Neonatal Intramuscular Injection of Plasmid DNA Encoding GLP-1 Reduces Serum Insulin level and Modifies Skeletal Muscle Myosin Heavy Chain Composition in Adult Rats

LINA WANG, FENG GUO, SHI WEI, RUQIAN ZHAO *

Key Laboratory of Animal & Biochemistry, Nanjing Agricultural University, Nanjing 210095, P. R. China

*Address proofs and correspondence to:

Dr. Ruqian Zhao

Key Laboratory of Animal Physiology & Biochemistry

Nanjing Agricultural University

Nanjing 210095

P. R. China

Email: zhao.ruqian@gmail.com

Tel. 00862584395047

Fax: 00862584398669

Short title: neonatal GLP-1 on adult insulin and MyHC

Summary

To test the hypothesis that neonatal GLP-1 exposure may program myosin heavy chain (MyHC) composition in adult skeletal muscle, two-day-old rats were transfected intramuscularly in vivo with vacant vector plasmid (VP), or recombinant plasmid expressing secretory GLP-1 at the doses of 60 μg (LG) and 120 μg (HG), respectively. Expression of GLP-1 mRNA was detected in muscles of both LG and HG rats 7 days after transfection, with more abundant GLP-1 transcript seen in LG rats. In accord with the GLP-1 expression, LG rats demonstrated more significant responses to neonatal GLP-1 exposure. Small yet significant growth retardation was observed in LG rats, which is accompanied with significantly reduced serum insulin concentration at 8 weeks of age compared to VP rats. The responses of skeletal muscle were muscle-type-dependent. Significant increase of PGC-1α and GLUT4 mRNA expression was detected in soleus of LG rats, whereas a MyHC type switch from ⅡB to Ⅰ was seen in gastrocnemius. These results indicate that neonatal exposure of healthy pups to ectopic GLP-1 cause growth retardation with decreased serum insulin, as well as muscle-type-dependent modifications in MyHC type composition and metabolic gene expression in adult rats.

Key words: GLP-1; suckling; skeletal muscle; insulin; myosin heavy chain (MyHC)

Introduction

Glucagon-like peptide (GLP-1) is produced through posttranslational processing of proglucagon in intestinal L-cells in response to nutrient ingestion (Aulinger *et al.* 2007), and is regarded as a potential agent for the treatment of type 2 diabetes, mainly due to its insulinotropic capacity and insulinomimetic actions (Kashima *et al.* 2001). Impaired GLP-1 secretion was observed in patients with type 2 diabetes (Toft-Nielsen *et al.* 2001). Administration of GLP-1 receptor (GLP-1R) agonists on diabetic (db/db) mice or a partial pancreatectomy rat model of type 2 diabetes could enhance β-cell proliferation, increase β-cell mass and attenuate the development of diabetes (Kim *et al.* 2003, Xu *et al.* 1999).

Besides its immediate effects, neonatal GLP-1 exposure has shown programming effects on adult pancreatic functions. Daily injection of GLP-1 or its long-acting analog exendin-4 (Ex-4) from day 2 to day 6 after birth led to improved β-cell mass and glucose homeostasis in adult GK rats, a genetic model of type 2 diabetes (Tourrel *et al.* 2002). Activation of GLP-1R signal transduction pathways by Ex-4 injection in the critical neonatal period is sufficient for sustained improvement in β-cell mass and function in adult intrauterine growth retarded (IUGR) rats (Stoffers *et al.* 2003). Therefore, GLP-1 is considered as a promising agent not only for the treatment, but also for the prevention of type 2 diabetes in susceptible individuals. As all the studies concerning the programming effects of GLP-1 or its anologs were carried out in diabetic or predisposed diabetic susceptible animal models, it is unknown whether and how neonatal exposure of high GLP-1 may program the adult glucose homeostasis in healthy animals.

Moreover, extrapancreatic tissues such as liver and skeletal muscle have been implicated

to be potential targets for GLP-1 action. For instance, GLP-1 was found to stimulate glycogen synthesis in human myocytes (Luque *et al.* 2002) and hepatic glycogen storage in mice (Knauf *et al.* 2005). Skeletal muscles account for almost 50% of the total body weight and their metabolic characteristics are closely related to the glucose homeostatic status (Sinacore and Gulve 1993). Furthermore, skeletal muscle has been shown to be one of the major target tissues for neonatal metabolic programming (Holness *et al.* 2000). The responses of skeletal muscles to nutritional or endocrine interventions in early life seem to be muscle type-dependent. A maternal low-protein diet during mid-pregnancy reduced total number and density of fast glycolytic fibers in soleus muscle of 4-week-old rats, whereas in gastrocnemius muscle, the density of slow oxidative fibers was reduced (Mallinson *et al.* 2007). Up to now, no data is available about neonatal programming effect of GLP-1 on adult myofiber type characteristics of different muscle types.

In order to circumvent the rapid turnover of exogenously injected GLP-1, we constructed a recombinant vector expressing secretory GLP-1, and transfected it intramuscularly in two-day-old rat pups to achieve continuous ectopic expression of GLP-1 during sucking period. Growth performance was monitored up to 8 weeks of age when the animals were sacrificed to determine insulin secretion, glucose concentration, as well as profiles of myosin heavy chain (MyHC) types and expression of metabolic-related genes in two types of skeletal muscle, soleus and gastrocnemius.

Methods

Plasmid construction

The recombinant plasmid expressing secretory GLP-1, sig-glp-1-pcDNA3, was

constructed containing signal peptide coding sequence and full length cDNA of rat glp-1. Briefly, upstream signal peptide coding sequence including restriction site Hind III (P1) and downstream signal peptide coding sequence (P2) were synthesized as two partly complementary single strands DNA. By mean of asymmetrical primer/template, frontal P1P2 was synthesized in vitro. Rat GLP-1 cDNA (P3P4) including part of P2 was cloned by RT-PCR with specific primers including restriction site (BamH I). At last P1P4 (sig-glp-1) was synthesized with P1P2 and P3P4 by mean of asymmetrical primer/template (Fig. 1). Sequenses of P1P4 were analysed using special software to confirm that splice site of signal peptide can still be identified after recombination. P1P4 (sig-glp-1) was inserted into the multiple cloning site of the pcDNA3 expression vector (sig-glp-1-pcDNA3). The empty pcDNA3 plasmid was used as a control. Plasmids were transformed in DH5α, extracted using Tiangen EndoFree plasmid Kits. Purified DNA was dissolved in phosphate-buffered saline (pH 7.4) and the quantity and quality were assessed by spectrophotometry.

Rats and In Vivo Electroporation

Adult male and female Wistar breeder rats were purchased from Shanghai Laboratory Animal Center (SLAC). Rats were housed in specific pathogen-free (SPF) facility with food and water provided *ad libitum*. The standard breeding protocol was followed and 34 newborn male rats from 16 mothers (two or three pups from each mother) were weighted at birth and divided at random into three groups: vacant plasmid group (VP, n = 11), low dose GLP-1 plasmid group (LG, n = 12) and high dose GLP-1 plasmid group (HG, n = 11). Electroporation was performed on the following day.

Aliquots of 120 μg (HG group) or 60 μg (LG group) sig-glp-1-pcDNA3 plasmid DNA

and 120 μg control pcDNA3 plasmid DNA (VP group) in 120 μl phosphate-buffered saline were injected respectively into the lateral side of left thigh of pups. Four electric pulses of 100 V at 50 ms followed by four more pulses of the opposite polarity were immediately delivered to the injected muscle. The electric pulse was delivered by an electric pulse generator (WJ-2002, Ningbo Scientz Biotechnology Co., Ltd).

Rats were maintained in specific pathogen-free facility following the standard protocol until 56 days of age. Weights of rats were recorded every two weeks and rats were weaning at day 21. As reported, plasmids may keep the expression ability for one month but the highest peak of expression is around 5-7 days after transfection (Aihara and Miyazaki 1998, Ataka *et al.* 2003). So, the whole leg muscle of five or six pups in each group was collected for confirming GLP-1 mRNA expression with RT-PCR using forward primer 5'-GCCAAGCTTATGAAGACCGTTTACATCGTG-3' and reverse primer 5'-TATGGATCCTCATCCTCGGCCTTTCACC-3' seven days after electroporation in our study. At 56 days of age, the remaining six rats in each group were weighed and euthanized by decapitation without fasting. Blood samples were collected just before sacrifice from carotid artery and sera were separated by centrifugation at 1300 × g at 4°C for 15 min and stored at -20°C until insulin and glucose assay. Soleus and gastrocnemius were dissected and weighted, rapidly frozen in liquid nitrogen, then stored at -80°C until analysis.

The use and treatment of the animals followed the European Community Guidelines as accepted principles for the use of experimental animals. The animal experiment protocol was approved by the Animal Ethics Committee of Nanjing Agricultural University.

Serum Analysis

125 Serum concentrations of insulin were measured in duplicate using commercially
126 available ^{125}I-RIA kit (Beijing Research Institute of Biotechnology, China) according to the
127 manufacturer's guideline. The kit was validated for measuring murine serum samples and the
128 detection limits were 2 μIU/ml. The intra and inter-assay coefficients of variation were 10%
129 and 15%, respectively.

130 Serum glucose concentrations were measured in duplicate using a commercial kit
131 (Nanjing Jiancheng, Nanjing, China) according to the manufacturer's guideline.

132 *MyHC electrophoresis and analysis*

133 MyHC isoforms were separated using a modification of the SDS-polyacrylamide gel
134 electrophoretic procedure developed by Talmadge and Roy (Talmadge and Roy 1993).
135 Briefly, frozen muscles were minced with scissors in 9 volumes of ice-cold homogenization
136 buffer (100 mM $Na_4P_2O_4$, 0.3 mM KCl, 5 mM EGTA, 5 mM $MgCl_2$ and 10 mM DTT), and
137 extracted for 30 min on ice, then centrifuged at 12000 g at 4 °C. Protein content of the
138 supernatant was estimated by Bradford assay. Samples were diluted in 2 × loading buffer (1%
139 β-mercaptoethanol, 4% SDS, 20% glycerol, 0.2% bromophenol blue, and 1 M Tris pH 6.8) to
140 a final concentration of 0.5 mg/mL. 2 μg total protein was loaded on the gel (Stacking: 30%
141 glycerol, 4% acrylamide:Bis (50:1),70 mM Tris (pH 6.8), 4 mM EDTA, 0.4% SDS;
142 Separating: 30% glycerol, 8% acrylamide:Bis (50:1), 0.2 M Tris (pH 8.8), 0.1 M glycine,
143 0.4% SDS) prepared using the Bio-Rad Mini-Protein II system. Separate upper (0.1 M Tris,
144 150 mM glycine, 0.1% SDS) and lower (50 mM Tris, 75 mM glycine, 0.05% SDS) running
145 buffers were used. The running conditions were 70 V (constant voltage) for 24 h at 4°C. The
146 MyHC isoforms were visualized after staining with Coomasie brilliant blue.

147 Band densities were analyzed with Kodak Digital Science 1D software (Eastman Kodak
148 Company Rochester, NY, USA). The ratio of each MyHC isoform density to the total was
149 calculated to represent the proportion of each MyHC isoform.

150 *Measuring mRNA expression by real-time PCR*

151 Total RNA were extracted from soleus and gastrocnemius with TRNzol total RNA Kit
152 (Tiangen Biotech Co., Ltd, Beijing, China), according to the manufacturer's instruction. Total
153 RNA concentration was then quantified by measuring the absorbance at 260 nm with a
154 photometer (Eppendorf Biophotometer). Ratios of absorption (260/280 nm) of all
155 preparations were between 1.9 and 2.1. 4.4 μg of each RNA samples were subjected to
156 electrophoresis through a 1.4% agarose formaldehyde gel to verify their integrity.

157 One microgram of total RNA was reverse transcribed by incubation at 37°C for 1 h for
158 the first-strand cDNA synthesis in a 25 μl mixture consisting of 100 U moloney murine
159 leukemia virus (M-MLV) reverse transcriptase (Cat. No. M1701, Promega, Shanghai, China),
160 10 U RNase Inhibitor (Cat. No. N2611, Promega, Shanghai, China), 12 μM random primers
161 (6mer) (Cat. No. D3801, TAKARA Biotechnology (Dalian) Co., Ltd., China), 50 mM
162 Tris-HCl (pH 8.3), 10 mM $MgCl_2$, 50 mM KCl, 10 mM DTT, 0.5 mM spermidine and 0.8 mM
163 each dNTP.

164 The synthesis of all primers was performed by Invitrogen Co. Shanghai, P. R. China.
165 Real-time PCR was performed in Mx3000P (Stratagene, USA) with specific primers: MyHC I
166 (NM-017240) F: 5'-TTGCTCTACCCAACCCTAAGGATG-3', R: 5'-TTGTGTTTCTGCCT
167 GAAGGTGC -3' ; MyHC 2A (L13606) F: 5'-CTCAGGCTTCAAGATTTGGTGG-3', R:
168 5'-TTGTGCCTCTCTTCGGTCATTC-3'; MyHC 2X (XM-213345) F: 5'-GGAGGAACAAT

CCAACGTCAACC-3', R: 5'-GGTCACTTTCCTGCTTTGGATCG-3'; MyHC ⅡB (NM-019325) F: 5'-TGAGCCAGACGGCACTGAA-3', R: 5'-GCTGCACTGTCTGGCCTTTG-3'; GLUT4 (NM-012751) F: 5'-CGTTGGCATGGGTTTCCAG-3', R: 5'-GCCTCTGGTTTCAGGCACTCTTAG-3'; PGC-1 α (AY-237127) F: 5'-CACTGACAGATGGAGCCGTGA-3', R: 5'-TGTTGGCTGGTGCCAGTAAGAG-3'; β-actin (NM-007393) F: 5'-CCCTGTGCTGCTCACCGA-3', R: 5'-ACAGTGTGGGTGACCCCGTC-3'. Mock RT and no template controls (NTC) were set to monitor the possible contamination of genomic DNA both at the stage of RT and RCR. The pooled sample made by mixing equal quantity of total RT products (cDNA) from all samples was used for optimizing the PCR condition and tailoring standard curve for each target gene, and melting curves were performed to insure a single specific PCR product for each gene. 4 μl of 40-fold dilution of each RT product was used for PCR in a final volume of 25 μl containing 0.4 μM primers and 12.5 μl SYBR Green Real-time PCR Master Mix (Cat. No. QPK-201, TOYOBO Ltd., Japan). Rodent β-actin was selected as reference gene. No marked difference of β-actin mRNA abundance was detected between groups. The method of $2^{-\Delta\Delta Ct}$ was used to analyze the real-time PCR data (Livak and Schmittgen 2001). The mRNA levels of LG and HG rats were expressed as the fold of change relative to the mean value of VP rats.

Statistical analysis

All data were presented as mean ± SEM, and analyzed using one-way analysis of variance (ANOVA) or General Linear Model for multivariate with SPSS 11.0 for Windows. Differences were considered significant when $P < 0.05$.

Results

Detection of GLP-1 mRNA expression in muscles injected with sig-glp-1-pcDNA3

Seven days after transfection, muscles received plasmid electroporation were taken from rats of all groups (five or six pups in each group) and used for detecting GLP-1 mRNA expression with RT-PCR. No GLP-1 transcript was detected in VP rats, whereas clear bands of expected size for GLP-1 were seen in muscles of both LG and HG rats (Fig. 2A). It is interesting to note that low dose of GLP-1 plasmid transfection induced more abundant GLP-1 mRNA expression in the muscle compared to high dose of GLP-1 plasmid transfection.

Body weight, muscle weight, and pancreas weight

As shown in Fig. 2B, LG treatment caused small yet significant growth retardation over the experimental period of 8 weeks ($P < 0.05$), when the general effects of treatment and age on body weight were tested with General Linear Model for multivariate. Nevertheless, no alterations were found in soleus and gastrocnemius muscle weight, as well as pancreas weight (data not shown).

Serum concentrations of insulin and glucose

At 56 days of age, both LG and HG rats demonstrated significantly reduced serum insulin concentration (Fig. 2C) compared to VP rats ($P < 0.05$). However, no alteration was detected for serum glucose concentration (Fig. 2D).

Expression of MyHC isoforms and metabolic genes in skeletal muscle

Alterations of mRNA expression for MyHC isoforms and metabolic genes were detected only in LG rats in response to neonatal GLP-1 exposure, in a muscle type dependent manner. In soleus muscle, expression of PGC-1α and GLUT4 mRNA was up-regulated significantly in

LG rats (Fig. 3), which is associated with increased MyHC IIB mRNA expression ($P < 0.05$). In gastrocnemius muscle, no changes were detected at the level of transcription for all the genes detected. However, a MyHC type switch from IIB to I was observed at the level of protein in LG rats, as shown with SDS-PAGE (Table 1, Fig. 4).

Discussion

"Gene therapy" approach has been used in preclinical studies for sustained therapeutic delivery of GLP-1R agonists. Systemic delivery of a GLP-1 minigene via an adenovirus improved plasma glucose homeostasis in both db/db mice and Zucker Diabetic Fatty (ZDF) rats (Parsons *et al.* 2007). Similarly, intramuscular injection of a hybrid cDNA encoding a GLP-1/Fc peptide normalized glucose tolerance by enhancing insulin secretion and suppressing glucagon release in db/db mice (Kumar *et al.* 2007). Delivery of GLP-1 via an adenoviral vector (Ad-GLP-1) was highly effective at controlling blood glucose in ob/ob mice. Ad-GLP-1 normalized blood glucose, improved β-cell function and insulin sensitivity and reduced hepatic glucose production (Lee *et al.* 2007). In our study a classic eukaryotic expression vector pcDNA3 was used for secretory GLP-1 expression, and robust expression of GLP-1 mRNA was detected in the muscle seven days after transfection.

It may be surprising that LG rats received low dose (60 μg) of plasmid DNA actually expressed higher GLP-1 mRNA, compared to those transfected with high dose (120 μg). Actually, expression efficiency is not always correlated positively with the amount of plasmid transfected, it is affected by numerous factors including species, age of animal, the type of the tissue, etc. Previous studies used 50 μg to 100 μg of plasmid DNA for intramuscular transfection in mice (Blomberg *et al.* 2002, Samakoglu *et al.* 2001), and more than 100 μg of

plasmid DNA was needed for adult rats (Watanabe *et al.* 2001). In the present study, higher expression efficiency was achieved with 60 μg of plasmid DNA in 2-day-old rat pups.

We observed significant growth retardation in LG group with higher ectopic GLP-1 expression in the muscle. Rats in HG group were also smaller compared to VP rats, but the difference did not reach statistical significance. This finding was in agreement with the previous report that daily subcutaneously injection of Ex-4 during first 6 days after birth caused significant lower body weight in both Intrauterine Growth Retarded (IUGR) and control Sprague Dawlay (SD) rats from 2 weeks of age through adulthood (Stoffers *et al.* 2003). However, the effects of neonatal treatment of GLP-1 or its analogs on animal growth are not consistent. Spontaneously diabetic GK rats (Tourrel *et al.* 2002) and streptozotocin (STZ)-induced diabetic rats (Tourrel *et al.* 2001) did not show body weight reduction in response to neonatal treatment of GLP-1 or Ex-4. Since GK rats and STZ-induced diabetic rats share the common feature of severely impaired insulin secretion, it is possible that the growth retardation caused by neonatal GLP-1 is mediated by alterations in serum insulin concentration.

In the present study, the growth retardation was accompanied with significantly reduced serum insulin concentration. This contradicts with the widely accepted notion that GLP-1 or Exendin-4 is potent secretogogues of insulin (Bai *et al.* 2005, D'Alessioo *et al.* 1994, Macdonald *et al.* 2002). It has to be mentioned, however, the insulinotropic capacity of neonatal GLP-1 is seen mostly on diabetic models. Our results indicate an opposite effect of neonatal GLP-1 on adult serum insulin levels in healthy rats. Interestingly, despite reduced serum insulin concentration, blood glucose level maintained stable in the present study. This

257 agrees with the previous report showing unaltered fasting blood glucose in adult SD rats
258 neonatally treated with Ex-4 (Stoffers *et al.* 2003). The ability to maintain glucose
259 homeostasis under reduced insulin may imply increased insulin sensitivity in peripheral
260 tissues (Gedulin *et al.* 2005).

261 Skeletal muscle is one of the most important target tissues for insulin action and plays an
262 important role in the regulation of glucose homeostasis. Increased insulin sensitivity in
263 skeletal muscle would mean enhanced glucose uptake and utilization (Lee *et al.* 2007). It is
264 well known that metabolic properties of skeletal muscle are closely associated with myofiber
265 type composition (Zierath and Hawley 2004). Muscles located at different part of the body
266 exhibit different myofiber type composition, i.e. different percentages of four major myofiber
267 types with type-specific expression of corresponding myosin heavy chain (MyHC) isoforms
268 (Rivero *et al.* 1998), namely slow-oxidative type I, fast-oxidative type IIA, fast
269 oxidative-glycolytic type IIX and fast-glycolytic IIB. We revealed, for the first time, muscle
270 type-dependent effects of neonatal GLP-1 on MyHC isoform composition and metabolic gene
271 expression. Slow oxidative soleus muscle of LG rats demonstrated significantly increased
272 PGC-1α and GLUT4 mRNA expression, implicating increased oxidative capacity, whereas
273 fast glycolytic gastrocnemius muscle of LG rats showing a MyHC type switch from ⅡB to
274 Ⅰ, indicating increased glucose uptake and improved insulin sensitivity which contributes to
275 the maintenance of glucose homeostasis. Nevertheless, as the function of genes is determined
276 by its protein rather than mRNA, and the steady-state mRNA levels do not always in line with
277 the contents of proteins, it awaits further investigation whether PGC-1α and GLUT4 are
278 increased also at the level of protein in soleus muscle of LG rats.

The mechanism by which neonatal GLP-1 affects gene expression of adult skeletal muscle is not clear. Since there is still controversy with respect to GLP-1 receptor expression in skeletal muscle, we can only speculate that GLP-1 may act directly on skeletal muscle via PI3K/PKB and MAPKs pathways (Acitores *et al.* 2004), to program the metabolic characteristics, or indirectly through the mediation of insulin, as previous studies hinted a link between MyHC composition and insulin concentration (Venojarvi *et al.* 2008, Oberbach *et al.* 2006).

In conclusion, the present study provided evidences that neonatal exposure of healthy pups to ectopic over expression of GLP-1 causes growth retardation with decreased serum insulin, as well as muscle type dependent modifications in MyHC type composition and metabolic gene expression in adult rats.

Acknowledgments

This work was supported by National Basic Research Program of China (2004CB117505).

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Table 1

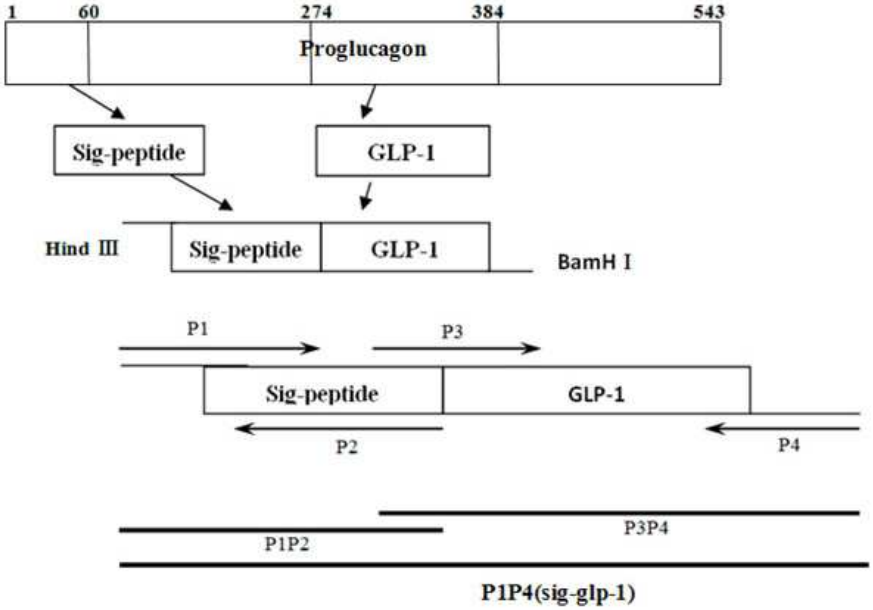
Myosin heavy chain composition in soleus and gastrocnemius muscle of 8-week-old rats neonatally treated with GLP-1

Muscle types		MyHC isoforms	VP	LG	HG
soleus	mRNA (fold change of control)	MyHC I	1.02±0.09	1.29±0.06	1.04±0.22
		MyHC 2A	1.01±0.16	0.78±0.14	0.74±0.11
		MyHC 2X	1.06±0.17	0.94±0.23	1.06±0.12
		MyHC 2B	1.00±0.02	1.25±0.07*	0.92±0.12
	Protein (%)	MyHC I	81.0±2.5	85.5±2.3	82.3±0.8
		MyHC 2A	19.0±2.5	14.4±2.3	17.7±0.8
gastrocnemius	mRNA (fold change of control)	MyHC I	1.07±0.20	1.08±0.15	0.69±0.17
		MyHC 2A	1.05±0.16	1.21±0.23	0.93±0.16
		MyHC 2X	1.00±0.04	0.99±0.06	0.99±0.09
		MyHC 2B	1.00±0.21	0.82±0.10	0.90±0.21
	Protein (%)	MyHC I	5.3±2.2	16.7±2.1*	6.0±3.8
		MyHC 2A/2X	30.7±1.6	42.0±3.8	26.8±8.1
		MyHC 2B	64.0±2.6	41.3±5.3*	67.2±11.6

Note: At 56 days of age, soleus and gastrocnemius of all rats were collected for detecting mRNA and protein level of MyHCs with real-time PCR and SDS-PAGE, respectively. The method of $2^{-\Delta\Delta Ct}$ was used to analyze the real-time PCR data, and the results were expressed as the fold of change relative to the mean value of VP rats. Result of SDS-PAGE was show in percentage of each MyHC isoforms. * $P < 0.05$ *vs* VP, n = 6.

411 Figure 1

Sequence
P1: 5'-GCCAAGCTTATGAAGACCGTTTACATCGTGGCTGGATTGTTTGTAATGCTGGTACAAG-3'
P2: 5'-AAGGTCCCTTCAGCATGCCTCTCAAATTCATCATGCTGCCAGCTGCCTTGTACCAGCA-3'
P3: 5'-GCAGCTGGCAGCATGATGAATTTGAGAGG-3'
P4: 5'-TATGGATCCTCATCCTCGGCCTTTCACC-3'



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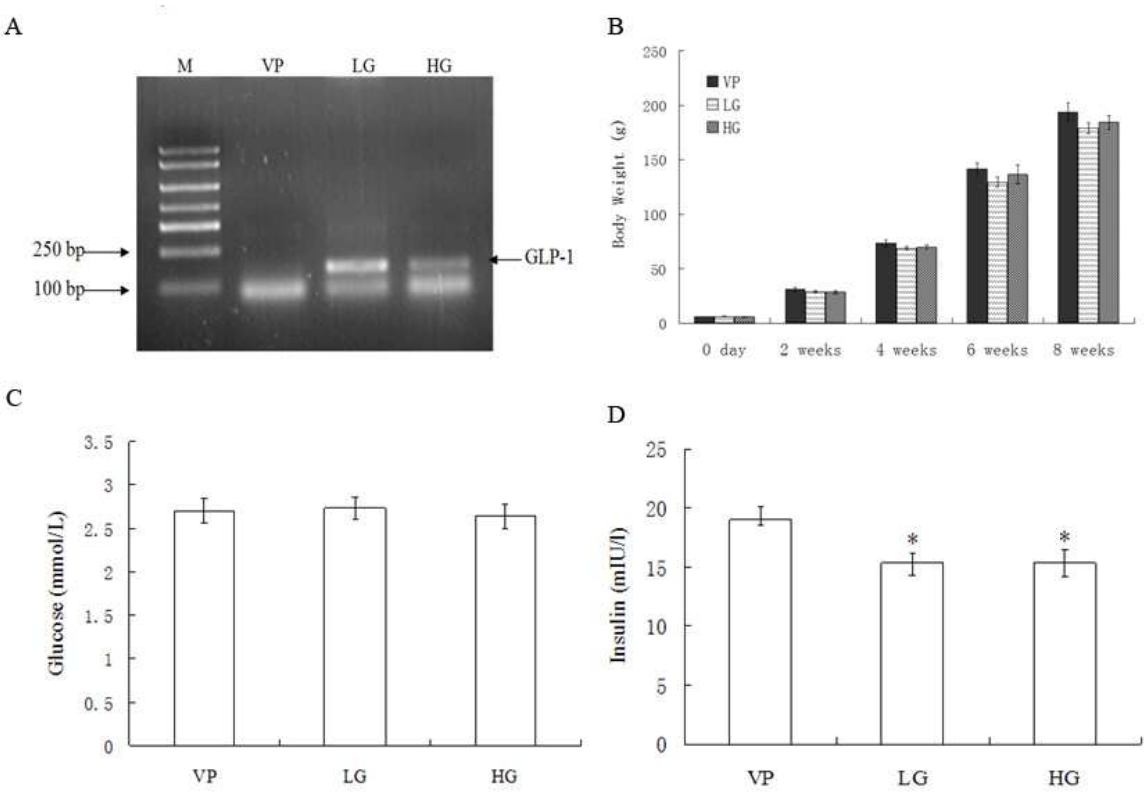
414 Figure 2



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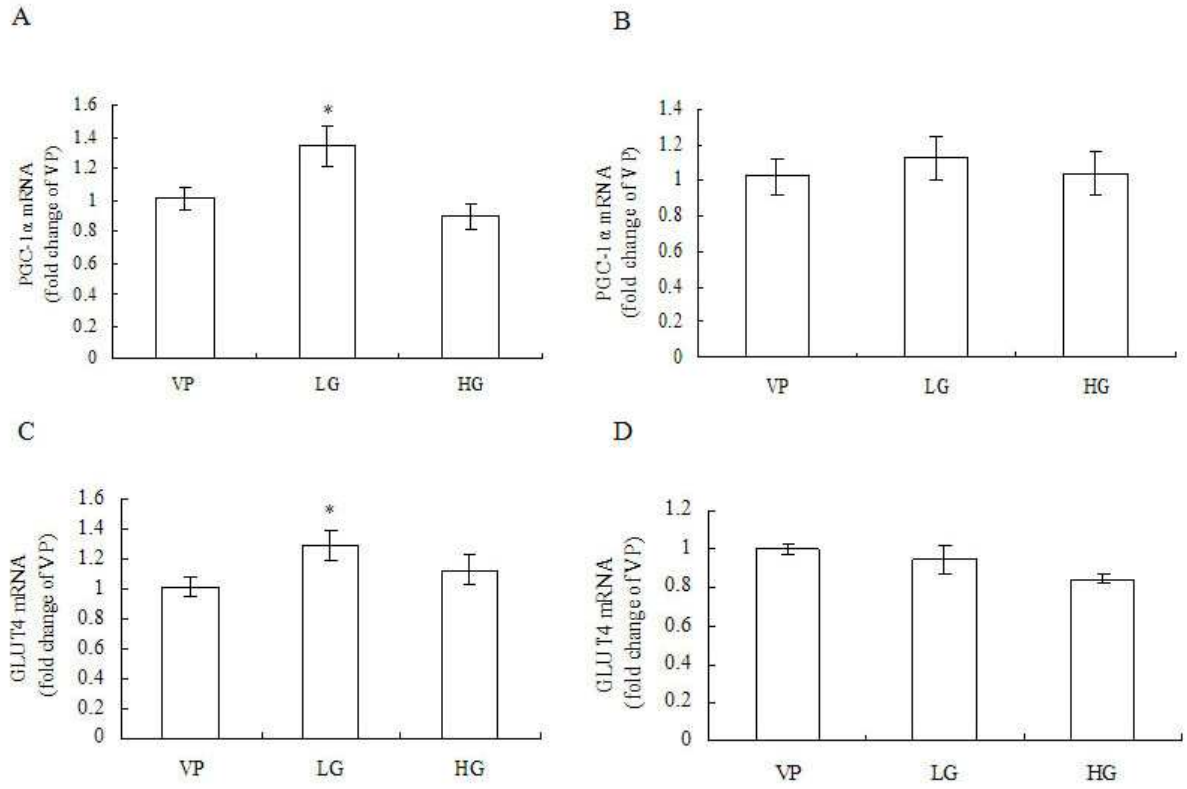
417 Figure 3



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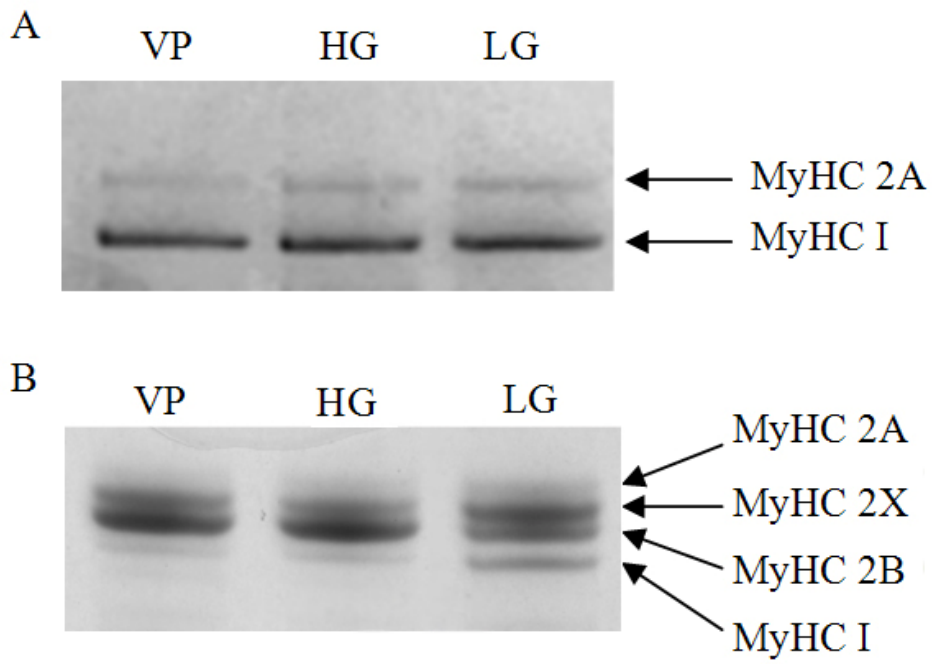
420 Figure 4



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Legend to Figures

Fig. 1 In vitro synthesis of DNA insert containing signal peptide coding sequence and full length cDNA of rat glp-1 (sig-glp-1).

Fig. 2 Effect of neonatal GLP-1 plasmid transfection on postnatal growth and adult serum glucose and insulin concentrations in rats. (A) GLP-1 mRNA expression. Muscle samples were taken from 5-6 pups per group seven days after transfection and pooled for detecting GLP-1 mRNA with RT-PCR; (B) Body weight. Body weight was recorded every two weeks from day 0 to 8 weeks in rats of different groups. The general effects of treatment and age on body weight were tested by General Linear Model for multivariate with SPSS 11.0 for Windows; (C) Serum glucose concentration; (D) Serum insulin level. Blood samples were collected at 56 days of age from carotid artery when 6 rats per group were sacrificed. Values are means ± SEM, * $P < 0.05$ *vs* VP, n = 6.

Fig. 3 Effect of neonatal GLP-1 plasmid transfection on expression of metabolic genes in skeletal muscle of rats. At 56 days of age, soleus and gastrocnemius muscles of six rats per group were collected after euthanasia. PGC-1α mRNA expression in soleus (A) and gastrocnemius (B); GLUT4 mRNA expression in soleus (C) and gastrocnemius (D) were measured with real-time PCR. The method of $2^{-\Delta\Delta Ct}$ was used to analyze the real-time PCR data. Values are means ± SEM, * $P < 0.05$ *vs* VP, n = 6.

Fig. 4 Representative photos of SDS-PAGE showing MyHC protein isoforms in soleus (A) and gastrocnemius (B) muscle of 56-day-old rats.