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Neonatal Exendin-4 Leads to Protection from Reperfusion Injury and Reduced Rates of Oxidative Phosphorylation in the Adult Rat Heart

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Abstract

Purpose—Glucagon like peptide-1 (7-36) amide (GLP-1) is an incretin hormone with multiple salutary cardiovascular effects. A short course of the GLP-1 analogue Exendin-4 (Ex-4) in the neonatal period prevents the development of mitochondrial dysfunction and oxidative stress in a rat prone to obesity and diabetes. We sought to evaluate whether neonatal Ex-4 can exert the same effect in the normal rat heart, as well as whether Ex-4 could affect susceptibility to cardiac reperfusion injury.

Methods—After birth, Sprague Dawley rat pups were given either Ex-4 (1 nmole/kg body weight) or vehicle (1% BSA in 0.9% saline) subcutaneously for 6 days. Animals were studied at juvenile (4–6 weeks) and adult (8–9 months) ages. Using the Langendorff isolated perfused heart, cardiovascular function was assessed at baseline and following ischemia-reperfusion. Mitochondria were isolated from fresh heart tissue, and oxidative phosphorylation and calcium sequestration were analyzed. TBARS, MnSOD activity, and non-enzymatic anti-oxidant capacity were measured to assess the degree of oxidative stress present in the two groups.

Results—Both at the juvenile and adult age, Ex-4 treated rats demonstrated improved recovery from an ischemic insult. Rates of oxidative phosphorylation were globally reduced in adult, but not juvenile Ex-4 treated animals. Furthermore, mitochondria isolated from adult Ex-4 treated rats

sequestered less calcium before undergoing the mitochondrial permeability transition. Oxidative stress did not differ between groups at any time point.

Conclusion—A short course of Exendin-4 in the neonatal period leads to protection from ischemic injury and a preconditioned mitochondrial phenotype in the adult rat.

Keywords

Cardioprotection; Mitochondria; Glucagon-like peptide; Oxidative phosphorylation

Introduction

Glucagon like peptide-1 (7-36) amide (GLP-1) is a member of the pro-glucagon family of incretin peptides, with insulinomimetic and insulinotropic actions that favor glucose uptake in many organs, including the heart. In addition to the growing enthusiasm for its use in the treatment of type 2 diabetes mellitus, GLP-1 has also been shown to have salutary cardiovascular effects that are independent of the presence of diabetes [1]. This implies that GLP-1 may have cardioprotective effects independent of those attributable to tight glycemic control.

Though also efficacious as an adjunctive treatment for non-ischemic cardiomyopathy [1, 2], GLP-1 has been studied most extensively in the setting of myocardial ischemia. The first study to demonstrate GLP-1's ability to minimize reperfusion injury involved the administration of GLP-1 to humans following acute myocardial infarction and successful reperfusion [3]. Subsequently, GLP-1 has also been shown to be protective when administered during the ischemic insult, and even when used as a preconditioning agent [4-7].

The cardioprotective properties of GLP-1 are likely mediated at least in part by an improvement in myocardial glucose uptake, since glycolytically derived ATP can maintain cell viability during ischemia while not requiring oxygen for its formation [8]. Mitochondria play a crucial role in triggering both necrotic and apoptotic cell death during reperfusion injury, and are also key participants in the mechanism of preconditioning, whether ischemia-induced or drug induced [9]. No study to date has evaluated the effect of GLP-1 or its analogues on mitochondrial function in heart.

In addition to its use in adult type 2 diabetes and cardiovascular disease, GLP-1 analogues have also been studied in the setting of intra-uterine growth restriction (IUGR). IUGR is associated with mitochondrial dysfunction and oxidative stress in a number of tissues, as well as an increased propensity to develop a number of diseases during adulthood. Remarkably, a short course of Exendin-4 (Ex-4, a long acting form of GLP-1 resistant to degradation by dipeptidyl peptidase-IV (DPP-IV)) in the neonatal period prevents several of the long-term sequelae of IUGR, including oxidative stress in the liver [10, 11]. These long-lasting effects imply that there is 'critical window' after birth during which Ex-4 is able to permanently modulate crucial pathways, leading to long-term beneficial sequelae.

Given the efficacy of this Ex-4 regimen in IUGR and the well established cardioprotective role of GLP-1 when given closer to the ischemic insult, we hypothesized that normal rats given Ex-4 in the neonatal period would be protected against reperfusion injury during adulthood. In addition, in light of the key role of mitochondria in reperfusion injury and preconditioning, we hypothesized that the cardioprotective phenotype of Ex-4 is mediated by the mitochondria.

Methods

Animal model

In brief, Sprague-Dawley pregnant rats (Charles River Laboratories, Wilmington, MA) were individually housed under standard conditions. After spontaneous delivery, pups were divided into two experimental groups: 1) Control rats treated with vehicle (1% BSA in 0.9% saline); 2) Experimental rats treated with Exendin-4 (Ex-4) (1 nmole/kg body weight) injected subcutaneously daily for 6 days starting on day 1 of life. The pups were weaned per usual protocol, and given free access to chow and water. Each experimental group was studied at two time points: juvenile age (4–6 weeks) and adult age (8–9 months). The 6-week time-point was chosen so that we could assess the perinatal phenotype at a time when the hearts were big enough to be studied in our experimental system (see Methods below). The 9 months time-point was chosen because this represents ‘middle age’, the time at which insulin resistance and the metabolic syndrome commonly become manifest in the human. These studies are approved by the Animal Care Committees of Children’s Hospital of Philadelphia and the University of Pennsylvania.

Langendorff isolated perfused heart preparation

The care and use of animals were conducted under the Guidelines on Human Use and Care of Laboratory Animals for Biomedical Research published by the National Institutes of Health and according to experimental protocol approved by the Institutional Animal Care Use Committee of Hospital of the University of Pennsylvania. Rats were anesthetized with inhaled isoflurane. After cessation of toe pinch reflex, median sternotomy was performed and heparin injected into the inferior vena cava under direct observation. Hearts were quickly excised, weighed and cannulated via ascending aorta. The hearts were then perfused in a retrograde fashion at 37°C under constant flow (15 ml/min for young and 20 ml/min for old age-groups) using KH buffer containing 130 mM NaCl, 5.4 mM KCl, 0.5 mM MgCl₂, 0.5 mM NaH₂PO₄, 2.0 mM CaCl₂, 25 mM NaHCO₃, 11 mM Dextrose, and 2 mM Sodium Pyruvate. All buffer components were obtained from Sigma Chemical (St. Louis, MO). A left atrial incision was made to expose the mitral annulus through which a water-filled latex balloon was passed into the left ventricle. The balloon was attached via polyethylene tubing to a pressure transducer (model Px 272; Baxter Scientific Products, Miami, FL) that was connected to Triton System I. The initial balloon volume was set to generate left ventricular end-diastolic pressure (LVEDP) ~10 mm Hg. Myocardial function was measured, including left ventricular developed pressure (LVDevP), left ventricular end diastolic pressure (LVEDP), first derivative of pressure development over time (+dP/dt), pressure relaxation over time (–dP/dt) and coronary perfusion pressure (CPP). LVDevP was calculated by subtracting LVEDP from the peak systolic pressure.

Ischemia Reperfusion (IR)

After 25 min of constant flow perfusion (15 ml/min for young and 20 ml/min for old age-groups) with KH buffer, hearts were subjected to low-flow global ischemia by reducing flow to 10% of baseline (1.5 ml/min for young and 2 ml/min for old age-groups) for 20 min. Baseline flow was reestablished over a 10 min period, and ventricular function was followed for an additional 30 min. Ventricular function was measured every 5 min during the protocol, including LVDevP, LVEDP, LV (+)dP/dt, LV (-) dP/dt and coronary perfusion pressure (CPP).

Mitochondrial isolation

Hearts were excised at euthanasia, weighed, and quickly immersed into ice-cold PBS. The tissue was minced finely with scissors, and suspended in 1:1 weight volume ice-cold Tris-KCl medium (0.1 M KCl, 0.05 M Trishydroxymethylaminomethane hydrochloride buffer pH7.4, 0.001 M sodium ATP, 0.005 M MgSO₄, and 0.001 M ethylenediaminetetraacetate). The minced tissue was homogenized with a tightly fitted Teflon:glass homogenizer (2 strokes) and incubated for 2 min (4°C) with trypsin (4 mg trypsin/g heart tissue) to facilitate the release of interfibrillar as well as subsarcolemmal mitochondria. The suspension was then diluted in Tris KCl medium (10 ml/g heart tissue) containing soybean trypsin inhibitor (4 mg/g heart tissue), and then re-homogenized (2 strokes). The suspension was then centrifuged at 1,000 g for 10 min at 4°C. The supernatant fluid was decanted and recentrifuged at 14,000 g for 10 min at 4°C. The mitochondria were washed once by resuspension in Tris-KCl medium, resedimented (10,000 g for 10 min at 4°C) and finally resuspended in medium containing 220 mM mannitol, 70 mM sucrose, 10 mM Hepes, pH7.2, and 0.2 mM EGTA. Mitochondrial protein was quantified using the BCA assay, with BSA as a standard.

Oxidative phosphorylation

Oxygen consumption in isolated myocardial mitochondria was measured polarographically using a Strathkelvin Instruments oxygen electrode in a magnetically stirred, thermostatically regulated chamber (30°C). Approximately 1 mg mitochondrial protein was suspended in a total volume of 0.15 mL of air-saturated isotonic MSTPE buffer, composed of 220 mM mannitol, 70 mM sucrose, 10 mM HEPES, 5 mM KH₂PO₄, and 0.2 mM EGTA, pH7.2. After equilibration of the mitochondria, indicated mitochondrial substrates were added. Respiration was initiated by the addition of 0.2 mM adenosine diphosphate (ADP), and oxygen consumption was measured during state 3 (ADP-stimulated) and state 4 (ADP-depleted) conditions by measuring the decrease in oxygen concentration in the chamber. State 3 and 4 rates of oxygen consumption were measured for each of the following substrates: 10 mM pyruvate, 10 mM glutamate, and 10 mM α -ketoglutarate, each with 2 mM malate, 10 mM succinate plus 2 μ M rotenone, and ascorbate (2.5 mM) + tetra-methyl phenylene diamine (TMPD, 0.25 mM). Fatty acid (FA) oxidation was examined using 0.05 mM C16 palmitoyl carnitine (a long chain FA) as the substrate in the presence of 2 mM malate. Respiratory control ratios were calculated from the rates of state 3/state 4 respiration. Rates of substrate oxidation were expressed as nanomoles oxygen consumed per minute per milligram of mitochondrial protein (nmolO/min/mg). Mean rates of state 3 and

4 oxygen consumption for each substrate were calculated for 2–3 consecutive additions of ADP. The uncoupler CICCP (1 μ M) was added following the final round of ADP to assess the maximal rate of O₂ consumption.

Calcium sequestration

Ca²⁺ uptake was measured with the fluorescence probe Calcium Green 5-N (Molecular Probes, Inc). Fluorescence was recorded continuously in a water-jacketed cuvette holder at 30°C using an Aminco Bowman series 2 spectrofluorometer with excitation and emission wave-lengths of 506 nm and 532 nm, respectively. Heart mitochondria (0.25 mg/mL) were added to a magnetically-stirred glass cuvette containing starting medium (100 mM NaCl, 25 mM Hepes pH7.4, 0.1% BSA, 5 mM NaH₂PO₄, 0.2 mM ADP, 10 mM succinate, 1 μ g/ml oligomycin, and 1 μ M rotenone, and 0.2 μ M CaG5N). While recording fluorescence, serial 20 nmol aliquots of CaCl₂ were added to the cuvette until the mitochondria underwent the permeability transition at which time an abrupt and sustained increase in the fluorescence was seen, indicating the release of matrix calcium as the MPTP opens. Measurements were performed in the presence and absence of inorganic phosphate (Pi), a physiologically important permeant anion that binds calcium within the mitochondrial matrix to form CaPO₄ salt, thus significantly increasing the capacity of the mitochondria to sequester calcium. The total capacity of the mitochondria to accumulate calcium was expressed as nmoles calcium/mg mitochondria.

TBARS

TBARS were measured in whole heart homogenate using a commercially available assay from Cayman Chemical (Ann Arbor, Michigan).

MnSOD activity

MnSOD activity was measured at 550 nm minus 540 nm with a DW2A spectrophotometer using an adaptation of the assay described by McCord and Fridovich [12], based on MnSOD's capacity to lower the rate of superoxide-mediated reduction of cytochrome c. The starting buffer was composed of 50 mM KPO₄ at pH7.8, 0.1 mM EDTA, 200 μ M xanthine, 1 M KCN, 10% VIV Triton X. The addition of KCN to the starting buffer inhibits CuZnSOD and therefore ensured that only MnSOD activity was measured. In a water jacketed cuvette (25°C), cytochrome c (50 μ M) was added to 1 mL of starting buffer, followed by xanthine oxidase (~0.4 Units). The rate of reduction of cytochrome c was measured spectrophotometrically at 550 nm in the absence (v0) and presence (v1) of 150–400 μ g mitochondrial protein. The measurement was repeated for 2–3 mitochondrial protein concentrations. One unit of SOD was defined as the amount required to inhibit the rate of reduction of cytochrome c by 50% ([SOD]0.5). The concentration of SOD in the sample was defined by: [SOD] = [(v0/v1)–1], with final activity expressed as Units/mg mitochondrial protein.

Non-enzymatic anti-oxidant capacity

Non-enzymatic (small molecule and protein) anti-oxidant capacity was measured in adult whole heart homogenate using a commercially available assay from Biovision (Mountain View, CA).

Statistics

All results were analyzed using unpaired student's *t*-tests, with the exception of post-ischemia hemodynamics. In this case, each hemodynamic variable was analyzed using an analysis of variance appropriate for a 2 factor experimental design with one repeated factor (time: 5, 10, 15, 20, 25, and 30 min of reperfusion) and one non-repeated factor (diagnosis: control vs. exendin). If a significant second order interaction existed between time and the variable of interest ($p < 0.20$), this indicated the presence of a significant effect modifier, thereby precluding the examination of the main effect of the diagnosis. In this case, time specific *t*-tests were performed at each time point during reperfusion.

Results

Juvenile age

Morphometrics—Heart weight (HW), HW/BW, and HW/Tibia length were similar in control and exendin-treated animals (Table 1). A high degree of variability was observed at this time-point, which is expected in rats of this age.

Hemodynamics—Table 2 demonstrates that baseline hemodynamics, including left ventricular developed pressure (LVDevP), coronary perfusion pressure (CPP), left ventricular end diastolic pressure (LVEDP), heart rate (HR), left ventricular dP/dt and -dP/dt were not different between control and exendin-treated animals.

Ischemia reperfusion experiments are displayed in Fig. 1a. Following 20 min of low flow ischemia (10% baseline), percent recovery of LVDevP was higher in exendin-treated animals ($p < 0.05$). Since a second order interaction was present between percent recovery and time, the analysis was repeated using *t*-tests at each time-point. At 20 min of reperfusion and all subsequent time points, there was improved percent recovery of LVDevP in Ex-4 treated rats ($p < 0.05$). LVEDP was significantly lower in exendin-treated animals at 20 and 25 min reperfusion. dP/dt, -dP/dt, and HR were comparable in the two groups. CPP, which reflects coronary vascular resistance, was also unchanged.

Mitochondrial function—To ascertain whether mitochondrial respiratory and bioenergetic defects are affected by exendin treatment, fresh mitochondria were isolated from heart and the rates of oxidative phosphorylation for different substrates were compared (Table 3). There was no difference in mitochondrial yield (expressed as mg mitochondrial protein/g heart weight). State 3 refers to oxygen consumption in the presence of both substrate and ADP; state 4 refers to the rate of oxygen consumption once all available ADP is converted to ATP. Rates of state 3 and state 4 respiration for all substrates tested were comparable in control and exendin-treated animals. This indicates that the two experimental

groups were equivalent with regard to rates of oxidative phosphorylation and mitochondrial coupling.

The ability to take up and sequester calcium is an integral function of the mitochondria. Under physiologic circumstances, a rise in mitochondrial matrix $[Ca^{2+}]$ activates several calcium-dependent Krebs cycle enzymes, elevating NADH, which increases mitochondrial ATP production. Under pathologic circumstances, calcium overload can trigger the mitochondrial permeability transition (MPT), which then stimulates apoptosis. This process is thought to be important in IR injury. Both in the presence and absence of inorganic phosphate (Pi), cardiac mitochondria from exendin-treated rats took up equivalent amounts of calcium: (–) Pi, C: 348.4 ± 48.7 , E: 274.8 ± 16.8 nmol/mg mitochondrial protein; (+) 5 mM Pi, C: 1187.2 ± 93.1 , E: 1091.2 ± 50.7 nmol/mg mitochondrial protein. This suggests that differences in postischemic LV function do not stem from differing sensitivity to calcium-induced triggering of the MPT.

Oxidative stress—GLP-1 and its analogues have been shown to decrease oxidative stress in the setting of myocardial ischemia and reperfusion (50). We used the TBARS assay, which quantifies the lipid peroxidation end product malondialdehyde (MDA), to measure oxidative stress in the myocardium. MDA in whole heart homogenate was the same in the two experimental groups at this time-point (C: 10.0 ± 1.1 /mg protein, E: 9.5 ± 0.9 /mg protein, $p=0.83$).

MnSOD is a matrix antioxidant enzyme that is upregulated in the setting of both acute and chronic production of ROS, and has been shown to be increased in hearts treated with Exenatide, a GLP-1 agonist (50). MnSOD activity was the same in control and exendin treated animals (C: 7.5 ± 0.5 , E: 7.5 ± 0.7 units/mg mitochondrial protein, $p=0.88$). Thus, the observed effects of Ex-4's on IR injury do not appear to be mediated by decreased oxidative stress or upregulation of MnSOD.

Adult age

Morphometrics—Body weight (BW), heart weight (HW), HW/BW, and HW/tibia length were similar between experimental groups (Table 1).

Hemodynamics—Baseline hemodynamics, including LVDevP, LVEDP, CPP, HR, dP/dt and –dP/dt were not different between control and exendin-treated rats (Table 2).

Ischemia reperfusion experiments are displayed in Fig. 1b. At the adult time-point, exendin-treated hearts continued to display improved recovery of LVDevP following 20 min of low flow ischemia (10% baseline) ($p<0.05$). Negative dP/dt was slightly but not significantly lower in exendin animals at an equal heart rate ($p=0.10$), and (+) dP/dt was similar between groups. LVEDP, which had been lower in exendin treated animals at the juvenile age, was the same between adult animals. CPP remained comparable between groups at this age.

Mitochondrial function—In adult animals, rates of state 3 respiration for all substrates tested except C16 carnitine were lower in exendin-treated animals (Table 3). Since these changes were present with multiple substrates, the defect is not likely to be at the level of

substrate oxidation, but instead is at the level of the electron transport chain (ETC). Rates of state 4 respiration did not differ between groups. There was no difference in mitochondrial yield (expressed in mg mitochondrial protein/g heart tissue).

Calcium sequestration was not significantly different between experimental groups when Pi was absent (no Pi C: 230 ± 28.5 , E: 182.0 ± 22.7 nmol/mg mitochondrial protein, $p=0.22$). However, in the presence of 5 mM Pi mitochondria from exendin treated animals took up significantly less calcium (with 5 mM Pi C: $1,032 \pm 106.9$, E: 752 ± 101.3 nmol/mg mitochondrial protein, $p=0.02$). This suggests that in the presence of a physiologic concentration of Pi, mitochondria from Ex-4 treated animals are more sensitive to the Ca^{2+} induced triggering of the MPT.

Oxidative stress—MDA concentrations were equivalent between groups at the adult time-point (C: 3.8 ± 0.7 /mg protein, E: 4.0 ± 0.9 /mg protein, $p=0.85$), as was MnSOD activity (C: 4.0 ± 0.3 , E: 3.9 ± 0.08 units/mg mitochondrial protein, $p=0.86$), and non-enzymatic antioxidant capacity (C: 0.55 ± 0.01 , E: 0.58 ± 0.02 Trilox equivalents, $p=0.83$).

Discussion

The major finding of our study was that a short treatment course of the GLP-1 analog, Exendin-4, in the newborn period leads to protection from cardiac reperfusion injury long into adulthood. Though GLP-1 and its analogues are known to be cardioprotective when given proximal to the ischemic insult, this is the first demonstration of the ability of Ex-4 to exert such long-term beneficial effects. In addition, this is the first study to examine the effect of Ex-4 on mitochondrial function: rates of oxidative phosphorylation were globally reduced in cardiomyocyte mitochondria from adult Ex-4 treated rats. The mitochondrial phenotype observed appears to be due to changes at the level of the electron transport chain (ETC) itself, since the findings were present with a wide variety of substrates donating reducing equivalents to several ETC complexes.

LVDevP is a global measure of cardiac function, reflecting both maximal systolic pressure achieved and the end-diastolic, or 'loading' pressure. dP/dt , on the other hand, is indicative of the rate of pressure development and dissipation, in part a reflection of calcium uptake and release from the SR. Our observations suggest that differences in recovery of function are due to changes at the level of the myofilament, and not due to alterations in the rate of SR calcium release.

Mitochondria play a key role in reperfusion injury as well as in preconditioning, both ischemia and drug-induced [9]. Though not present in juvenile animals, the reduction in rates of oxidative phosphorylation in adult animals is likely to account in part for the protection from reperfusion injury. ROS are important mediators of reperfusion injury, and interventions that reduce production or increase scavenging of ROS are associated with preconditioning, leading to improved tolerance to IR [13]. Since ROS are produced largely at ETC complexes I and III, decreasing flux through the ETC would thus limit the passage of reducing equivalents through the ETC, leading to a parallel decrease in ROS production. This has been demonstrated in numerous studies in which the addition of low doses of respiratory

chain inhibitors improve ventricular function and reduce infarct size following ischemia and reperfusion in rats [14-17]. In addition, it has been well documented that S-nitrosylation of Complex I in highly oxidative conditions leads to inhibition of this complex and to cardioprotection [18].

There are many mechanisms in addition to alterations in mitochondrial function by which protection from IR injury can take place. ROS, while harmful in excess, can trigger preconditioning if present in the pre-ischemic phase at the proper concentration [19]. In addition, MnSOD plays a prominent role in protection against oxidative injury, preventing both LV dysfunction and post-ischemic arrhythmias [20-23]. Neither oxidative stress (as measured by TBARS) nor MnSOD activity was different between control and Ex-4 treated animals, indicating that ROS-mediated and MnSOD-mediated preconditioning did not play prominent roles in our findings.

Calcium overload within the mitochondria is a hallmark of reperfusion injury, leading to mitochondrial swelling and eventual opening of the mitochondrial permeability transition pore (MPTP) [9]. We found that, compared to control, mitochondria from Ex-4-treated adult animals sequestered less Ca^{2+} in the presence of 5 mM inorganic phosphate (Pi) before the opening of the MPTP was triggered. One might have expected the apparently preconditioned mitochondria to be *less*, not *more* sensitive to Ca^{2+} induced opening of the MPTP. There have been few studies evaluating calcium sequestration in isolated mitochondria following preconditioning, and those that have been conducted have yielded inconsistent results [24, 25]. The significance of this finding is not clear given that it was not present in juvenile animals, which displayed the same phenotype.

There are several putative mechanisms for cardioprotective effects of GLP-1 in the acute and subacute setting. The first is via metabolic modulation: GLP-1 increases myocardial glucose uptake both at baseline and during ischemia, and does so in an Akt independent manner (making it independent of insulin signaling) [8]. This is beneficial in the setting of myocardial ischemia, when the heart is critically dependent on glycolytically-derived ATP for the maintenance of viability, and is paradoxically insulin resistant due to increased circulation of free fatty acids and secretion of catecholamines [26-29].

Despite these putative mechanisms of action in the acute or subacute settings, it is not yet clear precisely how a 7-day course of Ex-4 during the neonatal period can downregulate rates of oxidative phosphorylation and protect against reperfusion injury long into adulthood, nor is it clear whether these effects are direct or indirect. As for the mitochondrial phenotype, several ETC chain complexes are prone to oxidative modification and subsequent reductions in function [30-32], as is ubiquinone [33]. In addition, experimental models have shown that in highly oxidative environments mtDNA copy number decreases, as do mtDNA-encoded gene transcripts (including subunits of complex I, complex III, complex IV (cytochrome c oxidase) [34]. However, GLP-1 and its analogues have been shown to be protective against oxidative damage [35, 36], and our experiments found no difference in oxidative stress between experimental groups at either time-point.

It may be relevant to consider an epigenetic mechanism to explain our findings. Epigenetic changes refer to structural changes in genes that do not involve the nucleotide sequence itself but can have long-term effects on gene expression nonetheless. Fetal and neonatal environments can have important effects on the later development of cardiovascular and other disease [37-42], and epigenetic modifications have been implicated in this causal pathway [43-45]. Furthermore, an epigenetically modified phenotype can be silent until late in life [46], relevant to the current study in that the overt mitochondrial phenotype was not apparent until the adult time-point. Although the study of epigenetics has largely focused on the effect of adverse intra-uterine and neonatal environment on the development of later disease, the principles may still be relevant here. Another future consideration is whether structural and/or histologic changes contribute to cardioprotective effects of Exendin-treated animals.

In conclusion, we show for the first time that Ex-4 has longstanding cardioprotective effects when given during the neonatal period, adding to a growing body of literature citing the salutary effects of GLP-1 when given in proximity to the ischemic insult. In addition, we show that neonatal administration of Ex-4 leads to a 'preconditioned' phenotype in adult cardiac mitochondria, with globally reduced rates of oxidative phosphorylation.

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A. Juvenile Age

B. Adult Age:

Fig. 1.

Hemodynamic function during ischemia and reperfusion. Low flow ischemia (10% baseline) was induced for 20 min. Baseline flow was re-established over a period of 10 min, and reperfusion function was observed for an additional 30 min. **a.** Juvenile Age (C: $n=5$, E: $n=5$) * $p<0.05$ at 20, 25, and 30 min reperfusion. ** $p<0.05$ at 25 and 30 min reperfusion. # $p=0.06$. **b.** Adult Age: (C: $n=4$, E: $n=5$) * $p<0.05$ ** # $p=0.10$

Table 1

Morphometrics

	Juvenile		Adult	
	Control (n=10)	Exendin (n=10)	Control (n=9)	Exendin (n=10)
BW (g)	163.6±41.6	215±66.5	621.0±70.8	660±74.3
HW (g)	0.75±0.29	0.97±0.42	1.6±0.1	1.8±0.3
HW/BW (mg/g)	4.5±1.1	4.4±1.0	2.7±0.2	2.8±0.5
HW/Tibia length (mg/cm)	334.6±51.0	405.5±59.0	342.6±20.7	377.3±59.6

BW = body weight. HW = heart weight. Values are expressed as mean ± SEM

Table 2

Baseline hemodynamics

	<u>Juvenile</u>		<u>Adult</u>	
	Control (n=5)	Exendin (n=5)	Control (n=4)	Exendin (n=5)
LVDevP (mmHg)	110±7	111±7	107±6	98±9
LVEDP (mmHg)	9.5±0.9	9.6±1.3	9.1±0.4	8.8±0.5
CPP (mmHg)	99±17	59±4 [*]	76±8	70±10
LV -dP/dt (mmHg/sec)	2889±137	2956±211	2903±132	2672±159
LV -dP/dt (mmHg/sec)	-2225±136	-1957±182	-1904±133	-1844±187
HR (bpm)	317±13	332±15	299±19	285±16
LV dP/dt/HR (mmHg/sec/min)	9.2±0.7	8.9±0.5	9.8±0.6	9.5±0.9
LV -dP/dt/HR (mmHg/sec/min)	-7.1±0.6	-6.0±0.5	-6.4±0.5	-6.6±0.8

Values are expressed as mean ± SEM. LVDevP = Left ventricular developed pressure. LVEDP = Left ventricular end diastolic pressure. CPP = coronary perfusion pressure. LV dP/dt = Left ventricular dP/dt. LV -dP/dt = Left ventricular -dP/dt. HR = heart rate.

*
p=0.05

Table 3

Rate of oxygen consumption (natomsO/min/mg mitochondrial protein) and respiratory control ratios (RCR) in control and Ex-4

	<u>State 3</u>		<u>State 4</u>		<u>RCR</u>	
	Control	Exendin	Control	Exendin	Control	Exendin
A: Juvenile-aged rats (control $n=5$, Ex-4 $n=5$).						
Pyruvate	272.9±24.9	309.4±16.5	61.3±3.4	68.2±5.6	4.5±0.4	4.7±0.4
Glutamate	230.3±45.8	299.3±24.3	73.9±1.8	77.3±7.4	3.1±0.6	4.0±0.5
C16 Carnitine	217.6±26.1	237.4±15.2	87.6±2.7	102.4±13.4	2.5±0.3	2.5±0.4
Succinate	424.2±20.5	459.4±26.1	165.5±13.9	187.2±20.4	2.7±0.3	2.5±0.3
TMPD	848.2±60.4	933.3±44.9	552.3±39.8	587.2±30.6	1.5±0.07	1.6±0.2
B: Adult aged rats (control $n=4$, Ex-4 $n=5$). * $p<0.05$ control versus exendin						
Pyruvate	253.6±37.5	179.4±13.4*	46.7±0.9	42.8±5.9	5.5±0.9	4.7±1.0
Glutamate	258.5±13.9	204.6±15.9*	64.1±7.1	57.6±3.9	4.1±0.3	3.6±0.4
α -Keto Glutarate	259.6±15.5	191.3±18.8*	68.2±10.8	54.8±3.0	4.1±0.3	3.5±0.2
C16 Carnitine	147.9±8.4	116.3±25.7	77.4±7.1	63.0±6.6	2.0±0.2	1.8±0.3
Succinate	370.2±28.3	301.4±22.8*	142.6±11.5	157.9±14.3	2.6±0.04	2.0±0.2
TMPD	673.8±23.0	543.7±46.2*	438.0±36.3	351.1±24.9	1.6±0.1	1.6±0.9

Values are expressed as mean $\pm$ SEM