Impaired Cardiac Energy Metabolism in Embryos Lacking Adrenergic Stimulation

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Running Head: Norepinephrine stimulates embryonic cardiac metabolism

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As development proceeds from embryonic to fetal stages, cardiac energy demands increase substantially, and oxidative phosphorylation of ADP to ATP in mitochondria becomes vital. Relatively little, however, is known about the signaling mechanisms regulating the transition from anaerobic to aerobic metabolism that occurs during the embryonic period. The main objective of this study was to test the hypothesis that adrenergic hormones provide critical stimulation of energy metabolism during embryonic/fetal development. We examined ATP and ADP concentrations in mouse embryos lacking adrenergic hormones due to targeted disruption of the essential dopamine β-hydroxylase (Dbh) gene. Embryonic ATP concentrations decreased dramatically while ADP concentrations rose such that the ATP/ADP ratio in the adrenergic-deficient group was nearly 50-fold less than that found in littermate controls by embryonic day 11.5. We also found that cardiac extracellular acidification and oxygen consumption rates were significantly decreased, and mitochondria were significantly larger and more branched in adrenergic-deficient hearts. Notably, however, the mitochondria were intact with well-formed cristae, and there was no significant difference observed in mitochondrial membrane potential. Maternal administration of the adrenergic receptor agonists, isoproterenol or l-phenylephrine, significantly ameliorated the decreases in ATP observed in Dbh<sup>−/−</sup> embryos, suggesting that α- and β-adrenergic receptors were effective modulators of ATP concentrations in mouse embryos in vivo. These data demonstrate that adrenergic hormones stimulate cardiac energy metabolism during a critical period of embryonic development.
INTRODUCTION

The adrenergic hormones, epinephrine (EPI) and norepinephrine (NE), are key mediators of stress responses and sympathetic nervous system activities in adult mammals. NE, in particular, is also essential for embryonic development. Targeted disruption of the gene dopamine β-hydroxylase (Dbh), which codes for the enzyme that converts dopamine into NE, led to a loss of NE and EPI, and embryonic lethality due to heart failure in mice (54). In contrast, disruption of the subsequent enzymatic step catalyzed by phenylethanolamine n-methyltransferase (Pnmt) led to the loss of EPI without concomitant developmental phenotypes (21). Thus, while EPI may contribute to adrenergic activity in the embryo, NE is clearly of critical importance for heart development.

How NE influences heart development in utero is not fully understood. In Dbh⁻/⁻ embryos signs of cardiac distress begin to appear on embryonic day 10.5 (E10.5), and approximately 50% of the Dbh⁻/⁻ embryos die by E11.5 (54). Remarkably, the heart appears to develop and function normally up to this point, but then deteriorates rapidly into heart failure within 24h of the first signs, which include sluggish cardiac contractions, arrhythmia, and asynchrony as observed via echocardiography in utero (43). Recent work has shown that Dbh⁻/⁻ hearts display significantly delayed conduction speed across the atrioventricular junction relative to age-matched littermate controls (1). These results suggest important adrenergic influences on the development of cardiac structure and function, but do not fully explain how NE affects them.

At these early embryonic stages of development, there is no sympathetic innervation of the heart, and the adrenal glands have not yet formed. Instead, NE is produced in the embryonic heart itself, as well as outside of the heart in primordial sympathetic ganglia and brainstem neurons (20-22). With respect to heart function, NE appears to be acting primarily through β-
adrenergic receptors since $Dbh^{-/-}$ embryos could be rescued by providing the β-agonist, isoproterenol, in the maternal drinking water, whereas the α-agonist, l-phenylephrine, was partially effective at rescuing the heart failure and lethality (55). These results established that NE stimulates cardiovascular function during early embryonic development, but what are the important physiological targets regulated by adrenergic stimulation in the embryonic heart?

To gain insight about adrenergic actions in cardiac development, we recently performed a genome-wide expression screen of $Dbh^{-/-}$ and $Dbh^{+/+}$ embryonic hearts. A key finding from this screen demonstrated the largest category of differentially expressed genes (~31% of total) were those involved in metabolism (43). In adult mammals, adrenergic hormones are known to have profound and widespread influences on metabolism. In the liver, for example, β-adrenergic stimulation inhibits glycolysis, promotes gluconeogenesis, and stimulates breakdown of glycogen (6, 50). In cardiac and skeletal muscle, glycolysis and oxidative phosphorylation (OXPHOS) are increased in response to β-adrenergic stimulation to produce more available energy during stress. Free fatty acids are released from adipose tissue, and these serve as the primary fuel source for cardiac metabolism in adult mammals, but prior to birth the heart principally uses carbohydrates (8, 10, 17, 23, 26, 42, 48). The shift from carbohydrate to lipid metabolism in the heart mainly occurs at birth and is often referred to as the “fetal-shift” in cardiac metabolism (19, 36, 37). Surprisingly little, however, is known regarding adrenergic influences on cardiac metabolism during the embryonic period.

There is compelling evidence indicating that aerobic metabolism in the mitochondria becomes increasingly important as the heart transitions from embryonic to fetal stages of development. For example, genetic mutations that decrease or disrupt OXPHOS often result in heart failure and embryonic lethality (29, 34, 35). Furthermore, mutations that disrupt mitochondrial structure and function also interfered with cardiomyocyte differentiation and development (11, 12, 18, 58),
and many of these ultimately succumbed to heart failure and embryonic lethality. Thus, there appears to be an “embryonic-shift” from primarily anaerobic to aerobic metabolic mechanisms in the heart during the embryonic-fetal transition period of development (2).

We hypothesize that adrenergic hormones play a critical role in facilitating the metabolic shift towards aerobic oxidative phosphorylation in cardiac mitochondria during embryonic development. Here, we test this hypothesis by examining metabolic profiles of ATP, ADP, and ATP/ADP ratios in $Dbh^{-/-}$ and control embryos. In parallel, we also examine other metabolic indices, including cardiac oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) as estimators of aerobic and anaerobic metabolism, respectively. In addition, we performed detailed ultrastructural analysis of mitochondria in adrenergic-deficient and control hearts.
Glossary

Bpm, beats per minute
Dbh- dopamine β-hydroxylase
ECAR- extracellular acidification rate
EPI- epinephrine
ISO- isoproterenol
mtDNA- mitochondrial DNA
NE- norepinephrine
OCR- oxygen consumption rate
OXPHOS- oxidative phosphorylation
Pnmt- phenylethanolamine n-methyltransferase
TEM- transmission electron microscopy
METHODS

Mice

All procedures and handling of mice were conducted in accordance with the University of Central Florida Institutional Animal Care and Use Committees. The Dbh mouse strain was kindly provided by Dr. Richard Palmiter (University of Washington, Seattle, WA) (54) and maintained as previously described (1, 43). Timed pregnancies were determined by the presence of a vaginal plug (denoted as E0.5) and further confirmed by high-resolution ultrasound (Vevo 2100 instrument with 40 MHz transducer; Visualsonics, Inc.) at E8.5. For the dosing of maternal mice, drinking water was supplemented with ISO (0.02 mg/ml), l-phenylephrine (0.02 mg/ml), or timolol (1 mg/ml) beginning at E8.5, as previously described (49, 55). Vitamin C was added to the control and drug-containing drinking water bottles at a concentration of 2 mg/ml to help minimize oxidation of the drugs (55).

Embryonic Tissue Collections

All embryos used in this study appeared healthy and viable at the time of isolation as judged by their size (crown-rump lengths), color, texture, morphology, and overall appearance. Microscopic examination confirmed a beating heart and bright red blood coursing through the embryonic circulation. Unhealthy and dead embryos were discarded. From the living specimens collected, no apparent differences were observed between adrenergic-deficient and control embryos based on these gross examinations at the time of isolation. Upon isolation, the heads were removed and used for genotyping. For RNA and biochemical assays, hearts or trunks were flash-frozen in liquid nitrogen and stored at -80°C.

Reagents
Chemical reagents were purchased from Sigma-Aldrich, St. Louis, MO, except where noted otherwise. Electron microscopy-grade reagents for TEM were purchased from Electron Microscopy Sciences, Hatfield, PA. Cell culture reagents were purchased from Invitrogen, Carlsbad, CA, except for fetal bovine serum, which was obtained from Hyclone Labs (Logan, UT).

**ATP and ADP Measurements**

Briefly, embryonic tissue was homogenized in 6% trichloroacetic acid (TCA) for 1 minute then centrifuged at 6,000 g for 5 mins at 4ºC. The supernatant was then removed and TCA was neutralized with tris-acetate as previously described (16). ATP measurements were performed with ATPlite™ Bioluminescence Assay (Perkin Elmer) as instructed by the manufacturer’s protocol. ATP/ADP measurements were performed using ApoSENSOR™ ADP/ATP Ratio Bioluminescence Assay Kit (BioVision). Standard curves were generated with known concentrations of ATP and ADP. Luminescence was detected in an Envision Multilabel plate reader (Perkin Elmer), and ATP measurements were normalized to total protein concentrations.

**Lactate Measurements**

Embryos were homogenized in 8% perchloric acid for 1 min, and then centrifuged at 6,000 g for 4 mins. Absorbance readings at 340 nm before and after addition of L-lactate dehydrogenase were performed as described (5). The supernatants for lactate measurements were combined with nicotinamide adenine dinucleotide (NAD) solution (2.5 M NAD, 0.2 M Glycine buffer, and 100 µL ≥500 units/mg protein L-lactate dehydrogenase from bovine heart) in a 96-well plate. Increase in absorbance at 340 nm was compared to a standard curve of known lactate concentrations. Lactate measurements were done in triplicate and normalized to protein.

**Glucose Measurements**
Flash-frozen embryos were homogenized for 1 min, and then centrifuged at 12,000 g for 1 min. The supernatant was removed, and 2 µL was used to measure glucose concentrations using a WaveSense Presto blood glucose (AgaMatrix) monitoring system (15). Samples were compared to a standard curve of known D-glucose concentrations and normalized to total protein concentrations. Measurements were done in triplicate.

**Glycogen Measurements**

Flash-frozen embryos were homogenized for 30 sec in 1 M potassium hydroxide solution, and then incubated at 37ºC for 1 hr. Samples were then boiled for 5 mins and centrifuged at 13,000 g for 5 mins at room temperature (27). The supernatant was removed and glycogen was measured using a Glycogen Assay Kit (Sigma-Aldrich) as per the manufacturer’s protocol. Background glucose levels were subtracted from glycogen measurements and samples were normalized to total protein concentrations. Measurements were performed in duplicate.

**Oxygen Consumption Rate and Extracellular Acidification Rate Measurements**

E10.5 and E11.5 mouse hearts were isolated under aseptic conditions and cultured in Dulbecco’s modified eagle medium containing 10% fetal bovine serum (Hyclone Labs) that had been charcoal-stripped to remove catecholamine and steroid hormones (41). The media was additionally supplemented with penicillin G (100,000 U/L) and streptomycin (100 mg/L). Hearts were cultured in a Seahorse Biosciences XF24 Islet Capture Microplate with mesh grids placed on top of the specimen to prevent it from floating and from probe interference. Basal OCR and ECAR were simultaneously measured at 10min intervals over a period of 2 hrs using a Seahorse XF® Biosciences system. Rotenone (5 µM) and antimycin A (20 µM) were simultaneously added (61) to block mitochondrial electron transport.

**Gene Expression**
RNA was isolated from flash-frozen embryonic hearts using TRIzol reagent, and converted to
cDNA using High Capacity cDNA Reverse Transcription Kit (Invitrogen). Real-time PCR was
performed using SYBR Green Fast reagent in an AB7500 machine (Applied Biosystems).
Genes of interest were normalized to the housekeeping gene β-actin. Forward and reverse
primers were as follows: Pgc-1α, 5’-TATGGAGTGACATAGAGTGTGCT-3’ and 5’-
CCACTTCAATCCACCCAGAAAG-3’ Primer Bank ID 6679433a1, Tfam, 5’-
GAGCGTGCTAAAAGCAGTGTGAATTCCACCTTTTTCC-3’ (33), Sirt1, 5’-
TGTGAAGTTACTGAGGTGATGAAA-3’ and 5’-GCATAGATACGCTCTTGATCTGA-3’(33),
β-actin, 5’-CATCAGTATTGGCAACGAGC-3’ and 5’-ACGCAGCTCAGTAACAGTCC-3’ (24).

mtDNA Quantification

mtDNA content was measured as described previously (45). Briefly, total DNA was extracted
using TRIzol from flash-frozen embryonic hearts. Quantitative PCR of DNA was performed
using SYBR Green Fast reagent in an AB7500 machine (Applied Biosystems). The nuclear
gene, hexokinase 2, with forward and reverse primers 5’-GCCAGCCTCTCTGGATGT-3’ and 5’-
GGGAACACAAAAGACCTCTTCTGG-3’ was used to compare the mitochondrial encoded 16s
ribosomal RNA gene with forward and reverse primers 5’-CCGAAGGGAAAGATGAAAGA-
and 5’-TCGTTTGGTTTCCGGGTTC-3’. Measurements were performed in duplicate.

Transmission Electron Microscopy

Briefly, isolated embryonic hearts were placed in Karnovsky’s fixative (2% paraformaldehyde,
2.5% glutaraldehyde and 0.1 M sodium cacodylate, pH 7.3) for 1 hour. Samples were then
rinsed in 0.1 M sodium cacodylate with 3 µM CaCl₂ (pH 7.3), and post-fixed in a 1% osmium
tetroxide, 0.8% potassium ferrocyanide and 3 µM CaCl₂ solution. Samples were stained en bloc
with 2% uranyl acetate and dehydrated in graded ethanol solutions. Samples were embedded
in Durcupan, and sections were cut at 80 nm using a Leica UTC Ultramicrotome and diamond
knife, and then mounted on copper grids. Thin sections were stained with routine TEM double
stain: 4 mins in 4% uranyl acetate then 4-mins in Reynolds Lead Citrate. Thin sections were
examined using an FEI 268D TEM at 50Kv, and images recorded using an AMT XR-60 digital
camera. Mitochondrial morphometric and glycogen analyses were performed with ImageJ
software (NIH).

Oil Red O Staining
Oil Red O (5 mg/mL in isopropanol) stock stain was diluted to the working solution (60 percent
stock in distilled water), and used to stain for lipid droplets, as previously described (47).
Embryos were immediately placed in 2% fresh paraformaldehyde overnight and moved to 30%
sucrose and 0.02% sodium azide at 4ºC for storage. Frozen sections were made on a Leica
CM1850 cryostat at 12 µm thick at -20ºC. Briefly, the slides were air-dried, fixed in formalin,
and washed with running tap water for 1 min. They were then rinsed with 60% isopropanol,
stained with freshly prepared Oil Red O working solution for 15 mins. The staining solution was
then removed and the samples were rinsed with 60% isopropanol, rinsed further with distilled
water, and mounted using Vectashield mounting medium (Vector Labs). Digital micrographs
were obtained using a Leica DM2000 microscope, and images were analyzed for lipid droplets
using ImageJ software (NIH).

Free Fatty Acid Quantification
Fatty Acids were measured using the Free Fatty Acid Quantification Colorimetric/Fluorometric
Kit (Biovision) according to the manufacturer’s instructions. Briefly, flash-frozen embryos were
homogenized for 30 sec then centrifuged at 12,000 g for 1 min. 100 µL 1% Triton-X 100 in
chloroform was added to the supernatant. Samples were centrifuged at 12,000 g for 10 mins
and the lower phase was transferred and allowed to air dry, pellets were then resuspended in
fatty acid buffer. Fluorescence readings were performed using an Envision Multilabel plate
reader (Perkin Elmer) (Excitation: 535nm and Emission: 590 nm). Known concentrations of palmitic acid were used to generate a standard curve for this assay, and all samples were measured in duplicate.

**JC-1 dye**

Myocytes were isolated and cultured on coverglass (for microscopy) or 48-well plates (for flow cytometry) as described previously (38). Cardiomyocytes were cultured for 48 hours before staining with JC-1 dye (5 µg/ml; Invitrogen) for 20 mins. The dye was washed with PBS and samples were either viewed using a Perkin Elmer Spinning Disk confocal microscope or quantified using a BD FACs Canto flow cytometer (BD Biosciences, Inc.). Gates were set with unstained cells and carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP, 50 µM) treated controls and quantified emission filters appropriate for Alexa Fluor® 488 nm and R-phycoerythrin. Data were analyzed using FCSExpress software (DeNovo).

**Statistics**

Data are expressed as mean ± SEM. Student t-tests were performed to compare means between adrenergic-competent and adrenergic-deficient groups, with \( p < 0.05 \) required to reject the null hypothesis. One-way analysis of variance (ANOVA) was performed for multiple comparisions, with Bonferroni post-hoc testing for comparison between individual groups.
RESULTS

ATP is depleted in adrenergic-deficient embryos

To determine if adrenergic deficiency affects embryonic metabolism, we measured ATP and ADP concentrations in adrenergic-competent (Dbh\textsuperscript{+/+} and Dbh\textsuperscript{+/-}) and deficient (Dbh\textsuperscript{-/-}) embryos. Throughout this and previous studies, we observed no significant difference in Dbh\textsuperscript{+/+} and Dbh\textsuperscript{+/-} embryos for any assays employed, and these genotypes are phenotypically indistinguishable. Thus we have combined Dbh\textsuperscript{+/+} and Dbh\textsuperscript{+/-} mice into a single group that we will hereafter refer to as “adrenergic-competent”. Dbh\textsuperscript{-/-} mice fail to produce NE or EPI, and most will succumb to heart failure and embryonic lethality between E10.5-E15.5 (54) unless rescued by maternal supplementation of alternative catecholamine substrates (54) or β-adrenergic agonists, such as isoproterenol (ISO) (55).

ATP concentrations were significantly decreased in adrenergic-deficient embryos as early as E10.5, and declined precipitously by E11.5 (Figure 1A). Conversely, ADP concentrations increased over the same time period in the Dbh\textsuperscript{-/-} group relative to controls (Figure 1B). Notably, both ATP and ADP concentrations were unchanged at E9.5 relative to adrenergic-competent controls (Figure 1A, 1B). Steady-state ATP, ADP, and ATP/ADP values, generated from known ATP and ADP standard curve concentrations, are shown in Table I for adrenergic-competent and deficient embryos.

ATP/ADP ratios were virtually identical in adrenergic-competent and deficient embryos at E9.5, but then diverged dramatically over the next two days. In control embryos, the ATP/ADP ratio steadily increased over this time period, but in Dbh\textsuperscript{-/-} embryos, this ratio dropped by 50% at E10.5 and by >95% (~48-fold reduction) at E11.5 (Figure 1C). It is important to note that all embryos collected for these analyses appeared healthy and viable, as described in the
Materials and Methods section. Despite their outward appearance, however, *Dbh*−/− embryos exhibited an ATP/ADP energy deficit beginning around E10.5 that rapidly became much more severe by E11.5.

To determine if the observed energy depletion was due to the absence of β-adrenergic stimulation, we provided ISO in the maternal drinking water to rescue the phenotype. When the experiment was repeated under these conditions, the ATP deficits disappeared (Figure 1D). We observed a 1.8-fold increase in ATP concentrations at E10.5 and a 4.5-fold increase in E11.5 *Dbh*−/− ATP concentrations after ISO treatment compared to those observed without the addition of ISO. These results demonstrate that β-adrenergic stimulation was effective at preventing the energy loss resulting from adrenergic deficiency in developing mouse embryos.

Conversely, we applied the non-selective β-adrenergic receptor antagonist, timolol (1 mg/ml) in the maternal drinking water to determine if blockade of β-receptors would influence ATP concentrations in control (adrenergic-competent) embryos. E11.5 embryos from dams that received timolol contained significantly less (~22% reduction, p<0.01, n=16 for no drug controls, n=14 for timolol-treated group) ATP compared to age-matched controls that did not receive timolol (Figure 1E). Taken together, these results suggest that adrenergic stimulation of β-receptors is important for maintaining embryonic ATP concentrations.

We noticed, however, that while timolol was clearly effective at lowering ATP levels relative to controls during this period of development (Figure 1E), there was a greater decrease in ATP concentrations in the *Dbh*−/− age-matched embryos (compare panels A and E for E11.5, Figure 1). There are a number of possible explanations for this finding, including a potential role for α-adrenergic receptors in regulating embryonic energy metabolism. To test this, we applied the α-
adrenergic receptor agonist, l-phenylephrine, in the maternal drinking water beginning at E8.5, and again collected embryos at E11.5 to assess ATP concentrations. Remarkably, adrenergic-deficient embryos obtained from dams that received l-phenylephrine had significantly higher concentrations of ATP than adrenergic-deficient embryos from dams that did not receive any drug treatment as assessed by one-way ANOVA (Figure 1F). The recovery was not complete, but nevertheless achieved 50-60% of the ATP levels observed in controls. These results strongly suggest that embryonic ATP concentrations are positively regulated through $\alpha$- as well as $\beta$-adrenergic receptor stimulation.

Influence of adrenergic hormones on embryonic carbohydrate and lipid metabolism

Carbohydrate metabolism is the principal source of energy for the developing heart prior to birth, though lipid utilization by the heart also begins during the embryonic period as the heart gains oxidative metabolic capability (3, 17, 23, 48, 59). To determine if key carbohydrate metabolites were altered in adrenergic-deficient embryos, we measured glucose, glycogen, and lactate concentrations in embryos isolated at E10.5 and E11.5 (Table II). No significant differences were observed in the concentrations of these carbohydrate metabolites at these ages. However, lactate concentrations appeared slightly elevated, on average, in adrenergic-deficient embryos at both E10.5 and E11.5, but these differences were also not statistically significant. Glycogen concentrations trended lower in the deficient group, but the results were variable and again not found to be significantly different. The biochemical results for glycogen were corroborated by image analysis of glycogen granules from transmission electron microscopy (TEM) micrographs, which showed a similar insignificant downward trend in glycogen granules at E11.5 for the adrenergic-deficient group (Table II). We also measured free fatty acid concentrations and lipid droplets, but found no significant differences in these either, though there was a downward trend in the deficient group for lipid droplets, which were predominantly
located in the liver at these stages of development. Despite these trends, steady-state levels of free fatty acids as well as glucose, glycogen, and lactate were relatively unchanged (no significant differences) in adrenergic-deficient embryos compared to age-matched littermate controls at E10.5 and E11.5.

When we examined the rate of glycolysis, however, significant differences were observed. To perform these measurements, we isolated and cultured whole beating hearts from E10.5-E11.5 Dbh⁻/⁻ and littermate controls and recorded the extracellular acidification rate (ECAR) at various intervals over a 40-min period. At E10.5, there was little difference in ECAR from adrenergic-deficient and control samples over the entire 40-mins (Figure 2A). At E11.5, however, adrenergic-deficient and control ECARs were similar initially, but began to decline in the adrenergic-deficient group after about 10-mins and continued to decline further over the next 30-mins such that it was less than half the initial rate by 40-mins (Figure 2B). This decline was prevented by the addition of ISO in the maternal drinking water (Figure 2C). These results suggest that glycolytic rate was compromised in adrenergic-deficient hearts by E11.5.

**Oxygen consumption rate (OCR) decrease due to absence of β-adrenergic stimulation**

Mitochondria play an increasingly important role in the heart at these early embryonic stages of development. OCR measurements from isolated embryonic hearts can be used to estimate mitochondrial respiration. Prior to OCR measurements, beating rates were taken in isolated embryonic hearts to verify the viability of tissue collected. Similar beating rates were observed in the adrenergic-competent and deficient samples at both ages (Table III). To confirm the OCR was from mitochondrial function, we administered rotenone (5 µM) and antimycin A (20 µM) to control hearts to block complexes I and III, respectively. This treatment significantly decreased the overall OCR by 50-60% (Figure 3A and 3B), indicating that at least half of the observed OCR in E10.5-E11.5 mouse hearts was dependent on mitochondrial respiration. The remaining
portion likely reflects other oxidation reactions within the cells, as has been commonly observed in other systems (9, 28, 60).

We found that adrenergic-deficient hearts had lower rates of oxygen consumption, on average, compared with littermate controls (Figure 3C and D). Nevertheless, OCR increased similarly in adrenergic-deficient and competent hearts between E10.5 and E11.5 (compare panels C & D, Figure 3), but the adrenergic-deficient OCR continued to lag significantly below the control group through E11.5. As indicated above, the hearts appeared similar and were beating spontaneously at comparable slow but steady rates in both adrenergic-deficient and competent hearts during the ex vivo culture period (Table III). Thus, despite the lack of any clear differences in outward appearance or behavior, adrenergic-deficient hearts consumed oxygen at significantly lower rates than control hearts. To verify this effect was due to the absence of adrenergic hormones, we provided ISO in the maternal drinking water, which restored OCR to control levels in adrenergic-deficient hearts (Figure 3 panels E & F). Acute administration of ISO to the isolated heart in culture, however, did not result in any significant changes in OCR over a 1-hr recording period (not shown). These results suggest that longer term treatment with ISO in vivo is needed to effectively prevent the decline in OCR observed in adrenergic-deficient hearts.

Mitochondrial biogenesis not affected by the loss of adrenergic-hormones

In theory, the lowered OCR and ATP/ADP ratio could be due to fewer mitochondria resulting from compromised mitochondrial biogenesis in adrenergic-deficient hearts. To test this hypothesis, we measured expression of key mitochondrial biogenesis genes in isolated hearts, but no significant alterations in mRNA for *Pgc-1α* (p=0.58), *Tfam* (p=0.47), and *Sirt1* (p=0.65) were found in E10.5 isolated hearts when normalized to the housekeeping gene *β-actin* (Figure 4A). Similar results were found at E11.5. We also measured mitochondrial DNA (mtDNA)
content present in adrenergic-deficient and control hearts, but no significant differences were observed (Figure 4B). There was an apparent increase in mtDNA at E10.5, which could be related to the similar upward trend seen in Pgc-1α from this group (compare Figure 4A and 4B), but neither of these differences were significant, and both trends had disappeared by E11.5. These results suggest that mitochondrial biogenesis is not likely a limiting factor in adrenergic-deficient embryos at these early stages.

**Adrenergic-deficient myocytes have intact mitochondrial membranes**

The decreased OCR in adrenergic-deficient hearts suggests that mitochondrial function may be impaired. To properly function, mitochondria must maintain membrane potentials sufficient to drive the proton gradients necessary for OXPHOS in their inner membrane space. The fluorescent dye JC-1 can distinguish between mitochondria with intact membranes and those with compromised membrane potentials via differential fluorescence emissions (46). For example, red fluorescence (~590 nm) detects aggregates and is indicative of healthy intact membranes, while green fluorescence (~529 nm) detects monomers associated with perturbed mitochondrial potentials. We employed JC-1 staining with flow cytometry in combination with laser-scanning confocal fluorescence microscopy to assess mitochondrial membrane potential integrity in cardiomyocytes isolated from E10.5 and E11.5 adrenergic-competent and deficient hearts. Flow cytometry for red (aggregates) and green (monomers) showed no difference between adrenergic-deficient and control samples at E10.5 (Figure 5A) or E11.5 (Figure 5C). Quantification of the stained primary cardiomyocyte culture showed a red to green ratio of 3.4 ± 0.7; n=9 in the E10.5 deficient samples and 3.1 ± 0.6; n=9 in controls (Figure 5B). Similar results were observed in the E11.5 primary cardiomyocyte cultures with the red to green ratio of 3.4 ± 1.3; n=4 in the deficient group and 2.5 ± 0.8; n=4 in the control group (Figure 5D). Representative mitochondrial staining with JC-1 is shown in Figure 5E for adrenergic-competent and deficient myocytes isolated from E11.5 hearts. Despite the fact there were no significant
differences in the ratio of red/green staining in these specimens, the mitochondrial staining pattern in the adrenergic-deficient group appeared to be less densely clustered and less well-organized within myocytes than those typically found in age-matched adrenergic-competent littermates, indicating there may be structural abnormalities in mitochondria from adrenergic-deficient embryos. At this resolution, however, it was difficult to determine if there were truly structural anomalies in the mitochondria, so we employed transmission electron microscopy (TEM) for these evaluations as described below.

Mitochondrial morphology altered in adrenergic-deficient hearts

To obtain a detailed view and assessment of mitochondrial structure in adrenergic-deficient and control embryonic hearts, we analyzed cardiac tissue specimens using transmission electron microscopy (TEM). Our results show that mitochondria within E10.5 adrenergic-deficient myocardium were enlarged and appeared swollen relative to adrenergic-competent controls (Figure 6A and 6B). High magnification images show abnormal mitochondrial shapes in the adrenergic-deficient hearts (Figure 6C). Drawn-to-scale tracings of mitochondrial shapes from control and adrenergic-deficient hearts are shown for ease of comparison in panel D of Figure 6 at E10.5. Similar results were observed in E11.5 adrenergic-deficient hearts, demonstrating swollen and elongated mitochondria compared to those in adrenergic-competent samples (Figure 6E-H). Multiple mitochondria had branches and bulges in adrenergic-deficient hearts at E11.5 (Figure 6H). Adrenergic-deficient myocyte samples had fewer mitochondria per micrograph compared to adrenergic-competent hearts at E11.5, a trend that was evident at E10.5, though the numbers were more similar to control values at that age (Table IV). Mitochondrial length was significantly increased at E10.5 (p<0.05) and at E11.5 (p<0.0001) by nearly 30% in the adrenergic-deficient samples versus controls (Table IV). Mitochondrial surface area was also significantly increased at E10.5 (p<0.05) and at E11.5 (p<0.001) in adrenergic-deficient myocytes compared to controls (Table IV). These results clearly show that
mitochondrial structure was significantly altered in adrenergic-deficient embryos. On average, the mitochondria were longer, had greater surface area, and displayed more abnormal shapes such as branch points, curvatures, and budding bulges in their membranes compared with their control counterparts.
Anaerobic glycolysis is the predominant mode of metabolism of the heart during early embryonic development, but aerobic mitochondrial metabolism becomes an increasingly important and essential mode of ATP production at late embryonic to early fetal stages of development (7, 17, 23, 48). For example, classic studies showed that isolated embryonic rat hearts utilized glycolytic mechanisms through E11; however, glycolysis was not sufficient to maintain maximal heart rates at E12 or E13 (17). Further, energy metabolism was unaffected by the presence of oxygen at E11 in the rat, but was significantly and progressively elevated by oxygen in E12 and E13 hearts. This embryonic-shift in metabolic capability between E11-E12 in the rat roughly corresponds to the end of the organogenesis period of embryonic development and the beginning of fetal development. In the mouse, the equivalent stages of development are approximately E9.5-E10.5 (53). It is during this late embryonic period when metabolic deficiencies first become apparent in adrenergic-deficient (Dbh⁻/⁻) mouse embryos.

At E9.5, there were no discernible differences in ATP or ADP concentrations in adrenergic-deficient and control embryos, but ATP/ADP ratios began to decline at E10.5 and much more dramatically so at E11.5 in adrenergic-deficient embryos. This was mediated, in part, through β-adrenergic receptors since administration of the β-agonist, isoproterenol, was able to “rescue” the loss of ATP observed in Dbh⁻/⁻ embryos. Further, administration of the β-antagonist, timolol, induced significant decreases in ATP concentrations in adrenergic-competent control embryos. These results support a role for β-adrenergic receptor stimulation in regulation of ATP.

There also appears to be a role for α-adrenergic receptor involvement since the α-agonist, phenylephrine, was also able to “rescue” ATP concentrations in adrenergic-deficient embryos. In both rescue experiments, ATP depletion was partially rescued by either α- or β-agonists,
suggesting that both pathways likely contribute to adrenergic influence of ATP regulation in the developing embryo. It should be noted, however, that further work is needed to fully elucidate the molecular signaling pathways leading from receptor stimulation to regulation of ATP homeostasis. One must be cautious interpreting pharmacological data since partial effects could be attributed to a number of different factors including drug dosage, secondary or other indirect effects, drug distribution/penetrance in the embryo due to placental barrier, and other factors that may not be readily apparent. These caveats notwithstanding, the results of our pharmacological experiments are consistent with those observed in the genetic knockout experiments described in this study. In both cases, the results showed that adrenergic stimulation is needed to maintain embryonic ATP concentrations during critical early stages of development.

ATP is generated by glycolysis and mitochondrial oxidative phosphorylation. Our results suggest that the decline in ATP/ADP cannot be fully ascribed to glycolysis since no significant differences were observed in key glycolytic metabolites including glycogen, glucose, and lactate at E10.5 or E11.5. In addition, ECAR was virtually identical at E10.5 in adrenergic-deficient and control embryos. By E11.5, however, ECAR was selectively decreased in the adrenergic-deficient group, indicating that glycolytic rate may have been compromised. The decline was not apparent immediately, but developed over 15-30 mins, and was completely prevented by supplying the β-agonist, isoproterenol in the maternal drinking water. These results suggest that adrenergic stimulation is needed to maintain glycolytic rates after E10.5 in mouse embryos.

As we have shown, OCR was also significantly decreased in adrenergic-deficient hearts at E10.5 and E11.5 relative to littermate controls. These results suggest that aerobic metabolism was likely compromised in these embryos. It appeared that basal OCR in adrenergic-deficient
hearts lagged about a day behind adrenergic-competent hearts, and was only about 50% of control levels at E10.5 and E11.5. These effects were extinguished by supplying isoproterenol in the maternal drinking water, thereby indicating the absence of β-adrenergic stimulation was responsible for the declining OCRs in Dbh⁻/⁻ embryos. These results suggest that oxidative metabolism may have been impaired in adrenergic-deficient embryos.

Since oxidative phosphorylation occurs primarily in mitochondria, we examined mitochondrial ultrastructure directly in the embryonic heart using TEM, and found that it was significantly altered in adrenergic-deficient embryos. TEM analyses showed that mitochondria were enlarged and more frequently displayed branching or budding membranes in adrenergic-deficient hearts at E10.5 and E11.5. Despite the abnormal mitochondrial morphology in these hearts, the membranes appeared to be intact with well-formed cristae. Further, no significant alterations in red/green fluorescence ratios were observed following application of the mitochondrial membrane potential-sensitive dye, JC-1, in adrenergic-deficient hearts compared with controls. These findings suggest that although the mitochondria were larger and abnormally shaped, the observed structural changes may represent compensatory mechanisms to increase ATP production in energy-starved Dbh⁻/⁻ hearts (25, 39). Indeed, enlarged mitochondria have been associated with enhanced metabolic output while smaller fragmented mitochondria have generally been associated with decreased metabolic output (31, 44, 52). In the present study, however, we observed enlarged mitochondria with apparent decreased metabolic output. This phenomenon of elongated mitochondria with diminished function has been demonstrated previously in other models of mitochondrial dysfunction (4, 11, 14, 40).

We tested the hypothesis that Dbh⁻/⁻ embryos may be starved for substrate metabolites, but little change was observed in key carbohydrate or lipid substrates. This result was not entirely unexpected since glucose is known to pass freely from maternal blood supply to the embryo
These results suggest that metabolic substrates were not depleted in adrenergic-deficient embryos.

The pathophysiology of adult heart failure is commonly characterized by mitochondrial dysfunction, indicated by decreased energy levels (30, 56, 57). In the $Dbh^{-/-}$ embryos, however, signs of energy starvation were seen before any outward precursor of heart failure. Indeed, metabolic deficiencies were observed in $Dbh^{-/-}$ embryos that were otherwise phenotypically indistinguishable from adrenergic-competent controls at the time of isolation in terms of size, morphology, color, texture, and cardiac beating activity. These results suggest that metabolic defects likely contribute to the subsequent heart failure and embryonic lethality in this model. The depletion of ATP concentrations could be largely prevented by maternal administration of either an $\alpha$-adrenergic agonist (l-phenylephrine) or a $\beta$-adrenergic agonist (ISO), suggests that both of the major adrenergic signal transduction pathways play important roles to ensure adequate supplies of ATP available for embryonic/fetal growth and development (55).

In summary, we have shown that adrenergic hormones fulfill a critical developmental role by providing the growing embryo with sufficient chemical energy in the form of ATP to enable successful transition from embryonic to fetal stages. Further, our results demonstrate that adrenergic hormones are necessary to maintain sufficient ATP/ADP, ECAR, and OCR during late periods of embryonic development in preparation for the transition to the fetal period. The discovery of these influential regulatory connections between adrenergic hormones and embryonic energy metabolism opens new paths for the study of cardiovascular development that could give rise to novel therapeutic targets and strategies for treating congenital heart defects (13, 32) as well as adult forms of heart disease.
SOURCE OF FUNDING

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AUTHOR CONTRIBUTIONS: C.N.B. helped to design the experiments, performed most of the experiments and data analysis, and wrote the initial manuscript. G.P. sectioned embryonic heart specimens for electron microscopy, collected TEM images, and aided in the processing/analysis of TEM images. S.A.G. was primarily responsible for assisting in the lipid measurements, glycogen granule, and mtDNA content analysis. J.N.R.P. performed the timolol and l-phenylephrine treatment for ATP experiments, and helped with analysis of data. S.N.E. contributed to the experimental design, analysis, and writing of the manuscript. All co-authors reviewed and edited the final manuscript.

DISCLOSURES

None
REFERENCES


Figure 1. ATP, ADP and ATP/ADP measurements in adrenergic-deficient embryos compared to controls. (A) ATP, (B) ADP, and (C) ATP/ADP ratio measurements at E9.5, E10.5, and E11.5 in adrenergic-competent (black bars) and adrenergic-deficient littermates (white bars). (D) ATP measurements at E10.5 and E11.5 in adrenergic-deficient embryos with and without isoproterenol (0.02 mg/mL in maternal drinking water). (E) ATP measurements at E11.5 in adrenergic-competent embryos with (n=16) and without (n=14) timolol (1 mg/mL in maternal drinking water). (F) ATP measurements at E11.5 in adrenergic-competent (black bars) and adrenergic-deficient (white bars) embryos with and without l-phenylephrine (0.02 mg/mL in maternal drinking water). Numerical values in the columns refer to the number (n) of samples analyzed. For this experiment (F), one-way ANOVA was performed with Bonferroni’s Multiple Comparison Test used to evaluate differences between individual groups. All other comparisons in this figure (A-E) were analyzed using the Student’s T-test. Data are shown as fold-change compared to littermate controls. Fold-change calculations were generated from steady-state ATP and ADP concentrations (see also Table I). *, p<0.05; **p<0.005; ***p<0.001.

Figure 2. Effects of adrenergic-deficiency on Extracellular Acidification Rate (ECAR). (A-B) ECAR in E10.5 and E11.5 adrenergic-competent (closed circles) and deficient (open squares) isolated beating hearts, values are represented as percent E10.5 controls. For E10.5 adrenergic-competent, n=15 and adrenergic-deficient, n=9. For E11.5 adrenergic-competent, n=28 and adrenergic-deficient, n=8. (C) ECAR fold-change at 40-min between adrenergic-competent (black bars) and deficient (white bars) E11.5 isolated hearts with and without isoproterenol in maternal drinking water. Mean ± SEM values at the final (40-min) timepoint are shown. *, p<0.05.
Figure 3. Effects of adrenergic-deficiency on Oxygen Consumption Rate (OCR). (A) OCR E10.5 (circles) and E11.5 (squares) in adrenergic-competent hearts treated with rotenone and antimycin A (dashed line). (B) OCR in E10.5 (n=8) and E11.5 (n=10) adrenergic-competent hearts before (black bars) and after (grey bars) rotenone and antimycin A treatment. (A&B) Values are represented as percent E10.5 basal OCR. (C-D) Oxygen consumption rate (OCR) in E10.5 and E11.5 adrenergic-competent (closed circles) and deficient (open squares) hearts, values are represented as percent E10.5 controls. For E10.5 adrenergic-competent, n= 15 and adrenergic-deficient, n=9. For E11.5 adrenergic-competent, n=20 and adrenergic-deficient, n=9. (E-F) OCR fold-change at 15-min between adrenergic-competent (black bars) and deficient (white bars) E10.5 and E11.5 isolated hearts with and without isoproterenol. Data are represented as mean ± SEM. *, p<0.05; ***, p<0.001.

Figure 4. Mitochondrial biogenesis gene expression and mtDNA content in adrenergic-deficient versus control hearts. (A) Fold-change of key mitochondrial biogenesis genes between adrenergic-competent and deficient mRNA from E10.5 (n=5) and E11.5 (n=6) hearts as measured by quantitative RT-PCR analysis. (B) Mitochondrial DNA (mtDNA) content fold-change between E10.5 and E11.5 adrenergic-competent (black bars) and deficient (white bars) hearts. Numerical values in the columns refer to the number (n) of samples analyzed.

Figure 5. Analysis of mitochondrial membrane potentials in adrenergic-deficient and control myocytes using flow cytometry and fluorescence microscopy with JC-1 dye. (A and C) Representative scatter plots of flow cytometry with red and green fluorescence from JC-1 dye in adrenergic-deficient and adrenergic-competent E10.5 and E11.5 embryonic primary cardiomyocytes. Adrenergic-competent samples (red) and adrenergic-deficient samples (blue) in histogram overlay. (B and D) Ratio of red/green fluorescence in E10.5 and E11.5 adrenergic-competent (white bars) and deficient (black bars) samples. Numerical values in the columns
refer to the number (n) of samples analyzed. (E) Representative scanning laser confocal microscopy of JC-1 dye in E11.5 primary cardiomyocytes. Scale bar, 10 μm.

**Figure 6. Ultrastructural analysis of mitochondria in adrenergic-deficient and control hearts following evaluation of transmission electron microscopy (TEM) images.**

Mitochondrial morphology in adrenergic-competent and deficient E10.5 (compare A and B) and E11.5 (compare E and F) myocytes. (C and G) Branched (arrowheads) and swollen mitochondria in adrenergic-deficient samples. (D and H) Representative drawn-to-scale tracings of abnormally shaped mitochondria in adrenergic-deficient samples relative to adrenergic-competent control tracings at each age. Abbreviations: m=mitochondria; n=nucleus. *, p<0.05; **, p<0.01; ***, p<0.001. Data are represented as mean ± SEM. Scale bar= 500 nm.

**Table I. ATP, ADP, and ATP/ADP ratio values of adrenergic-deficient and control embryos.** Values obtained from standard curve of known ATP and ADP concentrations. Data are presented as mean ± SEM. *, p<0.05; **, p<0.01; ***, p<0.001

**Table II. Carbohydrate and lipid metabolite concentrations.** Values were obtained from standard curves of known metabolite concentrations (excluding lipid droplets), and are represented as mean ± SEM. Lipid droplets were counted in the liver and data are presented as number of droplets per square mm.

**Table III. Beating rates of embryonic mouse hearts after 24-hrs of ex vivo culture.** Data are presented as mean ± SEM.

**Table IV. Summary of TEM data in adrenergic-deficient and control hearts.** Data are presented as mean ± SEM. *, p<0.05; **, p<0.01; ***, p<0.001
Figure 1

A

ATP (Fold-Change)

E9.5  E10.5  E11.5

B

ADP (Fold-Change)

E9.5  E10.5  E11.5

C

ATP/ADP Ratio (Fold-Change)

E9.5  E10.5  E11.5

D

ATP (Fold-Change)

ISO  -  +  -  +
E9.5  E10.5  E11.5

E

ATP (Fold-Change)

Control  Timolol

F

ATP (Fold-Change)

No Drug  Phenylephrine
Figure 2

(A) E10.5

(B) E11.5

(C) E11.5

<table>
<thead>
<tr>
<th>ISO</th>
<th>Adrenergic-Competent</th>
<th>Adrenergic-Deficient</th>
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<tr>
<td>-</td>
<td>28</td>
<td>8</td>
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<tr>
<td>+</td>
<td>20</td>
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* *
Figure 3

A) Basal OCR (Percent E10.5 Control)

B) OCR (Percent E10.5 Basal)

C) E10.5

D) E11.5

E) E10.5

F) E11.5

- Adrenergic-Competent
- Adrenergic-Deficient

** * * * ***
**Figure 4**

**A**

```
<table>
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<tr>
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<td>Pgc-1α</td>
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<td>Sirt1</td>
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<td></td>
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<td>Tfam</td>
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**B**

```
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<tr>
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<tr>
<td>Adrenergic-Deficient</td>
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- Pgc-1α, Sirt1, Tfam
- E10.5, E11.5
- Adrenergic-Competent
- Adrenergic-Deficient

Figure 4 depicts the mRNA expression and mtDNA content changes between E10.5 and E11.5 in Adrenergic-Competent and Adrenergic-Deficient states.
Figure 5

A

Adrenergic-Competent

E10.5

Adrenergic-Deficient

Overlay

B

E10.5

C

E11.5

D

Ratio of aggregate/monomeric JC-1

E

Monomers

Aggregates

Merge

Adrenergic-Competent

Adrenergic-Deficient

0 2 4 6

Ratio of aggregate/monomeric JC-1

0 2 4 6

Figure 5
Figure 6
Table I  ATP, ADP, and ATP/ADP ratio values of adrenergic-deficient and control embryos

<table>
<thead>
<tr>
<th></th>
<th>[ATP] (nmol/mg protein)</th>
<th>[ADP] (nmol/mg protein)</th>
<th>ATP/ADP Ratio (AU)</th>
<th>Number of samples</th>
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<tbody>
<tr>
<td>E9.5</td>
<td>Competent</td>
<td>40.6 ± 10.7</td>
<td>19.8 ± 6.6</td>
<td>3.9 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>Deficient</td>
<td>32.4 ± 8.3</td>
<td>10.8 ± 2.9</td>
<td>3.8 ± 1.2</td>
</tr>
<tr>
<td>E10.5</td>
<td>Competent</td>
<td>26.8 ± 3.1</td>
<td>9.1 ± 1.9</td>
<td>5.1 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>Deficient</td>
<td>19.5 ± 7.33</td>
<td>9.9 ± 4.1</td>
<td>2.6 ± 0.8</td>
</tr>
<tr>
<td>E11.5</td>
<td>Competent</td>
<td>7.6 ± 0.5</td>
<td>0.8 ± 0.06</td>
<td>9.4 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>Deficient</td>
<td>1.0 ± 0.4***</td>
<td>4.3 ± 1.3**</td>
<td>0.2 ± 0.03***</td>
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Data are represented as mean±SEM. *, p<0.05; **, p<0.01; ***, p<0.001
Table II  Carbohydrate and Lipid Metabolite Concentrations

<table>
<thead>
<tr>
<th></th>
<th>E10.5</th>
<th>E11.5</th>
<th>p-value</th>
<th>E10.5</th>
<th>E11.5</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (nmol/mg protein)</td>
<td>Competent: 313±17 n=10</td>
<td>Deficient: 260±26 n=9</td>
<td>0.09</td>
<td>Competent: 436±27 n=8</td>
<td>Deficient: 421±44 n=7</td>
<td>0.77</td>
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<tr>
<td>Glycogen (nmol/mg protein)</td>
<td>Competent: 5.8±1.4 n=4</td>
<td>Deficient: 7.5±1.5 n=4</td>
<td>0.45</td>
<td>Competent: 9.9±6.5 n=4</td>
<td>Deficient: 4.3±2.2 n=3</td>
<td>0.51</td>
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<tr>
<td>Glycogen Granules (% per nm²)</td>
<td>Competent: 5.0±0.6 n=32</td>
<td>Deficient: 6.2±0.9 n=42</td>
<td>0.30</td>
<td>Competent: 4.8±1.0 n=18</td>
<td>Deficient: 3.3±0.7 n=23</td>
<td>0.20</td>
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<tr>
<td>Lactate (nmol/mg protein)</td>
<td>Competent: 250±50 n=6</td>
<td>Deficient: 422±107 n=6</td>
<td>0.17</td>
<td>Competent: 293±37 n=6</td>
<td>Deficient: 416±78 n=6</td>
<td>0.18</td>
</tr>
<tr>
<td>Lipid (liver) Droplets (per sq. mm)</td>
<td>Competent: 5015±677 n=10</td>
<td>Deficient: 3528±416 n=14</td>
<td>0.06</td>
<td>Competent: 5866±598 n=16</td>
<td>Deficient: 3988±869 n=16</td>
<td>0.09</td>
</tr>
<tr>
<td>Free Fatty Acids (nmol/mg protein)</td>
<td>Competent: 1.6±0.2 n=10</td>
<td>Deficient: 1.5±0.2 n=10</td>
<td>0.86</td>
<td>Competent: 1.5±0.1 n=8</td>
<td>Deficient: 1.5±0.3 n=7</td>
<td>0.92</td>
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Data are presented as mean±SEM.
Table III  Beating rates of isolated embryonic hearts

<table>
<thead>
<tr>
<th></th>
<th>Competent</th>
<th>Deficient</th>
<th>p-value</th>
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<tbody>
<tr>
<td>E10.5</td>
<td>88±11 bpm n=10</td>
<td>99±21 bpm n=4</td>
<td>0.62</td>
</tr>
<tr>
<td>E11.5</td>
<td>51±4 bpm n=39</td>
<td>47±8 bpm n=8</td>
<td>0.59</td>
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</tbody>
</table>

Data are presented as mean±SEM. bpm, beats per minute.
### Table IV  Quantification of Mitochondrial Numbers and Morphology from TEM

<table>
<thead>
<tr>
<th></th>
<th>E10.5 Adrenergic-Competent</th>
<th>E10.5 Adrenergic-Deficient</th>
<th>E11.5 Adrenergic-Competent</th>
<th>E11.5 Adrenergic-Deficient</th>
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<tbody>
<tr>
<td><strong>Number per 17.5 μm²</strong></td>
<td>11.7 ± 0.5 n=58</td>
<td>10.7 ± 0.4 n=103</td>
<td>13.9 ± 1.1 n=38</td>
<td>11.0 ± 0.7* n=38</td>
</tr>
<tr>
<td><strong>Length (nm)</strong></td>
<td>718.8 ± 13.8 n=669</td>
<td>772.4 ± 14.4* n=1106</td>
<td>637.5 ± 17.4 n=529</td>
<td>888.5 ± 27.9*** n=418</td>
</tr>
<tr>
<td><strong>Surface Area (μm²)</strong></td>
<td>257.5 ± 11.7 n=153</td>
<td>304.7 ± 19.0* n=158</td>
<td>204.8 ± 10.6 n=166</td>
<td>362.8 ± 28.7*** n=127</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM. *, p<0.05; **, p<0.01; ***, p<0.001