAE Agarose gel. Two microliters of a load buffer were added to each sample before loading into the gel well. Results were compared to a positive control of ovine skeletal muscle, fetal brain and fat and to a negative control of water. Base pair lengths were estimated using a standard ladder.

Receptor Concentrations

Using the iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories), blood concentrations of ADRα2A mRNA were quantified. SYBR Green PCR master mix (Applied Biosystems, Foster City, CA) was combined with cDNA from each sample and loaded into each well. Triplicates were run when cDNA concentrations permitted. Samples were run alongside a gene specific plasmid of varying, known concentrations to create a standard curve. Concentrations were then normalized using the reference gene S15 gene. (Limesand *et al.* 2007)

Biochemical Analysis

Blood oxygen and norepinephrine concentrations were measured at the time of collection as described in Leos *et al.* 2010. Oxygen concentrations were measured with ABL 720 with values temperature corrected at 39°C (Radiometer, Copenhagen, Denmark). Norepinephrine concentrations were measured an ELISA (Rocky Mountain Diagnostic, Colorado Springs, CO).

Results

Amplified PCR products for the ovine adrenergic receptors from fetal blood are shown in Figure 1. Two control and two placental insufficient fetal whole blood samples were compared to a negative control (milliQ water; no cDNA) and to a positive control containing a mixture of ovine skeletal muscle, fetal brain and fetal fat cDNA. The adrenergic receptor subtype α2A was highly expressed in both control and placental insufficient animal groups. Adrenergic receptor subtypes β1 and β2 were also expressed but PCR product intensities were lower than α2A, which indicates that mRNA concentrations may be lower. No PCR products were observed from adrenergic receptors α1A and β3. Adrenergic receptors α1B, α1D, α2B, and α2c were present; however they

appear to be in very low concentrations.

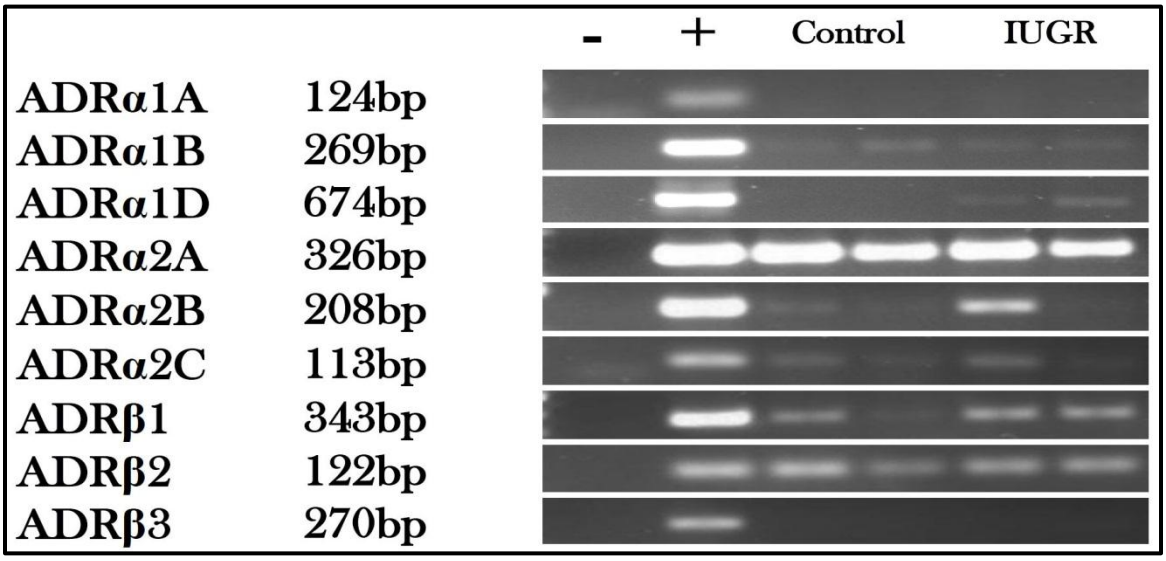

Figure 1: Gel electrophoresis for PCR amplified adrenergic receptor subtypes from fetal blood RNA. The product size is presented in base pairs (bp). For each gene a negative (-), positive (+), and two representative control and IUGR sheep samples were analyzed. ADRα2A was expressed robustly and used for subsequent analysis.

Blood oxygen concentrations are presented in Figure 2. Both groups of placental insufficient SHAMs and DeMeds had lower blood oxygen concentrations (30% and 31% respectively) when compared to the control groups.

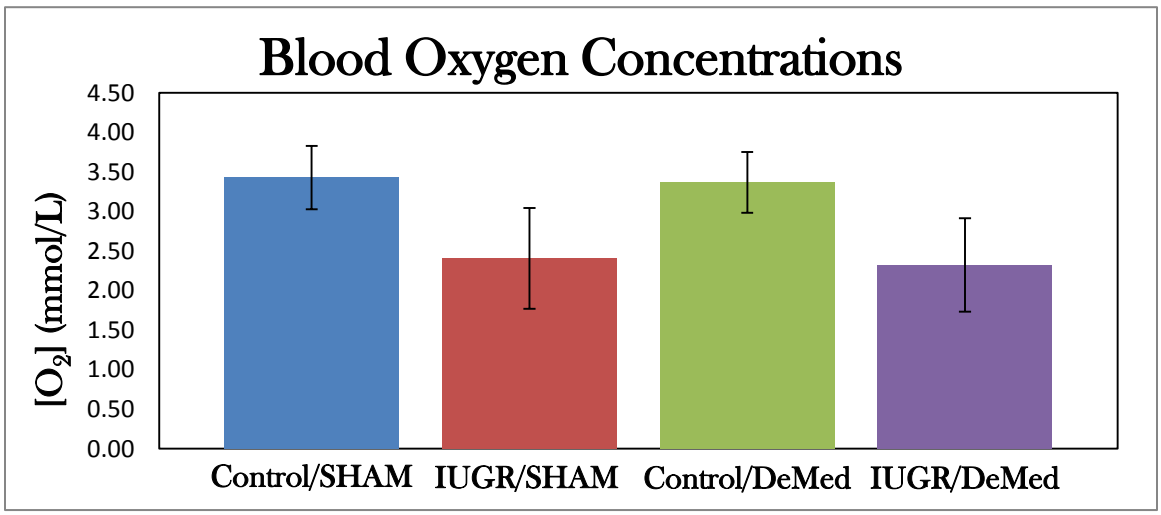

Figure 2: Mean blood oxygen concentrations of fetal treatment groups (± s.e.m.).

Plasma norepinephrine concentrations are presented in Figure 3. Placenta insufficient, SHAM fetuses showed a 4 fold increase in norepinephrine concentrations compared to control, SHAM fetuses. In IUGR fetuses, DeMeds had lower blood norepinephrine concentrations than the SHAMs and values were similar to control, SHAMs.

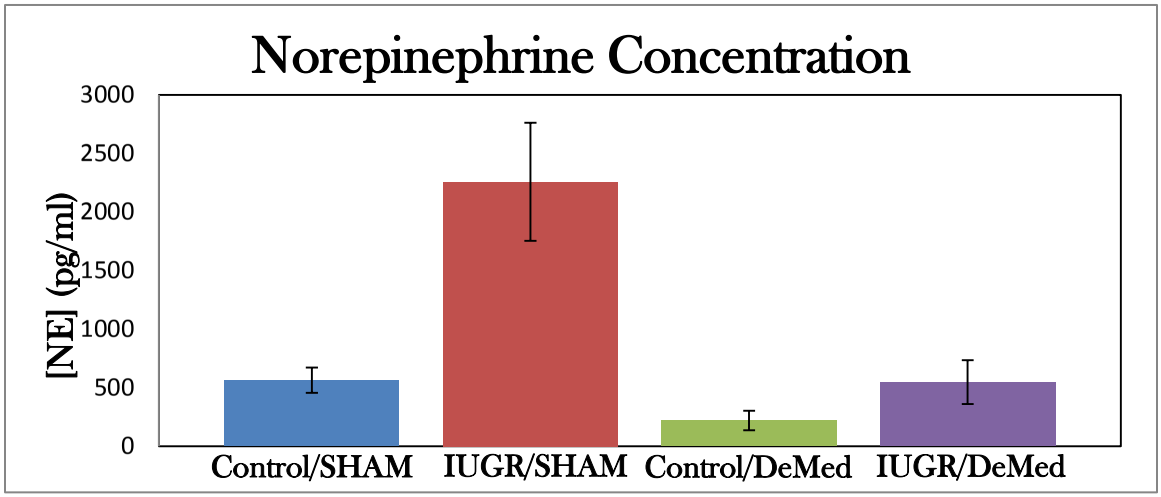

Figure 3: Mean plasma norepinephrine concentrations of fetal treatment groups (± s.e.m.).

The adrenergic receptor α2A mRNA concentrations in fetal blood are shown in Figure 4. Growth restricted, DeMeds showed a 73% decrease in receptor mRNA concentrations when compared to control, SHAM fetuses. The DeMed groups were less

effected that the IUGR, SHAM fetuses.

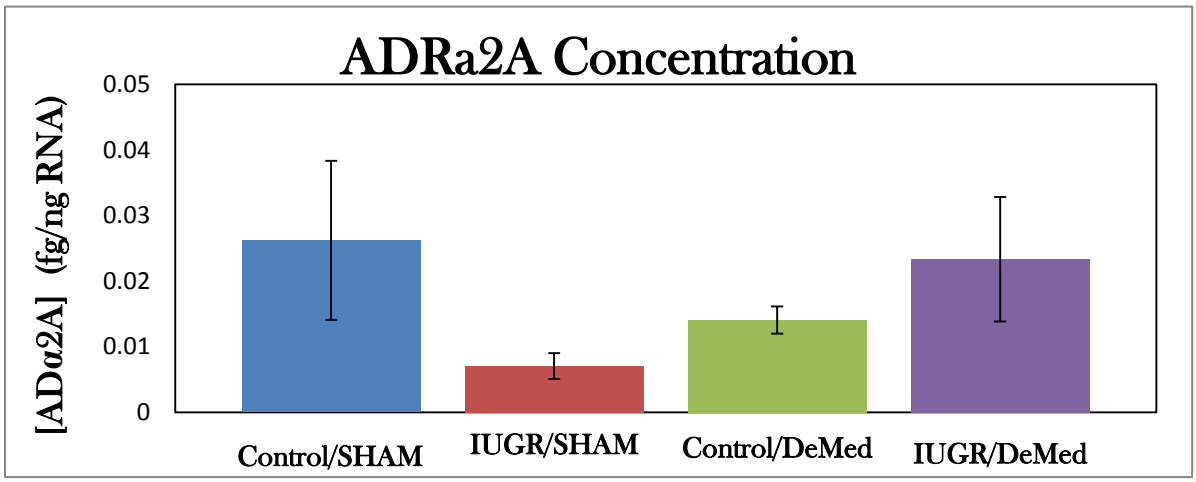

Figure 4: Mean mRNA concentrations of adrenergic receptor α2A of fetal whole blood (± s.e.m.).

Discussion

This study analyzed adrenergic receptor presence and concentrations in ovine fetal whole blood using the placenta insufficient IUGR model. The results show that tissue desensitization due to chronic norepinephrine exposure, as a result of fetal stress, is detectable in fetal whole blood. Furthermore, the findings show desensitization is a result of chronic norepinephrine exposure and not as a direct result of fetal hypoxemia.

We were able to verify the presence of seven adrenergic receptor sub types, through mRNA measurement, in both control and placenta insufficient, growth restricted groups. Concentration of adrenergic receptor α2A was chosen for further studies because of the high fluorescent signal expressed during the assay.

To induce periods of chronic stress, pregnant ewes were exposed to hyperthermic conditions during the second trimester, the period in which most placenta growth and development occurs. Lower blood oxygen concentrations in placenta insufficient IUGR

groups showed the successful induction of hypoxic conditions which were used to simulate chronic stress in the developing fetus.

The chronic stress caused a 4 fold increase in plasma norepinephrine concentrations of the IUGR, SHAM fetuses compared to the control group. The low norepinephrine concentrations measured in the Control, DeMed group indicate the success of the bilateral adrenal modulation performed on the fetuses. Also supporting this is the significantly lower norepinephrine concentrations measured in the IUGR, DeMed group when compared to the IUGR, SHAM group.

The elevated blood norepinephrine concentrations caused whole blood tissue desensitization in IUGR, SHAM fetuses. Fetuses in this group exhibited a 73% decrease in receptor mRNA concentrations when compared to the control, SHAMs. Other groups exhibited little or no differences. Because of this, we know that the desensitization was a result of the high norepinephrine concentrations and not a direct result of hypoxic conditions. If the reverse were true, we would expect to see desensitization in IUGR, DeMeds as well, which was not the case in this study.

Conclusion/Further Studies

Our findings indicate that fetal stress, associated with elevated norepinephrine, can be detected by adrenergic desensitization in fetal blood samples. Because hypoxemia stimulates norepinephrine secretion in the fetus, we may have identified a biomarker for determining fetal hypoxemia if lower ADRα2A expression persists in the neonate. Adrenergic receptors α1B, α1D, α2B, and α2c while present, were found at very low concentrations and do not appear to be good candidates for biomarkers. Future studies

will involve running the same tests on ADRβ1 and ADRβ2 to see if similar patterns exist as well as finding specific ranges of receptor concentrations associated with fetal stress.

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