Threshold effects of glucose transporter-4 (GLUT4) deficiency on cardiac glucose uptake and development of hypertrophy

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Abstract

The aim of this study was to investigate the metabolic and structural consequences of a decrease in glucose transporter-4 (GLUT4) levels on the heart. The CreLoxP system was utilised to delete GLUT4 in muscle tissue including heart. The presence of the PGK-neoR cassette in the GLUT4-Lox mice resulted in reduced expression in all tissues to levels 15–30% of wild-type control mice. In mice expressing Cre recombinase, there was a further reduction of GLUT4 in cardiac tissue to almost undetectable levels. Cardiac glucose uptake was measured basally and during a euglycaemic/hyperinsulinaemic clamp using 2-deoxy-[1-¹⁴C]glucose. Insulin-stimulated glucose uptake was normal in hearts expressing 15% of normal GLUT4 levels but markedly reduced in mice with more profound reduction in GLUT4. Cardiac enlargement occurred only when GLUT4 levels were less than 5% of normal values. In heart there is a threshold level of GLUT4 above which insulin-stimulated glucose uptake is maintained. As little as 5% of normal GLUT4 levels expressed in heart is sufficient to prevent the development of cardiac hypertrophy.

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Introduction

Diabetes is associated with cardiac pathology in the absence of coronary artery disease or high blood pressure (Nielsen *et al.* 1997, Hirayama *et al.* 2000) and diabetic patients have poorer outcomes following myocardial infarction (Sprafka *et al.* 1991). An autopsy study found that cardiac weight was significantly greater in patients with both hypertension and diabetes compared with patients with hypertension or diabetes alone (van Hoeven & Factor 1990).

The cause of this diabetic cardiac pathology remains unclear; however, it is known that manipulation of nutrient supply to the heart results in changes in cardiac morphology. Rats in which

mitochondrial free fatty acid oxidation is inhibited with the carnitine palmitoyl transferase-1 inhibitor, 2-tetradecylglycidic acid, develop cardiac hypertrophy in the absence of changes in blood pressure (Litwin *et al.* 1990b, Bressler & Goldman 1993). Although the heart preferentially metabolises fat, human studies have shown that glucose metabolism becomes important when there are high energy needs such as with exercise (Gertz *et al.* 1988). Cardiac glucose uptake is mediated by the glucose transporters GLUT1 and GLUT4 (Kraegen *et al.* 1993). Both insulin and exercise increase cardiac glucose uptake by translocating GLUT4 to the cell surface (Slot *et al.* 1991). Streptozotocin-induced diabetes results in a dilated cardiomyopathy that is reversed by insulin (Litwin *et al.* 1990a) and is

associated with a 50% decrease in the insulin-responsive glucose transporter GLUT4 protein and mRNA, accompanied by a 53% reduction in glucose transport into cardiac sarcolemmal vesicles (Garvey *et al.* 1993).

To investigate the impact of reduced GLUT4-mediated glucose uptake, several animal models have been produced in which GLUT4 expression is genetically suppressed. Whole-body global deletion of GLUT4 resulted in a 2.5-fold increase in heart/body weight ratio with myocyte hypertrophy and interstitial fibrosis (Katz *et al.* 1995, Stenbit *et al.* 2000). These animals exhibited marked elevation of plasma insulin and disturbances in plasma glucose regulation. However, cardiac glucose uptake was not diminished, apparently due to a coincident upregulation of the GLUT1 transporter. In cardiac-specific GLUT4 knockout mice, cardiac hypertrophy was more modest (heart weight/body weight ratio increased 1.43-fold in males and 1.25-fold in female mice compared with wild-type mice) and there was no evidence of fibrosis (Abel *et al.* 1999). In this model, plasma glucose and insulin levels were found to be normal.

To further investigate the cardiac effects of impaired glucose uptake, our goal was to generate a new experimental model in which disruption of the GLUT4 transporter in both skeletal and cardiac muscle was achieved. We produced mice with deletion of GLUT4 using the CreLoxP system, selecting the muscle-specific α -actin gene promoter to drive the GLUT4 ablation. In what is now a well-described occurrence (Meyers *et al.* 1998, Nagy 2000, Xu *et al.* 2001), the presence of the PGK-neoR cassette in the GLUT4-Lox gene caused the 'knockdown' phenomenon, reducing GLUT4 gene transcription in many tissues of the $Lox^{+/+}$ mice, producing animals with more widespread, but less marked reduction in GLUT4 levels. The presence of Cre recombinase in GLUT4-Lox^{+/+}Cre^{+/-} mice further reduced GLUT4 in the heart to an almost undetectable level. Thus, the use of the CreLoxP system in this instance has produced two phenotypes which may be contrasted with the wild-type – a generalised suppression of GLUT4 expression ($Lox^{+/+}$) upon which may be superimposed a cardiac-specific virtual ablation of GLUT4 ($Lox^{+/+}$ Cre^{+/-}). We report here threshold effects of GLUT4 levels on *in vivo* glucose uptake and on the development of cardiac hypertrophy.

Materials and methods

Construction of GLUT4-Lox transgenic mice

The glucose transporter gene GLUT4 was cloned from a 129 SvJ mouse genomic library constructed in the FixII phage vector (Stratagene, La Jolla, CA, USA) by screening with two oligonucleotide probes corresponding to either end of the GLUT4 gene. The full length GLUT4 gene including the promoter was sub-cloned into the BamHI site of pSP72 (Promega) (pSP72-G4) and characterised by restriction enzyme digestion, Southern blotting and sequencing. Double-stranded oligonucleotides encoding *LoxP* sites flanked by restriction enzyme sites were assembled after the synthesis of single-stranded oligonucleotides (Geneworks, Adelaide, SA, Australia). The *Lox-I* oligonucleotide was inserted into the *AvaI* restriction site in the intron between exons 6 and 7, and *Lox-II* was inserted into the 3' non-coding region (Acc I) of exon 11. The gene for neomycin resistance (neoR) with a PGK promoter was inserted 5' to the *Lox-II* site. The resulting targeting vector (pG4.4) was sequenced to confirm the correct orientation of the *LoxP* sites and that the GLUT4 gene had been reconstituted. This vector was designed so that after homologous recombination and gene replacement, Cre-recombinase would remove the 5 kb region between the two *LoxP* sites, which includes exons 7–11 together with the PGK-neoR selection cassette (Fig. 1A).

Introduction of the modified GLUT4 gene into embryonal stem (ES) cells, and the production of mice incorporating this modification, was carried out using standard homologous recombination methodology (Hogan *et al.* 1986, Lieschke *et al.* 1994). Briefly, ES.W9.5 cells (derived from 129 SvJ mice) growing on a primary embryonal fibroblast feeder layer, were transfected with the targeting vector pG4.4 by electroporation at 270 V, 500 F. One thousand two hundred neomycin-resistant colonies were screened using a locus-specific PCR primer set designed to identify targeted integration of the vector (Fig. 1A). Another set of primers in the unmodified portion of the GLUT4 gene was used as control. Eight independent ES cell colonies containing the GLUT4-Lox transgene inserted at the homologous site were identified by PCR and confirmed using Southern analysis (strategy described in legend to Fig. 1A). ES cells with the GLUT4-Lox modified gene were injected into

Figure 1 (A) The GLUT4 gene, showing coding exons ($\square$) and *Lox* sites ($\triangleright$). Homologous recombination of the targeting fragment XbaI-BamHI into ES cell DNA was detected by PCR using oligonucleotides A and B. Oligonucleotides C and D amplify the endogenous GLUT4 gene. Correct homologous recombination was confirmed by Southern blotting of ES cell genomic DNA digested with BamHI and probed with Neo DNA visualising a 12 kb band. The length of homologous DNA flanking the *LoxP* sites was 1 kb at the 5'-end and 1.2 kb at the 3'-end of the targeting fragment. (B) Northern analysis of tissues from *Lox*^{+/+} and *Lox*^{+/+}Cre^{+/-} mice. RNA (10 μ g per gel track) was transferred to Hybond N+ membrane (Amersham) and probed with the Neo-coding region. In *Lox*^{+/+} samples, the probe binds to a band the same size as the one binding to the GLUT4 probe. This is consistent with production of a transcript from the targeted allele, extending through the GLUT4 mRNA and continuing in an antisense direction through the Neo gene. As anticipated, this transcript is not present in *Lox*^{+/+}Cre^{+/-} mice in the tissues expressing Cre recombinase under the α -actin promoter, i.e. in diaphragm, heart and muscle. The Northern was re-probed with an 18S probe to show even RNA loading. B, brown adipose tissue; D, diaphragm; H, heart; M, muscle. (C) Northern blot probed with a cDNA probe spanning exons 3–11 of the GLUT4-coding region. The right-hand panel, with the *Lox*^{+/+} and *Lox*^{+/+}Cre^{+/-} samples, needed to be exposed 10 times longer than the wild-type (Wt) samples to show bands of an equivalent intensity to the Wt. The 4.0 kb GLUT4+Neo transcript was markedly reduced in the tissues in which Cre activity was present. The filters were re-hybridised with the control 18S probe. B, brown adipose tissue; D, diaphragm; H, heart; M, muscle.

blastocysts from C57BL/6 × BA mice. Chimeric pups were crossed with C57BL/6 mice and offspring screened as described above for the ES cell colonies to identify germline transmission of the GLUT4-Lox modified gene. Breeding heterozygous GLUT4-Lox^{+/-} produced homozygous (Lox^{+/+}) animals.

Construction of α -actin-CRE expression vector and transgenic mice

The pBS-185 plasmid (Du Pont, Wilmington, DE, USA) was linearised with XhoI and the 2.3 kb HindIII fragment containing the human skeletal muscle-specific α -actin gene promoter from pHSA2000 CAT (Muscat & Kedes 1987) inserted to drive Cre recombinase expression. The α -actin skeletal muscle promoter directs expression at the end of gestation and postnatally, specifically in skeletal and cardiac muscle (Brennan & Hardeman 1993, Miniou *et al.* 1999). The Cre transgene was introduced into fertilised oocytes from C57BL/6 × CBA mice by pronuclear injection. Three foster mothers were implanted with oocytes containing the injected transgene, resulting in the birth of 29 live pups.

Production and maintenance of GLUT4-Lox^{+/+}Cre^{+/-} mice

Cre^{+/-} mice were mated with homozygous GLUT4-Lox^{+/+} mice. The resulting double-heterozygous GLUT4-Lox^{+/+}Cre^{+/-} animals were mated with GLUT4-Lox^{+/+} mice and the GLUT4-Lox^{+/+}Cre^{+/-} mice identified by PCR and Southern blotting. The colony was maintained by breeding GLUT4-Lox^{+/+}Cre^{+/-} with GLUT4-Lox^{+/+} and identifying the Cre^{+/-} mice by PCR. The genetic background of the GLUT4-Lox^{+/+}Cre^{+/-} mice is 56.25% C57BL/6, 37.5% 129Sv and 6.25% CBA. The wild-type controls are 100% C57BL/6.

Mice were housed in The University of Melbourne Department of Medicine, Royal Melbourne Hospital, and maintained on a 12 h light:12 h darkness cycle with lights on at 0600 until 1800 h. Room temperature was kept constant at 20 °C. Animals were fed standard mouse chow consisting of 20% protein, 75.5% carbohydrate and 4.5% fat. All animals had free access to drinking water. The investigation conforms with the Guide

for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996) and was approved by the Royal Melbourne Hospital animal ethics committee.

Metabolic studies

Plasma glucose concentration (mmol/l) was determined on a 25 μ l aliquot of plasma using a glucose oxidase assay in an automated glucose analyser (YSI, Inc., Yellow Springs, OH, USA). Plasma insulin concentration (μ U/ml) was determined using a commercially available RIA kit (Pharmacia). Mice were fasted overnight and anaesthetised with 60 mg/kg pentobarbital sodium. Catheters were placed in the right jugular vein for infusion and the left carotid artery for blood sampling. A tracheotomy was performed to prevent upper airways obstruction. Temperature was monitored with a rectal probe and maintained at 37 °C using a heating lamp. Whole-body glucose turnover and cardiac, skeletal muscle and fat glucose uptake were measured fasting and during a hyperinsulinaemic euglycaemic clamp in anaesthetised mice using [6-³H]glucose and 2-deoxy-[1-¹⁴C]glucose respectively as previously described (Veroni *et al.* 1991).

Blood pressure measurements

Mice were anaesthetised by i.p. injection containing ketamine (100 mg/kg), xylazine (2.5 mg/kg) and heparin (5000 IU/kg). All surgical procedures were performed under a dissecting microscope. An incision was made from the mandible to the sternum, followed by blunt dissection of the thyroid. The carotid artery was exposed and dissected away from both the internal jugular vein and the vagus nerve. The rostral end was ligated using a 5-0 suture and the caudal end was temporarily blocked using an aneurysm clip. The carotid artery was punctured using a 26 G needle, followed by insertion of a polyethylene catheter (PE 10), attached to a pressure transducer (MLT 844; ADInstruments, Castle Hill, New South Wales, Australia). Two sutures were placed around the artery to secure the catheter, and the aneurysm clip was removed (Nemoto *et al.* 2003). Blood pressure was recorded using a MacLab data acquisition system and Chart (v4.2) analysis software (ADInstruments).

Molecular studies

Western analysis was performed using standard methods. A rabbit polyclonal antibody specific for GLUT4 (R1159) (Kelada *et al.* 1992) and a GLUT1 antibody purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) were used. RNA and DNA preparations, and Northern and Southern blotting were performed using standard molecular biological techniques (Sambrook *et al.* 1989; Fig. 1).

Preparation of cardiac tissue for histology

Hearts from 14-week-old male mice were excised under anaesthesia (pentobarbitone sodium, 100 mg/kg, i.p.), washed in 0.9% saline solution and weighed. The cardiac weight index (CWI) was calculated for all hearts by normalising heart weight to body weight (mg/g). Hearts for histological assessment were fixed for 24 h at room temperature in 10% neutral buffered formalin. A 2 mm transverse tissue slice was cut at the base–apex midpoint, processed and paraffin embedded. Sections (5 µm) were stained for collagen using a modified version of Van Gieson's technique. Slides were viewed at 1× and 10× magnification under bright-field conditions and digital images acquired by a SPOT camera and software (Diagnostic Instrument Inc, Michigan, USA). Chamber and wall dimensions were measured from images using ImagePro-Plus (Media Cybernetics, version 3.0).

Statistics

Unless otherwise specified, results are presented as means ± S.E.M. Student's two-tailed *t*-test and one- or two-way ANOVA were applied.

Results

Assessment of Cre expression and function in GLUT4-Lox^{+/+}Cre^{+/-} mice

Cre mRNA was shown by Northern analysis to be expressed only in skeletal muscle and heart. Cre expression in heart was less than 1% of that in skeletal muscle but was consistently detected (data not shown). Probing Northern blots for Neo, which should be deleted if Cre is active, showed that

Cre is functional in the tissues in which it is expressed (Fig. 1B).

Assessment for evidence of GLUT4 gene deletion

Expression of GLUT4 was assessed using Northern and Western blotting. Both methods demonstrated that GLUT4-Lox^{+/+} mice had reduced GLUT4 levels when compared with related wild-type controls. The mRNA transcript produced by GLUT4-Lox^{+/+} mice was larger than the wild-type transcript (2.8 kb), and probably comprised the wild-type messenger extended through the Neo gene in an antisense direction (Fig. 1B and C). The presence of the PGK-neoR selection cassette in the GLUT4 allele caused the gene expression to be 'knocked down' (Fig. 1C; Nagy 2000). The left panel in Fig. 1C shows that in Lox^{+/+} mice, GLUT4-Neo mRNA was transcribed at a much lower rate in all tissues tested compared with the GLUT4 expressed in the wild-type mice. The right panel in Fig. 1C underwent a longer exposure and GLUT4-Neo mRNA in Lox^{+/+} mice is shown again. In Lox^{+/+}Cre^{+/-} animals GLUT4-Neo mRNA is further reduced in tissues which express Cre recombinase (diaphragm, heart and skeletal muscle), but not in brown adipose tissue. Western blotting confirmed that in Lox^{+/+} mice, GLUT4 protein levels are reduced in muscle tissue (30% of normal) (Fig. 2A) and in white (16%) (Fig. 2B) and brown (20%) (data not shown) adipose tissue. Other studies have also reported that this phenomenon is caused by the presence of the PGK-neoR cassette (Meyers *et al.* 1998, Abel *et al.* 1999, Nagy 2000, Rucker *et al.* 2000, She *et al.* 2000).

In heart, the presence of the PGK-neoR cassette reduced GLUT4 protein levels to approximately 15% of wild-type mice. The presence of Cre recombinase reduced GLUT4 further to almost undetectable levels (Fig. 3A). In previous models, tissue-specific upregulation of GLUT1 (1.5- to 3-fold) coincident with GLUT4 downregulation has been reported in heart but not other tissues (Katz *et al.* 1995, Zisman *et al.* 2000). In our model there was no significant GLUT1 upregulation in either skeletal muscle (Lox^{+/+} 96 ± 16%, Lox^{+/+}Cre^{+/-} 96 ± 1%) or heart (Lox^{+/+} 110 ± 13%, Lox^{+/+}Cre^{+/-} 130 ± 20%) above wild-type (*n* = 18 in each group).

Figure 2 Western blot analysis of white quadriceps (A) and white adipose tissue (B) showing that in skeletal muscle and fat, both Lox^{+/+}Cre^{-/-} and Lox^{+/+}Cre^{+/-} mice have equal reduction in GLUT4 levels compared with C57BL/6 wild-type (Wt) mice. (Means±S.E.M., $n=6-12$ in each group.)

Physiological assessment of GLUT4-deficient animals

Fourteen-week-old male GLUT4-deficient mice were compared with age-matched wild-type C57BL/6 control mice. The GLUT4-deficient animals had normal body weights (28.6 ± 0.9 g, Lox^{+/+} 29.1 ± 1.4 g and Lox^{+/+}Cre^{+/-} 28.2 ± 0.9 g). Fasting glucose was not elevated but insulin levels were double in the GLUT4-deficient animals ($P=0.003$) indicating insulin resistance (Table 1).

Figure 3 (A) Western blots of heart tissue. Lox^{+/+} mice had profoundly reduced GLUT4 levels, to approximately 15% of normal. Cre expression caused a further reduction in GLUT4 to very low levels ($n=6-12$ in each group). (B) Basal ($n=3-4$) and insulin-stimulated ($n=6-9$) 2-deoxyglucose uptake in heart. * $P<0.05$ vs basal, # $P<0.05$ vs wild-type (Wt), + $P<0.05$ vs Lox^{+/+}. (Means±S.E.M.)

During the hyperinsulinaemic clamp, plasma glucose levels were higher in the GLUT4-deficient mice compared with wild-type but insulin levels were not different in the three groups (Table 1).

Whole-body glucose kinetics showed that the basal rate of glucose uptake and metabolic clearance rate (MCR) of glucose were not different in the three groups of mice (Table 1). However, in the insulin-stimulated state, MCR was lower in the Lox^{+/+} and Lox^{+/+}Cre^{+/-} mice (Table 1) indicating a defect in insulin-mediated glucose uptake in GLUT4-deficient mice.

Table 1 Glucose, insulin, rate of glucose disappearance (Rd) and metabolic clearance rate (MCR) in male wild-type (C57/BL/6J), GLUT4-Lox^{+/+} and Lox^{+/+}Cre^{+/-} mice during glucose turnover studies. Means±S.E.M.

Parameter	Basal (n=4)			Clamp (n=6–9)		
	Wild-type	Lox ^{+/+}	Lox ^{+/+} Cre ^{+/-}	Wild-type	Lox ^{+/+}	Lox ^{+/+} Cre ^{+/-}
Glucose (mmol/l)	7.1±0.5	7.6±0.4	7.6±1.3	6.1±0.6	10.1±0.9*	13.1±1.0*
Insulin (μU/ml)	19.1±3.2	51.6±4.6*	64.5±14.4*	255±39	215±47	201±30
Rd (μmol/kg per min)	64.7±8.3	54.8±4.0	63.9±14.7	146±18 [#]	119±15 [#]	122±14 [#]
MCR (ml/kg per min)	9.1±0.7	7.4±0.8	8.1±1.1	25.1±4.1 [#]	13.0±1.8**	11.4±0.8**

*P<0.05 vs wild-type; [#]*P*<0.05 vs respective basal.

Glucose uptake in muscle and white and brown adipose tissue is shown in Table 2. Basal glucose uptake tended to be higher in Lox^{+/+} and Lox^{+/+}Cre^{+/-} possibly in response to the higher fasting insulin in these animals. When insulin levels were equalised at a physiological hyperinsulinaemic level during the clamp, white quadriceps and white and brown adipose tissue showed reduced glucose uptake in mice expressing reduced GLUT4 levels (Table 2).

In the heart, basal glucose uptake was higher in GLUT4-Lox^{+/+} mice compared with wild-type, again reflecting the fasting hyperinsulinaemia in these animals. The threshold effect of GLUT4 on insulin-stimulated glucose uptake is clearly demonstrated in the heart. It can be seen that 15% of GLUT4 is sufficient to allow normal insulin-stimulated glucose uptake (Fig. 3B). However, the almost complete absence of GLUT4 in the Lox^{+/+}/Cre^{+/-} mice reduced insulin-stimulated glucose uptake.

Table 2 2-Deoxy-glucose uptake (μmol/100 g/min) in white quadriceps (WQ), white adipose tissue (WAT) and brown adipose tissue (BAT). Means±S.E.M.

	Wild-type	Lox ^{+/+}	Lox ^{+/+} Cre ^{+/-}
Basal (n=3–4)			
WQ	0.6±0.1	1.2±0.3	1.4±0.2*
WAT	0.2±0.1	0.5±0.2	1.2±0.1**
BAT	1.0±0.4	2.3±0.7	4.5±1.4*
Clamp (n=6–9)			
WQ	4.8±0.5	4.7±0.8	2.6±0.4**
WAT	3.1±0.6	1.4±0.4*	2.3±0.5
BAT	37.3±7.9	3.5±0.8*	5.9±2.4*

*P<0.05 vs wild-type; [#]*P*<0.05 vs Lox^{+/+}.

Blood pressure measurement

Blood pressure was measured in age- and sex-matched Lox^{+/+} (that have no hypertrophy) (*n*=11) and in Lox^{+/+}Cre^{+/-} mice (with cardiac hypertrophy) (*n*=9). Table 3 shows that cardiac hypertrophy was not associated with elevated mean arterial pressure. The values obtained for mean arterial pressure were comparable with those previously reported in anaesthetised wild-type C57BL6 mice at similar heart rate (Nemoto *et al.* 2002).

Threshold effects of GLUT4 deficiency on cardiac morphology

CWIs for the three animal groups (wild-type, Lox^{+/+} and Lox^{+/+}Cre^{+/-}) are shown in Fig. 4A. In Lox^{+/+} mice, a modest 15% increase in CWI is seen in association with the 85% reduction in GLUT4 level. In the Lox^{+/+}Cre^{+/-} mice there is a dramatic hypertrophy (approximately 80% increase in CWI) associated with a loss of 99% of GLUT4 protein. There was no difference in heart to body weight ratio between male and female animals and the data for these mice were pooled.

To explore the relationship between GLUT4 expression and CWI further, paired data were analysed. The plot of cardiac GLUT4 levels and CWI in Lox^{+/+} and Lox^{+/+}Cre^{+/-} mice (Fig 4B) shows that there is a threshold effect in this relationship. Suppression of GLUT4 protein levels in the heart to below 5% of wild-type levels is linked with a grossly hypertrophic phenotype. It is evident that there is no overlap between the Lox^{+/+} and Lox^{+/+}Cre^{+/-} distributions of these two variables.

Table 3 Age, heart weight to body weight ratio (HW/BW), maximal heart rate (HRmax) and mean arterial pressure measured maximal heart rate (MAP@HRmax) in $Lox^{+/+}$ and $Lox^{+/+}Cre^{+/-}$ mice. Means $\pm$ S.E.M.

Mice	Age (weeks)	HW/BW	HRmax (bpm)	MAP@HRmax (mmHg)
$Lox^{+/+}$ (n=11)	29.5 $\pm$ 6.1	5.5 $\pm$ 0.2	390 $\pm$ 25	86.7 $\pm$ 6.4
$Lox^{+/+}Cre^{+/-}$ (n=9)	33.0 $\pm$ 8.7	8.2 $\pm$ 0.3**	352 $\pm$ 27	74.8 $\pm$ 5.2

** $P<0.01$.

Histological evaluation of Van Gieson-stained sections showed that, compared with the wild-type and $Lox^{+/+}$ mice, the hypertrophic phenotype of the hearts from the $Lox^{+/+}Cre^{+/-}$ mice was accompanied by a marked interstitial fibrosis and fibroblast hyperplasia. Analysis of the chamber

dimensions demonstrated that there was a 65% increase in both the left and right ventricular wall thickness of the $Lox^{+/+}Cre^{+/-}$ mice ($P<0.001$), with a proportional increase in the left ventricular chamber (Fig. 5). These results indicate that the threshold effect of GLUT4 reduction below 5% of wild-type control level is associated with marked hypertrophic and fibrotic remodelling of the myocardium.

A

B

Figure 4 (A) Percent heart to body weight ratio in wild-type (Wt), $Lox^{+/+}$ and $Lox^{+/+}Cre^{+/-}$ mice. * $P<0.05$ vs wild-type, ** $P<0.001$ vs wild-type, # $P<0.001$ vs $Lox^{+/+}$. (B) Plot of GLUT4 levels expressed as arbitrary units vs CWI in wild-type (open squares), $Lox^{+/+}$ (grey squares) and $Lox^{+/+}Cre^{+/-}$ (black squares). (Means $\pm$ S.E.M.) (n=8–16).

Discussion

Diabetes, a condition that is estimated to affect over 100 million people worldwide and whose prevalence is increasing rapidly, is associated with cardiac abnormalities independently of associated hypertension and probably due to the metabolic derangement present. Both functional (Shapiro *et al.* 1981) and structural (van Hoeven & Factor 1990) abnormalities have been described. Although there may be several different mechanisms for these diabetes-related cardiac abnormalities, one possibility is a reduction in glucose availability secondarily to a marked reduction in the insulin-responsive glucose transporter (GLUT4) expression (Garvey *et al.* 1993). Consistent with the deficiency of energy substrates as the cause of cardiac abnormalities, is the fact that inhibition of free fatty acid oxidation, the heart’s major energy source, results in cardiac enlargement (Litwin *et al.* 1990*b*).

In this study we have investigated the relationship between GLUT4 transporter expression, glucose uptake and cardiac hypertrophy in a novel murine model produced using the CreLoxP system. In heart, where there was a difference in GLUT4 levels between the GLUT4- $Lox^{+/+}$ and GLUT4- $Lox^{+/+}Cre^{+/-}$ mice, insulin-stimulated glucose uptake was normal in mice with 15% of wild-type GLUT4 levels, but was reduced in mice with near

Figure 5 Collagen-specific Van Gieson-stained transverse sections of ventricles in wild-type (a), $Lox^{+/-}$ (b) and $Lox^{+/-}Cre^{+/-}$ (c) mice. Histology showing that excess fibrosis in ventricular tissues is present only in hearts from $Lox^{+/-}Cre^{+/-}$ mice (f), but not in wild-type (d) or $Lox^{+/-}$ (e) animals. LV, left ventricle.

complete absence of this transporter. These data suggest that in active cardiac muscle, there is a threshold above which GLUT4 deficiency does not impair insulin-stimulated glucose uptake. It has been reported that in the global knockout model, GLUT4-null hearts have the same rate of 2-deoxyglucose-6-phosphate accumulation as controls when measured in the isolated perfused state (Stenbit *et al.* 2000). In agreement with this, we could not detect any significant difference in basal glucose uptake in our mice with complete GLUT4 deficiency. A reduction in glucose uptake appeared in our GLUT4-null mice when the hearts were stimulated with insulin *in vivo* (Fig. 3B).

It is interesting that in the $Lox^{+/-}$ mice that respond normally to insulin, basal glucose uptake was elevated compared with wild-type, possibly reflecting a normal response to the hyperinsulinaemia present in these animals (Table 1). In contrast, in $Lox^{+/-}Cre^{+/-}$ mice that have no GLUT4 expression, basal glucose transport was not elevated. The normal cardiac basal glucose uptake in the $Lox^{+/-}Cre^{+/-}$ animals is consistent with the normal levels of GLUT1 detected. This is different to the heart-specific GLUT4 knockout model in which it was shown that a 3-fold increase in basal

glucose uptake was associated with a 3-fold increase in GLUT1 expression (Abel *et al.* 1999). Why our model does not show enhanced basal glucose uptake and increased expression of GLUT1 is not clear. A possible explanation is the different genetic background of the animals.

It is unlikely that anaesthesia influenced the relative findings of glucose uptake and insulin action in this study. The effects of anaesthesia on basal and insulin-stimulated glucose uptake have been investigated in rats. It was found that anaesthesia reduced insulin-stimulated glucose uptake in soleus and more modestly in plantaris, red quadriceps and red gastrocnemius but it did not reduce uptake in white quadriceps or in white adipose tissue (James *et al.* 1986). In addition any effects of anaesthesia would be the same in all three groups studied.

The threshold effects of GLUT4 levels are clearly demonstrated in the effects of GLUT4 deficiency on cardiac size. Previous models of complete GLUT4 deficiency, both global (Stenbit *et al.* 2000) and cardiac-specific (Abel *et al.* 1999), have demonstrated cardiac hypertrophy. In global knockout mice with no GLUT4 expression in any tissue, there was a 2.5-fold increase in cardiac size

with myocyte hypertrophy in all chambers (Katz *et al.* 1995). These mice, like those described in this report, were normotensive (Stenbit *et al.* 2000). In heart-specific GLUT4-deficient mice, the hypertrophy was more modest (1.4-fold increase) (Abel *et al.* 1999), leading the authors to speculate that the metabolic abnormalities present in mice with more widespread deficiency of GLUT4 might contribute to cardiac enlargement (Abel *et al.* 1999). However, our data do not support such a conclusion. $\text{Lox}^{+/-}$ and $\text{Lox}^{+/+}\text{Cre}^{+/-}$ animals had similar deficiency of GLUT4 levels in peripheral tissues with a consequent similar level of hyperinsulinaemia and a similar reduction in MCR compared with wild-type mice (Table 1). They had, however, a very different degree of cardiac enlargement, which appears to be related to the severity of GLUT4 deficiency in the heart. We show here that to induce marked cardiac hypertrophy, GLUT4 levels need to be reduced to less than 5% of normal (Fig. 3). In this situation, the cardiac hypertrophy is associated with a marked increase in interstitial collagen.

It is well known that the presence of the PGK-neoR cassette in a gene may create a 'knockdown' allele with low and variable levels of transcription (Nagy 2000). Several groups have reported this phenomenon. Thus, She *et al.* (2000), studying the role of phosphoenolpyruvate carboxykinase (PEPCK) in hepatic energy metabolism, produced both $\text{PEPCK}^{\text{Lox/Lox}}$ and $\text{PEPCK}^{\text{Lox+Neo/Lox+Neo}}$ mice and found that the presence of the Neo cassette between exons 3 and 4 reduced PEPCK expression in both liver and kidney to about 10–20% of normal. In the GLUT4-Lox mice used to produce cardiac- (Abel *et al.* 1999) and muscle- (Zisman *et al.* 2000) specific GLUT4 deletion, GLUT4 expression in white adipose tissue was reduced by up to 50%. It is not clear why in this model the Neo interfered with GLUT4 expression in fat but not in muscle as it did in our model. We have also demonstrated that Cre recombinase further reduces GLUT4 mRNA levels in the tissues it is expressed in (Fig. 1C). Despite the fact that there appears to be little difference in GLUT4 mRNA levels in these tissues (Fig. 1C), GLUT4 protein levels were further reduced in heart but not in other muscle tissues. This suggests that GLUT4 protein turnover may be faster in heart.

In conclusion, we have established that in cardiac muscle, GLUT4 levels need to be profoundly

reduced to impair insulin-stimulated glucose uptake when measured at physiological insulin levels in anaesthetised mice. Additionally we have demonstrated that gross cardiac enlargement will be evident only when GLUT4 levels are reduced to below 95% of normal values.

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