CHAPTER 3.

Acetylation of Cardiac Regulatory Proteins Increases Fatty Acid β-Oxidation and Represses Glycolysis During Maturation

My role in this work involved performing all experiments (except those noted below), as well as the writing of the manuscript. Cory Wagg perfused the rabbit's heart, and Sowndramalingam Sankaralingam performed perfusion data analysis.

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Abstract

Aims: Dramatic cardiac developmental changes in energy metabolism occur in neonate, with a shift from glycolytic to mitochondrial oxidative metabolism occurring shortly after birth. Lysine acetylation has recently been identified as a potentially important pathway involved in the control of energy metabolism. We therefore investigated the impact of changes in protein acetylation on the maturational changes in energy metabolism of the newborn rabbit heart.

Methods and results: One-day, 7-day, and 21-day old rabbit hearts were perfused retrogradely with Krebs-Henseleit solution to measure glycolysis, and the oxidation of lactate, glucose, and fatty acids. Cardiac fatty acid β-oxidation rates were significantly increased in 21-day vs 1-, and 7-day old rabbits (555±26 vs 299±10 and 364±24 nmol.g dry wt-1.min-1, $p<0.05$). Activities of the fatty acid β-oxidation enzymes, long chain acyl CoA dehydrogenase (LCAD) and β-hydroxyacyl CoA dehydrogenase (β-HAD) were increased in hearts from 7-, and 21-day vs 1-day old rabbits, and were associated with LCAD and β-HAD hyperacetylation. Increased overall myocardial proteins acetylation during maturation was associated

with increased GCN5L1 expression, while expression of the mitochondrial deacetylase, SIRT3, did not change. Increased expression of the nuclear deacetylase SIRT1 and the decreases in SIRT6 cardiac protein level during maturation was accompanied by decreased acetylation of PGC-1α and increased ATP production. Acetylation of the glycolytic enzymes hexokinase (HK) and phosphoglycerate mutase (PGM) were increased; scenarios consistent with the decrease in glycolytic activity seen in 7-, and 21-day vs. 1-day old hearts.

Conclusions: We conclude that increased lysine acetylation during maturation enhances cardiac fatty acid β-oxidation, and impairs glycolysis.

3.1 INTRODUCTION

Heart is a high energy demand organ which depends mainly on oxidation of fatty acids and glucose to produce adenosine triphosphate (ATP) required for maintenance of it is function (1). The reminder of ATP production is derived from glycolysis, and the oxidation of lactate, and ketone bodies (2). Interestingly, this energy substrate preference differs between the adult and neonatal heart (8). During the fetal life levels of fatty acids are very low and the heart relies mostly on glycolysis and lactate oxidation to meet its energy demands (9,8). Shortly after birth, major changes in energy metabolism occur, which is characterized by a shift in substrate preference from glycolysis to a rapid increase in fatty acid β-oxidation (5). The increase in fatty acid β-oxidation is related to maturational changes of transcription factors, and perhaps changes in posttranslational modifications that control key enzymes of fatty acid β-oxidation. Peroxisome proliferator activated receptor gamma coactivator 1-alpha (PGC-1α) is an important transcriptional regulator of genes involved in fatty acid metabolism, with activation is lead to increase in expression of genes involved in fatty acid uptake, storage, and oxidation (1). The expression of PGC-1α is increased with maturation which can lead to increase in fatty acid β-oxidation (78).

Lysine acetylation is an important post-translational modification which has recently been identified as a substantial pathway involved in

regulation of energy metabolism (191,192,194,200,235). Almost every enzyme in glycolysis, glucose oxidation, fatty acid β-oxidation, and tricarboxylic cycle is acetylated at least on one lysine residue (140,159,191,194). However, the impact of this post-translational modification on the activity of key enzymes involved in glucose and fatty acid metabolism is poorly understood. General control of amino acid synthesis 5 like 1(GCN5L1) is recently characterized as a mitochondrial acetyltransferase which plays important role in mitochondrial protein acetylation, respiration, and metabolism (211). Protein deacetylation is mediated by a family of seven enzymes called sirtuins (SIRT1-7) (148,165,177,236). SIRT3 is suggested as a major mitochondrial deacetylase which controls the activity of key enzymes involved in glucose and fatty acid β-oxidation (192,194,199,237). However, conflicting information has emerged regarding the role of acetylation in liver and muscle cell fatty acid β-oxidation. Studies by Hirschey *et al* suggested that deacetylation of fatty acid oxidative enzyme; long chain acyl CoA dehydrogenase (LCAD) by SIRT3 accelerates fatty acid β-oxidation (196,200,206). In contrast, Zhao *et al* showed that acetylation of the fatty acid β-oxidative enzyme; enoyl CoA hydratase/3-hydroxyacyl CoA dehydrogenase, results in an activation of enzyme activity (159). The impact of maturational changes of lysine acetylation on cardiac fatty acid β-oxidation has never been addressed before. SIRT6 plays an important role in glucose homeostasis and a protective role in cardiac hypertrophy

(238). The purpose of this study is to determine the role of lysine acetylation in regulation of cardiac energy metabolism and the contribution of this post-translational modification in the dramatic energy changes seen during maturation.

3.2 MATERIALS AND METHODS

Rabbits received care and were treated according to the guidelines of the Canadian Council on Animal Care, and all procedures performed on animals were approved by the University of Alberta Health Sciences Animal Welfare Committee. One-, 7-, and 21-day old New Zealand White rabbits were used in this study. Rabbits were sacrificed and hearts were retrogradely perfused with Krebs-Henseleit solution (2.5 mM Ca2+, 5 mM glucose, 0.4 mM palmitate prebound to 3% albumin, 1 mM lactate and 100 μU/mL insulin) to measure glycolysis, and the oxidation of glucose, lactate, and fatty acid, as described in details in **chapter 2**. At the end of the perfusion, hearts were immediately frozen in liquid N_2 and processed for acetylation status. Protocols for assessment of β-HAD activity, LCAD activity, immunoprecipitation, immunoblotting, and statistical analysis are described in details in **chapter 2** of this thesis.

3.4 RESULTS

3.4.1 Cardiac metabolic profile during maturation:

Glycolytic rates were significantly decreased in 21- and 7-day compared to 1-day old hearts (Figure 3-1A). In contrast, cardiac palmitate oxidation rates were markedly increased 3 weeks post-birth (Figure 3-1B). Glucose oxidation rates were significantly decreased in 21-day compared to 1-day old hearts (Figure 3-1C). Lactate oxidation rates were initially increased in 7-day old hearts. However, it declined markedly at 21 day old hearts (Figure 3-1D). Interestingly, there was a maturational increase in overall myocardial protein acetylation during the neonatal period 1–21 days of age (Figure 3-1E).

3.4.2 Age-dependent changes in PGC-1α acetylation:

Protein level of SIRT1 was significantly increased in 21-day compared to 1-, and 7-day old rabbit hearts (Figure 3-2A). In contrast, SIRT6 protein expression was significantly decreased with maturation (Figure 3-2B). There were no age-dependent changes in the level of PCG-1α protein expression, a key transcription factor involved in mitochondrial biogenesis (Figure 3-2C). However, levels of PCG-1α acetylation were significantly decreased in 21-day compared to 1-, and 7-day old rabbit hearts (Figure 3-2D). This decrease in PCG-1α acetylation was

accompanied by an overall increase in cardiac ATP production (Figure 3-2E).

3.4.4 Age-dependent changes in β-HAD acetylation and activity:

There were no changes in SIRT3 protein level (Figure 3-3A); however, the protein expression of GCN5L1 (mitochondrial acetyltransferase) was significantly increased with maturation (Figure 3-3B). Because of the marked increase in fatty acid β-oxidation during maturation, we examined whether the acetylation state was altered in hearts from 1-, 7-, and 21-day old rabbits. The levels of β-HAD protein was not changed during maturation (Figure 3-3C). However, the levels of β-HAD acetylation was significantly increased in hearts of 7-day compared to 1-, and 21-day old rabbits (Figure 3-3D) accompanied with age-dependent increase in β-HAD activity (Figure 3-3E).

3.4.5 Age-dependent changes in LCAD acetylation and activity:

Similar to what was observed with β-HAD, the level of LCAD protein expression was the same in all groups (Figure 3-4A). However, a significant age-dependent increase in LCAD acetylation was observed (Figure 3-4B) accompanied by a marked increase in enzyme activity (Figure 3-4C). Collectively, our results suggest that acetylation is activating fatty acid β-oxidation.

3.4.6 Age-dependent changes in PDH acetylation:

Pyruvate dehydrogenase (PDH) is the rate limiting enzyme in the glucose oxidation pathway. Western blot analysis showed that cardiac expression of PDH was not change with maturation (Figure 3-5A). No differences in PDH acetylation/PDH expression were found between hearts from 1-, 7-, and 21-day old rabbits (Figure 3-5B).

3.4.7 Age-dependent changes in Hexokinase (HK) acetylation:

The cytoplasmic deacetylase SIRT2 cardiac protein level was decreased in 7-day old rabbits; however, its level in 21-day old hearts was comparable to 1-day old hearts (Figure 3-6A). Glycolytic rates significantly declined in the heart with maturation. HK is a key enzyme that catalyzes the first step of glycolysis. The level of HK protein expression was not changed between groups (Figure 3-6B). HK acetylation was significantly increased 7- and 21-days post-birth compared to 1-day old hearts (Figure 3-6C).

3.4.8 Age-dependent changes in Phosphoglycerate Mutase (PGM) acetylation:

To further investigate the role of acetylation in glycolysis, we assessed the acetylation level of another glycolytic enzyme PGM. The level of PGM protein expression was similar in all groups (Figure 3-7A). cardiac PGM acetylation levels were significantly increased in 7- and 21-

day compared to 1-day old rabbits (Figure 3-7B). Hypoxia inducible factor one alpha (HIF-1α) is a transcriptional factor which upregulates glycolytic enzyme expression. Interestingly, HIF-1α protein expression was significantly declined 3 weeks post-birth (Figure 3-7C).

Figure 3-1: Steady-state rates of glycolysis, glucose, lactate, and palmitate oxidation in isolated langendorff perfused hearts from 1-, 7-, and 21-day old rabbits.

A) Glycolysis **(B)**, Palmitate oxidation **(C)**, Glucose oxidation **(D)**, Lactate oxidation **(E)**, Total protein acetylation. Values represent mean±SEM; (n=6). *Significant difference ($P<0.05$) vs. 1-day old. †significant difference vs. 7-day old.

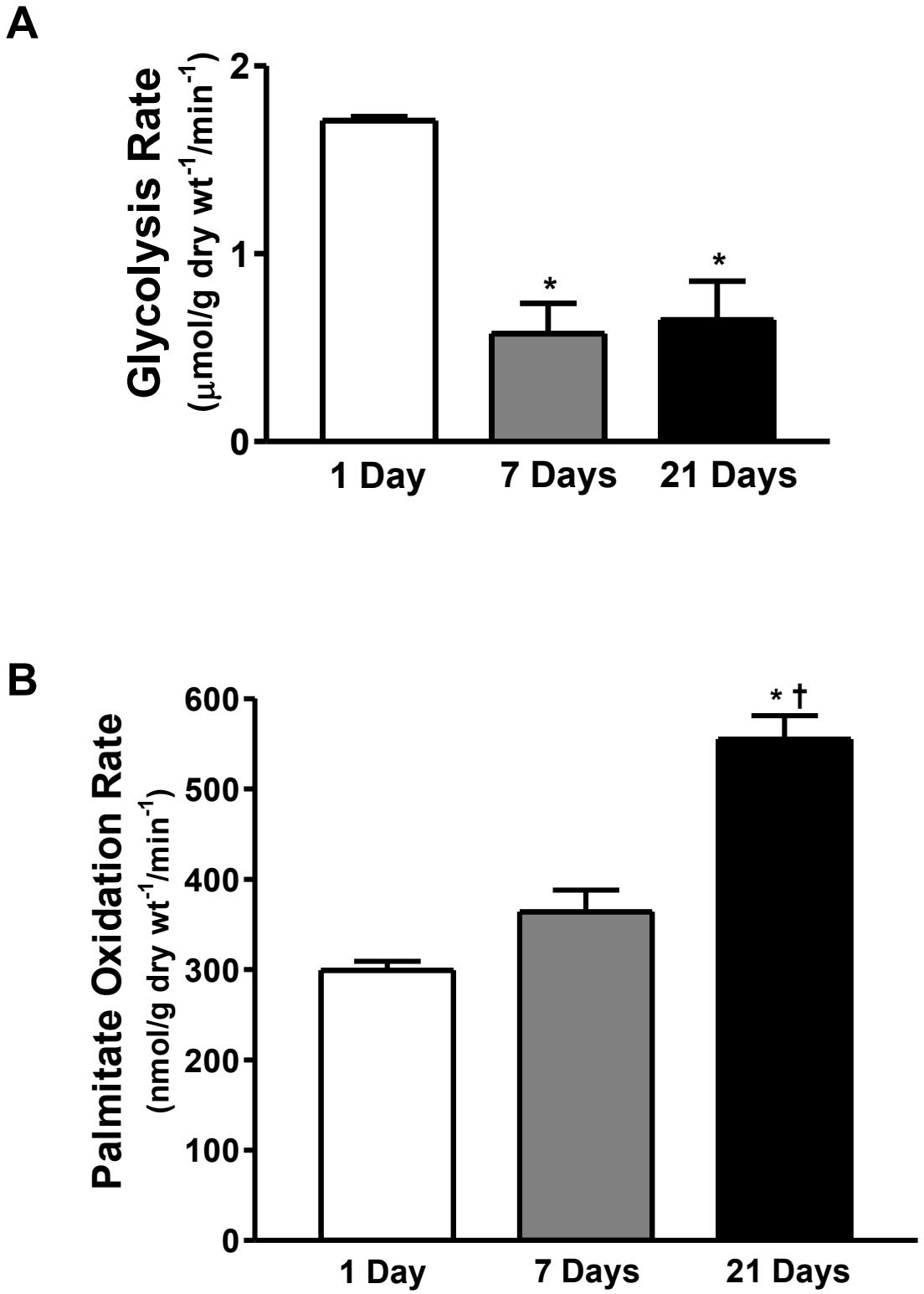
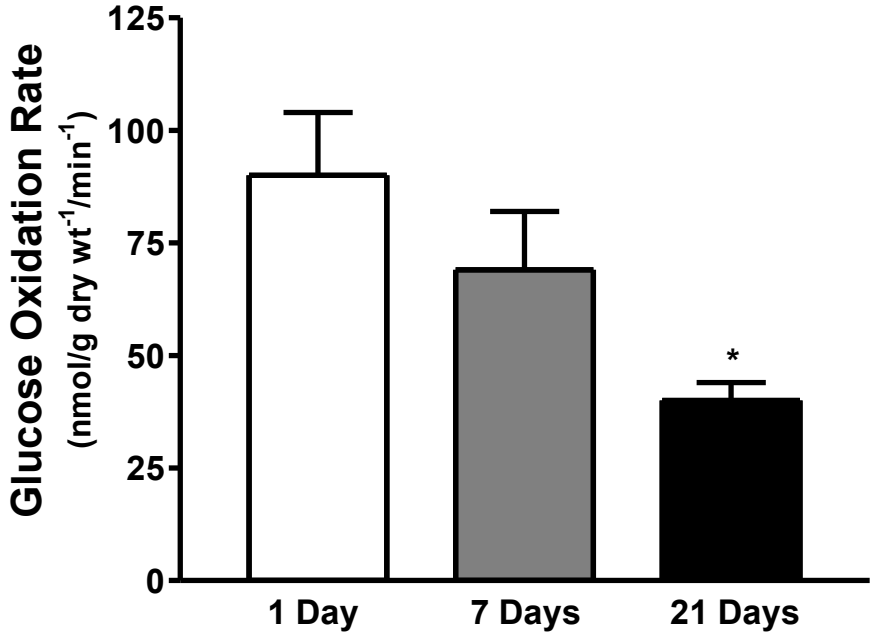

A
Glycolysis Rate
(µmol/g dry wt-1/min-1)
2
1
0
*
*
1 Day
7 Days
21 Days
B
Palmitate Oxidation Rate
(nmol/g dry wt-1/min-1)
600
500
400
300
200
100
0
* †
1 Day
7 Days
21 Days

Figure 3-1

C


Glucose Oxidation Rate
(nmol/g dry wt-1/min-1)
125
100
75
50
25
0
*
1 Day
7 Days
21 Days

D

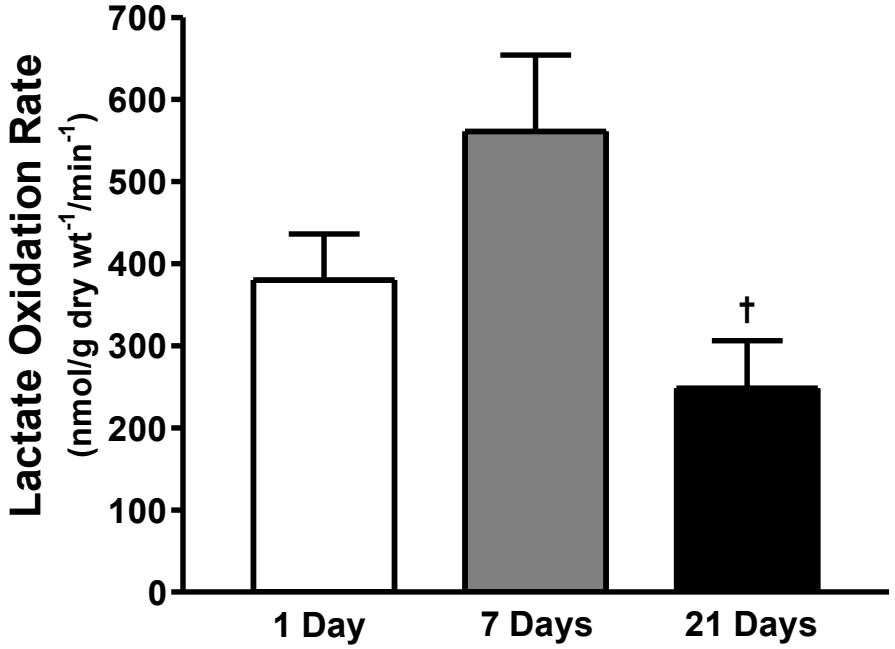

Lactate Oxidation Rate
(nmol/g dry wt-1/min-1)
700
600
500
400
300
200
100
0
†
1 Day
7 Days
21 Days

Figure 3-1

E

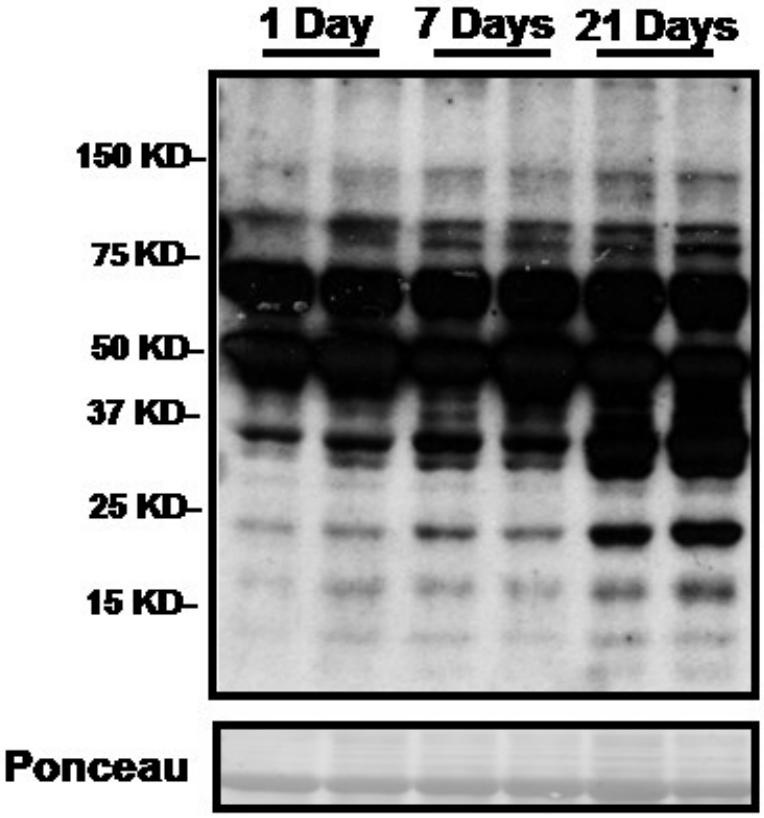


Figure 3-1

Figure 3-2: Level of cardiac PGC-1α acetylation and protein expression of its upstream sirtuins regulator in 1-, 7-, and 21-day old rabbits.

Protein expressions of SIRT1 **(A)**, SIRT6 **(B)**, and PGC-1α **(C)**. **D)** Level of PGC-1α acetylation. Total lysate from 1-, 7-, and 21-day perfused rabbit's hearts were immunoprecipitated with anti-acetyl-K antibodies and then immunoblotted with antibodies specific for PGC-1α. **E)** Calculated steady-state ATP production rate (n=6). Values represent mean±SEM; (n=4). *Significant difference ($P<0.05$) vs. 1-day old. †significant difference vs. 7-day old. Steady-state rates of glycolysis, glucose, lactate, and palmitate oxidation in isolated langendorff perfused hearts from 1-, 7-, and 21-day old rabbits.

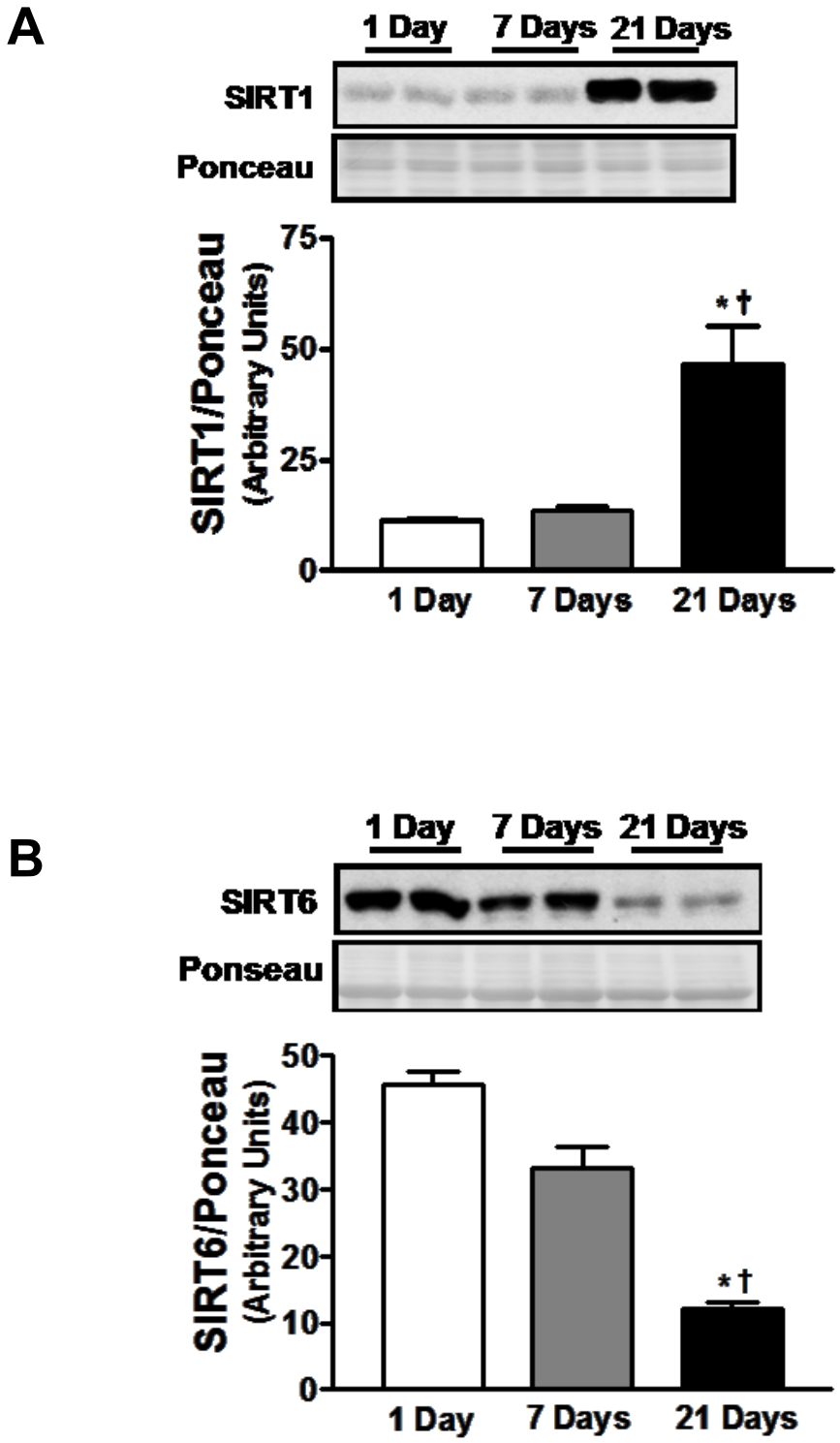
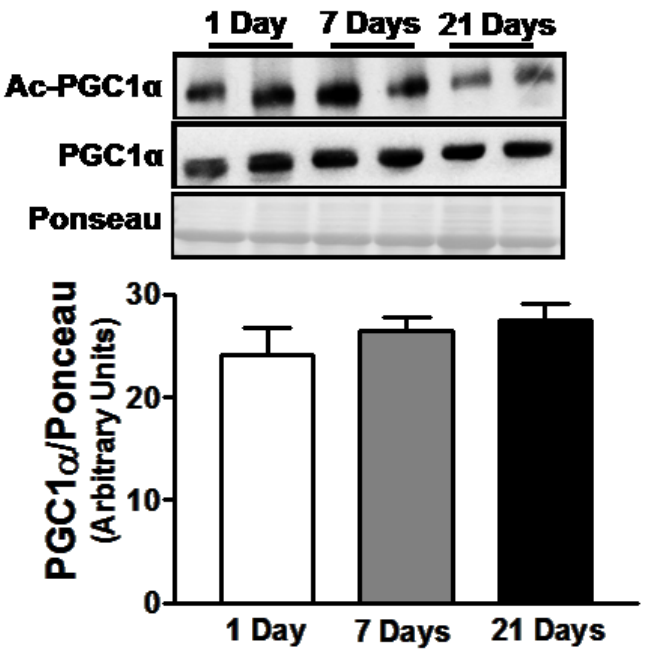

A
1 Day
7 Days
21 Days
SIRT1
Ponceau
SIRT1/Ponceau
(Arbitrary Units)
75
50
25
0
*†
1 Day
7 Days
21 Days
B
1 Day
7 Days
21 Days
SIRT6
Ponseau
SIRT6/Ponceau
(Arbitrary Units)
50
40
30
20
10
0
*†
1 Day
7 Days
21 Days

Figure 3-2

C


1 Day
7 Days
21 Days
Ac-PGC1α
PGC1α
Ponseau
PGC1α/Ponceau
(Arbitrary Units)
30
20
10
0
1 Day
7 Days
21 Days

D

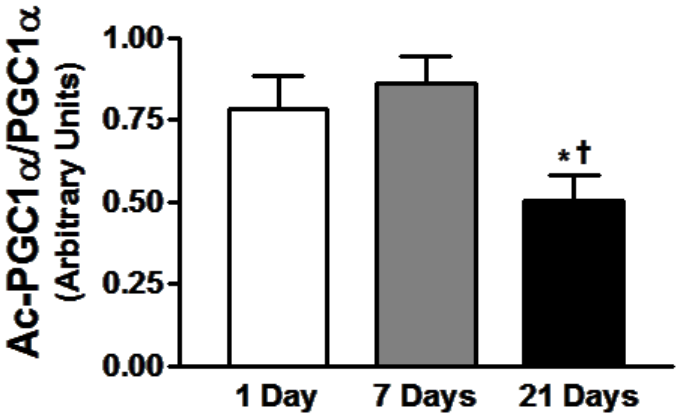

Ac-PGC1α/PGC1α
(Arbitrary Units)
1.00
0.75
0.50
0.25
0.00
*†
1 Day
7 Days
21 Days

Figure 3-2

E

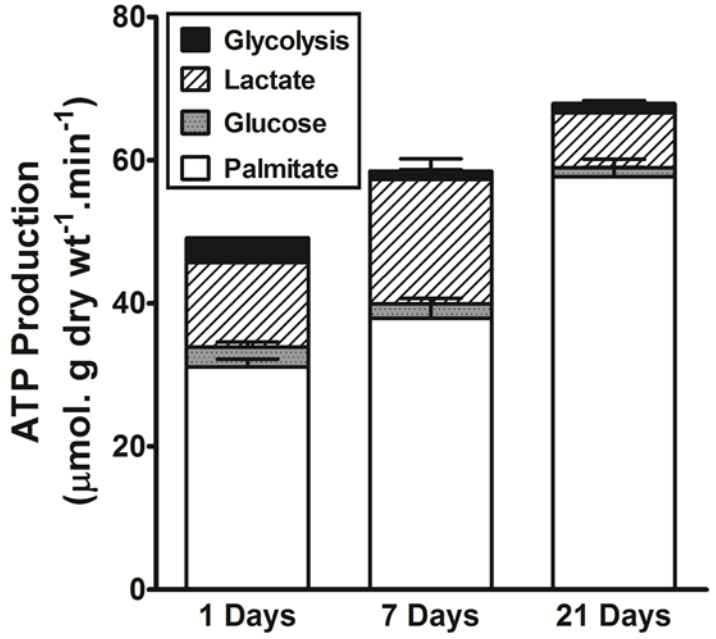
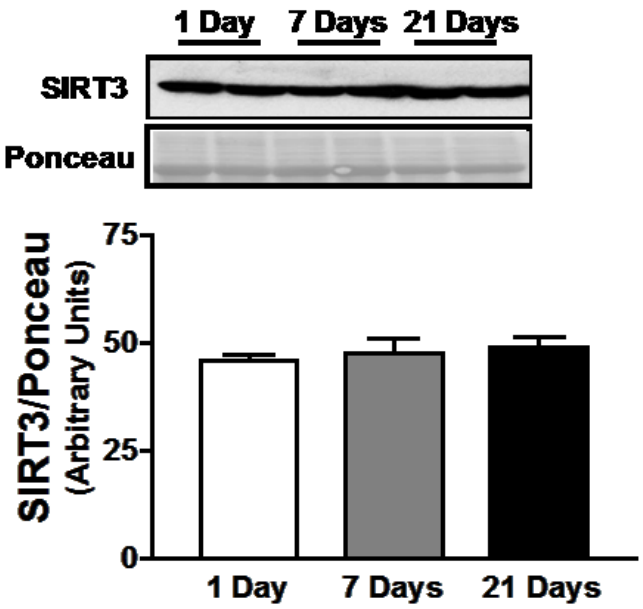

Glycolysis
Lactate
Glucose
Palmitate
ATP Production
(μmol. g dry wt-1.min-1)
80
60
40
20
0
1 Days
7 Days
21 Days

Figure 3-2

Figure 3-3: Protein expression, acetylation, and activity of β-HAD in hearts from 1-, 7-, and 21-day old rabbits.

Protein expressions of SIRT3 **(A)**, GCN5L1 **(B)**, and β-HAD **(C)**. **D)** Level of β-HAD acetylation. Total lysate from 1-, 7-, and 21-day old perfused rabbit's hearts were immunoprecipitated with anti-acetyl-K antibodies and then immunoblotted with antibodies specific for β-HAD. **E)** β-HAD enzyme activity (n=6). Values represent mean±SEM; (n=4). *Significant difference ($P<0.05$) vs. 1-day old. †significant difference vs. 7-day old.

A


1 Day
7 Days
21 Days
SIRT3
Ponceau
SIRT3/Ponceau
(Arbitrary Units)
75
50
25
0
1 Day
7 Days
21 Days

B

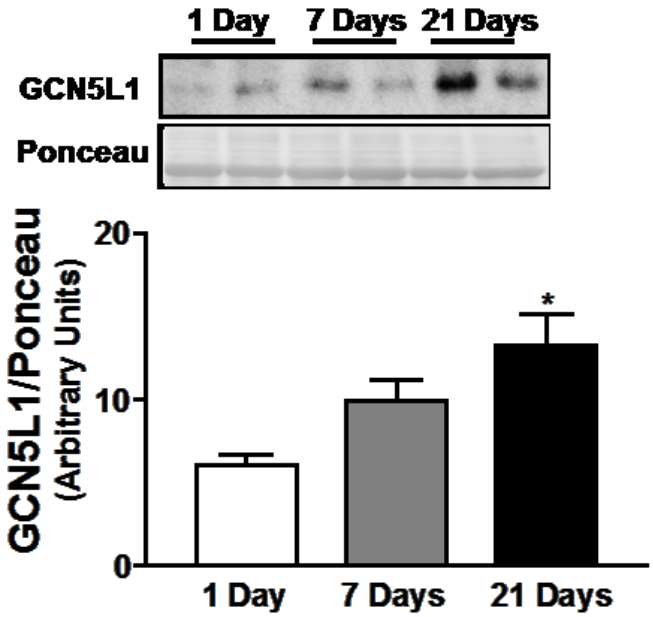
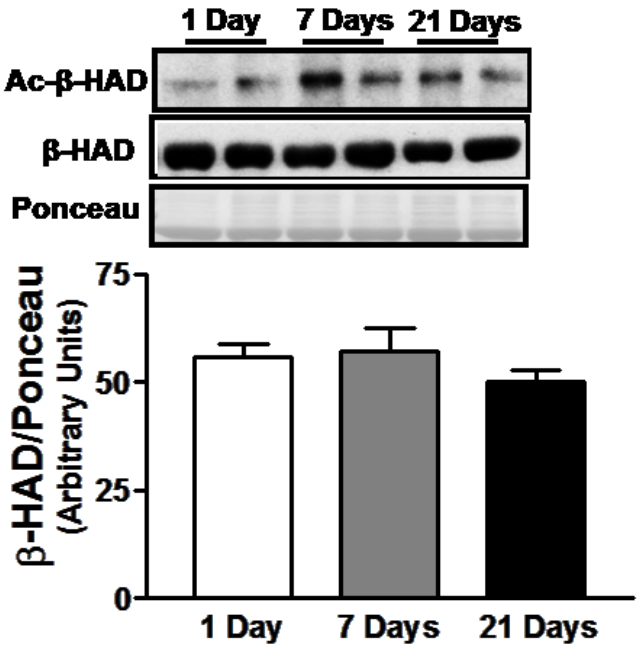

1 Day
7 Days
21 Days
GCN5L1
Ponceau
GCN5L1/Ponceau
(Arbitrary Units)
20
10
0
*
1 Day
7 Days
21 Days

Figure 3-3

C


1 Day
7 Days
21 Days
Ac-β-HAD
β-HAD
Ponceau
β-HAD/Ponceau
(Arbitrary Units)
75
50
25
0
1 Day
7 Days
21 Days

D

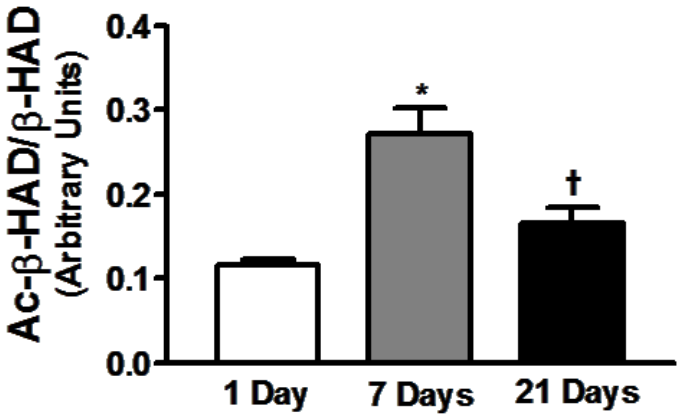

Ac-β-HAD/β-HAD
(Arbitrary Units)
0.4
0.3
0.2
0.1
0.0
*
†
1 Day
7 Days
21 Days

Figure 3-3

E

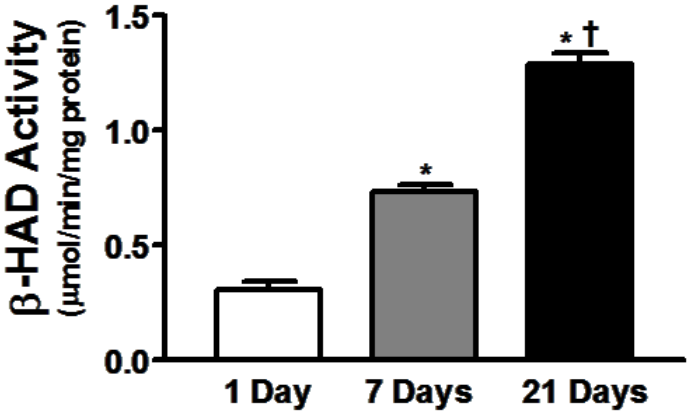
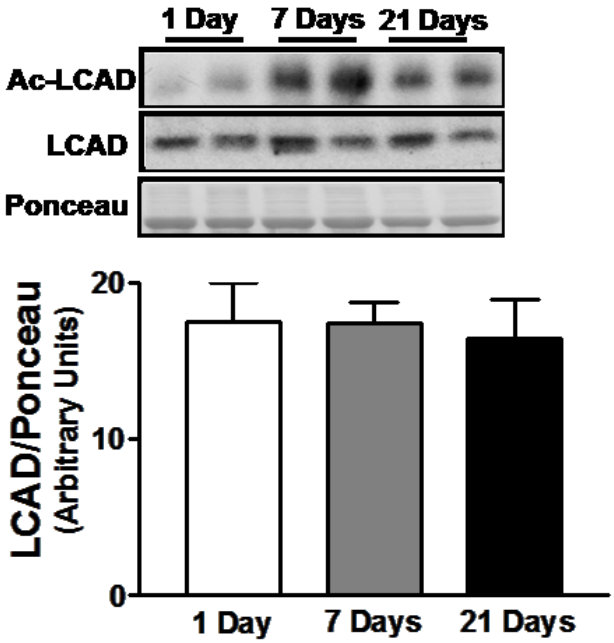

β-HAD Activity
(µmol/min/mg protein)
1.5
1.0
0.5
0.0
*
* †
1 Day
7 Days
21 Days

Figure 3-3

Figure 3-4: Levels of cardiac LCAD acetylation and activity during maturation.

A) LCAD expression. **B)** Level of LCAD acetylation. Total lysate from 1-, 7-, and 21-day old perfused rabbit's hearts were immunoprecipitated with anti-acetyl-K antibodies and then immunoblotted with antibodies specific for LCAD. **C)** LCAD activity (n=6). Values represent mean±SEM; (n=4). *Significant difference ($P<0.05$) vs. 1-day old. †significant difference vs. 7-day old.

A


1 Day
7 Days
21 Days
Ac-LCAD
LCAD
Ponceau
LCAD/Ponceau
(Arbitrary Units)
20
10
0
1 Day
7 Days
21 Days

B

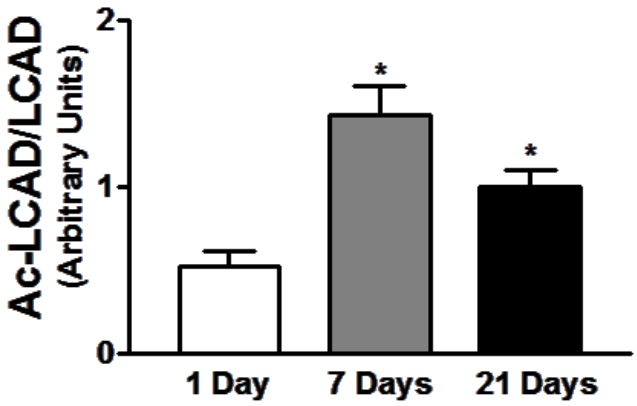

Ac-LCAD/LCAD
(Arbitrary Units)
2
1
0
*
*
1 Day
7 Days
21 Days

Figure 3-4

C

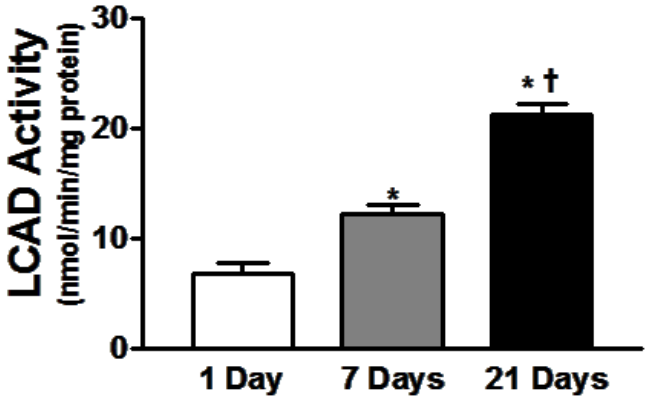
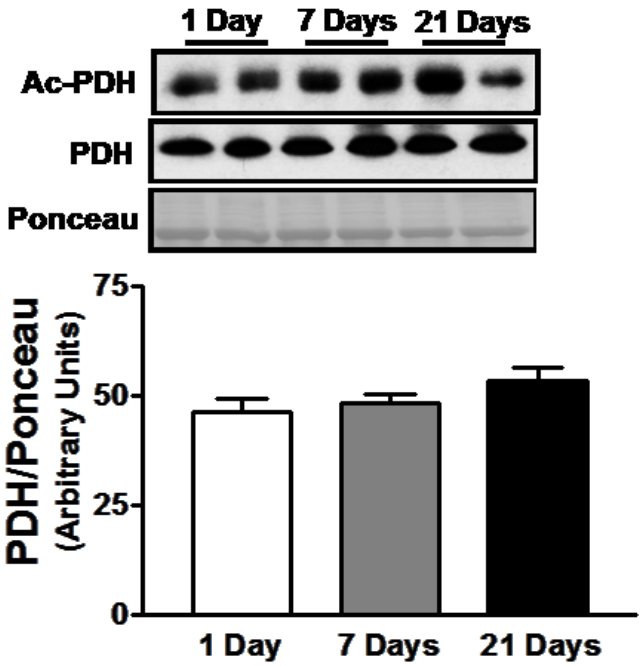

LCAD Activity
(nmol/min/mg protein)
30
20
10
0
*
* †
1 Day
7 Days
21 Days

Figure 3-4

Figure 3-5: PDH acetylation levels in hearts from 1-, 7-, and 21-day old rabbits.

A) PDH expression. **B)** Level of PDH acetylation. Total lysate from 1-, 7-, and 21-day old perfused rabbit's hearts were immunoprecipitated with anti-acetyl-K antibodies and then immunoblotted with antibodies specific for PDH. Values represent mean±SEM; (n=4).

A


1 Day
7 Days
21 Days
Ac-PDH
PDH
Ponceau
PDH/Ponceau
(Arbitrary Units)
75
50
25
0
1 Day
7 Days
21 Days

B

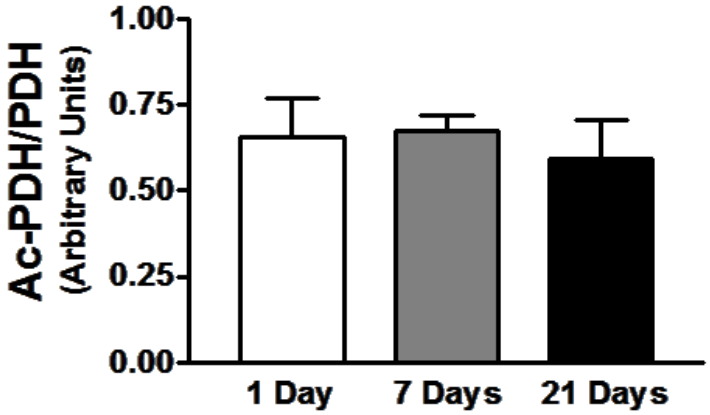
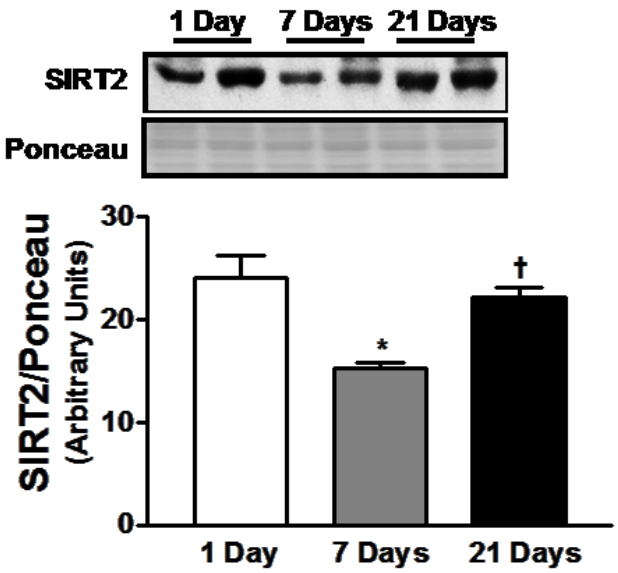

Ac-PDH/PDH
(Arbitrary Units)
1.00
0.75
0.50
0.25
0.00
1 Day
7 Days
21 Days

Figure 3-5

Figure 3-6: Acetylation levels of the glycolytic enzyme HK in hearts from 1-, 7-, and 21-day old rabbits.

Protein expressions of SIRT2 **(A)**, and HK **(B)**. **C)** Level of HK acetylation. Total lysate from 1-, 7-, and 21-day old perfused rabbit's hearts were immunoprecipitated with anti-acetyl-K antibodies and then immunoblotted with antibodies specific for HK. Values represent mean±SEM; (n=4). *Significant difference ($P<0.05$) vs. 1-day old. †significant difference vs. 7-day old.

A


1 Day
7 Days
21 Days
SIRT2
Ponceau
SIRT2/Ponceau
(Arbitrary Units)
30
20
10
0
*
†
1 Day
7 Days
21 Days

B

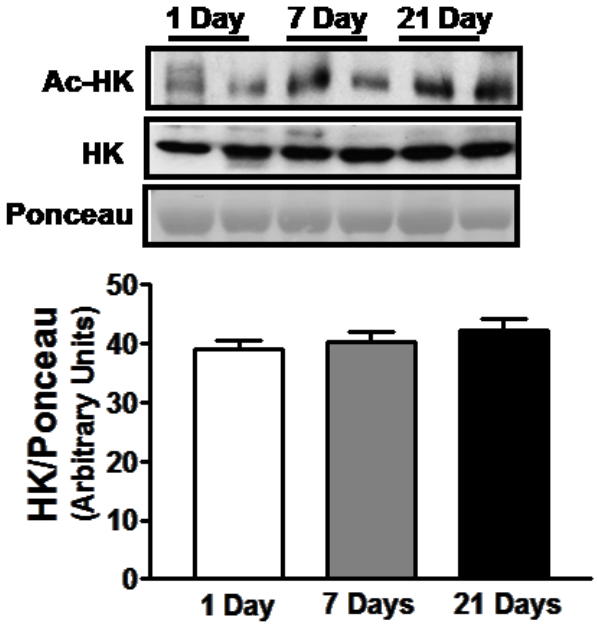

1 Day
7 Day
21 Day
Ac-HK
HK
Ponceau
HK/Ponceau
(Arbitrary Units)
50
40
30
20
10
0
1 Day
7 Days
21 Days

Figure 3-6

C

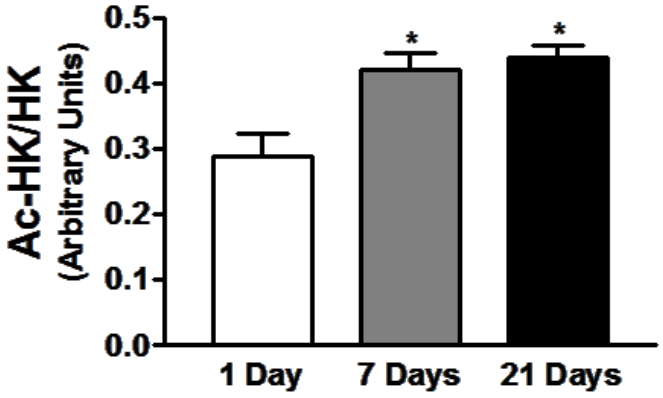
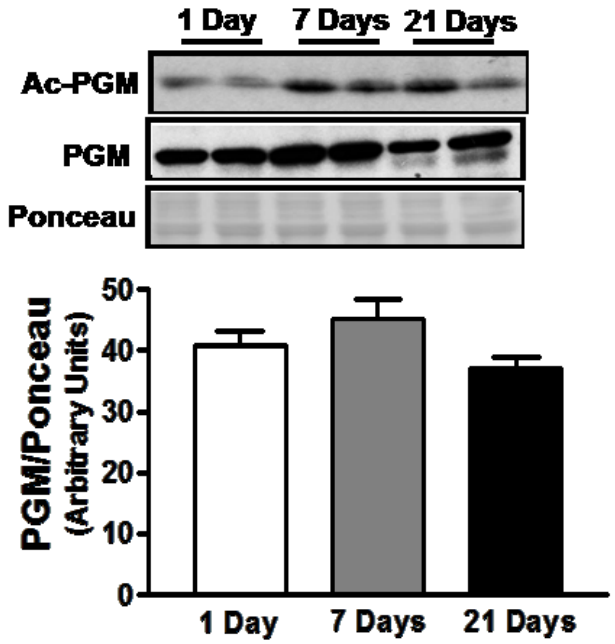


Figure 3-6

Figure 3-7: Acetylation levels of the glycolytic enzyme PGM in hearts from 1-, 7-, and 21-day old rabbits.

A) PGM expression. **B)** Level of PGM acetylation. Total lysate from 1-, 7-, and 21-day old perfused rabbit's hearts were immunoprecipitated with anti-acetyl-K antibodies and then immunoblotted with antibodies specific for PGM.. **C)** HIF-1α expression. Values represent mean±SEM; (n=4). *Significant difference ($P<0.05$) vs. 1-day old. †significant difference vs. 7-day old.

A


1 Day
7 Days
21 Days
Ac-PGM
PGM
Ponceau
PGM/Ponceau
(Arbitrary Units)
50
40
30
20
10
0
1 Day
7 Days
21 Days

B

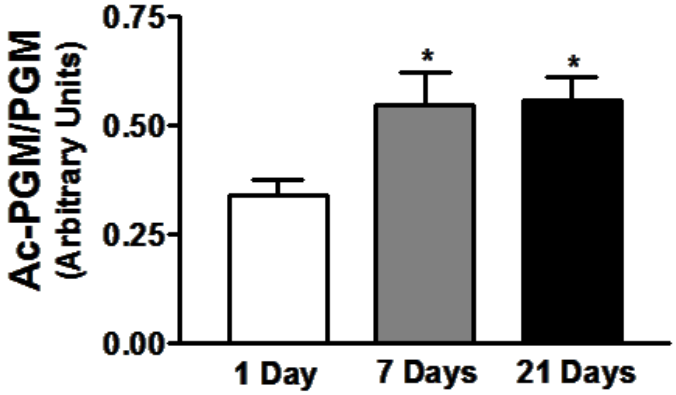

Ac-PGM/PGM
(Arbitrary Units)
0.75
0.50
0.25
0.00
*
*
1 Day
7 Days
21 Days

Figure 3-7

C

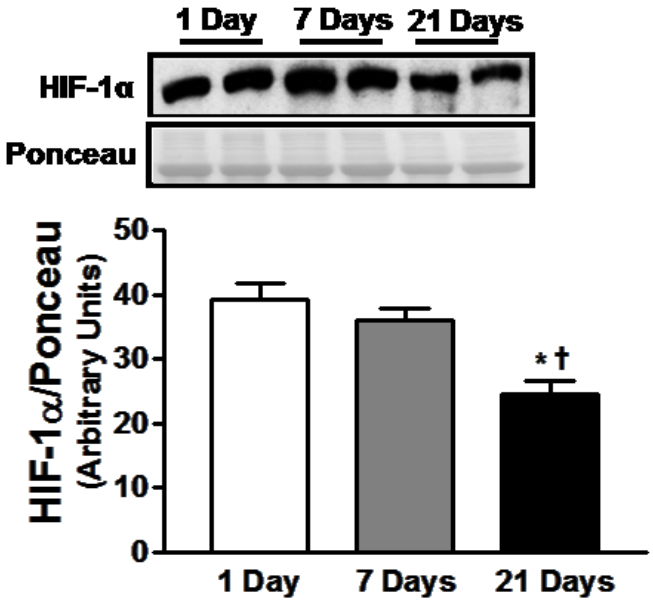

1 Day
7 Days
21 Days
HIF-1α
Ponceau
HIF-1α/Ponceau
(Arbitrary Units)
50
40
30
20
10
0
*†
1 Day
7 Days
21 Days

Figure 3-7

3.5 DISCUSSION

This study investigated the effects of acetylation on activity of enzymes involved in glycolysis, glucose, and fatty acid β-oxidation during maturation of newborn rabbit's heart. Our study provides a number of novel observations regarding age-dependent changes in acetylation. First, there was an age-dependent increase in the overall acetylation of cardiac proteins along with an increase in GCN5L1 and no change in SIRT3 protein expression. Second, acetylation of PGC-1α was significantly decreased with maturation, concomitant with decreased SIRT6 and increased SIRT1 expression. Third, a significant increase in fatty acid β-oxidation during maturation was accompanied by an increased acetylation and activity of the fatty acid β-oxidation enzymes, β-HAD and LCAD. Finally, acetylation of the glycolytic enzymes HK and PGM were increased; scenarios consistent with the decrease in glycolytic activity seen in 21-day vs. 1-, 7-day old hearts. Collectively, these data suggest an important role of lysine acetylation in regulating cardiac energy metabolism during maturation.

Examination of PGC-1α acetylation revealed an age-dependent decrease in acetylation following birth. Previous studies showed that PGC-1α acetylation by the nuclear acetyltransferase GCN5 inhibits its activity (178,190). SIRT1 can deacetylate and activate PGC-1α (33-35). We showed that increased SIRT1 expression 3 weeks post-birth leads to a

robust decrease in PGC-1α acetylation and enhanced mitochondrial oxidative capacity and ATP production. SIRT6 binds to and activates GCN5, a nuclear acetyltransferase that acetylates and inhibits PGC-1α activity (179). Interestingly, cardiac protein level of SIRT6 was significantly decreased in 21-day compared to 1-, and 7-day old rabbits. The decrease in SIRT6 can lead to inhibition of GCN5 and hence decreased PGC-1α acetylation in 21-day old hearts. In acute inflammatory response, SIRT1 and SIRT6 coordinate the switch from glucose to fatty acid β-oxidation (239). Results from these studies support our findings that increased SIRT1 and decreased SIRT6 coordinate PGC-1α acetylation to improve mitochondrial oxidative capacity during maturation (174,176,178,179,190,239).

Fatty acid β-oxidation rates were significantly increased during maturation. There was an age-dependent increase in β-HAD and LCAD acetylation levels. Since hearts from 7-, 21- day have a greater reliance on fatty acid β-oxidation as a source of energy, our results suggest a stimulatory effect of acetylation on β-HAD and LCAD activity. These, effects can be mediated by the upregulation of mitochondrial acetyltransferase GCN5L1. Our findings are in agreement with the study by Zhao *et al* where they showed that lysine acetylation activates fatty acid β-oxidation in muscle cells (159). In liver, enhanced fatty acid β-oxidation increases mitochondrial and peroxisomal protein acetylation (210). The increases in fatty acid β-oxidation during maturation enhanced overall

cardiac protein acetylation. SIRT3 is identified as a major deacetylase and a key regulator of mitochondrial oxidative metabolism (191,192,199,200). The stable expression of SIRT3 during maturation suggested that SIRT3 is pivotal for cardiac development and function. PDH activity can be regulated by covalent modifications, such as phosphorylation and acetylation as identified recently (201,202,234). Hyperacetylation of PDH inhibits enzyme activity in skeletal muscles and human cancer cells (201,202). We demonstrated that neither PDH protein levels, nor PDH acetylation were altered during maturation. Our results suggest that mechanisms other than acetylation primarily regulate PDH activity during cardiac maturation. The increase in fatty acid β-oxidation can predominate and inhibit glucose oxidation during maturation (90). Moreover, increased PGC-1α activity upregulates pyruvate dehydrogenase kinase 4 resulting in hyper-phosphorylation and inactivation of PDH during maturation (79,240).

Lactate is an important fuel during maturation, as fetal hearts can readily extract and oxidize lactate (56). We found that cardiac lactate oxidation was high till 7-day post-birth, however, 3 weeks later rates of lactate oxidation significantly dropped. As circulating lactate levels are very high in the fetus (241), lactate oxidation accounts for the majority of myocardial oxygen consumption (9,241). Following birth, blood levels of lactate decreases significantly, and the contribution of lactate oxidation to ATP production in the neonatal period markedly decreases (8,9,242).

SIRT2 is a cytoplasmic deacetylase and a key regulator of heart necrosis and metabolism (154-156). SIRT2 is a critical regulator for Akt and GSK3β activation in mouse and human embryonic cells (145,167). We showed that SIRT2 protein level was initially decreased 7 days post-birth, and then increased 3 weeks post-birth to baseline. A number of glycolytic enzymes are acetylated, and inhibition of glycolysis is associated with a reduction in SIRT1 expression (172). In contrast, a recent study suggested that SIRT1 activation inhibits the activity of the glycolytic enzyme phosphoglycerate mutase-1, suggesting that acetylation actually stimulates glycolysis (161). We showed that glycolysis rates markedly declined with maturation along with an increase in acetylation of the glycolytic enzymes; HK and PGM. Our data suggest that acetylation of the glycolytic enzymes HK and PGM inhibit glycolysis. Acetylation of the glycolytic enzyme pyruvate kinase at lysine 305 inhibits its activity in muscle (163). At the transcriptional level, Hypoxia inducible factor alpha (HIF-1α) is a transcription factor that upregulates the expression of genes involved in anaerobic glycolysis (80). It was shown previously that HIF-1α level of expression is significantly decreased post-birth (54). Indeed, cardiac protein level of HIF-1α was significantly decreased 3 weeks post-birth. Our data suggest that both, the decrease in HIF-1α expression and increased glycolytic enzymes acetylation could account for the age-dependent decrease in glycolysis.

In conclusion, we demonstrate that decreased acetylation of PGC-1α, and increased acetylation of β-HAD, LCAD, HK, and PGM may account for the dramatic shift in energy metabolism from glycolysis to fatty acid β-oxidation seen during maturation. Our results suggest that lysine acetylation enhances cardiac fatty acid β-oxidation and inhibits glycolysis during maturation.