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Changes in myoblast responsiveness to TNFα and IL-6 contribute to decreased skeletal muscle mass in intrauterine growth restricted fetal sheep

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Changes in myoblast responsiveness to TNFα and IL-6 contribute to decreased skeletal muscle mass in intrauterine growth restricted fetal sheep[1]

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INTRODUCTION

Intrauterine growth restriction (**IUGR**) is a leading cause of perinatal morbidity and mortality (Alisi et al., 2011). Skeletal muscle growth is disproportionately reduced in IUGR fetuses and offspring (Padoan et al. 2004; Yates et al. 2014). These individuals present with reduced muscle mass and increased risk for metabolic disorders at all stages of life (Godfrey and Barker, 2000; Yates et al. 2016.). Muscle growth requires proliferation, differentiation, and fusion of myoblasts (muscle stem cells) to form muscle fibers early in gestation and to increase myonuclear content of existing fibers during late gestation and after birth (Yates et al., 2014). These processes can be disrupted by inflammation, which is a potential factor in impaired muscle development in the IUGR fetus (Yates et al., 2012; Cadaret et al., 2017). Tumor necrosis factor-alpha (**TNFα**) and interleukin 6 (**IL-6**) are potent multifunctional cytokines involved in inflammatory and noninflammatory skeletal muscle disorders (Tüzün et al., 2006). We recently found that changes in gene expression of these cytokines and muscle sensitivity to them differed between IUGR and control rats (Cadaret et al., 2017), and that maternal inflammation induced fetal leukocyte adaptations, increasing gene expression of TNFα and its receptor TNFR1, but decreasing gene expression of IL-6 receptor. Both cytokines also regulate myoblast proliferation and differentiation outside of inflammatory states (Al-Shanti et al., 2008). These findings indicate TNFα and IL-6 are essential factors in proper growth and development of muscle, and thus, we postulate that expression and sensitivity changes contribute to decreased muscle growth capacity in IUGR fetuses. The objective of this study was to determine the effects of cytokines on fetal myoblast function and to determine if altered responsiveness is intrinsic in IUGR myoblasts, which would represent a potential adaptive mechanism for reduced muscle mass in IUGR offspring.

MATERIALS AND METHODS

Animals and Myoblast Isolation

The following experiments were approved by the Institutional Animal Care and Use Committees at the University of Nebraska-Lincoln and The University of Arizona which are both are accredited by AAALAC International. Columbia-Rambouillet ewes carrying singleton pregnancies were used to

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create IUGR fetuses ($n = 7$); placental insufficiency was induced by exposing ewes to elevated ambient temperatures (35–40 °C, 35% RH) from 40 to 95 d of gestational age (**dGA**), as previously described (Yates et al., 2014). Control fetuses ($n = 7$) were from pair-fed ewes maintained at 25 °C. Ewes were necropsied at 134 ± 1 dGA and myoblasts were isolated from fetal hindlimb muscle as described (Yates et al., 2014). Briefly, muscle was finely minced, digested (Protease type XIV from *Strept. griseus*; Sigma), and serial-centrifuged to isolate myoblasts, which were stored in liquid nitrogen until used.

Myoblast Functional Studies

Myoblast isolates were preplated in DMEM (Gibco) + 10% fetal bovine serum (**FBS**, Atlas Biologicals) for 2 h (37 °C; 95% O_2, 5% CO_2) to yield ≥96% pure populations (Yates et al., 2014). Cells were plated at 5,000 cells well^{-1} on fibronectin-coated six-well culture plates, grown for 3 d in growth media (DMEM + 20% FBS), and then incubated for 24 h in growth media containing either no additive (basal), TNFα (20 ng/mL; Sigma), or IL-6 (1 ng/mL; Sigma). Myoblasts were then pulse-labeled with EdU for 2 h and fixed in 4% PFA for 10 min. A second set of myoblasts (10,000 cells well^{-1}) were plated as described above and cultured for 4 d in differentiation media (DMEM + 2% FBS) with the same three treatments described above.

Immunocytochemistry

Myoblasts incorporating EdU (i.e., replicating) were identified by staining in suspension (ClickIT EdU). Differentiated myoblasts were identified by staining for myogenin (F5B; 1:50; BD Pharmingen) or desmin (DE-U-10; 1:250; GeneTex). Primary antibodies diluted in Permeablize/Block Buffer (PBS + 5% FBS 0.5% Saponin + 2% bovine serum albumin) were applied for 1 h at room temperature, followed by 1-h incubation at room temperature with secondary affinity purified anti-mouse IgG PE-Conjugate antibody (1:250; Cell Signaling). All samples were analyzed on a zEPI flow cytometer (ORFLO Technologies), with gates set from negative controls.

Statistical Analysis

Data were analyzed for effects due to treatment, incubation condition, and their interaction using the Mixed procedure in SAS (SAS Institute, Cary NC) with culture condition as a repeated variable. Two replicates per incubation condition were performed for each fetus and averaged. Values are expressed as mean percentages ± SE and fetus is the experimental unit.

RESULTS

Myoblast Proliferation

A treatment × incubation condition interaction was observed ($P < 0.05$) for proliferation rates (Figure 1). Proliferation was greater ($P < 0.05$) in control compared to IUGR myoblasts in basal and IL-6 spiked media but was similar between control and IUGR myoblasts in TNFα-spiked media. In control myoblasts, proliferation was decreased ($P < 0.05$) after incubation in media spiked with either TNFα or IL-6 compared to basal media. However, proliferation rates in IUGR myoblasts were similar among basal, IL-6, and TNFα-spiked media.

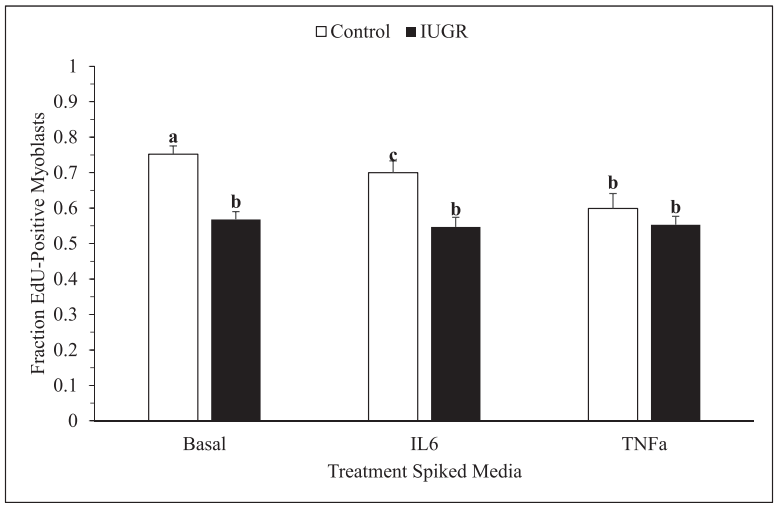

Figure 1. Proliferation in IUGR fetal myoblasts incubated with TNFα (20 ng mL^{-1}) or IL-6 (1 ng mL^{-1}). Cells were pulsed with EdU for 2 h. [a,b,c]means with different superscripts differ ($P < 0.05$).

Myoblast Differentiation

The percentages of myogenin-positive myoblasts were less ($P < 0.05$) for IUGR populations than controls regardless of incubation condition (Figure 2). Myogenin-positive percentages were similar between basal and IL-6–spiked media and were decreased ($P < 0.05$) by TNFα-spiked media regardless of treatment group. A treatment × incubation condition interaction was observed ($P < 0.05$) for desmin-positive myoblasts (Figure 3). Percentages of desmin-positive myoblasts were less ($P < 0.05$) for IUGR populations compared to controls when incubated in basal media but did not differ when incubated in IL-6– or TNFα-spiked media. For control myoblasts, desmin-positive percentages were greatest ($P < 0.05$) in basal media and least ($P < 0.05$) in TNFα-spiked media. For IUGR myoblasts, desmin-positive percentages did not differ among media conditions.

DISCUSSION

In this study, we show that TNFα and IL-6 have multifunctional effects on fetal myoblast dynamics, and that IUGR fetal myoblasts have inherent changes in responsiveness to these cytokines. Both cytokines decreased proliferation in control myoblasts but not in IUGR myoblasts, which indicates a

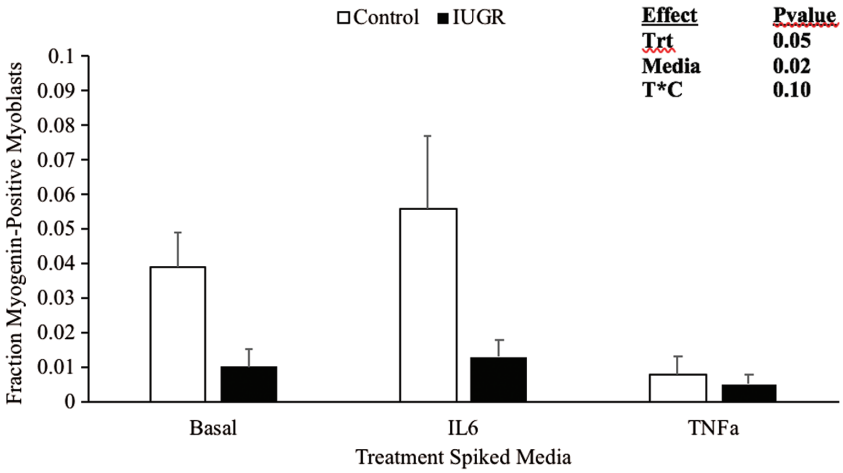

Figure 2. Myogenin expression in IUGR myoblasts after 3 d in differentiation media (2% FBS). Myoblasts were differentiated in media containing no additive (basal), TNFα (20 ng mL^{-1}), or IL-6 (1 ng mL^{-1}). [a,b,c]Means with different superscripts differ ($P < 0.05$).

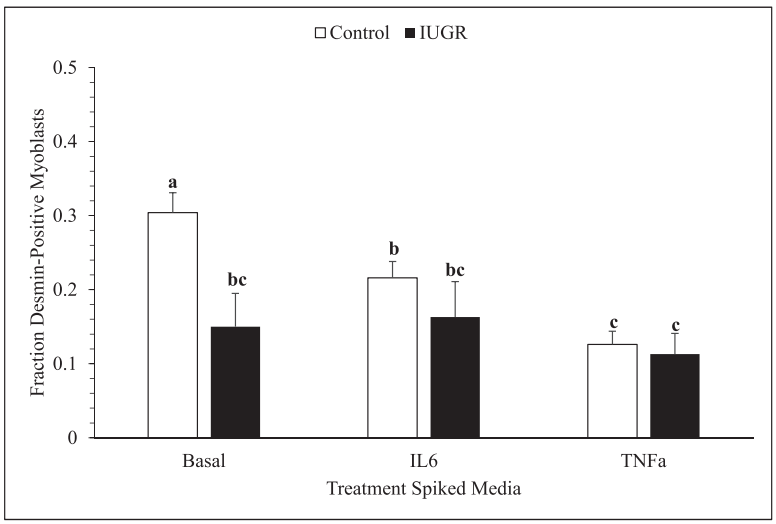

Figure 3. Desmin expression in IUGR myoblasts after 3 d in differentiation media (2% FBS). Myoblasts were differentiated in media containing no additive (basal), TNFα (20 ng mL^{-1}), or IL-6 (1 ng mL^{-1}). [a,b,c]Means with different superscripts differ ($P < 0.05$).

lost capacity to respond to these functional regulators and helps to explain their intrinsic functional deficits (Yates et al., 2012, 2016; Brown, 2014). Cytokine effects on differentiation were variable, with IL-6 not affecting myogenin-positive myoblasts but reducing desmin-positive myoblasts in controls. Conversely, TNFα reduced both myogenin and desmin-positive control myoblasts, indicating that it might be a more potent regulator of fetal myoblasts. Neither cytokine affected differentiation in IUGR myoblasts, however, which further demonstrates desensitization to cytokine regulation. These data indicate that TNFα and IL-6 contribute to dynamic changes in myoblast proliferation and differentiation rates.

Intrinsically reduced function in IUGR myoblasts controls muscle growth, which is beneficial under the hostile conditions of maternal stress but detrimental after birth. This adaptation allows the growing fetus to selectively mitigate the effects of cytokines, which are increased in the IUGR intrauterine environment (Cadaret et al., 2017). After birth, however, IUGR muscle is unable to respond adequately to the (noninflammatory) regulation by these cytokines that is essential for proper growth and development (Al-Shanti et al., 2008). TNFα is a particularly critical factor in muscle growth regulation (Collins and Grounds, 2001), and differential responsiveness in IUGR myoblasts may help to explain reduced muscle mass and smaller fibers observed with IUGR (Yates et al., 2016), as well as impaired glucose metabolism (Godfrey and Barker, 2000; Cadaret et al., 2017). Decreased proliferation and differentiation in uncompromised myoblasts by IL-6 is likely due to its ability to augment substrate delivery by interacting with the IGF system and to inhibit TNFα production by peripheral tissues (Pedersen et al., 2008; Al-Shanti et al., 2008; Akash et al., 2018), which is lost in IUGR myoblasts.

IMPLICATIONS

Fetal adaptations that result in IUGR also impair the responsiveness of skeletal muscle stem cells to critical regulation by inflammatory cytokines, mostly likely due to chronic overexposure in utero. Responses to TNFα and IL-6 are selectively absent in IUGR myoblasts as a protective response to mitigate the effects of maternofetal stress. Our findings show that IUGR fetal skeletal muscle adaptations change the ability of myoblasts to respond to these regulatory cytokines. These adaptations restrict skeletal muscle growth which, although helpful to fetal survival, contributes to reduced muscle mass and insulin resistance in IUGR offspring.

LITERATURE CITED

Akash, M. S. H., K. Rehman, and A. Liaqat. 2018. Tumor necrosis factor-alpha: role in development of insulin resistance and pathogenesis of type 2 diabetes mellitus. J. Cell. Biochem. 119:105–110. doi:10.1002/jcb.26174

Alisi, A., N. Panera, C. Agostoni, and V. Nobili. 2011. Intrauterine growth retardation and nonalcoholic fatty liver disease in children. Int. J. Endocrinol. 2011:269853. doi:10.1155/2011/269853

Al-Shanti, N., A. Saini, S. H. Faulkner, and C. E. Stewart. 2008. Beneficial synergistic interactions of TNF-alpha and IL-6 in C2 skeletal myoblasts–potential cross-talk with IGF system. Growth Factors 26:61–73. doi:10.1080/08977190802025024

Brown, L. D. 2014. Endocrine regulation of fetal skeletal muscle growth: impact on future metabolic health. J. Endocrinol. 221:R13–R29. doi:10.1530/JOE-13-0567

Cadaret, C. N., K. A. Beede, H. E. Riley, and D. T. Yates. 2017. Acute exposure of primary rat soleus muscle to zilpaterol HCl (β2 adrenergic agonist), TNFα, or IL-6 in culture increases glucose oxidation rates independent of the impact on insulin signaling or glucose uptake. Cytokine 96:107–113. doi:10.1016/j.cyto.2017.03.014

Collins, R. A., and M. D. Grounds. 2001. The role of tumor necrosis factor-alpha (TNF-alpha) in skeletal muscle regeneration. Studies in TNF-alpha(-/-) and TNF-alpha(-/-)/LT-alpha(-/-) mice. J. Histochem. Cytochem. 49:989–1001. doi:10.1177/002215540104900807

Godfrey, K. M., and D. J. Barker. 2000. Fetal nutrition and adult disease. Am. J. Clin. Nutr. 71 (5 Suppl):1344S–1352S.

Padoan, A., S. Rigano, E. Ferrazzi, B. L. Beaty, F. C. Battaglia, and H. L. Galan. 2004. Differences in fat and lean mass proportions in normal and growth-restricted fetuses. Am. J. Obstet. Gynecol. 191:1459–1464. doi:10.1016/j.ajog.2004.06.045

Pedersen, B. K., A. Steensberg, P. Keller, C. Keller, C. Fischer, N. Hiscock, G. van Hall, P. Plomgaard, and M. A. Febbriao. 2008. Muscle-derived interleukin-6: lypolytic, anti-inflammatory and immune regulatory effects. Pflugers Arch. 446:9–16. doi:10.1007/s00424-002-0981-z

Tüzün, E., J. Li, N. Wanasen, L. Soong, and P. Christadoss. 2006. Immunization of mice with T cell-dependent antigens promotes IL-6 and TNF-alpha production in muscle cells. Cytokine 35:100–106. doi:10.1016/j.cyto.2006.05.009

Yates, D. T., C. N. Cadaret, K. A. Beede, H. E. Riley, A. R. Macko, M. J. Anderson, L. E. Camacho, and S. W. Limesand. 2016. Intrauterine growth-restricted sheep fetuses exhibit smaller hindlimb muscle fibers and lower proportions of insulin-sensitive type I fibers near term. Am. J. Physiol. Regul. Integr. Comp. Physiol. 310:R1020–R1029. doi:10.1152/ajpregu.00528.2015

Yates, D. T., D. S. Clarke, A. R. Macko, M. J. Anderson, L. A. Shelton, M. Nearing, R. E. Allen, R. P. Rhoads, and S. W. Limesand. 2014. Myoblasts form intrauterine growth-restricted sheep fetuses exhibit intrinsic deficiencies in proliferation that contribute to smaller semitendinosus myofibres. J. Physiol. 14:3113–3125. doi:10.1113/jphysiol.2014.272591

Yates, D. T., A. R. Macko, M. Nearing, X. Chen, R. P. Rhoads, and S. W. Limesand. 2012. Developmental programming in response to intrauterine growth restriction impairs myoblast function and skeletal muscle metabolism. J. Pregnancy 2012:631038. doi:10.1155/2012/631038