



# Metabolomics reveals critical adrenergic regulatory checkpoints in glycolysis and pentose–phosphate pathways in embryonic heart

Received for publication, February 21, 2018. Published, Papers in Press, March 14, 2018, DOI 10.1074/jbc.RA118.002566

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Edited by Jeffrey E. Pessin

Cardiac energy demands during early embryonic periods are sufficiently met through glycolysis, but as development proceeds, the oxidative phosphorylation in mitochondria becomes increasingly vital. Adrenergic hormones are known to stimulate metabolism in adult mammals and are essential for embryonic development, but relatively little is known about their effects on metabolism in the embryonic heart. Here, we show that embryos lacking adrenergic stimulation have ~10-fold less cardiac ATP compared with littermate controls. Despite this deficit in steady-state ATP, neither the rates of ATP formation nor degradation was affected in adrenergic hormone-deficient hearts, suggesting that ATP synthesis and hydrolysis mechanisms were fully operational. We thus hypothesized that adrenergic hormones stimulate metabolism of glucose to provide chemical substrates for oxidation in mitochondria. To test this hypothesis, we employed a metabolomics-based approach using LC/MS. Our results showed glucose 1-phosphate and glucose 6-phosphate concentrations were not significantly altered, but several downstream metabolites in both glycolytic and pentose–phosphate pathways were significantly lower compared with controls. Furthermore, we identified glyceraldehyde-3-phosphate dehydrogenase and glucose-6-phosphate dehydrogenase as key enzymes in those respective metabolic pathways whose activity was significantly ( $p < 0.05$ ) and substantially (80 and 40%, respectively) lower in adrenergic hormone-deficient hearts. Addition of pyruvate and to a lesser extent ribose led to significant recovery of steady-state ATP concentrations. These results demonstrate that without adrenergic stimulation, glucose metabolism in the embryonic heart is severely impaired in multiple pathways, ultimately leading to insufficient metabolic substrate

availability for successful transition to aerobic respiration needed for survival.

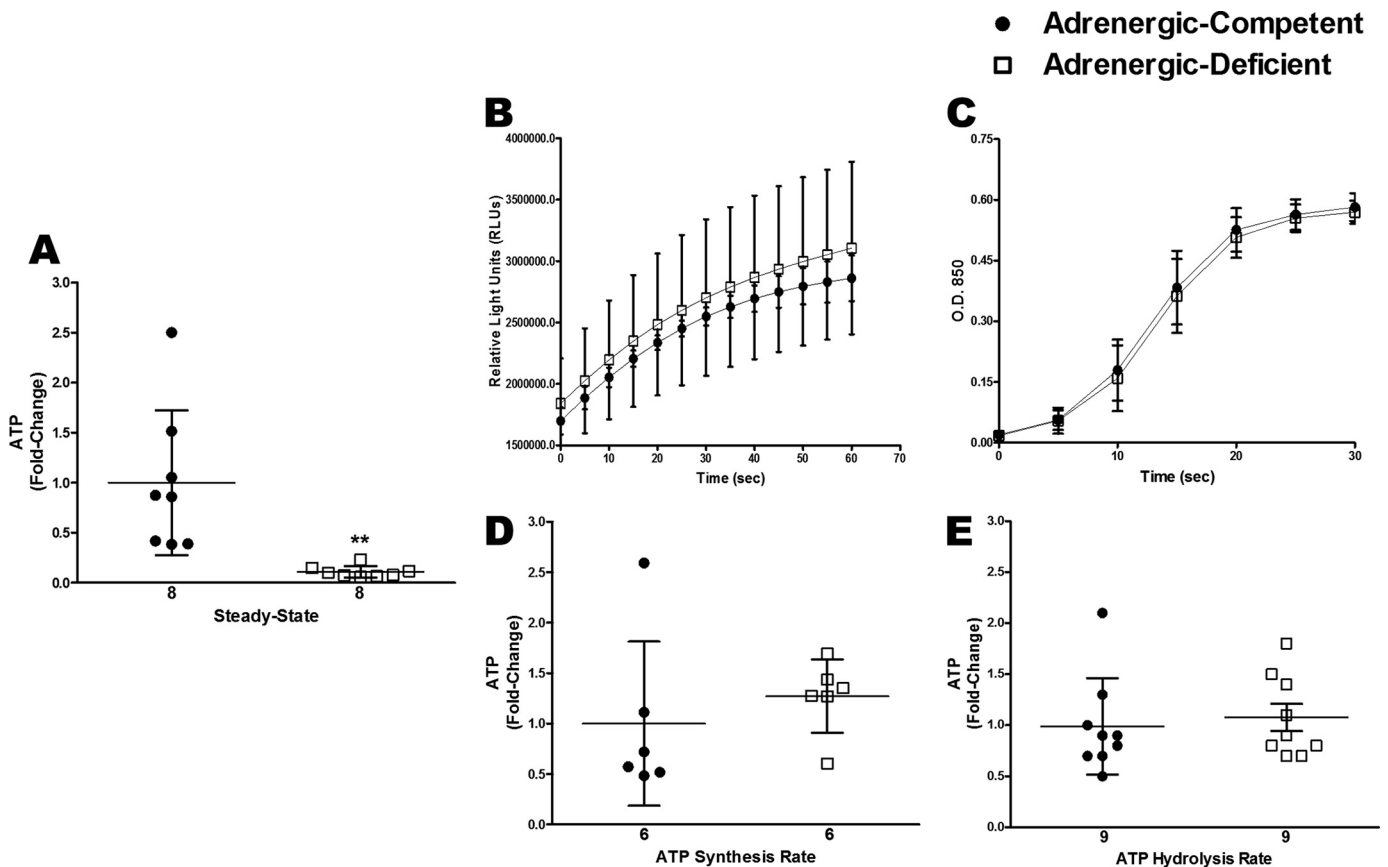
The developing heart experiences a number of physiological and structural changes while transitioning through developmental stages. Metabolic shifts occur during embryonic heart development as cardiac energy demands increase to promote oxidative phosphorylation of ADP to ATP in mitochondria. Early embryonic heart development is characterized by a glycolytic metabolism with carbohydrates as the primary energy source (1–4). However, as energy demands increase, cardiac metabolism undergoes an “embryonic shift” in metabolism from anaerobic to aerobic (5). The mechanisms underlying the embryonic shift initiating mitochondrial metabolism during heart development are not well-understood. The adrenergic hormones, epinephrine (EPI)<sup>2</sup> and norepinephrine (NE), are regulators of stress and the sympathetic nervous system in adult mammals. Adrenergic hormones, produced in the heart as early as embryonic day 8.5 (E8.5), are also active and essential during embryonic heart development (5–7). Targeted disruption of the essential dopamine  $\beta$ -hydroxylase (*Dbh*) gene prevents NE and EPI biosynthesis and leads to embryonic lethality due to heart failure in mice (6, 8). Subsequent disruption of the phenylethanolamine *N*-methyltransferase (*Pnmt*) gene leads to loss of EPI, but it does not impede prenatal development (9, 10). Thus, NE is crucial for embryonic heart development and survival, but the regulatory roles and physiological targets of adrenergic stimulation during embryonic heart development remain unknown.

Adrenergic hormone-deficient (*Dbh*<sup>−/−</sup>) embryos appear to develop and function normally through approximately embryonic day 9.5 (E9.5), but they begin to show cardiac distress

This work was supported by University of Central Florida College of Medicine (to S. N. E.), National Institutes of Health Grant R01-HL-071158 (to P. S. B.), and American Heart Association Founder's Affiliate Grant 12GRNT12060233 (to G. A. P.). The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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<sup>2</sup> The abbreviations used are: EPI, epinephrine; AMPK, AMP-activated protein kinase; *Dbh*, dopamine  $\beta$ -hydroxylase; EC, energy charge; G-6-PDH, glucose-6-phosphate dehydrogenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NE, norepinephrine; OCR, oxygen consumption rate; PDH, pyruvate dehydrogenase; PKC, protein kinase C; PPP, pentose–phosphate pathway; PRPP, phosphoribosyl pyrophosphate; TCA, tricarboxylic acid cycle; DMEM, Dulbecco's modified Eagle's medium; Ap5A, *P*<sup>1</sup>,*P*<sup>5</sup>-di(adenosine 5')-pentaphosphate; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.



**Figure 1. Steady-state ATP concentrations compared with rates of ATP synthesis and hydrolysis in E11.5 isolated adrenergic hormone-deficient hearts compared with controls.** *A*, steady-state ATP concentrations in adrenergic hormone-competent (●) and adrenergic hormone-deficient (□) embryonic hearts at embryonic day 11.5. *B* and *D*, ATP synthesis rate in adrenergic hormone-competent (●) and adrenergic hormone-deficient (□) embryonic hearts, expressed as relative light units (RLUs) produced/s and fold-change, respectively. *C* and *E*, ATP hydrolysis rates in adrenergic hormone-competent (●) and adrenergic hormone-deficient (□) embryonic hearts expressed as absorbance/s and fold-change, respectively. Numerical values below the x axes refer to the number (*n*) of samples analyzed. Data are presented as fold-change compared with competent control littermates. Student's *t* test was used to compare means between competent and deficient groups. \*\*, *p* < 0.01.

between E10.5 and E12 (8). These adrenergic hormone-deficient (*Dbh*<sup>-/-</sup>) embryos undergo steady deterioration that ultimately ends in death within 24 h of initial symptoms. To gain insight into transcriptional changes between *Dbh*<sup>+/+</sup> and *Dbh*<sup>-/-</sup>, a genome-wide expression screen showed that the largest set of differentially expressed genes are involved in metabolism (11). Further evidence demonstrated that *Dbh*<sup>-/-</sup> embryos are energy-depleted with >95% ATP/ADP reduction by E11.5 (12). Oxygen consumption rates (OCR), an indicator of mitochondrial respiration, are established in E10.5 hearts; however, *Dbh*<sup>-/-</sup> hearts lag behind by ~24 h later compared with age-matched littermate controls. These results established that NE and EPI influence embryonic heart energy metabolism; however, the mechanism(s) and metabolic targets regulated by adrenergic hormones remain unknown.

Aerobic metabolism in mitochondria becomes increasingly important as the heart transitions from embryonic to fetal stages. Several studies using genetic mutations to disrupt aerobic respiration and/or mitochondrial structure/function have resulted in embryonic lethality due to heart failure (5, 13–16). Similarly, lack of adrenergic hormones leads to delayed transition to aerobic metabolism in mitochondria and is associated with embryonic lethality due to apparent heart failure. We thus hypothesized that adrenergic hormones facilitate the embry-

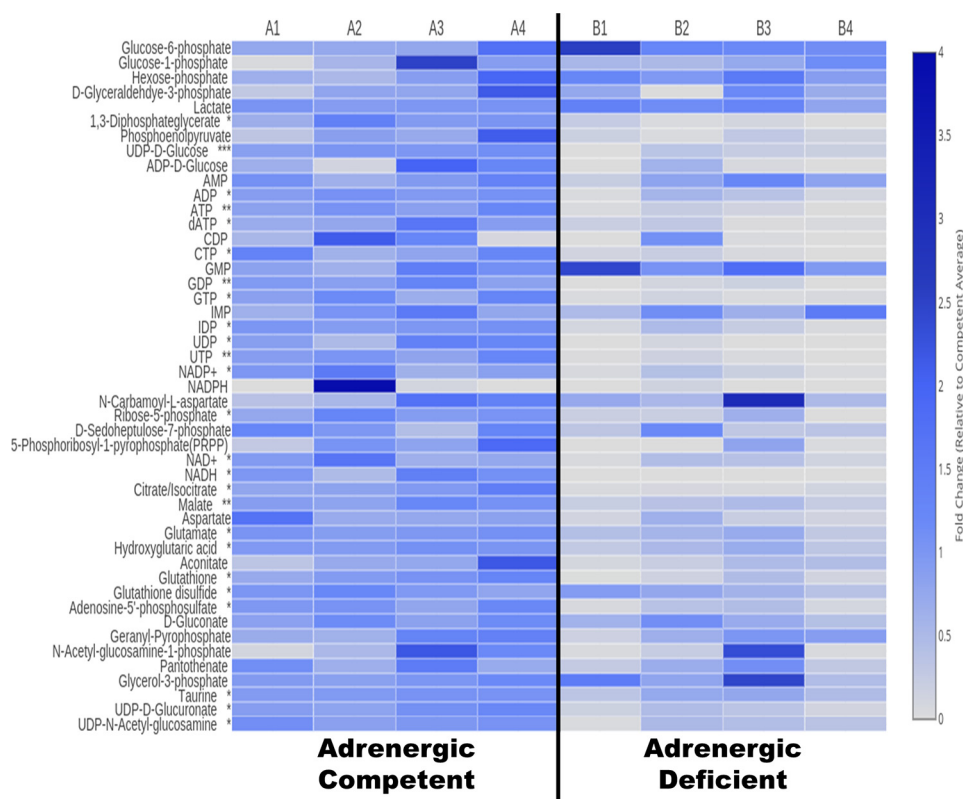
onic shift in metabolism from anaerobic glycolysis toward aerobic oxidative phosphorylation in mitochondria. This study focuses primarily on carbohydrate metabolism because lipid metabolism is not fully established until late fetal and neonatal stages (17–19). We tested our hypothesis using metabolomics analysis and measurements of key enzymatic reaction rates to evaluate metabolic changes in response to adrenergic deficiency in embryonic mouse hearts. In addition, we were able to show that metabolic blockade could be overcome, in part, by addition of downstream metabolites, leading to significant recovery of steady-state ATP in adrenergic hormone-deficient embryonic hearts.

## Results

### Influence of adrenergic hormones on embryonic heart metabolism

To determine whether cardiac energy levels were impaired in adrenergic hormone-deficient embryonic hearts, we first isolated E11.5 mouse hearts to measure steady-state ATP concentrations. Our results show a dramatic and significant 10-fold decrease (*p* < 0.01) in steady-state ATP concentrations in adrenergic hormone-deficient hearts compared with adrenergic hormone-competent littermate controls (Fig. 1A). To deter-

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**Figure 2. LC-MS metabolomics analysis of isolated adrenergic hormone-deficient embryonic hearts.** Columns A1–A4 represent the adrenergic hormone-competent controls with three embryonic hearts analyzed per group. Columns B1–B4 represent the adrenergic hormone-deficient group with three embryonic hearts analyzed per group. Data are presented as fold-change compared with competent control littermates. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .

mine whether adrenergic deficiency slows ATP production or increases ATP utilization/degradation as a potential cause of energy depletion, we measured rates of ATP synthesis and hydrolysis in adrenergic hormone-competent and -deficient embryonic hearts (Fig. 1, B–E). Embryonic heart lysates were incubated with pyruvate and malate prior to measurements to provide substrates for ATP synthesis. We observed no significant difference in ATP synthesis rates in adrenergic hormone-competent and -deficient hearts, suggesting that ATP synthesis rates were not impaired when the hearts were provided with necessary substrates and conditions. Similarly, no significant differences were observed in ATP hydrolysis rates in adrenergic hormone-competent and -deficient hearts, suggesting that the observed depletion of steady-state ATP concentrations may not necessarily be the result of increased rates of ATP hydrolysis. These data suggest ATP synthesis and hydrolysis rates were not impaired in adrenergic hormone-deficient embryonic hearts when supplied with sufficient substrates.

To test whether metabolic substrates were limited in adrenergic hormone-deficient embryonic hearts, we utilized LC/MS to generate metabolomic profiles. The results are shown in the Fig. 2 heat map depicting relative concentrations of 47 key metabolites involved in carbohydrate, amino acid, and nucleotide metabolism. Other metabolites in these pathways, such as acetyl-CoA, were also evaluated but are not included here because we did not obtain reliable measurements for them due to limiting concentrations in both adrenergic hormone-competent control and adrenergic hormone-deficient hearts at this embryonic stage of development (E11.5). Initial analysis of

the heat map data clearly reveals that many but not all of the measured metabolites were substantially decreased in adrenergic hormone-deficient hearts relative to littermate controls (Fig. 2). Remarkably, adrenergic hormone-deficient hearts show significant decreases in several metabolites from pentose–phosphate pathway (PPP), glycolysis, and the TCA cycle, suggesting that glucose metabolism was severely compromised (Fig. 2). Despite this, adrenergic hormone-deficient hearts had comparable glucose 6-phosphate, glucose 1-phosphate, and glyceraldehyde 3-phosphate concentrations compared with adrenergic hormone-competent controls, indicating that there was sufficient starting substrate (glucose). Nevertheless, there were ~10-fold decreases in downstream glycolytic metabolites such as 1,3-diphosphoglycerate ( $p < 0.05$ ) and phosphoenolpyruvate, indicating the “pay-off” phase of glycolysis was likely impaired. In contrast, lactate concentrations did not differ between adrenergic hormone-deficient and -competent hearts suggesting that some glycolytic activity remains or that lactate levels may be stabilized from embryonic/maternal circulation.

Another pathway that was clearly abrogated in adrenergic hormone-deficient hearts was the PPP as evidenced by the 5-fold decreases in critically important intermediate metabolites, including ribose 5-phosphate ( $p < 0.05$ ) and phosphoribosyl pyrophosphate (PRPP) as well as 2-fold decreases in sedoheptulose 7-phosphate. Consistent with these results, we also found major decreases in cardiac concentrations of many nucleotides, which rely in large part on the PPP for crucial substrates for their biosynthesis.



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Furthermore, adrenergic hormone-deficient hearts had significantly lower concentrations of several tricarboxylic acid (TCA) cycles and related intermediates, including citrate/isocitrate, aconitate, malate, glutamate, and aspartate, that likely contributed to impaired mitochondrial metabolism (Fig. 2). In addition, adrenergic hormone-deficient hearts had 2-fold lower pantothenate concentrations (not significant) compared with adrenergic hormone-competent controls suggesting that CoA biosynthesis, an essential precursor for acetyl-CoA and succinyl-CoA, may also be compromised (20). These results demonstrate that entry and phosphorylation of glucose are functional; however, further utilization to produce downstream metabolic intermediates for glycolysis/glucose oxidation and PPP may be compromised in adrenergic hormone-deficient hearts.

In addition, several metabolites involved in oxidation/reduction were significantly decreased in adrenergic hormone-deficient hearts (Fig. 2). Notably, adrenergic hormone-deficient hearts had a significant 10-fold decrease in GSH ( $p < 0.05$ ), 1.5-fold decrease in GSH disulfide ( $p < 0.05$ ), and 2-fold decrease in taurine ( $p < 0.05$ ) suggesting that these hearts may be experiencing some oxidative stress due to limited antioxidants (21–23). In addition, NAD<sup>+</sup> and NADH concentrations were significantly decreased (5-fold,  $p < 0.05$  and 500-fold,  $p < 0.05$ , respectively) in adrenergic hormone-deficient hearts. NADP<sup>+</sup> and NADPH concentrations were also significantly decreased (10-fold,  $p < 0.05$  and greater than 30-fold, respectively) in adrenergic hormone-deficient hearts. These data thus suggest that there is increased oxidative and decreased reducing capacity in adrenergic hormone-deficient hearts, indicating that metabolic pathways with redox enzymes, such as dehydrogenases, may have limited activities due to limitations in essential NAD<sup>+</sup>/NADH and NADP<sup>+</sup>/NADPH cofactors (24).

Based on these metabolomic data, the redox state of the embryonic heart appeared to be substantially less reduced in the absence of adrenergic stimulation. To quantify this, we calculated the ratios of NAD<sup>+</sup>/NADH, NADP<sup>+</sup>/NADPH, and GSSG/GSH from the LC/MS data. Remarkably, all three ratios were clearly elevated in adrenergic hormone-deficient hearts compared with competent controls (Fig. 3). It may be important to also note that in each of these examples, the oxidized forms (*i.e.* NAD<sup>+</sup>, NADP<sup>+</sup>, and GSSG) were also decreased in adrenergic hormone-deficient hearts compared with controls (Fig. 2), and as seen from the ratiometric displays in Fig. 3, the reduced forms were decreased even further, resulting in a shift to a more oxidized state that is less favorable for energy production. These results indicate that an adrenergic hormone-deficient state leads to an overall shift toward increased oxidized and corresponding decreased reduced forms of critical enzymatic co-factors necessary for metabolic oxidation–reduction reactions.

Consistent with these findings, we found that the ratios of ATP/ADP, ADP/AMP, and ATP/AMP were all decreased in adrenergic hormone-deficient E11.5 hearts relative to competent controls (Fig. 4, A–C, respectively). Furthermore, the overall “Energy Charge” or “EC” ( $EC = ([ATP] + 0.5[ADP])/([ATP] + [ADP] + [AMP])$ ) (25, 26) in E11.5 mouse hearts showed that adrenergic hormone-competent control hearts had an EC of  $0.90 \pm 0.005$ , consistent with normal, healthy

tissues. In contrast, the EC of adrenergic hormone-deficient hearts was significantly lower ( $0.66 \pm 0.091$ ,  $p < 0.05$ ; Fig. 4D). It is important to note that the redox and energy ratios in Figs. 3 and 4 are not based on absolute values, but instead they are relative comparisons calculated from the metabolite pools measured by LC/MS. These results nevertheless indicate that adrenergic hormone-deficient hearts had significantly diminished energy status by E11.5 compared with adrenergic hormone-competent controls.

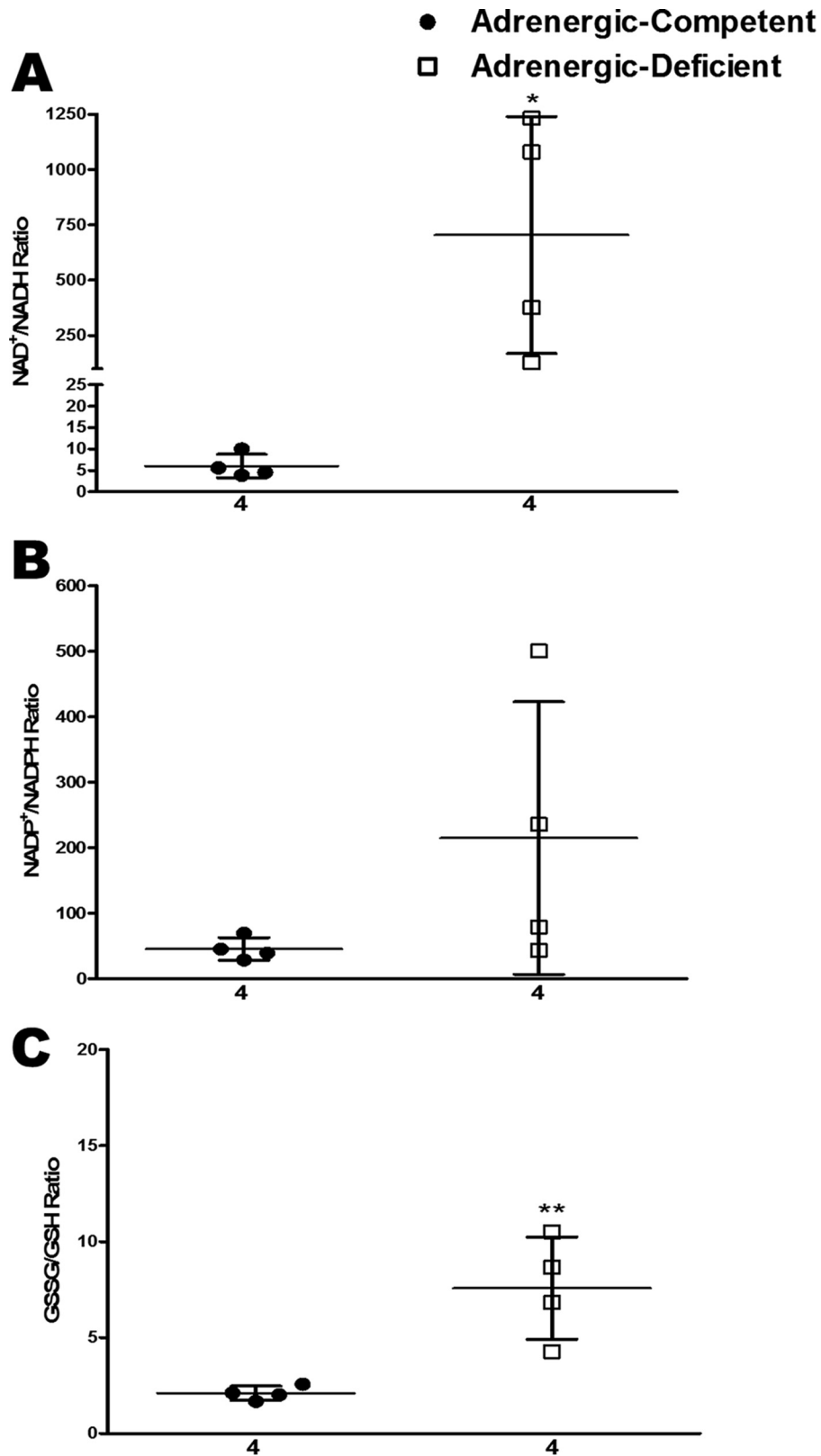
### AMPK activation compromised in absence of adrenergic hormones

Significant decreases in ATP/AMP and EC in adrenergic hormone-deficient hearts suggested potential involvement of AMPK (27–29). We measured protein expression of the catalytic subunit,  $\alpha$ -AMPK, and its phosphorylated form, pAMPK (Thr-172) in adrenergic hormone-deficient embryonic trunks containing the heart to assess AMPK activity. Adrenergic hormone-deficient embryos had significantly decreased  $\alpha$ -AMPK protein concentrations compared with adrenergic hormone-competent controls. Interestingly, pAMPK protein concentrations were not significantly affected in adrenergic hormone-deficient embryos (Fig. 5, A and B). pAMPK normalized to total  $\alpha$ -AMPK also indicated that adrenergic hormone-deficient embryos have slightly more activated AMPK compared with adrenergic hormone-competent embryos (Fig. 5C, not significant). These data suggest that AMPK activation is intact in adrenergic hormone-deficient embryos; however, this signaling is not sufficient for survival, possibly due to lower overall  $\alpha$ -AMPK protein.

### Glycolysis and the pentose–phosphate pathway are impaired in the absence of adrenergic stimulation

Using the metabolomics data, we examined relative amounts of reactants (substrates) and products for known metabolic reactions to identify other potential targets of adrenergic stimulation. Adrenergic hormone-deficient hearts had comparable glyceraldehyde-3-phosphate concentrations but decreased 1,3-diphosphoglycerate concentrations compared with adrenergic hormone-competent controls suggesting impairment of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Fig. 6A, see *yellow highlights*). To determine whether the lack of adrenergic stimulation affected GAPDH activity, we measured GAPDH rate kinetics. Our results showed that adrenergic hormone-deficient hearts had significantly decreased GAPDH activity (80% reduction,  $p < 0.05$ ) compared with adrenergic hormone-competent controls (Fig. 6B). GAPDH protein concentrations were similar in adrenergic hormone-deficient and -competent embryos, suggesting adrenergic control of GAPDH likely occurs through post-translational mechanisms (Fig. 6, C and D). These results identify glycolysis and GAPDH as a metabolic pathway and checkpoint, respectively, impaired by adrenergic deficiency.

Adrenergic hormone-deficient hearts also had significantly lower concentrations of ribose -phosphate and PRPP but comparable levels of glucose 6-phosphate in adrenergic hormone-deficient hearts suggesting that glucose-6-phosphate dehydrogenase (G-6-PDH), a key rate-limiting enzymatic reaction in

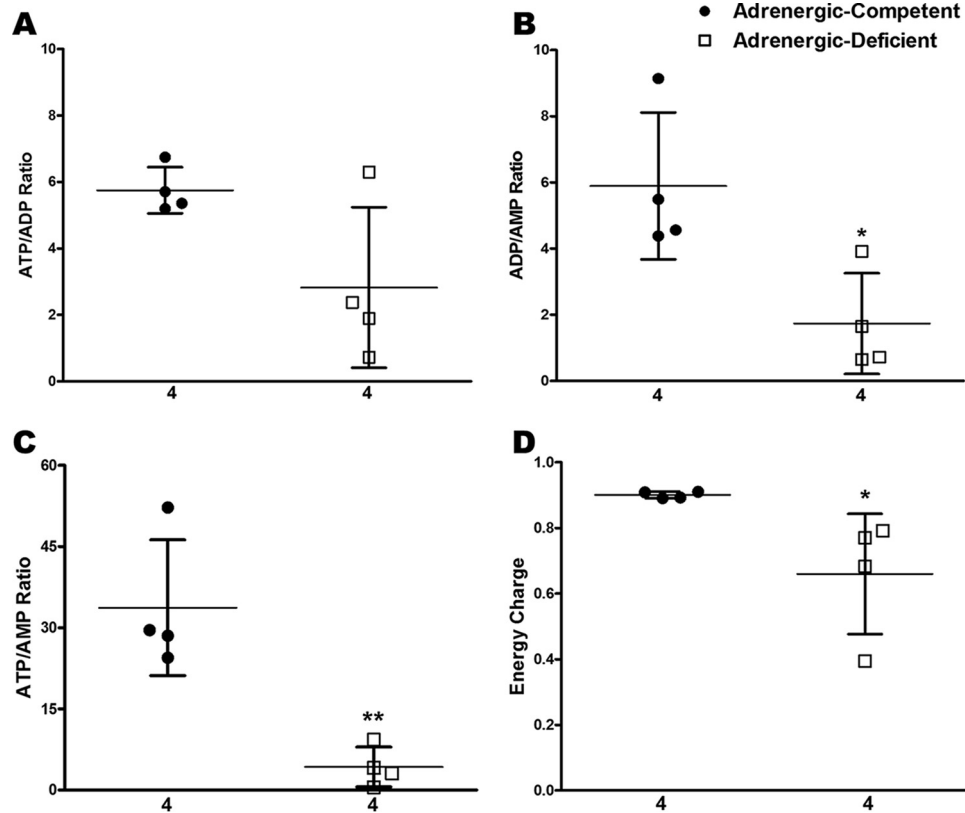


**Figure 3. Estimation of oxidative stress in adrenergic hormone-deficient embryonic hearts compared with controls.** A–C, steady-state NAD<sup>+</sup>/NADH, NADP<sup>+</sup>/NADPH, and GSSG/GSH at E11.5 in adrenergic hormone-competent (●) and adrenergic hormone-deficient littermates (□). Numerical values below the x axes refer to the number (n) of samples analyzed. Student's t test was used to compare means between competent and deficient groups. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ .

the PPP, may also be impaired (Fig. 7A, see yellow highlights). G-6-PDH activity was significantly lower (40% reduction,  $p < 0.05$ ) in adrenergic hormone-deficient hearts (Fig. 7B); how-

ever, G-6-PDH protein concentrations did not differ compared with adrenergic hormone-competent controls (Fig. 7, C and D). Consequently, this result suggests G-6-PDH protein concen-

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**Figure 4. Estimation of energy charge in adrenergic hormone-deficient embryonic hearts compared with controls.** A–D, steady-state ATP/ADP, ADP/AMP, ATP/AMP, and EC =  $([ATP] + 0.5 [ADP]) / ([ATP] + [ADP] + [AMP])$  at E11.5 in adrenergic hormone-competent (●) and adrenergic hormone-deficient littermates (□). Numerical values below the x axes refer to the number (n) of samples analyzed. Student's *t* test was used to compare means between competent and deficient groups. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ .

trations are not limiting, but rather that G-6-PDH activity may be lower due to lack of post-translational regulation in the absence of adrenergic hormone stimulation. Many nucleotides, including GTP, UTP, CTP, and dATP, were also significantly decreased in adrenergic hormone-deficient hearts suggesting that impaired G-6-PDH activity may also impact nucleotide biosynthesis. Thus, G-6-PDH was another major metabolic checkpoint affected by adrenergic deficiency.

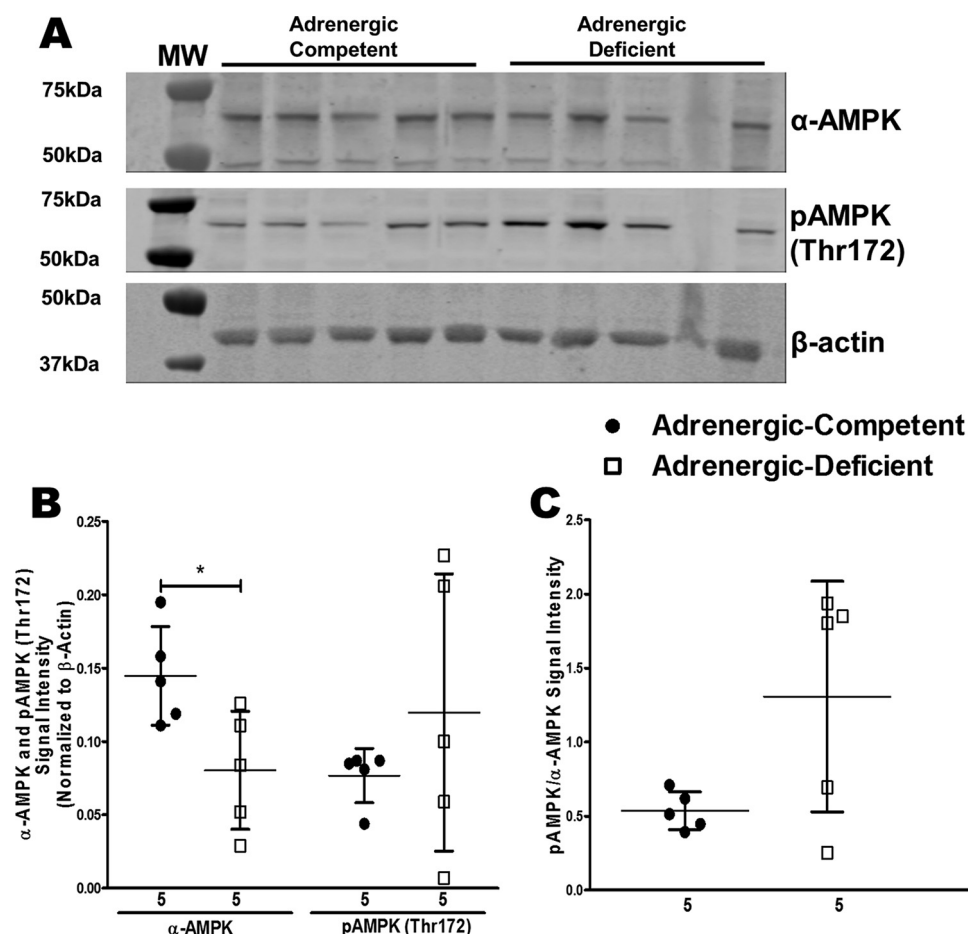
### Adrenergic deficiency changes phosphorylation status of GAPDH and G-6-PDH

Significant decreases in GAPDH and G-6-PDH activity but similar protein concentrations in adrenergic hormone-deficient embryos compared with adrenergic hormone-competent controls suggested that adrenergic hormones regulate GAPDH and G-6-PDH activity via post-translational mechanisms. To identify post-translational changes in adrenergic hormone-deficient embryos, we immunoprecipitated phosphoserine and acetyl-lysine proteins, respectively, and phosphorylated/acetylated GAPDH and G-6-PDH were detected using Western blotting (Fig. 8). When comparing the total amounts of GAPDH, phospho-GAPDH, and acetylated GAPDH for adrenergic hormone-deficient and -competent controls, no significant differences were observed (Fig. 8A). The same was true for G-6-PDH when similar analysis was performed (Fig. 8B). In contrast, the ratio of pGAPDH relative to total GAPDH protein was found to be significantly decreased in adrenergic hormone-deficient embryos compared with adrenergic hormone-competent

controls, although there was no change in the ratio of acetylated GAPDH to total GAPDH protein in these groups (Fig. 8C). There was a similar lack of significant change in the ratio of acetylated G-6-PDH to total G-6-PDH, although a slight tendency toward increased acetylated G-6-PDH was apparent in the adrenergic hormone-deficient group (Fig. 8D). Moreover, there was a significant increase in the ratio of phosphorylated G-6-PDH to total G-6-PDH protein in the adrenergic hormone-deficient group relative to adrenergic hormone-competent controls ( $p < 0.01$ ; Fig. 8D). These results show that lack of adrenergic stimulation alters the phosphorylation status of GAPDH and G-6-PDH during embryonic development, thereby providing a potential mechanistic explanation for how adrenergic hormones may regulate the activity of these enzymes during embryonic development.

### Lack of adrenergic stimulation does not affect pyruvate dehydrogenase

TCA cycle intermediates, citrate/isocitrate, aconitate, and malate, were significantly decreased in adrenergic hormone-deficient hearts implicating impaired mitochondrial metabolism (Fig. 9A). We measured pyruvate dehydrogenase (PDH) activity to determine whether entry into the TCA cycle was impaired in adrenergic hormone-deficient hearts. PDH activity did not differ in adrenergic hormone-deficient and -competent hearts (Fig. 9B). Adrenergic hormone-deficient hearts appeared to have slightly increased PDH activity (not significant) compared with controls, indicating that PDH clearly is not



**Figure 5. Effects of adrenergic deficiency on (p)AMPK protein concentrations.** A and B,  $\alpha$ -AMPK (62 kDa) and pAMPK (62 kDa) protein concentrations in E11.5 adrenergic hormone-competent (●) and adrenergic hormone-deficient (□) embryonic trunks containing the heart (one embryo per lane) normalized to  $\beta$ -actin. C, pAMPK/ $\alpha$ -AMPK in adrenergic hormone-competent (●) and adrenergic hormone-deficient embryonic trunks. Numerical values below the x axes refer to the number (n) of samples analyzed. Student's t test was used to compare means between competent and deficient groups. \*,  $p < 0.05$ .

impaired by lack of adrenergic stimulation. PDH concentrations were also not affected between adrenergic hormone-deficient and -competent hearts (Fig. 9, C and D). These results indicate that adrenergic deficiency had little influence on PDH activity, thereby suggesting that addition of pyruvate substrate may aid in rescuing the observed metabolic deficiency.

#### Pyruvate rescues adrenergic hormone-deficient OCR and steady-state ATP

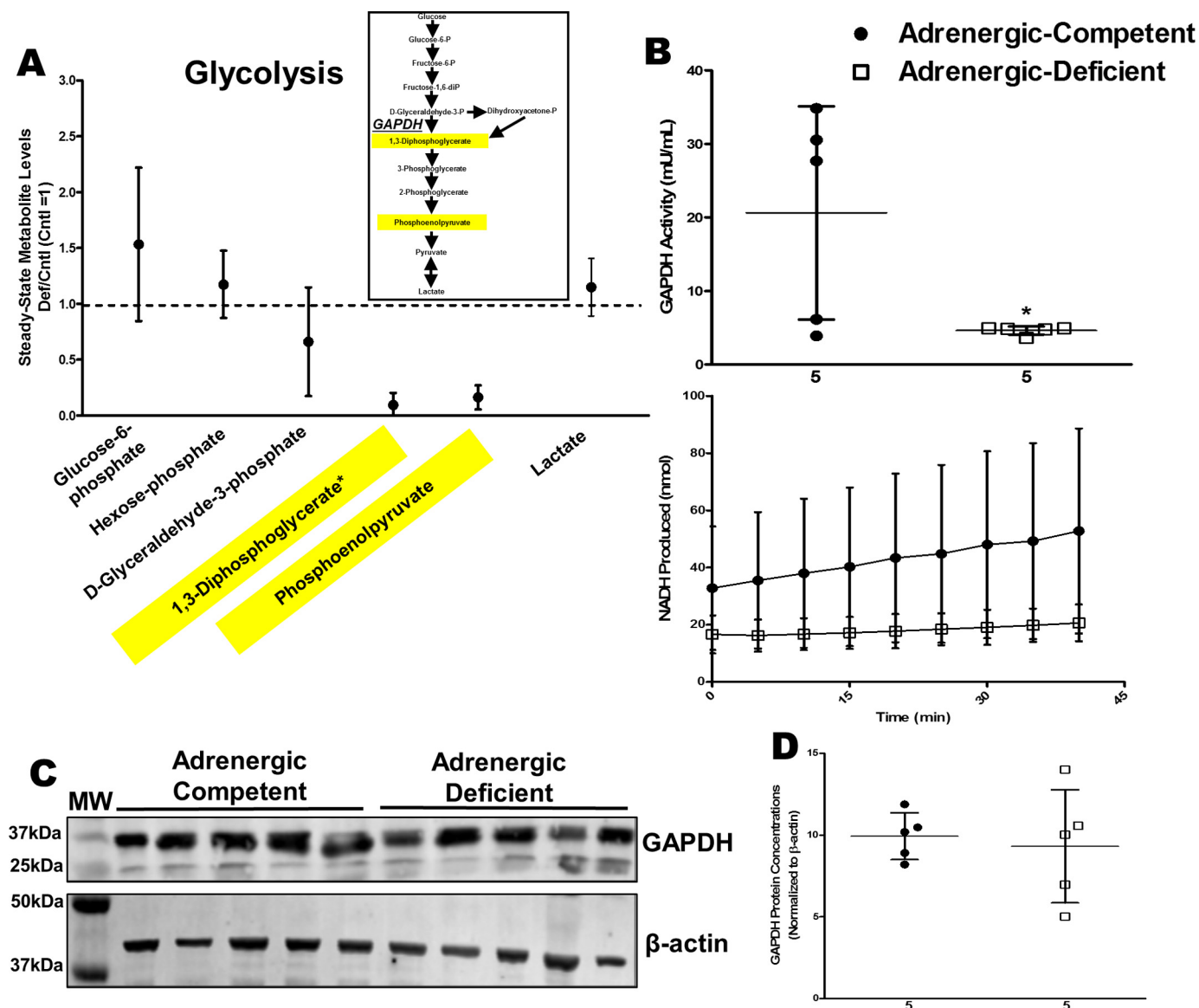
By E11.5, adrenergic hormone-deficient embryonic hearts had significantly lower amounts of metabolites needed to produce ATP and NADH, indicating that at this developmental stage, these embryos may be metabolically starved relative to adrenergic hormone-competent controls. We thus attempted to rescue cardiac energy levels by providing essential metabolites that were limiting, such as pyruvate (for TCA cycle) and ribose (for PPP) to embryonic hearts *ex vivo*. Pyruvate significantly rescued OCR (~50% increase,  $p < 0.05$ ) and steady-state ATP concentrations (~40% increase,  $p < 0.001$ ) compared with untreated adrenergic hormone-deficient controls. Ribose had no effect on OCR in adrenergic hormone-deficient hearts, but it significantly rescued steady-state ATP concentrations (~25% increase,  $p < 0.05$ ; Fig. 10, A–C). Addition of pyruvate and ribose did not have an additive effect on ATP concentra-

tions in adrenergic hormone-deficient hearts (Fig. 10C) suggesting that energy deficiency is not further rescued with both pyruvate and ribose. These results support the hypothesis that adrenergic hormone-deficient embryonic hearts are unable to produce sufficient chemical substrates (e.g. pyruvate and ribose) for glucose oxidation and nucleotide synthesis, respectively.

#### Discussion

The aim of this study was to identify metabolic targets of adrenergic stimulation necessary for the embryonic shift from anaerobic to aerobic metabolism during a critical period of cardiac development. Recent evidence demonstrated that the electron transport chain is activated beginning at E11.5 via closing of the mitochondrial transition pore (30). Therefore, impaired flux from glycolysis to the TCA cycle would disrupt essential ATP production from the mitochondria that normally would occur during this critical transition period in cardiac development (5). Our metabolomics analysis revealed adrenergic hormone-deficient embryonic hearts have a severely compromised glucose metabolism impeding ATP production. We also demonstrated that adrenergic hormone-deficient hearts appear to harbor a more oxidized intracellular environment compared with adrenergic hormone-competent controls and thus may

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**Figure 6. Effects of adrenergic deficiency on GAPDH activity and protein concentrations.** *A*, steady-state metabolites involved in glycolysis in E11.5 adrenergic hormone-deficient hearts compared with controls (*dotted line*). Schematic representation of glycolysis pathway provided as reference. *B*, GAPDH activity in adrenergic hormone-competent (●) and adrenergic hormone-deficient (□) embryonic hearts, expressed as milliunits of enzyme per ml of sample and NADH produced per min, respectively. Milliunits/ml are calculated as ((nmol of NADH)/(min)(ml of sample)). *C* and *D*, GAPDH protein concentrations (36 kDa) in adrenergic hormone-deficient embryonic trunks (□) compared with controls (●), normalized to  $\beta$ -actin. The  $\beta$ -actin panel is re-used as a representative image of six replicated experiments probing for GAPDH, G-6-PDH, and PDH. Numerical values below the x axes refer to the number (*n*) of samples analyzed. Student's *t* test was used to compare means between competent and deficient groups. \*,  $p < 0.05$ .

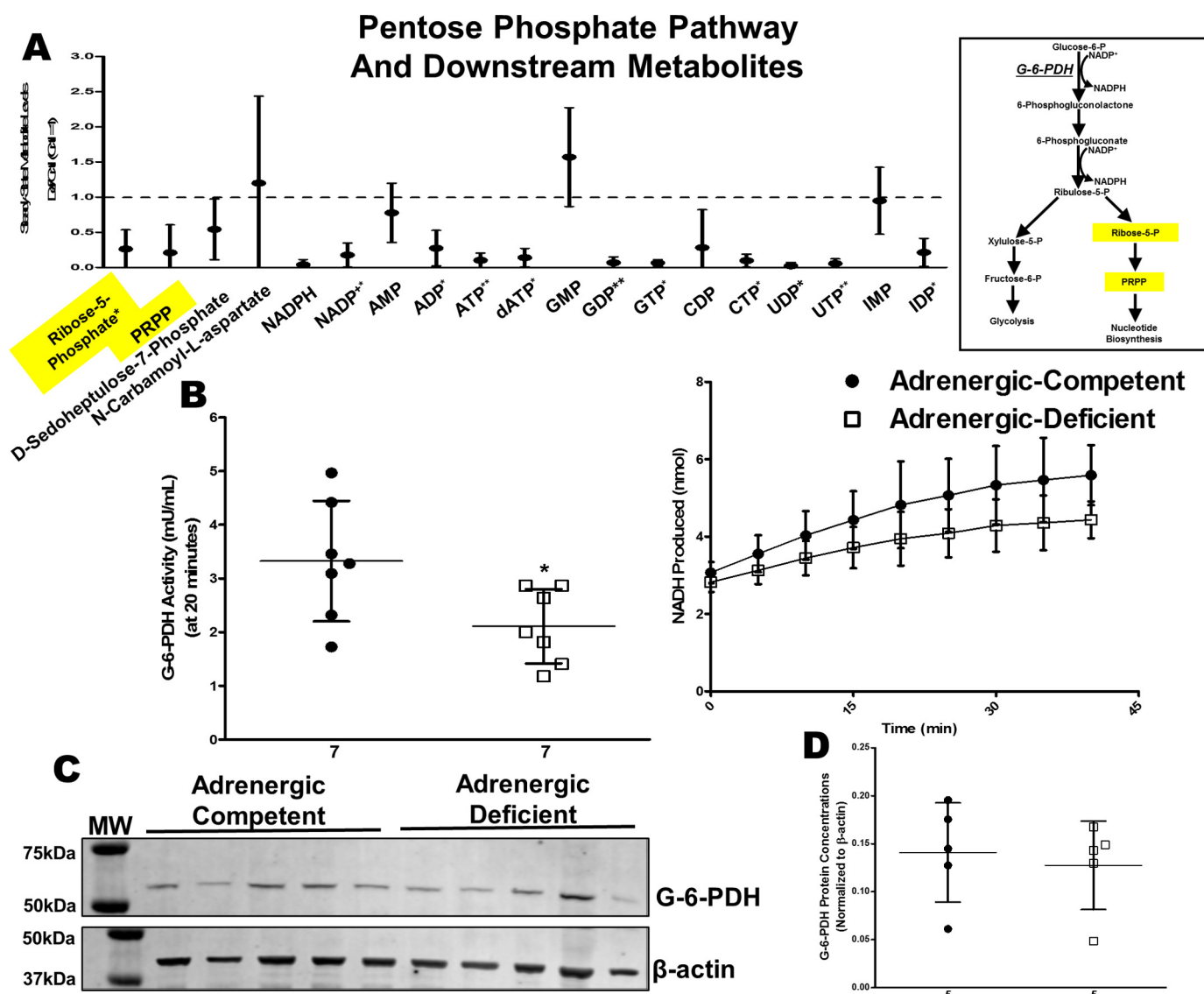
have impairments in anaplerotic processes, including nucleotide, amino acid, and other biosynthesis pathways. Our results also indicated an increased trend of AMPK activation via phosphorylation in adrenergic hormone-deficient embryos; however, energy status is not sufficiently restored likely due to decreased total  $\alpha$ -AMPK. Furthermore, we have identified two key enzymes impaired as a consequence of adrenergic deficiency that impact two major avenues of ATP production: (i) GAPDH of glycolysis/aerobic respiration and (ii) G-6-PDH of PPP.

### Redox shift during embryonic development

Normal developmental processes, such as differentiation and organ formation, require gradual increases in cellular oxidation

and redox signaling. However, excessive increases in oxidation can lead to abnormal development and embryonic lethality (31–34). NADPH, GSH, and other antioxidants have essential roles preventing excessive oxidized levels during development (35–37). GSSG is formed as GSH peroxidase reduces reactive oxygen species to a more stable species, H<sub>2</sub>O. NADPH produced from G-6-PDH is required to regenerate GSH from GSSG via GSH reductase thus maintaining low GSSG levels (33, 38). Relative ratios of NAD(H) also serve as an indicator of the cellular redox and metabolic states (33, 39). Our results show significant increases in NAD<sup>+</sup>/NADH and GSSG/GSH ratios as well as nonsignificant increases in NADP<sup>+</sup>/NADPH ratios suggesting that the redox state in adrenergic hormone-deficient hearts is shifted to a more oxidized state with less reducing



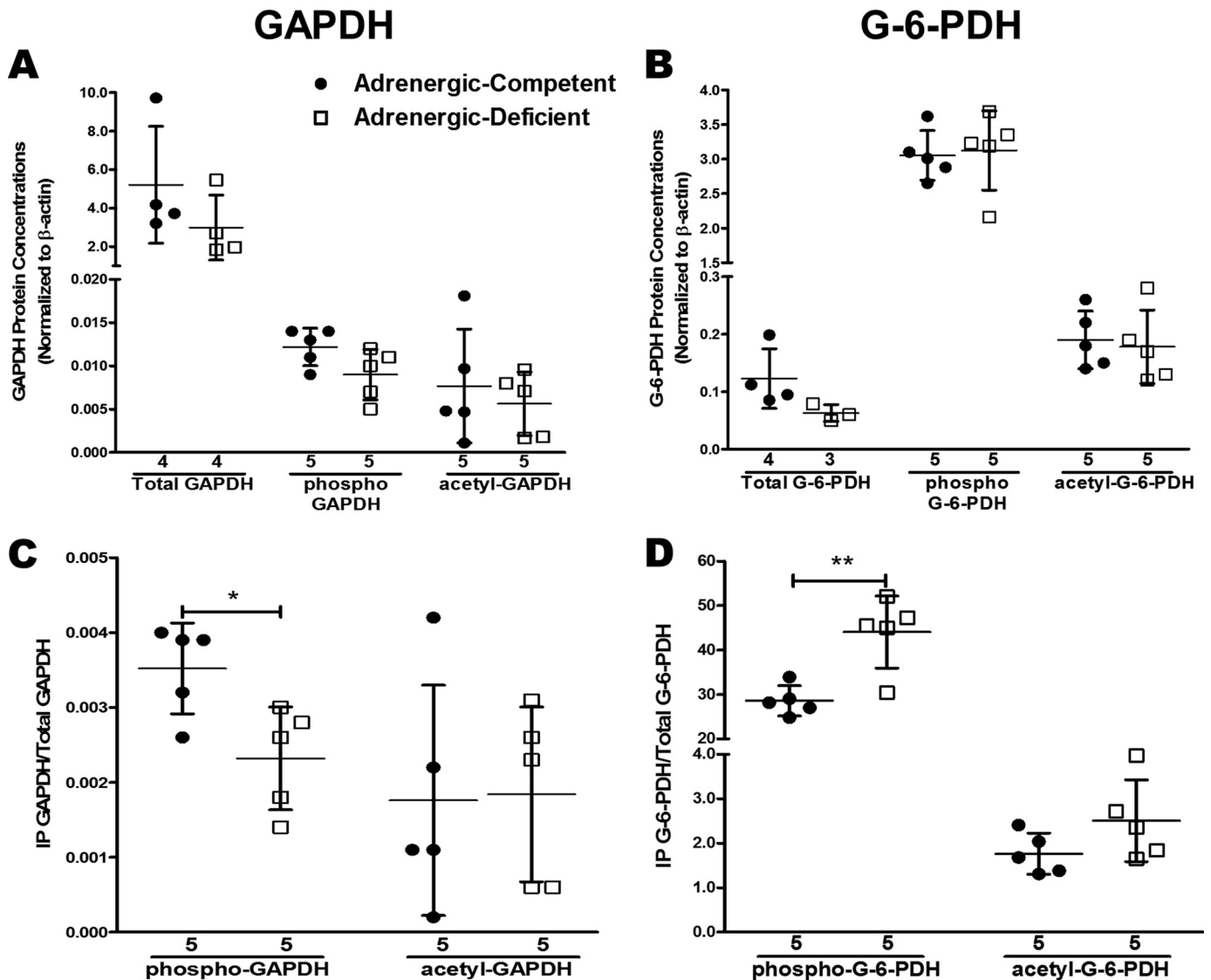


potential needed to drive metabolic reactions, including glycolysis, PPP, and TCA cycle pathways.

### GAPDH

GAPDH, an essential enzyme of glycolysis that converts glyceraldehyde 3-phosphate to 1,3-diphosphoglycerate, was identified as an important target affected by the absence of adrenergic hormone influence. Decreased GAPDH activity in adrenergic hormone-deficient hearts was accompanied by decreased phosphorylation of GAPDH and decreased 1,3-diphosphoglycerate and phosphoenolpyruvate concentrations implicating adrenergic regulation of GAPDH. One potential regulatory mechanism following adrenergic stimulation is phosphorylation of GAPDH to increase activity (40, 41). Studies have shown several kinases regulated by adrenergic stimu-

lation, including Akt/PKB, CaMKII $\beta$ , PKC, and AMPK, phosphorylate GAPDH to enhance activity (42–45). Thus, the observed decrease in the ratio of phosphorylated to total GAPDH protein in adrenergic hormone-deficient embryos is consistent with the decreased enzymatic activity observed for GAPDH in these embryos. Although activation of AMPK appears to be intact in adrenergic hormone-deficient embryos, decreased  $\alpha$ -AMPK protein concentrations may be preventing sufficient GAPDH phosphorylation and activation leading to disruption of the glycolysis pathway. Evidence also suggests that GAPDH activity is highly sensitive to oxidative stress (37, 46–50), and hence, the limitations in important NAD<sup>+</sup>/NADH co-factors likely also contributed to the observed decreases in GAPDH activity in adrenergic hormone-deficient hearts.



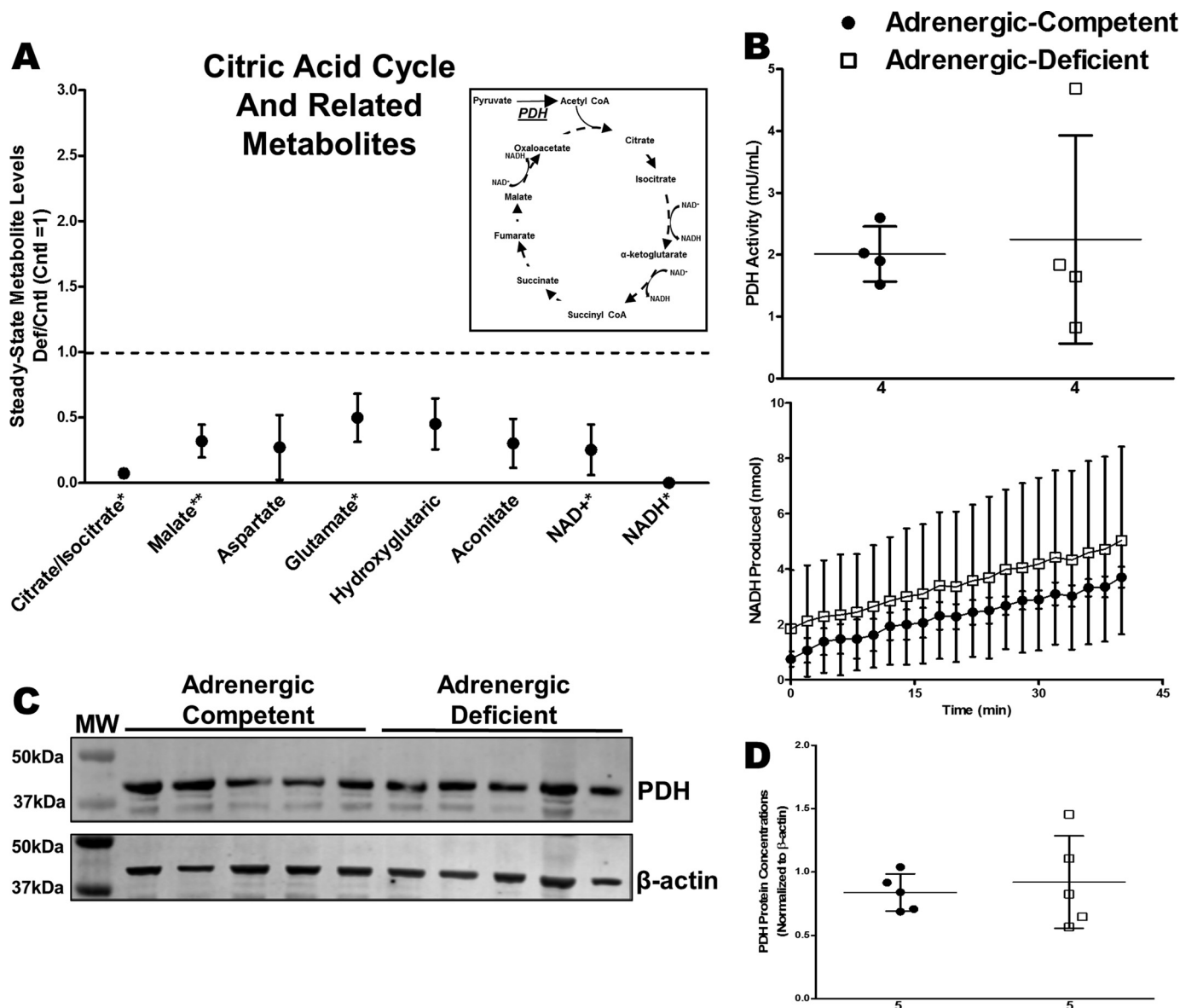
**Figure 8. Effects of adrenergic deficiency on phosphorylation and acetylation of GAPDH and G-6-PDH.** A and B, total, phospho-, and acetyl-GAPDH and G-6-PDH protein concentrations in E11.5 adrenergic hormone-competent (●) and adrenergic hormone-deficient (□) embryonic trunks, normalized  $\beta$ -actin. C and D, relative phospho- and acetyl-GAPDH and G-6-PDH protein concentrations in adrenergic hormone-competent (●) and adrenergic hormone-deficient (□) embryonic trunks, normalized to total GAPDH protein or G-6-PDH protein. Numerical values below the x axes refer to the number (n) of samples analyzed. Student's *t* test was used to compare means between competent and deficient groups. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ .

Interestingly, however, cardiac lactate concentrations were not significantly affected by adrenergic deficiency. Possible explanations for this finding could be minimal GAPDH activity is compensated by extra-cardiac (including maternal circulation) lactate or other pyruvate sources, such as alanine, glycine, and/or glycerol 3-phosphate (which was not diminished in concentration in adrenergic hormone-deficient hearts, see Fig. 2, *heat map*), that help maintain lactate concentrations under anaerobic conditions (51–53). This would indicate that adrenergic hormones are necessary to shift the fate of glucose from anaerobic lactate formation to glucose oxidation and aerobic respiration during this phase of embryonic development (embryonic shift) (5). Another explanation is lactate produced from glucose feeds into the TCA cycle (54). Because the TCA cycle is compromised in adrenergic hormone-deficient embryonic hearts, this may also contribute to the relatively unaffected

lactate concentrations in these hearts compared with those found in adrenergic hormone-competent controls.

### G-6-PDH

Our metabolomic results also identified G-6-PDH, the rate-limiting enzyme in PPP that converts glucose 6-phosphate to 6-phosphogluconolactone, as another target of adrenergic stimulation. We show that adrenergic hormone-deficient embryos have decreased G-6-PDH activity, increased ratios of phosphorylated to total G-6-PDH protein, and decreased ribose 5-phosphate, PRPP, and nucleotide concentrations indicating G-6-PDH and PPP are effectively regulated by adrenergic stimulation during embryonic development. Other studies have also shown adrenergic regulation of PPP in working rat hearts exposed to NE and other adrenergic agonists with increased G-6-PDH activity, PRPP concentrations, and adenine



**Figure 9. Effects of adrenergic deficiency on PDH activity and protein concentrations.** *A*, steady-state metabolites involved in TCA cycle in E11.5 adrenergic hormone-deficient hearts compared with controls (dotted line). Schematic representation of TCA cycle and related pathways provided as reference. *B*, PDH activity in adrenergic hormone-competent (●) and adrenergic hormone-deficient (□) embryonic hearts, expressed as milliunits of enzyme per ml of sample and NADH produced per min, respectively. Milliunits/ml are calculated as ((nmol of NADH)/(min)(ml of sample)). *C* and *D*, PDH protein concentrations (39 kDa) in adrenergic hormone-deficient embryonic trunks (□) compared with controls (●), normalized to  $\beta$ -actin. The  $\beta$ -actin panel is re-used as a representative image of six replicated experiments probing for GAPDH, G-6-PDH, and PDH. Numerical values below the *x* axes refer to the number (*n*) of samples analyzed. Student's *t* test was used to compare means between competent and deficient groups. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ .

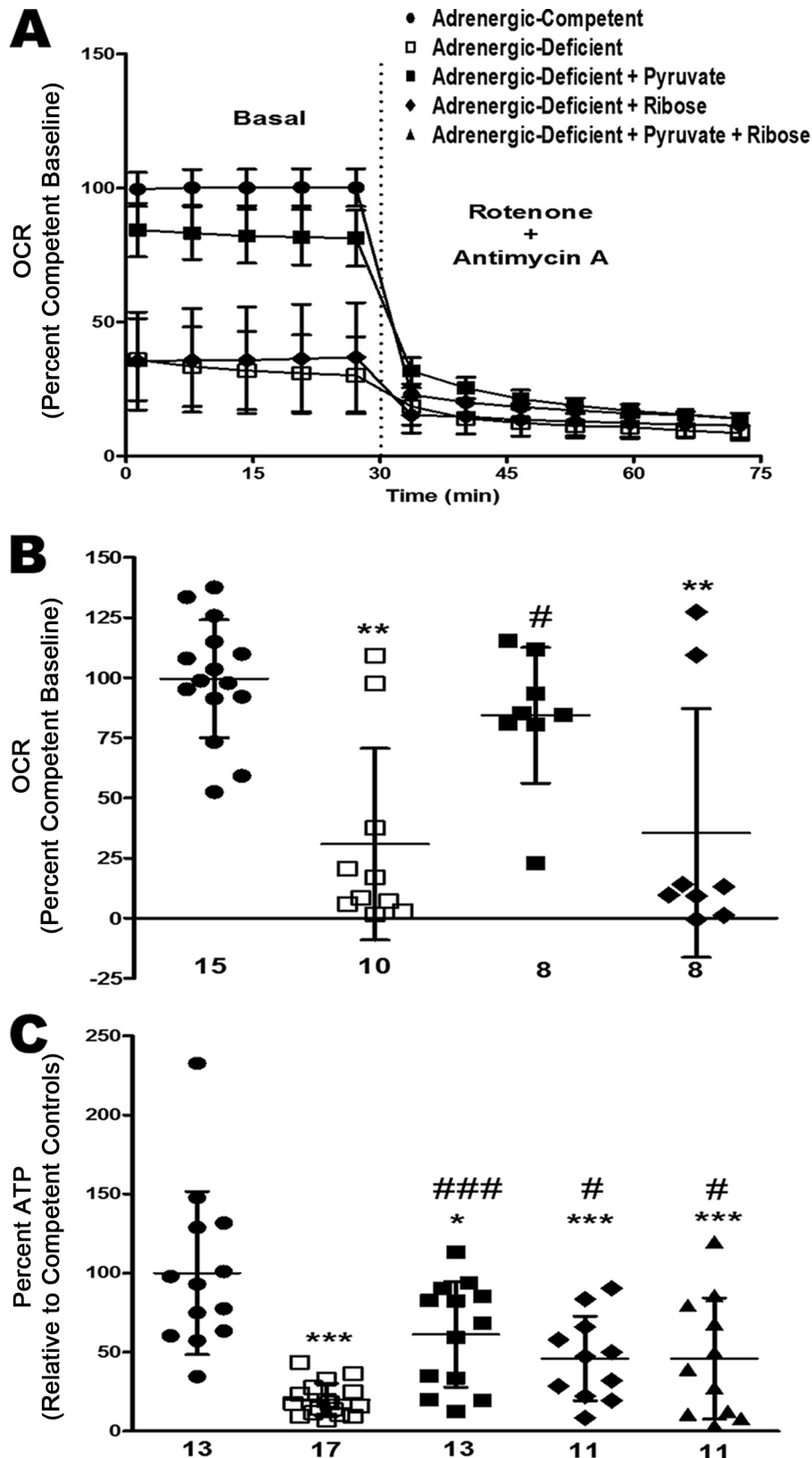
nucleotide biosynthesis (59–61). During development, G-6-PDH has been shown to be protective against oxidative stress resulting from placental circulation and nutrient/respiratory exchange (31, 38). Additionally, phosphorylation of G-6-PDH has been shown to decrease activity leading to increased oxidative stress (62, 63). The decreased G-6-PDH activity and increased  $NAD^+/NADH$  and  $NADP^+/NADPH$  indicate adrenergic hormone-deficient embryos have shifted to a more oxidized status with consequently less reducing power that could contribute to oxidative stress. Therefore, our results suggest that adrenergic stimulation may regulate G-6-PDH by reducing phosphorylation status, which in turn leads to increased enzymatic activity thereby allowing for protection against oxidative stresses in the embryonic heart.

Furthermore, observed deficits in ribose 5-phosphate concentrations in adrenergic hormone-deficient hearts suggested that addition of D-ribose may also be an effective rescue strategy to bypass the G-6-PDH reaction and improve energy status. Consistent with this hypothesis, we showed that addition of ribose significantly improved ATP concentrations in adrenergic hormone-deficient hearts, although it was interestingly neither additive nor synergistic when applied in conjunction with pyruvate, possibly reflecting the overall increase in oxidized status under conditions when adrenergic hormones are not present.

#### Metabolic rescue of energy metabolism

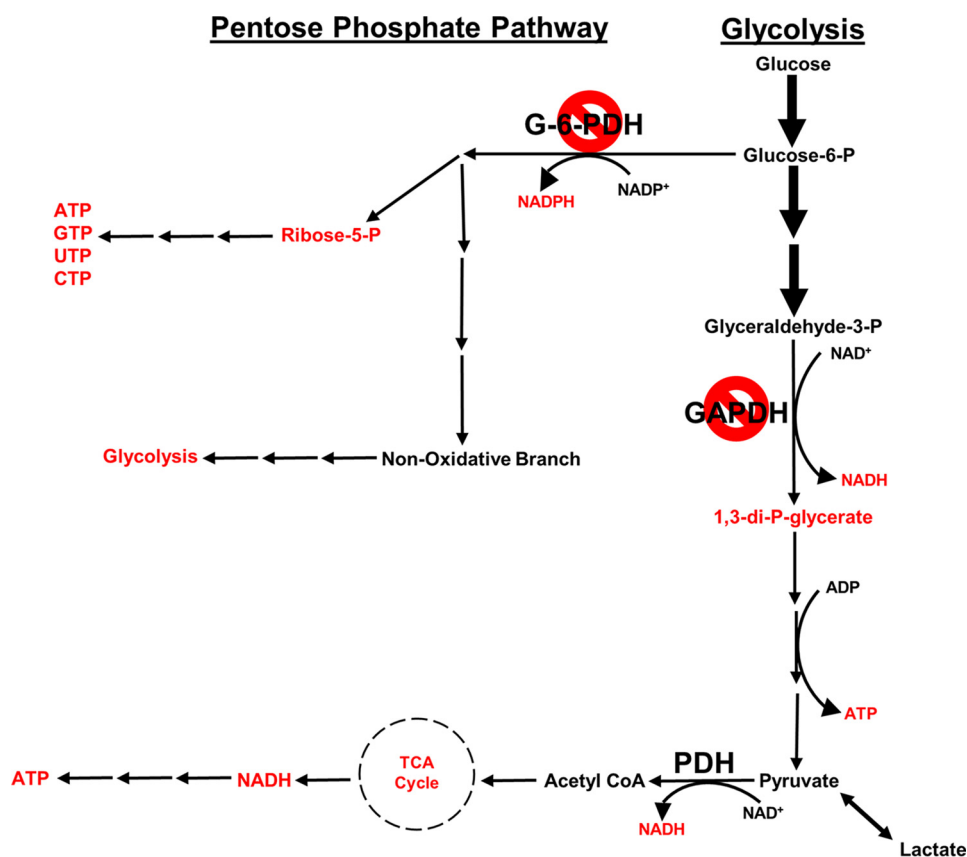
Our observations of comparable glucose 6-phosphate, but decreased citrate/isocitrate and malate, suggested that pyru-

## Adrenergic hormones stimulate embryonic heart metabolism



**Figure 10. Rescue of OCR and steady-state ATP in adrenergic hormone-deficient embryonic hearts with pyruvate or ribose.** A and B, oxygen consumption rates in E11.5 adrenergic hormone-competent (●) and adrenergic hormone-deficient (□) embryonic hearts after addition of pyruvate (■), ribose (◆), and rotenone/antimycin A treatment, expressed as percentage compared with adrenergic hormone-competent control baseline. C, steady-state ATP concentrations in adrenergic hormone-competent and adrenergic hormone-deficient embryonic hearts after addition of pyruvate and ribose. Numerical values below the x axes refer to the number (n) of samples analyzed. Data are represented as a percentage compared with competent controls. One-way analysis of variance was performed with Dunnett's multiple comparison test used to evaluate differences between treated groups and control groups. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$  compared with adrenergic hormone-competent controls. #,  $p < 0.05$ ; ###,  $p < 0.001$  compared with adrenergic hormone-deficient controls.





**Figure 11. Summary of metabolic pathways and targets affected by adrenergic deficiency during embryonic heart development.** Shown is a schematic representation of metabolites and enzymes of PPP, glycolysis, and TCA cycle. Metabolites and enzymes are significantly lower in adrenergic hormone-deficient embryos compared with competent controls as indicated in red or by the red symbol, respectively.

vate oxidation and PDH activity may have been impaired in adrenergic hormone-deficient hearts. Intriguingly, however, PDH activity was not affected in adrenergic hormone-deficient hearts, indicating that not all metabolic dehydrogenase enzymes were equally affected by the absence of adrenergic stimulation possibly due to differing sensitivities to oxidation (55, 56). For example, GAPDH and G-6-PDH have been shown to be sensitive to oxidative stress, whereas oxidative environments have lesser effects on PDH (57, 58).

Because PDH rates were similar to adrenergic hormone-competent controls, addition of the metabolic substrate pyruvate to adrenergic hormone-deficient hearts should provide effective rescue that could facilitate restoration of cellular energy levels. *Ex vivo* delivery of pyruvate to adrenergic hormone-deficient embryonic hearts substantially improved OCR and steady-state ATP levels, demonstrating that apparent metabolic starvation could be overcome by addition of the appropriate substrate(s). We previously showed that adrenergic hormone-deficient embryos have larger and more elongated mitochondria compared with controls, consistent with a “starvation” phenotype (12, 64, 65). Taken together, these results suggest that mitochondrial metabolism is capable of functioning in adrenergic hormone-deficient embryos, but impaired GAPDH activity may limit the amount of pyruvate substrate entering mitochondria to fuel aerobic respiration.

### Summary

Adrenergic hormone-deficient embryos have severely compromised glucose metabolism, specifically involving glycolysis, PPP, and the TCA cycle (Fig. 11). We have shown that the absence of adrenergic hormones selectively affects GAPDH and G-6-PDH during embryonic cardiac development, suggesting that adrenergic stimulation is necessary to enable GAPDH and G-6-PDH to produce sufficient metabolic fuel and anaplerotic substrates, respectively, for successful transition from anaerobic to aerobic metabolism during a crucial phase of embryonic development.

### Experimental procedures

#### Mice

Female (2–6 months) and male (2–12 months) C57BL/6J mice mated in the present studies were housed in a 12-h light/12-h dark cycle and given *ad libitum* access to food and water. All procedures and handling of mice were conducted in accordance with and approved by the University of Central Florida Institutional Animal Care and Use Committee and the guidelines established by the National Institutes of Health for vertebrate animal research (66, 67). The *Dbh* mouse colony was kindly provided by the Palmiter laboratory (University of Washington, Seattle) (8) and maintained as described previously (11, 12, 68). Timed pregnancies were determined by the presence of a vaginal plug at E0.5. Preg-

## Adrenergic hormones stimulate embryonic heart metabolism

nancies were confirmed by high-resolution ultrasound at E8.5 using a Visual Sonics Vevo3100 instrument with an MX250 transducer.

### Embryonic tissue collections

All embryos appearing healthy in color, size (crown–rump length = 6–7 mm), texture, morphology, heartbeat, and blood circulation during microscopic examination were used for this study. All unhealthy and dead embryos were discarded. No differences were seen upon gross examination of adrenergic hormone-deficient (*Dbh*<sup>-/-</sup>) and adrenergic hormone-competent control (*Dbh*<sup>+/+</sup> and *Dbh*<sup>+/-</sup>) embryonic hearts at time of isolation. Embryonic heads were isolated for genotyping. For LC-MS metabolomics analysis, enzyme activity assays, and immunoblotting E11.5 hearts or trunks containing the heart were flash-frozen in liquid nitrogen and stored at -80 °C for further analysis.

### Ex vivo embryonic heart culture

E11.5 hearts were isolated under aseptic conditions and cultured overnight in Dulbecco's modified Eagle's medium (DMEM) (no glucose, no glutamine, no phenol red) (Thermo Fisher Scientific) containing 5% fetal bovine serum (Hyclone Laboratories) that was charcoal-stripped of catecholamines and steroid hormones. The medium was additionally supplemented with penicillin G (100,000 units/liter) and streptomycin (100 mg/liter). Cultures were incubated overnight prior to next day steady-state ATP measurements.

### Reagents

Chemical reagents were purchased from Sigma, except where noted otherwise.

### Steady-state ATP measurements

Isolated E11.5 embryonic hearts were flash-frozen in liquid nitrogen and stored at -80 °C. Samples were homogenized with 6% TCA. *Ex vivo* embryonic heart cultures were placed in DMEM supplemented with pyruvate (11 mM), ribose (6.6 mM), or control (no supplement) following overnight incubation. Cultures were incubated for 2 h, then collected and sonicated in 50  $\mu$ l of media and 10  $\mu$ l of 6% TCA.

All samples were centrifuged at 6000  $\times$  g for 5 min at 4 °C. Supernatant was collected and neutralized with 1 $\times$  Tris acetate, as described previously (12, 69). ATP measurements were performed with the ATPlite Bioluminescence Assay (Perkin-Elmer Life Sciences) according to the manufacturer's protocol. Standard curves were generated with known concentrations of ATP. Luminescence was measured with Envision Multilabel plate reader, and ATP measurements were normalized to total protein concentrations determined using Thermo Fisher Scientific NanoDrop 8000 spectrophotometer.

### ATP synthesis/hydrolysis assays

Isolated E11.5 embryonic hearts were flash-frozen in liquid nitrogen and stored at -80 °C. Samples were homogenized for 30 s in a buffer containing 25 mM Tris-HCl, 40 mM KCl, 2 mM EGTA, 5 mM MgCl<sub>2</sub>, 0.8 M sucrose, and 200  $\mu$ M phenylmethylsulfonyl fluoride. Samples went through two freeze/thaw cycles

and vortexing before centrifugation at 600  $\times$  g for 10 min at 4 °C. Supernatant was collected and solubilized in 50  $\mu$ g/ $\mu$ l digitonin.

ATP synthesis standards were generated with known concentrations of ATP. Synthesis activity was measured in 96-well white wall plates in 100- $\mu$ l reactions containing 1 mM pyruvate, 1 mM malate, 30  $\mu$ M *P*<sup>1</sup>,*P*<sup>5</sup>-di(adenosine 5')-pentaphosphate (Ap5A, adenylate kinase inhibitor), 0.5  $\mu$ g/ml luciferase, 40  $\mu$ M luciferin, and 100  $\mu$ M ADP (70, 71). Luminescence was measured from 5- $\mu$ l sample extracts in 5-s intervals for 1 min.

ATP hydrolysis standards were generated with known concentrations of monopotassium phosphate. Hydrolysis activity was measured in 1-ml reactions containing 1 mM ATP, 80 mM sulfuric acid, 10 mM ascorbic acid, 50  $\mu$ M tartrate, and 700  $\mu$ M ammonium molybdate (72). Absorbance was measured at 850 nm in 2- $\mu$ l extracts in quartz cuvettes in 5-s intervals for 30 s.

### Liquid chromatography-mass spectrometry

Isolated E11.5 embryonic hearts were flash-frozen in liquid nitrogen and stored at -80 °C. Samples were prepared as previously described (73). Briefly, metabolites were serially extracted from tissue powder of each heart in 80% methanol (1 then 0.5 ml) and extracts were dried under nitrogen gas and stored at -80 °C. Samples were suspended in 120  $\mu$ l of 50% methanol and, for amino acid detection, were derivatized with 2% (v/v) triethylamine and benzyl chloroformate. Metabolites were separated by reverse phase HPLC (Shimadzu) and identified by single reaction monitoring on a triple-quadrupole mass spectrometer (Thermo Fisher Scientific QuantumUltra). Data were analyzed using a publicly available MzRock machine learning tool kit (<http://code.google.com/p/mzrock/>), which automated metabolite identification based on retention time, whole-molecule mass, collision energy, and fragment mass. Selected metabolites were also analyzed using XCalibur Qual Browser (Thermo Fisher Scientific). We attempted to measure pyruvate and acetyl-CoA by LC/MS and other biochemical techniques; however, detection was limited in both adrenergic hormone-competent as well as deficient controls, possibly due to rapid flux from pyruvate to acetyl-CoA to citrate (74, 75).

### GAPDH, G-6-PDH, and PDH activities

GAPDH activity assay kit (catalogue number ab204732) was purchased from Abcam. G-6-PDH and PDH activity assay kits (catalogue numbers MAK105 and MAK183) were purchased from Sigma. Standard curves were generated for these activity assays with known concentrations of NADH. Flash-frozen embryonic hearts were sonicated for 5 s in cold PBS or buffer specified in manufacturers' protocols.

For the GAPDH assay, extracts were incubated on ice for 10 min and centrifuged at 10,000  $\times$  g for 5 min at 4 °C, and supernatant was collected. Absorbance was measured at 450 nm in 25- $\mu$ l extracts in a clear 96-well plate in 5-min intervals for 40 min, according to the manufacturer's protocol.

For the G-6-PDH assay, extracts were centrifuged at 15,000  $\times$  g for 10-min at 4 °C, and the supernatant was collected. Absorbance was measured at 450 nm in 25- $\mu$ l extracts in a clear 96-well plate in 5-min intervals for 40 min, according to the manufacturer's protocol.

For the PDH assay, extracts were incubated on ice for 10 min and centrifuged at  $10,000 \times g$  for 5 min at 4 °C, and the supernatant was collected. Absorbance was measured at 450 nm in 25- $\mu$ l extracts in a clear 96-well plate in 2-min intervals for 40 min, according to the manufacturer's protocol.

### Western blotting

Flash-frozen embryonic trunks were sonicated in  $1 \times$  RIPA Buffer supplemented with  $100 \times$  Halt<sup>TM</sup> Protease Inhibitor Mixture and  $100 \times$  Halt<sup>TM</sup> Phosphatase Inhibitor Single Use Mixture (ThermoFisher Scientific). Protein was quantified using a Bradford assay (Sigma). Protein lysates (50  $\mu$ g/lane) were separated on an 4–12% BisTris Precast gel (Thermo Fisher Scientific) followed by transfer to PVDF membrane (Thermo Fisher Scientific). Membranes were incubated in TBS blocking buffer for 1 h at 4 °C (LICOR Odyssey). Membranes were then incubated overnight at 4 °C in primary antibodies. Antibodies used included the following: phospho-AMPK (Thr-172) (rabbit polyclonal IgG, 1:1000, Cell Signaling Technology (CST), catalogue number 2531S, lot number 15);  $\alpha$ -AMPK (rabbit polyclonal IgG, 1:1000, CST, catalogue number 2532S, lot number 19); GAPDH (rabbit polyclonal IgG, 1:2500, Thermo Fisher Scientific, catalogue number PA1–987, lot number RF233990); G-6-PDH (rabbit polyclonal IgG, 1:1000, (CST), catalogue number 8866, lot number 3); and PDH (rabbit polyclonal IgG, 1:500, CST, catalogue number 2784, lot number 2).  $\beta$ -Actin (monoclonal mouse IgG2b, 1:5000, Thermo Fisher Scientific catalogue number MA5-15739, lot number RH236746) was used as a loading control. Following three TBS-T washes, membranes were incubated for 1 h at room temperature in secondary antibodies (LICOR Odyssey goat anti-rabbit or anti-mouse IRDye 680 or 800, catalogue number 926-32210, lot number C40528-02, and catalogue number 926-68021, lot number C40415-04). This procedure was replicated by probing six individual blots for GAPDH, G-6-PDH, and  $\beta$ -actin, then stripping each blot with Restore Fluorescent Western blotting Stripping Buffer (Thermo Fisher Scientific), and re-probing for PDH. pAMPK and  $\beta$ -actin were also probed together, then stripped and re-probed for AMPK. Blots were visualized using a LICOR Odyssey IR imaging system. All blots were analyzed using ImageStudio software and normalized to  $\beta$ -actin.

### Immunoprecipitations

Flash-frozen embryonic trunks were sonicated in  $1 \times$  Tris-HCl supplemented with  $100 \times$  Protease Inhibitor Mixture,  $100 \times$  Phosphatase Inhibitor Mixture, and  $100 \times$  sodium butyrate. Protein was quantified using a Bradford assay. Antibodies were incubated with 100  $\mu$ l of Pierce protein A-agarose (Thermo Fisher Scientific) for 1 h at room temperature. Antibodies used to precipitate phosphoproteins and acetylated proteins were anti-phosphoserine (pSer) (rabbit polyclonal IgG, 20  $\mu$ g/mg lysate, Abcam, catalogue number ab9332, lot number GR3179704-4) and anti-acetylated lysine (rabbit polyclonal IgG, 1:100, CST, catalogue number 9441S, lot number 13). Embryonic trunk lysates (50  $\mu$ g) were incubated with antibody/agarose conjugates overnight at 4 °C. Bound protein was washed three times in  $1 \times$  Tris-HCl and eluted with 1:1  $1 \times$

Tris-HCl and  $2 \times$  Laemmli sample buffer (4% SDS, 10% DTT, 20% glycerol, 0.004% bromphenol blue, and 0.125 M Tris-HCl, pH 6.8) at 95 °C for 5 min. Lysates were analyzed for phosphorylation and acetylation of G-6-PDH and GAPDH by Western blotting.

### OCR measurements

*Ex vivo* E11.5 mouse hearts were isolated as mentioned above and incubated overnight in a Seahorse Biosciences XF24 Islet Capture Microplate with mesh grids placed on top of the specimen to prevent floating and probe interference. The next day, hearts were incubated in Seahorse XF Base Medium Minimal DMEM (0 mM glucose) supplemented with pyruvate (1 mg/ml), ribose (1 mg/ml), or control (no supplement) for 2 h. Basal OCR was measured at 6-min intervals over 72 min using a Seahorse XF<sup>e</sup> Biosciences system. Rotenone (5  $\mu$ M) and antimycin A (20  $\mu$ M) were added simultaneously to block mitochondrial electron transport (76).

### Statistics

Data are expressed as means  $\pm$  S.D. Student's *t* tests were performed, unless otherwise noted, to compare means between adrenergic hormone-competent and adrenergic hormone-deficient groups, with *p* < 0.05 required to reject the null hypothesis.

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*Author contributions*—J. N. P., P. S. B., G. A. P., and S. N. E. conceptualization; J. N. P. data curation; J. N. P., T. M., Q. L., G. A. P., and S. N. E. formal analysis; J. N. P. validation; J. N. P., T. M., Q. L., S. M. N., P. S. B., G. A. P., V. L. D., and S. N. E. investigation; J. N. P., T. M., Q. L., S. M. N., P. S. B., and V. L. D. methodology; J. N. P. writing-original draft; J. N. P., T. M., Q. L., P. S. B., G. A. P., V. L. D., and S. N. E. writing-review and editing; P. S. B., V. L. D., and S. N. E. supervision; G. A. P. and S. N. E. resources; S. N. E. funding acquisition; S. N. E. project administration.

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