Effects of neonatal dexamethasone administration on cardiac recovery ability under ischemia-reperfusion in 24-wk-old rats

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BACKGROUND: Evaluations of stress-induced cardiac functional alterations in adults after neonatal glucocorticoid (GC) treatment have been limited. In the present study, we evaluated adult cardiac functional recovery during postischemic reperfusion and measured cardiac gene expression involved energy metabolism in rats neonatally treated with dexamethasone (DEX).

METHOD: Male Wistar rats were injected DEX in first 3 d after birth and controls were received saline (SAL). At 24 wk of age, insulin tolerance tests were performed, plasma lipid levels were measured, and left ventricular function and myocardial infarct size were evaluated. Expressions of genes involved in cardiac energy metabolism were measured by quantitative real-time polymerase chain reaction (PCR) and western blot.

RESULTS: In 24-wk-old rats, neonatal DEX administration caused dyslipidemia, impaired cardiac recovery function and increased size of infarction, decreased cardiac expression of glucose transporter 4 (GLUT4), peroxisome proliferative-activated receptor gamma coactivator 1 α (PGC-1 α) and ratios of phospho-forkhead box O1/forkhead box O1 (p-FoxO1/FoxO1) and phospho AMP-activated protein kinase/AMP-activated protein kinase (p-AMPK/AMPK) but increased pyruvate dehydrogenase kinase isoenzyme 4 (PDK4) expression compared with controls.

CONCLUSION: Neonatal DEX administration impairs cardiac functional recovery during reperfusion following ischemia in 24-wk-old rats. Reduced cardiac glucose utilization may contribute to the long-term detrimental effects caused by neonatal DEX treatment.

Glucocorticoids (GCs) exhibit anti-inflammatory effects and have been widely used in western countries to treat chronic lung disease in premature infants since the 1970s. In spite of their short-term benefits, concerns have emerged regarding their long-term effects, including growth retardation (1), neurologic defect (2), increased blood pressure, and renal damage (1).

Extensive data indicate that cardiovascular diseases can be “programmed” by early life factors. Previous studies in

rats have demonstrated that neonatal dexamethasone (DEX) administration may cause cardiac dilation and depressed cardiac systolic function at 4 wk of age (5), and cardiac dysfunction at about 14 wk of age (3), suggesting that neonatal GC administration may have long-term negative effects on cardiac function. Functional evaluation of the heart of adult animals neonatally treated with GCs has been performed by Niu *et al.* (3) previously but data are still limited.

The heart requires a continuous energy supply to maintain its ability to pump. Mammalian hearts derive about 70% of their energy from fatty acid oxidation and about 30% from glucose after birth. Disruptions in energy supply and alterations in energy substrate utilization have been found correlated with cardiac pathophysiological conditions, which combined with impaired cardiac functionality, increased susceptibility to ischemia-reperfusion (I/R) injury and impaired functional recovery following I/R (6). Mitochondria are the site of energy metabolism, and alterations in mitochondrial architecture and mitochondrial dysfunction have been linked to several heart disorders (7). Previous data also indicate that cardiac mitochondrial disorders may be “programmed” by early life factors such as intrauterine growth restriction or maternal overnutrition (8), predisposing cardiac abnormalities in later life. The effects of neonatal GC administration on cardiac energy metabolism have been studied in rats from 2 to 14 d of age (9), but little research has been undertaken regarding the effects of this therapeutic strategy on cardiac energy metabolism in adult rats.

Therefore, we hypothesize that neonatal GC administration impairs cardiac function in adults by disturbing cardiac energy metabolism.

RESULTS

The Effects of Neonatal DEX Administration on Body Weight, Heart Weight, and Serum Levels of Glucose and Lipids

In the present study, we observed that neonatal DEX administration caused growth retardation in rats (Figure 1). Significant reductions in body weight were noted before 14 wk of age in the group treated with DEX compared with age-matched SAL-treated controls (DEX vs. SAL: $P < 0.05$). The body weights of

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Figure 1. Effects of neonatal DEX treatment on body weight. Neonatal DEX treatment induces growth retardation. SAL: white bar; DEX: black bar. $n = 20$ per group. Values are means $\pm$ SD. * $P < 0.05$ vs. control. DEX, dexamethasone; SAL, saline.

Table 1. The basic characteristics of 24-wk-old rats

	SAL	DEX
Body weight (g)	420.2 $\pm$ 56.8	399.1 $\pm$ 52.4
Heart weight (g)	1.46 $\pm$ 0.17	1.43 $\pm$ 0.17
Heart weight/body weight (%)	0.35 $\pm$ 0.04	0.36 $\pm$ 0.04
Cholesterol (mmol/l)	1.85 $\pm$ 0.33	2.36 $\pm$ 0.44*
Triglyceride (mmol/l)	1.96 $\pm$ 0.40	2.30 $\pm$ 0.80
NEFA (mmol/l)	0.50 $\pm$ 0.07	0.76 $\pm$ 0.20*
Fasted blood glucose (mmol/l)	5.29 $\pm$ 0.25	5.03 $\pm$ 0.47

Values are expressed as the means $\pm$ SD ($n = 6$ per treatment group). Student's *T*-test was used to check the differences between two groups.

* $P < 0.05$ DEX vs. age-matched SAL-treated controls.

DEX, dexamethasone; NEFA, nonesterified fatty acids; SAL, saline.

the DEX-treated animals appeared to be lower than those of the controls after 14 wk but this difference was not significant.

In the present experiment, there were no statistical differences found in body weight, total and relative heart weight at 24 wk of age between saline (SAL) and DEX-treated rats (Table 1, DEX vs. SAL: all $P > 0.05$), indicating neonatal DEX treatment could not cause permanent growth retardation and apparent cardiac hypertrophy.

Neonatal DEX administration caused significant increases in serum total cholesterol and nonesterified fatty acid levels in 24-wk-old rats compared with the control group (Table 1, DEX vs. SAL: $P < 0.05$) but did not affect the levels of fasting blood glucose or serum triglycerides (Table 1, DEX vs. SAL: $P > 0.05$).

The Effects of Neonatal DEX Administration on Insulin Testing in 24-wk-Old Rats

Insulin tolerance testing was performed to check insulin resistance. With the exception of higher glucose levels noted at 1 h following insulin injections (Figure 2; DEX vs. SAL: $P < 0.05$), neonatal DEX treatment did not result in altered glucose levels at either 30 min or 2 h compared with SAL-treated controls.

Figure 2. The effects of neonatal DEX treatment on glucose levels during the insulin tolerance test. At 24 wk of age, rats were fasted overnight and intraperitoneally injected with regular insulin (1 IU/kg). Blood glucose levels were measured before and 30, 60, and 120 min after insulin injection. Neonatal DEX treatment caused significantly higher blood glucose levels at 60 min following insulin injection. $n = 6$ per group. Values are means $\pm$ SD. Student's *T*-test was used to check the differences between two groups at the same time point. * $P < 0.05$ DEX vs. SAL. SAL: open symbols; DEX: closed symbols. DEX, dexamethasone; SAL, saline.

The Effects of Neonatal DEX Administration on Ventricular Function and Cardiac Infarct Size in 24-wk-Old Rats

Compared with SAL-treated animals, the hearts of neonatal DEX-treated rats exhibited impaired recovery following ischemia. Basal Left ventricular developing pressure (LVDP), maximum rate of rise of left ventricular developed pressure ($+(LVdP/dt)_{max}$), maximum rate of decline of left ventricular developed pressure ($-(LVdP/dt)_{max}$) and left ventricular end diastolic pressure (LVEDP) values were not significantly different between SAL- and DEX-treated rats (Figure 3). During I/R, LVDP, $+(LVdP/dt)_{max}$ and $-(LVdP/dt)_{max}$ values were decreased (Figure 3a,c,d), whereas LVEDP was increased in both groups (Figure 3b), suggesting that left ventricular function was decreased. However, the changes in left ventricular function were significantly aggravated in the DEX group compared with the control group. Recovery of LVDP, $+(LVdP/dt)_{max}$, $-(LVdP/dt)_{max}$ and LVEDP following I/R was less successful in DEX-treated rats than in SAL-treated rats (Figure 3) (DEX vs. SAL: $P < 0.05$ or $P < 0.01$), suggesting greater functional recovery impairment in DEX-treated rats. Each of these findings suggests that DEX treatment has detrimental long-term effects on the heart. Infarct sizes induced by I/R were found larger in DEX-treated animals when compared with age-matched controls (Figure 4a,b).

Neonatal DEX Treatment Causes Mitochondrial Destruction in the Cardiomyocytes of 24-wk-Old Rats

Ultrastructural analyses of cardiomyocytes via transmission electron microscope (TEM) revealed that neonatal DEX treatment caused mitochondrial structural abnormalities at 24 wk of age. These abnormalities were characterized by marked changes in the architecture of the cristae, including fragmented and tubular cristae, as well as the loss of cristae (Figure 5).

Figure 3. Effects of neonatal DEX administration on cardiac function in isolated rat hearts subjected to 30 min of global ischemia followed by 60 min of reperfusion. (a) LVDP; (b) LVEDP; (c) $+(LVdp/dt)_{max}$; (d) $-(LVdp/dt)_{max}$; $n = 8$ per group. Values are means $\pm$ SD. Student's *T*-test was used to check the differences between two groups at same time point. * $P < 0.05$ DEX vs. SAL group. SAL: open symbols; DEX: closed symbols. DEX, dexamethasone; SAL, saline.

Figure 4. The effects of neonatal DEX treatment on infarct sizes of isolated hearts following I/R in the SAL and DEX groups. (a) Representative 2,3,5-triphenyl-tetrazolium chloride staining of different groups of hearts following I/R. (b) Relative sizes of cardiac infarcts in SAL- and DEX-treated rats following I/R. $n = 11$ per group. All data are expressed as the means $\pm$ SD. Student's *T*-test was used to check the differences between two groups. * $P < 0.05$ DEX vs. SAL-treated control. SAL: white bar; DEX: black bar. DEX, dexamethasone; SAL, saline.

Figure 5. Ultrastructural alterations of cardiomyocytes observed by TEM in 24-wk-old rats after neonatal DEX treatment. Neonatal DEX administration caused defects in the architecture of mitochondria. Arrow: mitochondria with loss of cristae. Magnification: $\times 15,000$; $n = 3$ per group. Scale bar: 667 nm. DEX, dexamethasone; TEM, transmission electron microscopy.

The Effects of Neonatal DEX Administration on Relative Gene Expression Levels in the Heart

Neonatal DEX administration significantly increased cardiac *pyruvate dehydrogenase kinase isoenzyme 4* (*pdk4*) but decreased *glucose transporter4* (*glut4*) gene expression at both mRNA (Figure 6; DEX vs. SAL: $P < 0.05$) and protein levels (Figure 7; DEX vs. SAL: $P < 0.05$). Increased cardiac *carnitine palmitoyltransferase 1b* (*cpt1b*) gene expression at the mRNA level was observed in DEX-treated rats (Figure 6), but no apparent alterations in CPT1B protein content were noted between the two groups (Figure 7; DEX vs. SAL: $P > 0.05$).

Compared with age-matched controls, DEX treatment resulted in slightly higher cardiac forkhead box O1 (FoxO1) expression at both mRNA (Figure 6; DEX vs. SAL: $P < 0.05$) and protein levels (Figure 7; DEX vs. SAL: $P < 0.05$), but no differences were noted in phospho-FoxO1 (p-FoxO1) levels between the two groups (Figure 7a), resulting in significantly reduced ratios of p-FoxO1/FoxO1 (Figure 7b; DEX vs. SAL: $P < 0.05$).

Figure 6. Effects of neonatal DEX treatment on relative expression of cardiac genes involved in energy metabolism at 24 wk of age at mRNA level. $n = 6$ per group. Gene expression levels were calculated by $2^{-\Delta\Delta C_t}$ method and standardized against *gapdh* mRNA. Student's *T*-test was used to check the differences between two groups. * $P < 0.05$ DEX vs. SAL-treated control. SAL: white bar; DEX: black bar. DEX, dexamethasone; SAL, saline.

Neonatal DEX administration also caused significant reductions in cardiac peroxisome proliferative activated receptor, gamma, coactivator 1α (PGC-1α) expression at both mRNA (Figure 6; DEX vs. SAL: $P < 0.05$) and protein levels (Figure 7; DEX vs. SAL: $P < 0.05$) compared with SAL-treated controls.

Neonatal DEX treatment had no effects on cardiac AMP-activated protein kinase (*ampk*) gene expression at either the mRNA (Figure 6) or protein levels compared with SAL-treated rats (Figure 7; DEX vs. SAL: $P > 0.05$). Significantly, reductions in cardiac phospho-AMPK (p-AMPK) protein content were noted in DEX-treated rats (Figure 7; DEX vs. SAL: $P < 0.05$) and these reductions resulted in significantly lower cardiac ratios of p-AMPK/AMPK (Figure 7b; DEX vs. SAL: $P < 0.05$).

DISCUSSION

Synthetic glucocorticoid DEX has been used to treat chronic lung disease in preterm infants with respiratory distress syndrome since the 1970s, and great concern has emerged regarding its long-term negative effects (10). Growth retardation is one of the consequences of early-life DEX exposure and has been observed both clinically and experimentally (1,11). Our results indicated that DEX-treated rats had significantly lower body weights than SAL-treated rats up to 14 wk of age, and no reduced body weight mentioned afterwards, indicating neonatal DEX administration may cause transient growth retardation, and there might exit a catch-up body weight gain in adult life. Previous studies described lower heart weights in rats aged 50 wk following neonatal DEX administration (12). However, in the present experiment, no alterations in relative

Figure 7. Effects of neonatal DEX administration on cardiac gene expression involved in energy metabolism at 24 wk of age at protein level. (a) Western blot images of the expression of cardiac genes involved in energy metabolism in 24-wk-old rats. (b) Neonatal DEX administration caused significant reductions in cardiac GLUT4 levels but increased PDK4 protein levels. No significant changes were noted in CPT1B protein levels in the hearts of DEX-treated animals compared with SAL-treated controls. Neonatal DEX treatment resulted in increased cardiac FoxO1 protein levels but decreased ratios of p-FoxO1/FoxO1 and decreased cardiac PGC-1α protein levels. Neonatal DEX administration caused no changes in cardiac AMPK protein levels but significantly decreased p-AMPK content and the ratios of p-AMPK/AMPK compared to the control group. $n = 4$ per group. Relative densitometric values are presented as the means $\pm$ SD. Student's *T*-test was used to check the differences between two groups. * $P < 0.05$ DEX vs. SAL-treated control. SAL: white bar; DEX: black bar. DEX, dexamethasone; SAL, saline.

heart weight were observed following neonatal DEX treatment at 24 wk of age, indicating there might no apparent cardiac hypertrophy or relative cardiac growth retardation were noted in this age group.

Metabolic disorders are one of the long-term negative consequences of early GC treatment. Prenatal GC administration may cause metabolic disorders such as insulin resistance (13) and diabetes (14), in the offspring. However, data regarding the effects of neonatal GC treatment on glucose levels, lipid levels, and insulin sensitivity in adult animal models are rare. In this study, we observed that neonatal DEX treatment caused significantly higher serum nonesterified fatty acid and total cholesterol levels in 24-wk-old rats, suggesting that neonatal GC treatment may cause dyslipidemia in adult. However, no hyperglycemia was observed among 24-wk-old DEX-treated rats in the present study. In combination with similar blood glucose level observations in a previous study of the same animal model at 50 wk of age (1), these results suggest that neonatal DEX administration may not cause alterations in fasting blood glucose levels during adulthood.

Insulin tolerance tests performed in 24-wk-old rats revealed higher blood glucose levels at 1 h after insulin injection in DEX-treated animals, indicating that insulin resistance may occur at this age. Adigun *et al.* (15) reported that neonatal DEX treatment might increase hepatic levels of adenylyl cyclase in rats at 75 d of age. Adenylyl cyclase synthesizes cyclic AMP from ATP and functions as a second messenger controlling hepatic gluconeogenesis and glycogenolysis. Therefore, hepatic insulin resistance may occur in this model, but further study is warranted.

Recent studies have indicated that cardiac susceptibility to I/R injury in adults can be “programmed” by unfavorable maternal factors such as low protein diet (16) and maternal obesity (17). Studies evaluating cardiac function during adulthood after neonatal GC administration are quite limited. Human follow-up studies in school-age children have not found any cardiac functional abnormalities (18), but the long-term effects appear to be noteworthy (19). In previous studies, reduced systolic function was noted in 4-wk-old rats (20) and impaired cardiac function was noted in adult rats (3) after neonatal DEX treatment. In the present study, we found that although the function of hearts isolated from DEX-treated rats and SAL-treated rats was comparable before I/R, the cardiac recovery ability during I/R was drastically impaired in the DEX-treated group. Therefore, although neonatal DEX administration does not cause significant alterations in cardiac performance under “normal” physiological conditions at 24 wk of age, it may increase cardiac vulnerability to I/R injury and reduce cardiac function under stress. Previous study performed by Niu *et al.* (3) indicated that neonatal DEX administration caused reduced cardiac output response to afterload in adult rats. Combined with the results from this study, there might be a big possibility that neonatal DEX administration may impair cardiac working potential under stress such as overload and I/R.

The beating heart requires a continuous energy supply to maintain its contractile function, and unfavorable energy

substrate utilization correlates with the development of cardiomyopathy (21). In the present study, neonatal DEX administration decreased cardiac GLUT4 expression but increased PDK4 expression at the protein level, suggesting reduced cardiac glucose uptake and oxidation. Diabetic hearts are characterized by reduced glucose oxidation and increased fatty acid utilization and are more prone to I/R injury (6). Post-I/R cardiac functional recovery may be improved by partially inhibiting fatty acid oxidation or by stimulating glucose oxidation (22,23). Therefore, decreased cardiac glucose oxidation caused by neonatal DEX treatment may contribute to reduced cardiac post-I/R functional recovery in 24-wk-old rats.

FoxO1 is a transcription factor involved in many cellular processes via the targeting of downstream genes such as PDK4 (24). Phosphorylated FoxO1 can export from the nucleus and loses its transcriptional activity. Elevated cardiac FoxO1 levels may exert protective effects in some cardiomyopathies (25) but appear to be detrimental in diabetic hearts due to blunted cardiac insulin sensitivity and increased PDK4 gene expression (26). In the present study, we observed that neonatal DEX administration resulted in increased cardiac total FoxO1 and decreased p-FoxO1 protein content in 24-wk-old rats, indicating that elevated levels of activated FoxO1 may contribute to elevated levels of downstream PDK4 expression.

AMPK orchestrates cellular responses to a variety of stressors and regulates metabolism and organelle functions. Recent investigations have shed light on the cardioprotective role of AMPK during I/R injury and pathological hypertrophy and heart failure (27). AMPK is activated by phosphorylation of Thr172, which leads to the activation of downstream target genes such as FoxO and PGC-1 α (28) and triggering the signaling pathways. Data from the present experiment demonstrated that neonatal DEX administration did not cause alterations in AMPK gene expression lead to decreased cardiac p-AMPK/AMPK ratio in rats, which may contribute to decreases in PGC-1 α and p-FoxO1/FoxO1 protein levels.

Mitochondria are responsible for energy metabolism. Metabolic defects such as hyperlipidemia and insulin resistance may impair cardiac functional recovery post-I/R by attenuating mitochondrial biogenesis (29), enhancing oxidative stress, (30) and causing intracellular Ca(2+)-overload (31). Mitochondria may also be affected by early-life factors (32). In the present study, neonatal DEX administration caused mitochondrial architectural defects in 24-wk-old rats. PGC-1 α is an important energy regulator in the heart and may upregulate cardiac mitochondrial antioxidants such as SOD2 and Trx2 (33) and participate in mitochondriogenesis (34). Repressed PGC-1 α expression has been noted in cardiac hypertrophy, heart failure, (35,36) and the latter stages of diabetes, in which changes in mitochondrial architecture have also been noted (37). Impaired cardiac AMPK/PGC-1 α signaling caused by hypoadiponectinemia may result in reduced mitochondrial biogenesis, rendering hearts more vulnerable to ischemic injury (29). Metformin, an antidiabetes drug, may protect the heart against I/R damage by increasing the expression of phosphorylated AMPK and PGC-1 α , which are associated with

more organized and elongated mitochondria (38). Turdi *et al.* (8) observed that prenatal and postnatal high-fat diets may cause suppression of AMPK and PGC-1 α , reduced glucose uptake, and mitochondrial ultrastructural abnormalities in cardiomyocytes of adult offspring, contributing to cardiac contractility defects, which are similar to the results found in this experiment. Therefore, we assume that neonatal DEX administration may cause mitochondrial structural abnormalities by disrupting the AMPK/PGC-1 α pathway, partially contributing to impaired cardiac functional recovery following I/R.

In conclusion, neonatal DEX administration leads to dyslipidemia and impairs cardiac functional recovery during postischemic reperfusion in 24-wk-old rats. Increased cardiac PDK4 and reduced GLUT4, p-AMPK, and PGC-1 α protein levels, as well as altered cardiomyocyte mitochondrial architecture, may lead to decreased cardiac glucose uptake and oxidation and mitochondrial dysfunction, contributing to the long-term detrimental effects of neonatal DEX treatment on the heart.

METHODS

Animals

Pregnant Wistar rats (270–300 g) were obtained from the Experimental Animal Center of Hebei Province and housed individually under conventional conditions, with free access to food and water. Pups were born naturally at 21–22 d gestation. At birth (day 0), male pups were selected and randomly assigned to the treatment group or to the control group. Temperature and humidity remained constant, and the dams were allowed free access to food and water. Artificial 12-h light/12-h dark cycles were used. Rat pups in the treatment group were injected intraperitoneally with dexamethasone 21-phosphate (DEX) on days 1, 2, and 3 (0.5, 0.3, and 0.1 μ g/g body weight, respectively) after birth between 9:00 and 10:00 in the morning. The dosages of DEX used in the present study were similar to those used in immature human babies with chronic lung disease, which indicate that in most cases, DEX is given in a dosage of 0.5 mg/kg of body weight per day for 3 d, followed by a tapering course of 0.3, 0.1, and 0.05 mg/kg of body weight per day each for 3 d (39). Controls were injected with an equal volume (10 μ l/g) of sterile pyrogen-free saline (SAL). Body weights were measured once per week.

At 24 wk of age, insulin tolerance tests were performed. Rats were sacrificed under anesthesia between 9:00 AM and 12:00 PM. Blood samples were collected from fasted rats. Hearts were collected quickly for measurements of ventricular function during I/R and cardiac infarction sizes following I/R. Separated heart tissues were collected from rats for ultrastructural analysis and gene expression analysis.

The use of animals for experimentation was approved by the Ethics Committee of Hebei Medical University. All experiments were performed under anesthesia, with the exception of the insulin tolerance test, and all efforts were made to minimize animal suffering.

Blood Glucose and Lipid Measurements

Rats aged 24 wk were fasted overnight (12 h) and anesthetized using sodium pentobarbital (40 mg/kg, i.p.). Blood samples ($n = 6$ per group) were collected from the abdominal artery, and blood glucose levels were measured using the ACCU-CHEK Performa glucose meter (Roche Diagnostics, Mannheim, Germany) immediately after blood sampling. Serum was separated via centrifugation and stored at -20°C until analysis. Serum concentrations of total cholesterol, triglycerides and nonesterified fatty acids were measured using commercially available kits.

Simplified Insulin Tolerance Test

Rats aged 24 wk were fasted overnight ($n = 6$ per group) and injected intraperitoneally with regular insulin (1 IU/kg). Blood samples were taken from the tip of the tail before (0 min) and after (30, 60, and 120 min) the insulin injection and used for blood glucose analyses,

which were performed using the ACCU-CHEK Performa glucose meter.

Measurements of Ventricular Function of Isolated Rat Hearts

Rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) ($n = 8$ per group), and hearts were quickly removed and mounted on a Langendorff apparatus via the aorta for retrograde perfusion with Krebs–Henseleit (K–H) buffer at a constant pressure (10 kPa) and constant temperature (37°C). The K–H buffer (in mmol/l) was composed of the following: NaCl 118, KCl 4.7, MgSO_4 1.2, CaCl_2 2.5, KH_2PO_4 1.2, NaHCO_3 25, and glucose 11. The buffer was saturated with 95% O_2 /5% CO_2 (pH 7.4). A water-filled latex balloon-tipped catheter was placed into the left ventricle through the left atrium and adjusted to a LVDP of 3–10 mm Hg during the initial equilibration. The distal end of the catheter was connected to a PowerLab system (AD Instrument, Castle Hill, Australia) via a pressure transducer (model Gould P23Db). After 20 min of stabilization with K–H buffer, hearts were subjected to 30 min of no-flow global ischemia, followed by 60 min of reperfusion. LVDP, LVEDP, and $-(\text{LVdp}/\text{dt})_{\text{max}}$ were continuously recorded by the PowerLab system. Data were analyzed using Chart software (AD Instrument).

Determination of Myocardial Infarct Size

Isolated rat hearts were subjected to 30 min of ischemia followed by 120 min of reperfusion ($n = 11$ per group). Following reperfusion, the hearts were quickly removed and frozen at -20°C . Frozen hearts were cut into thin slices with a thickness of approximately 1.5 mm perpendicular to the septum and extended from the apex to the base. The slices were then incubated in sodium phosphate buffer containing 1% (wt/vol) 2,3,5-triphenyl-tetrazolium chloride for 10 min to visualize the unstained infarct regions. Infarcted myocardium not stained by 2,3,5-triphenyl-tetrazolium chloride appeared pale. Microphotographs were acquired with a digital camera and analyzed using an image processing system (Motic MED 6.0, Xiamen, China). The amounts of infarcted myocardium were expressed as percentages of infarct size to ventricular size.

RNA Isolation and Quantitative Real-Time PCR

Rats were anesthetized using sodium pentobarbital (40 mg/kg, i.p.), and their hearts were quickly removed ($n = 6$ per group). Tissues used for RNA isolation were rapidly excised and frozen in liquid nitrogen. RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA) method according to the manufacturer's protocol. Total RNA integrity was assessed using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE). RNA was reverse transcribed using Invitrogen 2-step RT kits and a Superscript II first strand synthesis kit (Invitrogen) according to the manufacturer's instructions.

cDNA was diluted 1:20 and used for real-time polymerase chain reaction (PCR). Platinum SYBR Green qPCR SuperMix-UDG with ROX kits (Invitrogen) were used to measure gene expression using the following specific primer sets: *pdk4* (Forward primer 5' to 3': CGACAGATTCTTTGGTTCCTTACTT, reverse primer 5' to 3': CCCCTTGGCTGGTTTGGTTAC), *cpt1b* (Forward primer 5' to 3': CCTGGGATGCGTGTAGTGTGAA, reverse primer 5' to 3': GTGACTGGTGGGAAGAGTACGTC), *foxO1* (Forward primer 5' to 3': CGCAAGACCATTTCGTCGCGC, reverse primer 5' to 3': CTTATCCTTGAAGTAGGCGAC), *glut4* (Forward primer 5' to 3': TCAAAGA TGCTGGTTGAATAGTAGAA, reverse primer 5' to 3': GATGAGAAA CGGAAGTTGGAAAG), *ampk* (Forward primer 5' to 3': TCCCGTGTTTACAGATGTAGTCG, reverse primer 5' to 3': AAGATTCGCAGTTTAGATGTTGTTG), *pgc-1 α* (Forward primer 5' to 3': GAA TGACGCGCTCTTAGC, reverse primer 5' to 3': GCTTTTG CTGTTTGACAAATG), and *glyceraldehyde-3-phosphate dehydrogenase* (*gapdh*) (Forward primer 5' to 3': GGACCAGGTTGTCTCCTGTG, reverse primer 5' to 3': ATTCGAGAGAAGGGAGGGCT), (Invitrogen). Dissociation curves were constructed following real-time PCR to ensure the detection of the desired amplicon and exclude the presence of contaminating products. All reactions were performed using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Gene expression was normalized to *gapdh* and the data were analyzed using the comparative $2^{-\Delta\Delta\text{Ct}}$ method.

Western Blot

Heart tissues ($n = 4$ per group) used for western blot analysis were rapidly excised and frozen in liquid nitrogen. Proteins were extracted in lysis buffer containing 50 mmol/l Tris-HCl (pH 8.0), and a complete protease inhibitor cocktail tablet. Fifty micrograms of total protein extract from rat heart tissue were separated by electrophoresis in a polyacrylamide/SDS gel and transferred via electroblotting onto a PVDF membrane. The membrane was blocked for 1 h in 5% (w/v) nonfat milk before incubation with appropriate dilutions of antibodies against PDK4 (1:1,500, Proteintech, Chicago, IL), CPT1B (1:1,000, Aviva Systems Biology, San Diego, CA), AMPK α 1 (1:1,000, Empitomics, Burlingame, CA), Phospho-AMPK alpha (Thr172) (1:1,000, Affinity biosciences, Cincinnati, OH), PGC-1 α (1:100, Biorbyt, Cambridge, UK), GLUT4 (1:1,000, Empitomics), FoxO1 (1:500, Empitomics), phospho-FoxO1 (S256) (1:500, Bioworld Technology, Minneapolis, MN), and glyceraldehyde-3-phosphate dehydrogenase (1:5,000, Abcam, Cambridge, MA) at 4 °C overnight, followed by incubation with the corresponding secondary antibodies (1:5,000, KPL, Gaithersburg, MD). The blots were developed using the ECL system (Immobilon Western, Millipore, Bedford, MA) and analyzed using Quantity One Software (Bio-Rad, Hercules, CA). Protein content was normalized against GAPDH levels.

Cardiomyocyte Ultrastructural Analysis via TEM

Fresh heart tissues were collected from left ventricular samples after the heart was harvested and subsequently prepared for TEM analysis according to a previously developed protocol ($n = 3$ per group). Briefly, heart tissues were fixed in 4% glutaraldehyde for 24 h. Following two washings with 0.1 M PB, the tissues were postfixed with 1% osmium tetroxide aqueous solution for 2 h and then dehydrated in acetone and embedded in Epon 812. Ultrathin sections were cut using a Leica UCT ultramicrotome, double-stained with uranyl acetate and lead citrate and observed via TEM (Hitachi H-7500, Tokyo, Japan).

Data Analysis

Data were expressed as the means $\pm$ SD. Statistical analysis was performed using Student's *t*-test. The level of significance was set at $P < 0.05$. Analyses were performed using SPSS software for Windows (SPSS, Chicago, IL).

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