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Impact of Stress Hormones and IUGR Fetal Conditions on Myoblast Function

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Impact of Stress Hormones and IUGR Fetal Conditions on Myoblast Function

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Abstract

Chronic fetal stress causes adaptive responses that result in intrauterine growth restriction (IUGR). Maternal stressors including heat stress, illness, and obesity cause placental dysfunction that harshens the intrauterine environment by inducing hypoxia and nutrient restriction. IUGR fetuses have restricted growth through the last trimester and after birth. The objective of this study was to test the effects of stress hormones on myoblast proliferation rates. L6 cells and primary myoblasts that were isolated from IUGR fetal sheep in a previous study were used to study the effects of stress hormones on myoblast proliferation and myoblast gene expression. Incubation in epinephrine-spiked media initially reduced L6 cell proliferation at 4 hours but then increased proliferation at 48 and 96 hours. TNFa, IL-6 and TWEAK all increased proliferation rates, but the quickest and most profound increase was with IL-6. Although IUGR fetal myoblasts showed greater proliferation rates than controls, proliferation rates didn't change with increasing amounts of norepinephrine or insulin in controls or IUGR myoblasts. Together our findings show that increased exposure to stress hormones have a positive effect on myoblast proliferation, especially in relatively short durations (48-96 hours).

Introduction

Research has shown that skeletal muscle is the major tissue involved in adaptive fetal growth restriction. IUGR fetuses diagnosed by ultrasound have less muscle in late gestation and offspring have slower muscle growth and accrue fat faster during childhood. Late gestation and after birth, skeletal muscle growth relies on myoblast proliferation and incorporation into existing fibers instead of increasing fiber numbers. Recent studies have shown stress hormones may regulate myoblast function even at sub acute levels. By using an in vitro and ex vivo system, we were able to investigate the effects of hypercatecholaminemia and inflammatory actions on skeletal muscle proliferation. We hypothesize that L6 extended culture with stress hormones will affect proliferation rates.

Materials and Methods

L6 Myoblast Proliferation

- 1.) L6 myoblasts were plated in 6-well fibronectin-coated plates at a density that yielded ~60k cells at collection.
- 2.) Myoblasts were grown in common growth media spiked with the following treatments (n=8) for either 4, 48, or 96 hrs:
Control: no additive, Insulin: 1 mU/ml, Epinephrine: 10 nM, IL-6: 1 ng/ml, TNFa: 20 ng/ml, TWEAK: 10 ng/ml
- 3.) Proliferation rates were determined by EdU pulse for the final 2 hours of the incubation period.
- 4.) Myoblasts were lifted off the plate and then fixed using 4% PFA. Cells were washed, permeabilized, and stained using a Click-iT Alexa Fluor 555 kit (Thermo Scientific).
- 5.) Cells were suspended in Moxi Cyte Reagent (Orflo) and analyzed using a zEPI Flow Cytometer (Orflo) for percent positive staining for EdU as a measure of proliferation rate.

L6 Myoblast Gene Expression

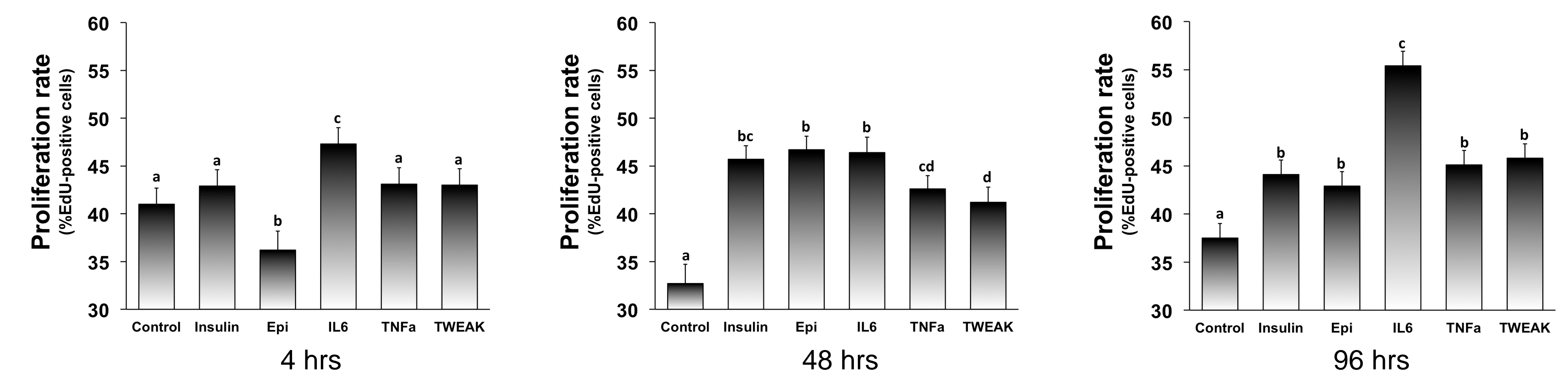
- 1.) RNA was extracted from 96 hrs cultured myoblasts that we treated the same as the L6 proliferation study using a RNeasy Mini Kit (Qiagen) and was reverse transcribed to cDNA using QuantiTect Reverse Transcription Kit (Qiagen).
- 2.) Validated oligonucleotide primers for TWEAK receptor (Fn14), TNF receptor 1 (TNFR1), IL-6 receptor (IL6R), adrenergic receptor $\beta 2$ (ADR $\beta 2$), and the housekeeping gene Ywhaz (Bio-Rad) were measured by droplet digital PCR.
- 3.) Droplets were generated from a 20- μ l reaction of EvaGreen supermix, primers, and cDNA template with 70 μ l of droplet generation oil. The resulting droplets were transferred to a heat-sealed 96-well plate for thermal cycling under manufacturer-recommended conditions.
- 4.) Number of copies were determined using a QX200 Droplet Digital PCR system (Bio-Rad Laboratories) and copy number was determined with QuantaSoft 1.7.4. software (Bio-Rad Laboratories).

IUGR Fetal Myoblast Proliferation

- 1.) IUGR myoblasts cells were isolated from fetal semitendinosus muscle from ewes that were euthanized at 134 ± 1 day of gestational age (dGA). These ewes had been exposed to elevated ambient temperatures (35-40°C) from the 40th to 95th dGA. Control ewes were housed at 25°C. After cells were isolated and purified, they were stored in liquid nitrogen until the proliferation study was performed.
- 2.) For this study - cells were plated at a density of 4,000 cells per well in 6-well fibronectin-coated plates (n=2 per treatment).
- 3.) Cells were grown in complete growth media for 4 days, with one media change on day 2.
- 4.) On the 4th day, cells were incubated in treatment media for 4 hours. For the first 2 hours cells were incubated in treatment media only, but for the final 2 hours cells were incubated in treatment media + 10 μ M EdU.
Treatments Medias: Media A: no additive, Media B: 50 pM Norepinephrine (NE), Media C: 500 pM NE, Media D: 5 mU/mL Insulin (Ins), Media E: 5 mU/mL Ins + 50 pM NE, Media F: 5 mU/mL Ins + 500 pM NE
- 5.) Following the 4 hour incubation period cells were accutased off the plate, fixed with 4% PFA and stained the same way as steps 5-8 from the L6 myoblast proliferation protocol above.

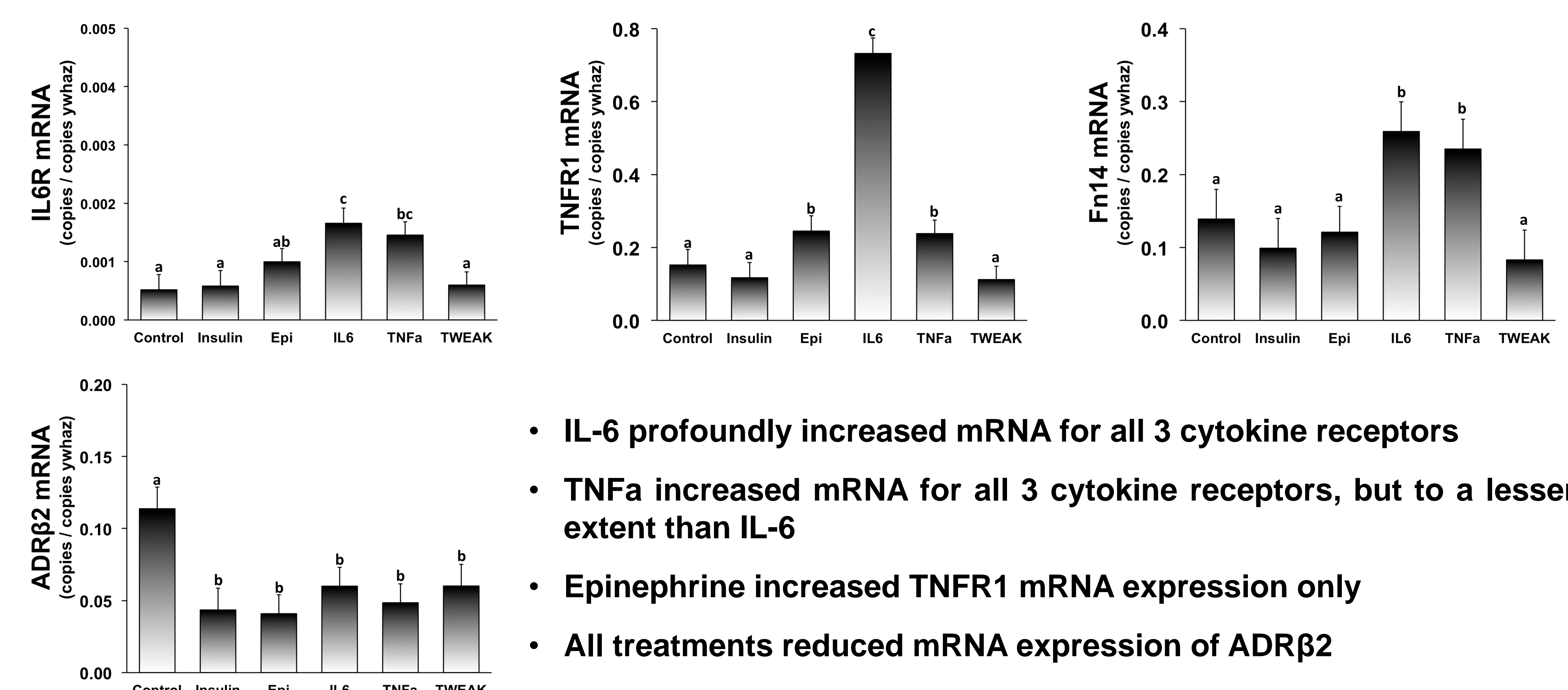
Results

Figure 1. L6 Myoblast Proliferation



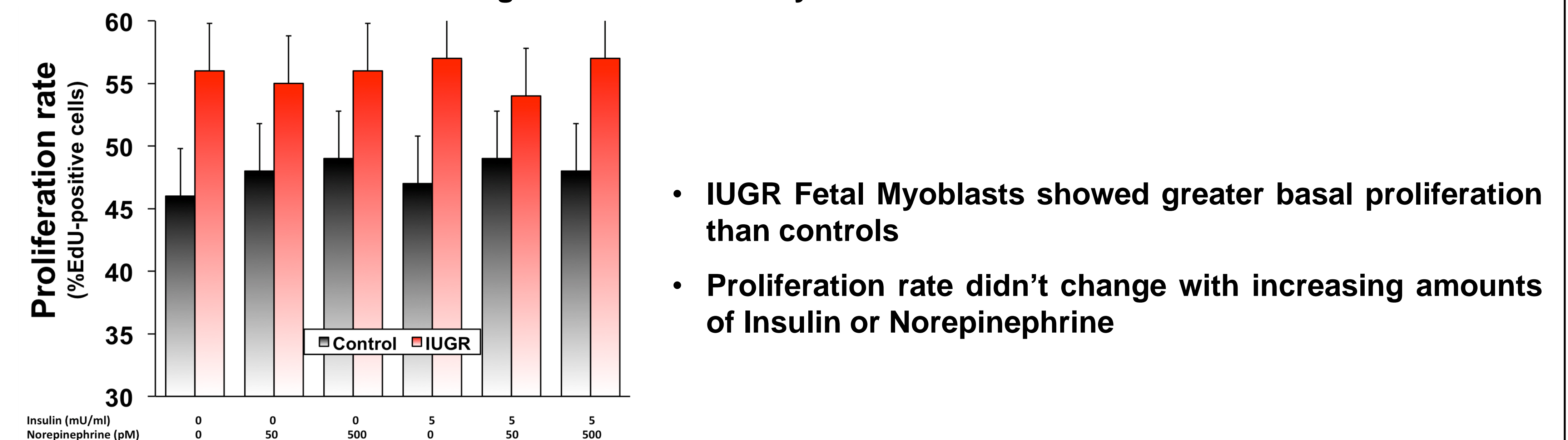
- Epinephrine initially reduced and ultimately increased L6 myoblast proliferation
- Pro-inflammatory cytokines increased proliferation, with the quickest and most profound effect from IL-6

Figure 2. L6 Myoblast Gene Expression



- IL-6 profoundly increased mRNA for all 3 cytokine receptors
- TNFa increased mRNA for all 3 cytokine receptors, but to a lesser extent than IL-6
- Epinephrine increased TNFR1 mRNA expression only
- All treatments reduced mRNA expression of ADR $\beta 2$

Figure 3. IUGR Fetal Myoblast Proliferation



Summary

48 to 96-hour exposure to pro-inflammatory cytokines and epinephrine has a positive effect on myoblast proliferation and may explain greater proliferation in IUGR fetal myoblasts, as IUGR fetuses are known to be hypercatecholaminemic.

Acknowledgements

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