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Developmental programming of mitochondrial substrate metabolism in skeletal muscle of adult sheep by cortisol exposure before birth

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Original Article

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Abstract

Prenatal glucocorticoid overexposure causes adult metabolic dysfunction in several species but its effects on adult mitochondrial function remain largely unknown. Using respirometry, this study examined mitochondrial substrate metabolism of fetal and adult ovine *biceps femoris* (BF) and *semitendinosus* (ST) muscles after cortisol infusion before birth. Physiological increases in fetal cortisol concentrations pre-term induced muscle- and substrate-specific changes in mitochondrial oxidative phosphorylation capacity in adulthood. These changes were accompanied by muscle-specific alterations in protein content, fibre composition and abundance of the mitochondrial electron transfer system (ETS) complexes. In adult ST, respiration using palmitoyl-carnitine and malate was increased after fetal cortisol treatment but not with other substrate combinations. There were also significant increases in protein content and reductions in the abundance of all four ETS complexes, but not ATP synthase, in the ST of adults receiving cortisol prenatally. In adult BF, intrauterine cortisol treatment had no effect on protein content, respiratory rates, ETS complex abundances or ATP synthase. Activity of citrate synthase, a marker of mitochondrial content, was unaffected by intrauterine treatment in both adult muscles. In the ST but not BF, respiratory rates using all substrate combinations were significantly lower in the adults than fetuses, predominantly in the saline-infused controls. The ontogenic and cortisol-induced changes in mitochondrial function were, therefore, more pronounced in the ST than BF muscle. Collectively, the results show that fetal cortisol overexposure programmes mitochondrial substrate metabolism in specific adult muscles with potential consequences for adult metabolism and energetics.

Introduction

Glucocorticoids are important regulatory signals during intrauterine development.[1] In common with their adult role [2,3], they can act as stress hormones prenatally and adapt fetal development in response to adverse intrauterine conditions such as hypoglycaemia and hypoxaemia.[1,4] However, in normal conditions, fetal glucocorticoid concentrations rise naturally towards term as a signal of impending delivery.[4] This prepartum cortisol surge switches fetal tissues from accretion to differentiation in preparation for birth and thereby activates functions that have little or no role before birth but are essential for neonatal survival, such as pulmonary respiration, thermoregulation, glucose production and, in precocial species, locomotion.[1,4] Glucocorticoids, therefore, act as both environmental cues and maturational signals in the fetus but, in optimising fitness for intrauterine and perinatal survival, these hormones can programme phenotype with functional consequences much later in postnatal life.[1,5,6] Indeed, glucocorticoid overexposure before term induced either by stressful intrauterine conditions or by maternal glucocorticoid treatment have been shown to lead to metabolic and cardiovascular dysfunction in the adult offspring of several species. [5–7]

Many of the survival mechanisms activated by glucocorticoids require extra energy in the form of ATP, which is produced predominantly by mitochondrial oxidative phosphorylation (OXPHOS).[3,8,9] In adult tissues, mitochondria are dynamic organelles that respond to altered energy demands by biogenesis, fusion/fission and changes in the abundance of the electron transfer system (ETS) complexes and other proteins regulating OXPHOS.[3,10,11] Mitochondrial ATP production is controlled by ATP synthase using the proton gradient that is generated across the mitochondrial membranes by the ETS complexes with oxygen acting as the final electron acceptor.[9,11] The efficiency of OXPHOS also depends on uncoupling proteins (UCPs) that dissipate the proton gradient and adenine nucleotide transporters (ANTs) that shuttle ADP and ATP across the mitochondrial membranes.[11–13]

Glucocorticoids are known to affect many of these mitochondrial processes in adult tissues including skeletal muscle.[9,13–17]

Administration of the synthetic glucocorticoid, dexamethasone, to pregnant rodent dams near term affects mitochondrial proteins in several fetal tissues including the heart and brain.[18–20] Elevating maternal cortisol concentrations during late pregnancy also alters expression of mitochondrial genes in the heart and skeletal muscle of fetal sheep.[21] In addition, recent studies have shown that the normal prepartum increment in cortisol in fetal sheep is responsible for increasing the mitochondrial OXPHOS capacity of specific skeletal muscles in anticipation of the extra locomotive demands for energy after birth.[22] Furthermore, adverse conditions during pregnancy that raise fetal glucocorticoid concentrations are known to alter abundance of specific mitochondrial proteins in the liver, heart, adipose tissue and skeletal muscle of fetuses and neonates of several different species.[23–29] However, whether direct elevations in the glucocorticoid concentration of the fetus affect its adult mitochondrial function remain largely unknown. Thus, this study examined mitochondrial OXPHOS capacity of skeletal muscle from adult sheep infused with cortisol before term.

Methods

Animals

A total of 30 time-mated pregnant Welsh Mountain ewes with single fetuses were used in this study. Of their offspring, 12 were studied as fetuses in late gestation while the remaining 18 were born at term and studied as young adults. Pregnant ewes were group housed in barns before surgery and single housed within sight and sound of other sheep after surgery until either fetal tissue collection or spontaneous labour and delivery. The ewes with newborn lambs were barn housed for a further 4–6 weeks before being turned out to grazing. The 18 offspring studied as young adults were weaned at 12 weeks of postnatal age and then kept at grazing with addition of vitamin and mineral supplements available *ad libitum*. A week before surgery as young adults, they were returned to single housing within sight and sound of other sheep until the end of the experimental protocol. When housed indoors, the pregnant ewes and young adult sheep had free access to hay and water, except for 12–18 h before surgery when food was withheld. All animal procedures were carried out under the UK Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012 following ethical review by the Animal Welfare and Ethical Review Body of the University of Cambridge.

Surgical procedures

Between 114–119 days of gestational age (dGA, term approximately 145 dGA), surgery was carried out on the ewes under isofluorane anaesthesia (1.5%–2% in 5:1 O_2:N_2O mixture) with positive pressure ventilation. Catheters were inserted into the maternal dorsal aorta and the fetal caudal vena cava, via the maternal femoral artery and two branches of the fetal tarsal vein respectively, and then exteriorised through the maternal flank.[30] In the young adult offspring, a catheter was inserted into the dorsal aorta and two catheters were placed into the caudal vena cava using the femoral vessels. The ewes and adults were monitored throughout surgery using a capnograph and pulse oximeter. At surgery, they were given antibiotics (oxytetracycline, 20 mg/kg i.m., Allamycin, Norbrook Laboratories, Newry, UK and penicillin, Depocillin, Intervet international, Milton Keynes, UK, 15 mg/kg i.m. to adults and i.v. to fetus) and analgesia (1 mg/kg carprofen, s.c. to the adults Rimadyl, Zoetis, London UK). Penicillin treatment to the ewes and adults continued for 2 days post-operatively.

Experimental procedures

After catheterisation, all animals were sampled daily to maintain catheter patency and to collect blood samples to measure blood gases and metabolite concentrations, and plasma hormone concentrations. Following post-operative recovery for at least 5 days, the catheterised fetuses were assigned randomly to receive a 5-day intravenous infusion of either saline (0.9% NaCl, 3ml/day, $n = 15$, controls, 7 male [M]: 8 female [F]) or cortisol (2–3 mg/kg/day Solu-Cortef; Pharmacia, $n = 15$, 8M:7F) with respect to balancing the numbers of males and females in each treatment group. At the end of infusion (128–131 dGA), the ewes were either killed for fetal tissue collection ($n = 12$) or allowed to deliver their offspring for study later in life as young adults ($n = 18$). All ewes and fetuses were killed by administration of a lethal dose of anaesthetic (200 mg/kg sodium pentobarbitone i.v., Pentoject, Animalcare Ltd, York, UK). The saline- (3M:3F) and cortisol- (4M:2F) infused fetuses were euthanised before biometric measurements were made and a range of tissues collected for subsequent analyses. Ewes allowed to deliver were killed after weaning of their lambs as described above.

In ewes destined to deliver spontaneously at term, the maternal polytetrafluoroethylene catheter was removed by gentle traction without further surgery or general anaesthesia and the fetal catheters were shortened, sealed and, after disinfection, internalised at the end of the infusion. At birth, the lambs were weighed and measured ($n = 9$ saline-infused, 4M:5F; $n = 9$ cortisol-infused, 4M:5F). Any catheters remaining *in situ* in the lambs were removed under local anaesthetic and all but one of the ram lambs were castrated by ringing the scrotum within 5 days of birth. The remaining ram lamb had undescended testes at birth and was surgically castrated under anaesthesia post-weaning by the Named Veterinary Surgeon. The lambs were weighed weekly until weaned at 12 weeks (w) and were catheterised as young adults at a mean postnatal age that did not differ significantly with intrauterine treatment (saline-infused, 43.8 ± 1.8 w, $n = 9$; cortisol-infused, 44.9 ± 1.3 w, $n = 9$). On average, 8 days after completing of a series of metabolic and endocrine measurements carried out as part of another study, an arterial blood sample was collected from the adult animals in the fed state before euthanasia for tissue collection using a lethal dose of anaesthetic as described above.

Samples of two skeletal muscles from the hind leg, the *biceps femoris* (BF) and *semitendinosus* (ST), were collected from all fetal and adult animals. These two muscles are both weight baring and generate mechanical power by fibre shortening for flexion and rotation of the lower limb.[31,32] However, they differ in innervation, blood supply and fibre-type composition.[32,33] After weighing the whole muscle, a portion from the central region was immediately placed into ice cold biopsy preservation medium (BIOPS; pH 7.1 solution containing 2.77 mm CaK_2EGTA, 7.23 mm K_2EGTA, 20 mm imidazole, 20 mm taurine, 50 mm MES, 0.5 mm dithiothreitol, 6.56 mm $MgCl_2.H_2O$, 5.77 mm Na_2ATP and 15 mm phosphocreatine[34]) for respiratory measurements. Samples of the remaining tissue were snap-frozen in liquid nitrogen and stored at −80°C until required for further biochemical and molecular analyses. All blood samples were placed into

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heparin-coated tubes and, after centrifugation, the plasma was stored at −20°C for cortisol and thyroid hormone measurements.

Respirometry

Respirometry measurements were made on saponin-permeabilised skeletal muscle fibre bundles from the fetal and adult sheep using the protocol described.[34,35] Briefly, 2–3 mg pieces of tissue were dissected in BIOPS and bundles of 6–8 fibres were teased apart before incubating with saponin for 20 min to permeabilise the plasma membrane (100 µg saponin/ml BIOPS). Samples were transferred into an isotonic respiration medium maintained at 37°C (MiR05; pH7.1 solution containing 20 mm HEPES, 0.5 mm EGTA, 3 mm $MgCl_2.6H_2O$, 10 mm KH_2PO_4, 20 mm taurine, 110 mm sucrose, 60 mm K-lactobionate and 1 g/l BSA;[34,36]) in order to measure oxygen (O_2) consumption using Clark-type oxygen electrodes (Strathkelvin Instruments, Glasgow, UK). Substrates were added into the chambers at saturating concentrations according to three protocols as previously described.[37] Malate (2 mm), glutamate (10 mm), ADP (10 mm) and succinate (10 mm) were added in sequence to give a measure of maximal ADP-coupled oxygen consumption when electron entry to both complex I and II of the ETS is saturated (GMS respiration). The second protocol involving the addition of malate (2 mm), pyruvate (5 mm) and ADP (10 mm) was used to obtain a measure of oxidative capacity for pyruvate, a derivative of glucose (PyM respiration). In the third protocol, malate (2 mm), palmitoyl-carnitine (40 µm) and ADP (10 mm) were added to provide a measure of fatty acid oxidation capacity (PCM respiration). In all protocols, leak state was measured in the presence of substrates before the addition of ADP, and the experiment concluded with the addition of cytochrome *c* (10 µm) to check outer mitochondrial membrane integrity. Results were excluded if there was a ≥15% increase in O_2 consumption following cytochrome *c* addition. Additionally, data were excluded if the rate of O_2 uptake over the baseline period before substrates were added, exceeded 0.001 µmolO_2/min as this suggests insufficient plasma membrane permeabilisation.[35] Following respirometry, muscle fibres were extracted from chambers and dried for 48 h before being weighed, and results are presented as the rate of O_2 consumption normalised to citrate synthase (CS) activity.

Biochemical and molecular analyses

Hormone assays

Plasma cortisol concentrations were measured using a human ELISA (RE52061, Tecan, Männedorf, Switzerland), previously validated for sheep plasma.[38] Intra- and inter-assay coefficients of variation for the cortisol assay were 3% and 5% respectively and the limit of detection was 5.2 ng/ml. Total plasma concentrations of thyroxine (T_4) and triiodothyronine (T_3) were measured using radioimmunoassays (MP Biochemical, Santa Ana, CA, USA) validated for use with sheep plasma.[30] For T_4, inter-assay variation was 4.6% and intra-assay variation was 3%. For T_3, inter-assay variation was 7.6% and intra-assay variation was 2.3%. The limits of detection for T_4 and T_3 were 11.3 ng/ml and 0.14 ng/ml, respectively.

Biochemical composition

Muscle water content was calculated as a percentage by weighing, freeze-drying overnight and then re-weighing samples of frozen muscles. Following extraction from homogenised frozen tissue, protein content was measured using a bicinchoninic acid assay and expressed as mg protein per gram tissue wet weight.

Activity of CS is a putative marker of muscle mitochondrial content and was assayed spectrophotometrically in the skeletal muscles.[37,39] After homogenisation of the muscle samples (10–30 mg 100 mm KH_2PO_4, 5 mm EDTA, 0.1% Triton X-100), aliquots of the homogenate were added to the assay buffer (pH8) containing 0.1 mm 5,5'-Dithio-bis(2-nitrobenzoic acid), DTNB, 1 mm oxaloacetate and 0.3 mm acetyl-CoA. CS activity was determined in duplicate as the maximal rate of absorbance change at 412 nm over 3 min (a measure of the rate of 5-thio-2-nitrobenzoic acid production). CS activity is expressed as µmol/min/mg dry tissue weight to allow for normalisation of the respiratory rates.

Western blotting

Frozen muscle samples were homogenised and diluted to 2.5 µg protein/µl in 8% SDS solution after measurement of the total protein content. Protein was electrophoresed on a 12% polyacrylamide gel, transferred to a nitrocellulose membrane and stained with Ponceau S to allow for normalisation of protein loading. Membranes were incubated with primary antibodies to ETS complexes I-IV and ATP synthase (OXPHOS antibody cocktail; 458099; Life Technologies; 1:1000), followed by an HRP-linked anti-mouse secondary antibody (NIF82; GE Healthcare; 1:5000). Enhanced chemiluminescence was used to visualise protein bands and quantified using ImageJ (http://rsb.info.nih.gov/ij/).

qRT-PCR

Frozen skeletal muscle samples were powdered and RNA extracted using TRIzol (Thermo Fisher) and chloroform, and the aqueous phase used in the RNeasy Plus kit (Qiagen, Hilden, Germany). RNA concentration was measured using a NanoDrop ND-1000 spectrophotometer, diluted to 50 ng/µl and used for cDNA synthesis (High Capacity cDNA Reverse Transcription Kit; Applied Biosystems, Foster City, CA, USA). qRT-PCR was performed using a MESA BLUE Mastermix (Eurogentec, Liège, Belgium) following the manufacturers recommended protocol (5 min at 95°C followed by 40 amplification cycles of 15 s at 95°C and 1 min at 60°C). The genes assayed, their encoded protein and function together with the primer sequences used are given in Table 1. In order to compare mRNA abundance of target genes between the treatment groups, cycle thresholds (Ct) were expressed relative to the geometric mean of S15 and 18S housekeeper genes and analysed using the delta-delta-Ct (ΔΔCt) method.[37,45] Housekeeper gene expression did not differ significantly between groups.

Statistical Analyses

Data are presented as mean ± standard error of the mean (SEM) with Sigma Stat 3.5 used for statistical analyses (Systat Software Inc., Point Richmond, USA). Differences between cortisol and saline treated animals at each age and between fetal and adult values in each treatment group were analysed by *t*-test or non-parametric Mann–Whitney U test, as appropriate, in the two muscles independently with the males and females in each treatment group combined. When the sample size and differences between treatment allowed sufficient power, a two-way ANOVA was used to assess the effects of treatment and sex and their interaction using the Tukey *post doc* test.

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Table 1. Forward and reverse primer sequences used for SYBR qRT-PCR

Target Gene, Encoded Protein and Function	Primer Sequences	References
Ribosomal protein S15 (RPS15)	F: ATCATTCTGCCCGAGATGGTG R: TGCTTCACGGGCTTGTAGGTG	Yates et al.[40]
18S rRNA	F: GTAACCCGTTGAACCCCATT R: CCATCCAATCGGTAGTAGCG	Byrne et al.[41]
Peroxisome proliferator-activated receptor gamma coactivator 1 Alpha (PPARGC1A); PGC1α protein; Regulator of Mitochondrial biogenesis	F: GAGATGTGACCACCGAGAATGAG R: GCTGTTGACAAATGCTCTTCGC	Myers et al.[42]
Mitofusin 1 (MFN1); MFN1 protein; Regulator of mitochondrial membrane fusion	F: TGGTGAGGTGCTATCTCGGA R: AACAGAGCTCTTCCCACTGC	This paper
Mitofusin 2 (MFN2); MFN2 protein; Regulator of mitochondrial membrane fusion	F: CATCAGCTATACTGGCTCCAACT R: AATGAGCAAAAGTCCCAGACA	Davies et al.[22]
Dynamin-related protein1 (DRP1); DRP1 protein; Regulator of mitochondrial membrane fission	F: ATGCCAGCAAGTCCACAGAA R: TGTTCTCGGGCAGACAGTTT	Reddy et al.[43]
Uncoupling protein 2 (UCP2); UCP2 protein; Mitochondrial uncoupling	F: AAGGCCCACCTAATGACAGA R: CCCAGGGCAGAGTTCATGT	Davies et al.[22]
Uncoupling protein 3 (UCP3); UCP3 protein; Mitochondrial uncoupling	F: GAAAGGAATTCTGCCCAACA R: TCCAAAGGCAGAGACGAAGT	Kelly et al.[44]
SLC25A4; Adenine nucleotide translocase 1 (ANT1) protein; Transport of ADP and ATP across mitochondrial membranes. Mild mitochondrial uncoupling	F: TGGTGTCCTACCCCTTTGAC R: CAGGCGCCTTTGAAGAAAGC	Kelly et al.[44]
Myosin heavy chain 7 (MHY7); MHCI protein; Muscle contraction	F: GAGATGGCCGCGTTTGGGGAG R: GGCTCGTGCAGGAAGGTCAGC	Yates et al.[40]
MHY2; MHCIIa protein; Muscle contraction	F: ACCGAAGGAGGGGCGACTCTG R: GGCTCGTGCAGGTGGGTCATC	Yates et al.[40]
MHY1; MHCIIx protein; Muscle contraction	F: AAAGCGACCGTGCAGAGCAGG R: GGCTCGTGCAGGTGGGTCATC	Yates et al.[40]

A two-way ANOVA was appropriate only for BF fat content. $P < 0.05$ was considered significant throughout.

Results

Cortisol infusion, biometry and muscle biochemical composition

Cortisol infusion before 130 dGA increased fetal cortisol concentrations to values similar to those seen closer to term and in response to hypoglycaemia and/or hypoxaemia earlier in gestation induced by adverse intrauterine conditions such as cord compression, maternal dietary manipulation or hypoxaemia.[1,4,6,7] Mean plasma cortisol concentrations were significantly higher in cortisol- than saline-infused fetuses throughout the 5-day infusion period (Fig. 1). Fetal cortisol infusion had no significant effect on gestational length in the ewes allowed to give birth compared with their saline-infused counterparts (saline-infused, 147.0 ± 0.8 dGA, $n = 9$; cortisol-infused, 146.0 ± 1.0 dGA, $n = 9$). Plasma cortisol concentrations of the adult sheep on the day of tissue collection did not differ significantly with intrauterine treatment (saline-infused, 52.3 ± 5.3 ng/ml, $n = 8$; cortisol-infused, 57.4 ± 9.9 ng/ml, $n = 9$). Although cortisol infusion of the fetus is known to increase its T_3 concentrations,[22] there were no significant differences in the plasma concentrations of T_4 and T_3 with intrauterine treatment at the time of tissue collection in adulthood (saline-infused; T_4, 70.3 ± 4.3 ng/ml, T_3, 1.44 ± 0.10 ng/ml, $n = 9$: cortisol-infused; T_4, 66.9 ± 4.2 ng/ml, T_3, 1.31 ± 0.07 ngml, $n = 8$).

No significant differences in body weight were observed between the two treatment groups either at tissue collection at 130 dGA (Table 2) or at birth (saline-infused, 3.37 ± 0.18 kg, $n = 9$; cortisol-infused, 3.68 ± 0.13 kg, $n = 9$). Body weight of the two treatment groups in adulthood was also similar at the time of tissue collection (Table 2). In both the fetuses and adults, weights of the BF and ST muscles did not differ significantly with intrauterine treatment either in absolute value or when expressed relative to body weight (Table 2). As expected, absolute and relative muscle weights were significantly greater in the adults than fetuses, irrespective of intrauterine treatment (Table 2).

In line with previous studies infusing cortisol into fetal sheep,[46,47] there was no significant difference in fetal blood pO2 between treatments during infusion (data not shown) whereas, blood glucose concentrations were higher in the cortisol than saline treated fetuses at the end of infusion (saline, 0.643 ± 0.050 mmol/l, $n = 14$; cortisol, 0.817 ± 0.045 mmol/l, $n = 14$, $P < 0.02$, t-test). Adult blood glucose concentrations were unaffected by prenatal

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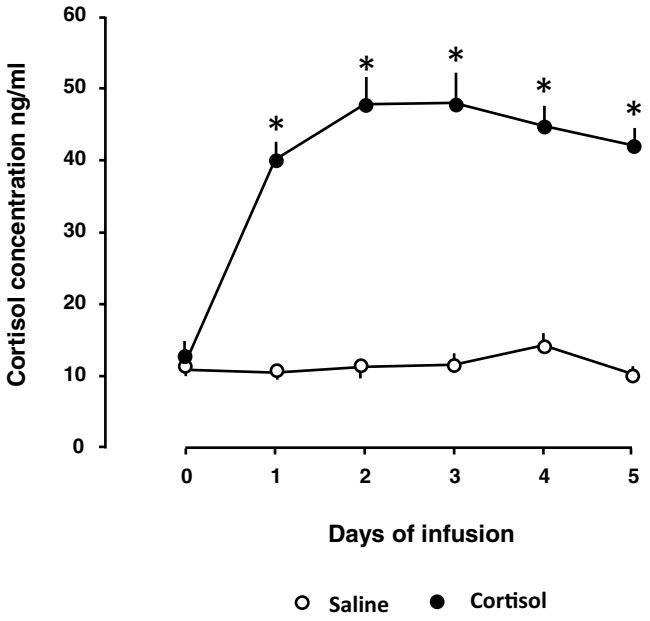

Fig. 1. Mean (±SEM) fetal concentrations of plasma cortisol during a 5-day intravenous infusion of either saline (open symbols, $n = 15$) or cortisol (filled symbols, $n = 15$) into the fetus. *Significantly different from the value in the saline-infused animals, $P < 0.05$ (*t*-test).

treatment (saline, 2.88 ± 0.11 mmol/l, $n = 9$; cortisol, 2.94 ± 0.12 mmol/l, $n = 9$, $P > 0.05$, *t*-test). In both fetuses and adults, there was no significant effect of fetal cortisol infusion on the glycogen content of either muscle relative to the corresponding saline-infused values (Table 2). Glycogen content of both the BF and ST decreased significantly with age, irrespective of intrauterine treatment (Table 2). Fat content of the ST, but not the BF, was significantly higher in cortisol- than saline-infused fetuses (Table 2).

In adulthood, there was no effect of fetal cortisol infusion on the fat content of either the ST or BF muscle when the two sexes were combined in each treatment group (*t*-test, Table 2). However, using a two-way ANOVA with sex and treatment as factors, there was a significant interaction between treatment and sex in determining BF fat content ($P < 0.01$), but no overall effect of treatment or sex. A significantly lower BF fat content was observed in the adult males infused with cortisol prenatally (25.0 ± 3.1 mg/g, $n = 4$) relative to their saline-infused counterparts (41.6 ± 7.2 mg/g, $n = 4$, $P < 0.05$), with no significant difference with treatment in the adult females ($P > 0.05$, $n = 5$, both groups). Cortisol infusion had no effect on the protein content of either the BF or ST in the fetuses (Table 2). In adulthood, protein content of the ST but not the BF muscle was significantly higher in the animals that received cortisol prenatally than in their saline-infused counterparts (Table 2). In both muscles, protein content was significantly higher in the adults than fetuses, irrespective of treatment (Table 2).

In both muscles, there was no effect of intrauterine treatment on mitochondrial CS activity in either the fetuses or adults (Table 2). There was also no significant effect of age on CS activity in the ST. In the BF, CS activity was significantly higher in the adult than fetal animals receiving saline but not cortisol prenatally (Table 2). The BF differences in CS activity between fetal and adult animals suggested differences in mitochondrial content so all respiration rates were corrected to CS activity to explore intrinsic changes in respiratory capacity that were independent of the confounding effect of differing mitochondrial contents between groups.

The effects of cortisol on muscle fibre composition were assessed by quantifying gene expression of the myosin heavy chain (*MHC*) isoforms for the type I slow-twitch oxidative fibres with abundant mitochondria (*MHCI*) and the type II fast-twitch fibres with fewer mitochondria that are either oxidative/glycolytic (*MHCIIa*) or predominantly glycolytic, (*MHCIIx*.)[33,40] In the BF, cortisol infusion significantly increased *MHCIIx* expression in the fetuses but not in the adults, relative to their respective saline-infused counterparts (Fig. 2A, 2B respectively). None of the other *MHC* isoforms were significantly altered by intrauterine treatment in either the fetal or adult BF, although there was a tendency for increased *MHCIIa* expression in the BF of cortisol-infused fetuses and adults ($P = 0.097$ and $P = 0.051$, Fig. 2A, 2B, respectively). In the ST, cortisol infusion had no significant effect on expression of any of the *MHC* isoforms in either the fetal or adult animals, although there was a tendency for increased *MHCIIx* in the cortisol-infused fetuses ($P = 0.096$, Fig. 2A).

Mitochondrial respiratory rates

Cortisol infusion had no significant effect on any of the respiratory rates in the fetal BF or ST relative to their saline-infused counterparts (Fig. 3), although there was a tendency for a higher PyM-supported respiration rate in the cortisol- than saline-infused fetal BF (Fig. 3B). Similarly, there were no significant differences in the GMS- or PyM-supported rates of respiration with intrauterine treatment in either the adult BF or ST (Fig. 3A, 3B). However, the rate of PCM-linked respiration was significantly higher in the ST, but not the BF, of adults treated with cortisol prenatally relative to their saline-infused counterparts (Fig. 3C).

In the BF, there was no significant effect of age on any of the respiratory rates, irrespective of intrauterine treatment (Fig. 3). In contrast, in the ST, age was a significant factor in determining all three respiratory rates with lower rates in the adults than fetuses, primarily in the saline treated animals (Fig. 3).

In both muscles, there was no significant effect of treatment on any of the substrate-specific rates of leak respiration normalised to CS activity in either the fetal or adult animals (Table 3). Using the pyruvate and malate substrate combination, there were significant decreases in leak respiration with age in the BF of the saline-infused animals and in the ST of those cortisol-treated prenatally but not in the other treatment groups (Table 3). In both muscles, there were no age-related changes in leak respiration rates with any of the other substrates, irrespective of treatment (Table 3).

Mitochondrial ETS proteins

Muscle expression of the ETS complexes and ATP synthase were assessed only in the adult muscles. There were no significant effects of intrauterine treatment on the abundance of any of the ETS complexes or ATP synthase in the adult BF (Fig. 4). In contrast, compared to saline treatment, fetal cortisol infusion significantly reduced abundance of all four ETS complexes, but not ATP synthase, in the adult ST (Fig. 4).

Mitochondrial biogenesis and OXPHOS regulatory genes

Expression of genes encoding proteins involved in mitochondrial biogenesis (*PGC1α*), fusion (*MFN1*, *MFN2*) and fission (*DRP1)* of the mitochondrial membranes and in the regulation of OXPHOS

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Table 2. Mean ± SEM measurements of bodyweight and of weights and composition of the *biceps femoris* and *semitendinosus* muscles in fetal and adult sheep infused with saline or cortisol *in utero* at the time of tissue collection

	Fetus		Adult	
	Saline ($n = 6$)	Cortisol ($n = 6$)	Saline ($n = 8$–9)	Cortisol ($n = 8$–9)
Body weight (kg)	3.05 ± 0.12	2.96 ± 0.15	36.8 ± 1.6[a]	38.6 ± 1.7[a]
Biceps femoris muscle				
Weight (g)	13.07 ± 0.61	12.30 ± 0.87	228.3 ± 16.6[a]	233.6 ± 7.4[a]
(g/kg body weight)	4.28 ± 0.09	4.14 ± 0.14	6.57 ± 0.40[a]	6.10 ± 0.40[a]
Water content (%)	82.1 ± 0.2	80.5 ± 0.2*	75.1 ± 0.2[a]	75.5 ± 0.4[a]
Protein content (mg/g wet wt	44.0 ± 1.2	47.5 ± 2.6	64.8 ± 1.0[a]	68.5 ± 3.4[a]
Glycogen content (mg/g wet wt	34.6 ± 2.0	37.8 ± 0.8	6.0 ± 0.8[a]	5.4 ± 0.5[a]
Fat content (mg/g wet wt)[b]	38.8 ± 0.5	39.5 ± 1.1	37.3 ± 3.6	36.2 ± 4.1
Citrate synthase activity (μmol/min/mg dry wt)	0.043 ± 0.003	0.045 ± 0.005	0.074 ± 0.007[a]	0.066 ± 0.009
Semitendinosus muscle				
Weight (g)	4.78 ± 0.29	4.36 ± 0.30	79.1 ± 6.4[a]	96.0 ± 5.8[a]
(g/kg body weight)	1.56 ± 0.05	1.47 ± 0.07	2.29 ± 0.18[a]	2.59 ± 0.08[a]
Water content (%)	81.7 ± 0.1	79.6 ± 0.4**	72.5 ± 1.2[a]	73.6 ± 1.2[a]
Protein content (mg/g wet wt)	47.7 ± 1.4	48.1 ± 1.7	109.1 ± 2.7[a]	120.1 ± 2.0*[a]
Glycogen content (mg/g wet wt)	19.9 ± 0.5	25.0 ± 2.5	4.6 ± 0.6[a]	3.6 ± 0.4[a]
Fat content (mg/g wet wt)	27.7 ± 2.1	42.1 ± 2.7*	38.1 ± 4.6	37.4 ± 2.5
Citrate synthase activity (μmol/min/mg dry wt)	0.053 ± 0.005	0.058 ± 0.010	0.070 ± 0.008	0.063 ± 0.007

For adult muscle weights $n = 6$. Wt = weight. Significantly different from the saline-infused group at the same age *$P < 0.05$, **$P < 0.01$ (*t*-test).
[a]Significantly different from the fetal group with the same treatment ($P < 0.01$, *t*-test).
[b]Significant interaction between sex and treatment in the adults ($P < 0.01$, interaction, two-way ANOVA with no significant effect of either treatment or sex overall).

efficiency (*UCP2*, *UCP3* and *ANT1*) were quantified for both adult muscles. There was no significant effect of intrauterine cortisol treatment on the relative expression of any of these genes in either the adult BF or ST, although there was a tendency for increased *UCP3* in the BF ($P = 0.072$, Supplemental Figure 1).

Discussion

The results demonstrate that a physiological increase in the plasma cortisol concentration in fetal sheep before term alters the biochemical composition and mitochondrial substrate metabolism of its hind limb skeletal muscles in adulthood. More specifically, there were changes in the protein content, PC-supported respiration and abundance of the ETS complexes of adult muscle after raising cortisol concentrations prenatally, which were specific to the different muscles. Muscle composition and mitochondrial OXPHOS capacity also differed between fetal and adult life in a manner that depended, in part, on the prenatal treatment. Collectively, the ontogenic and cortisol-induced changes in muscle mitochondrial phenotype were more pronounced in the ST than BF.

Neither CS activity nor mitochondrial OXPHOS capacity normalised to CS activity were affected by cortisol infusion in the fetuses, regardless of the specific substrate or muscle, consistent with previous measurements of CS activity per mg BF protein in cortisol-infused fetal sheep.[22] However, by adulthood, PCM-supported respiration was 60–70% higher than control values in the ST of animals that received cortisol as fetuses. Fetal exposure to higher than normal levels of cortisol in late gestation can, therefore, have longer term effects on mitochondrial substrate metabolism and may enhance the capacity of adult sheep to use fatty acids for OXPHOS in selective muscles. In adult sheep, fatty acid uptake by the hind limb makes a significant contribution to its whole-body O_2 consumption, particularly during exercise, whereas, in fetuses, there is little if any whole-body oxidative use of fatty acids, despite the higher capacity for PCM respiration in the control fetal than adult ST in the current study and the known availability of free fatty acids in the fetal circulation.[48,49] Since glucose is not absorbed from the ruminant gut, a switch to greater fatty acid utilisation for energy production in adult exercising muscles would help conserve endogenously produced glucose for use by other tissues with an essential glucose requirement like the brain, erythrocytes and mammary glands.[48] In addition, the ready availability of volatile fatty acids like acetate from rumen fermentation in adult sheep would provide an alternative substrate for Complex I- and II-linked respiration by metabolism to acetyl-CoA and then entry into the Krebs cycle.[48,50] However, in the adult BF treated with cortisol prenatally, there was no change in mitochondrial OXPHOS capacity with any of the substrates. Instead, there was a trend for an increase in the relative abundance of Type 2 *MHCIIa* fibres in the BF, which was not observed in the adult ST. The two muscles therefore, appear to adopt different strategies in altering fuel metabolism after intrauterine cortisol overexposure with the ST increasing the potential

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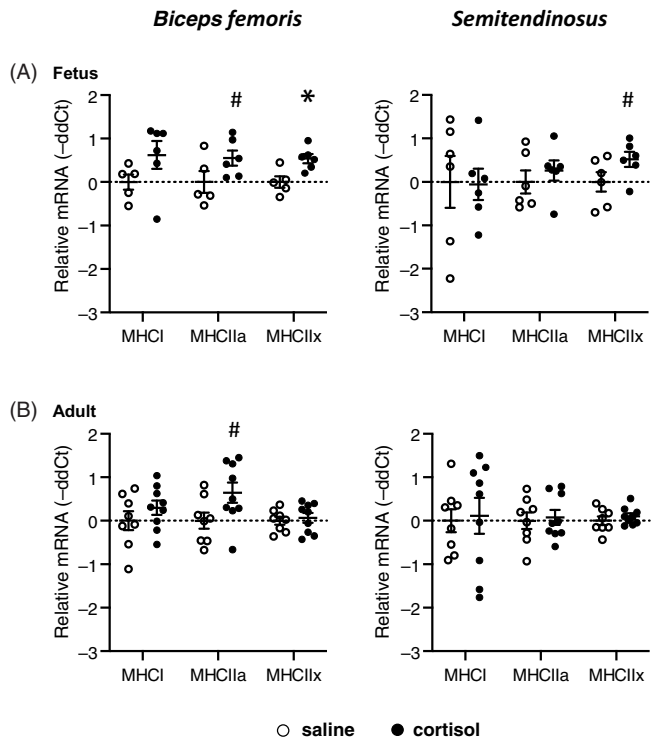

Fig. 2. Individual and mean (±SEM) relative gene expression of *MHCI*, *MHCIIa* and *MHCIIx* in the *biceps femoris* (BF) and *semitendinosus* (ST) muscles from (A) fetuses and (B) adult sheep (BF infused prenatally with either saline (open symbols) or cortisol (filled symbols)). Number of animals: (A) BF, $n = 5$ saline, $n = 6$ cortisol; ST, $n = 6$ saline and cortisol: (B) BF and ST $n = 8$ saline, $n = 9$ cortisol. Different from the value in the saline-infused animals,* $P < 0.05$, # $P < 0.10$ (*t*-test). Data on the fetal BF from reference 22.

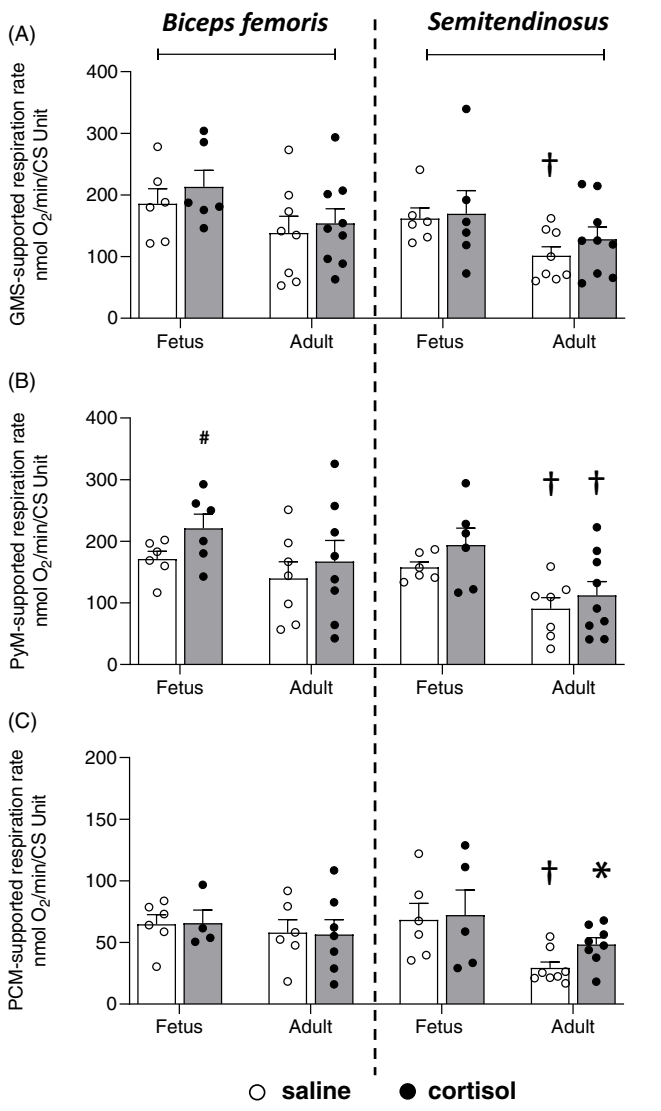

Fig. 3. Individual and mean (±SEM) rates of respiration supported by (A) glutamate, malate and succinate (GMS), (B) pyruvate and malate (PyM) and (C) palmitoyl-carnitine and malate (PCM) in the *biceps femoris* and *semitendinosus* muscles of fetal and adult sheep infused prenatally with either saline (open symbols and columns) or cortisol (filled symbols and columns). Number of animals: (A) Fetuses, $n = 6$ in each treatment group; Adults, $n = 8$ saline-infused, $n = 9$ cortisol-infused, (B) Fetuses, $n = 6$ in each treatment group; Adults, $n = 7$ saline-infused, $n = 8$ cortisol-infused, (C) Fetuses, $n = 6$ saline-infused, $n = 4$ cortisol-infused; Adults, $n = 6$ saline-infused, $n = 7$ cortisol-infused. * Significantly different from the value in the saline-infused group at the same age ($P < 0.05$, *t*-test or Mann–Whitney U test). † Significantly different from the fetal value in the same treatment group ($P < 0.05$, *t*-test or Mann–Whitney U test).

for fatty acid oxidation while the BF may show a greater capacity to switch between oxidative and glycolytic pathways for ATP production.

In the adult ST, the increment in PCM-supported O_2 consumption normalised to CS activity seen after intrauterine cortisol treatment occurred despite a 20–30% decrease in the relative abundance of all four ETS Complexes. Decreases in gene expression and protein abundance for the ETS Complexes have also been observed in cardiac and several skeletal muscles from adult offspring of rodent pregnancies compromised by conditions that can alter fetal glucocorticoid bioavailability such as reduced uterine blood flow, placental insufficiency, maternal hypoxia and diet-induced maternal obesity.[23,28,51–58] These changes in ETS Complex availability were often accompanied by a reduced Complex I-II mitochondrial OXPHOS capacity and mitochondrial dysmorphia with disorganised membranes and decreased *Pgc1α, Mfn2* and *Drp1* gene expression, in some instances.[51,55,58] There were no changes in *PGC1*α, *MFN*2, *DRP*1, *UCP*2, *UCP*3 or *ANT*1 gene expression in the ST of adult sheep treated with cortisol prenatally that might contribute to the increased PCM-linked mitochondrial OXPHOS capacity observed in the current study. There were also no changes in the adult concentrations of the thyroid hormones, which are known to regulate mitochondrial function and ETS Complex abundance in a range of fetal and adult tissues including skeletal muscle.[8,10,25,37,59] However, there may have been alterations in deiodinase activity or thyroid hormone receptor abundance in the adult muscle that affected cellular thyroid hormone bioavailability as intrauterine cortisol infusion has been shown to alter deiodinase activity in other fetal tissues.[59] Total muscle protein content was increased specifically in the adult ST after fetal cortisol treatment, which would partially ameliorate the reduced Complex abundance per unit of ST protein. Collectively, the results on the adult ST suggest that there may be changes in other regulatory elements of the adult ETS and/or in the biochemical pathways of mitochondrial fatty acid transport and β-oxidation after fetal cortisol treatment. Activity of the β-hydroxyacyl-CoA dehydrogenase (HOAD), the rate limiting enzyme of β-oxidation, is known to rise towards term in the BF in parallel with the prepartum increase in cortisol concentration in fetal sheep.[22] Increased expression of mitochondrial carnitine

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Table 3. Mean (±SEM) substrate-specific rates of leak respiration normalised to citrate synthase (CS) activity (nmols O_2/min/CS Unit) in the *Biceps femoris* and *Semitendinosus* muscles of fetal and adult sheep infused with cortisol or saline *in utero*

	Fetus		Adult	
	Saline (n = 5–6)	Cortisol (n = 4–6)	Saline (n = 6–9)	Cortisol (n = 7–9)
Biceps femoris				
Glutamate/malate/succinate (GMS)	36.2 ± 4.9	43.0 ± 14.0	28.8 ± 6.3	28.7 ± 6.1
Pyruvate/malate (PyM)	40.1 ± 5.7	32.8 ± 11.6	19.8 ± 1.9[a]	24.6 ± 4.1
Palmitoyl-carnitine/malate (PCM)	19.1 ± 3.2	30.7 ± 11.1	23.5 ± 4.1	21.5 ± 3.6
Semitendinosus				
Glutamate/malate/succinate (GMS)	28.7 ± 6.5	22.1 ± 6.3	26.0 ± 3.7	27.8 ± 4.9
Pyruvate/malate (PyM)	49.5 ± 14.8	81.5 ± 25.3	28.9 ± 9.3	33.3 ± 7.9[a]
Palmitoyl-carnitine/malate (PCM)	21.9 ± 5.8	28.6 ± 13.2	28.7 ± 3.7	35.8 ± 5.7

[a]Significantly different from the value in the fetuses with the same treatment, t-test, $P < 0.05$.

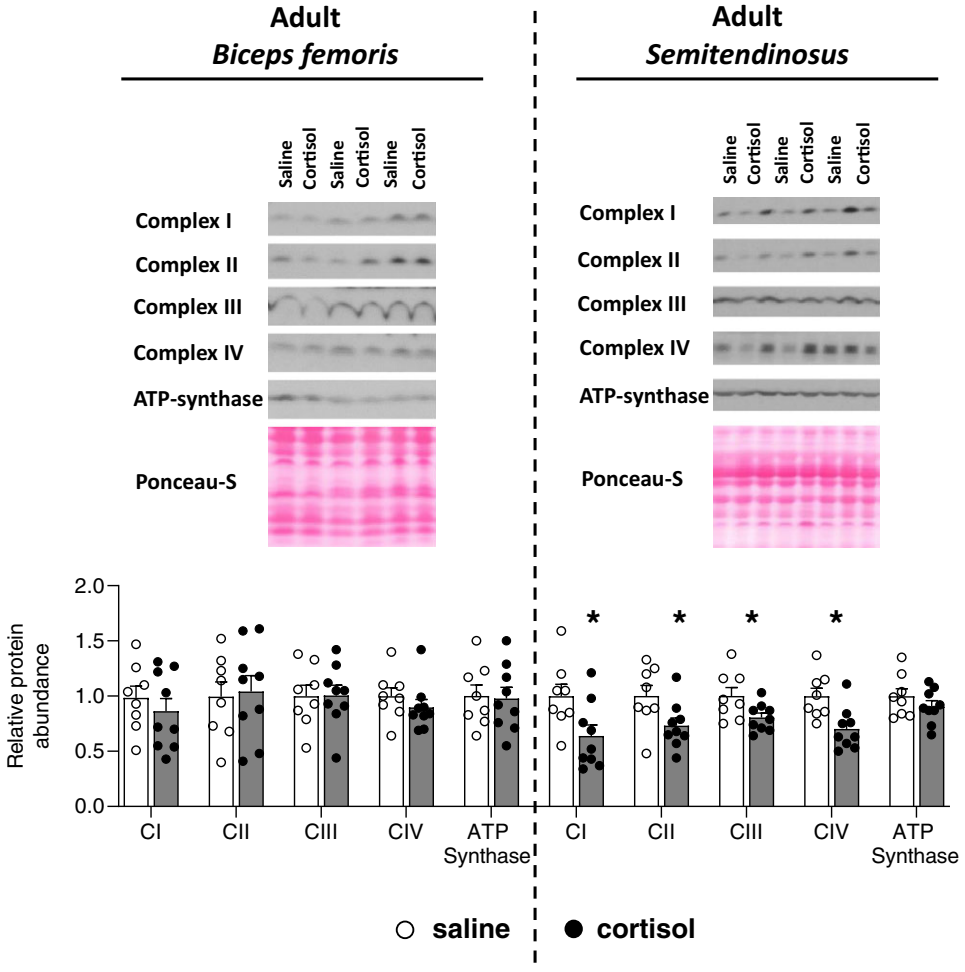

Fig. 4. Representative Western blots with individual and mean (±SEM) relative abundance of electron transfer system complexes, CI, CII, CIII, and CIV, and of ATP synthase in the *biceps femoris* and *semitendinosus* muscles of adult sheep infused prenatally with either saline (open symbols and columns, $n = 8$, both muscles) or cortisol (filled symbols and columns, $n = 9$, both muscles). *Significantly different from the value in the corresponding saline-infused group ($P < 0.05$, t-test).

palmitoyltransferase and HOAD has also been observed in skeletal muscle from juvenile rat pups after intrauterine growth restriction induced by uterine artery ligation.[24,60]

In rodents, the consequences of altering maternal diet during pregnancy on mitochondrial function of cardiac and skeletal muscle of the adult offspring appear to be sex-linked, with earlier and more pronounced changes in males than females.[23,51–55] By old age, muscle mitochondria of both sexes appear to be adversely affected by maternal nutritional manipulations during pregnancy, although the specific abnormalities in mitochondrial OXPHOS and ETS Complex abundance tend to differ between the sexes.[23,52,56] Compared to the sex-linked developmental programming of mitochondria seen in studies of intact male rodents [51,55,56], sexual dimorphism of mitochondrial function in adult ovine

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muscle may be less evident in the current study using castrated males due to lower than normal testosterone levels in adulthood. Testosterone is known to affect mitochondrial function in skeletal muscle and other tissues in adulthood and recent studies have also shown that prenatal testosterone overexposure causes mitochondrial dysfunction in skeletal muscle of female sheep later in postnatal life.[61–63]

In the current study, mitochondrial OXPHOS capacity decreased between fetal and adult life in the ST, but not the BF, in the absence of any change in the ST mitochondrial content measured as CS activity. This ST decrement in mitochondrial OXPHOS capacity was dependent, in part, on the respiratory substrate and the intrauterine treatment, and was more consistent in the saline- than cortisol-treated groups. With the additional challenges of extrauterine life,[4] neonatal energy requirements are high and are more dependent on fatty acid oxidation than seen prenatally or post-weaning, consistent with the high fat content of the milk.[22,49] The lack of a significant change in PCM-supported respiration in the ST between fetal and adult life in the cortisol-treated animals suggests that, rather than stimulating PCM-linked respiration, intrauterine cortisol overexposure may prevent or slow the normal postnatal decline in dependence on fatty acid oxidation for energy production.

In summary, exposure to higher than normal cortisol concentrations in fetal sheep during late gestation causes changes in mitochondrial OXPHOS capacity and abundance of the ETS Complexes in specific adult muscles. The cortisol-induced changes in mitochondrial respiration in the adult ST were also substrate-specific and related to an increased capacity for fat oxidation. Inappropriate early exposure to elevated cortisol concentrations *in utero* can, therefore, programme muscle mitochondrial substrate utilisation with potential long term consequences for metabolism and energy production later in life. Thus, cortisol acts not only as an environmental and maturational cue *in utero*, but also as a programming signal in preparing muscle mitochondria for future energy demands. These programming actions of cortisol *in utero* may be direct on the developing muscle or mediated via other known cortisol-dependent changes in the fetal endocrine environment and/or in the intrauterine availability of metabolic substrates such as glucose.[4,37,46,47,59] They may also provide a mechanism for the developmental programming of adult mitochondrial function seen in response to nutritional and other environmental challenges during pregnancy that cause fetal glucocorticoid overexposure.[5–7,64,65] However, the precise nature of the interplay between cortisol, nutrition and other endocrine factors that may mediate the effects of cortisol in developmental programming still remains unclear. Further studies are needed establish the specific cellular and molecular mechanisms involved in the muscle and substrate specificity of the ontogenic and cortisol-induced changes in ovine mitochondrial OXPHOS capacity seen in the current study.

Data availability. The data is stored in the University of Cambridge data repository (https://www.cam.ac.uk/respository) and is assigned a reference number obtainable from the corresponding author on publication.

Supplementary materials. For supplementary material for this article, please visit https://doi.org/10.1017/S204017442200040X.

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Author contributions. AJM, AJF and ALF conceived and designed the study. KLD and EJC generated the *in vivo* animal data. KLD DJS and JM produced the *in vitro* data on the tissues. KLD, AJM and ALF analysed and interpreted all the data. All authors were involved in drafting and revising the manuscript.

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Conflicts of interest. There are no conflicts of interest for any of the authors.

Ethical standards. All animal procedures were carried out under the UK Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012 following ethical approval by the Animal Welfare and Ethical Review Body of the University of Cambridge.

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