RESEARCH

Fructose bisphosphatase 2 overexpression increases glucose uptake in skeletal muscle

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Abstract

Skeletal muscle is a major tissue for glucose metabolism and can store glucose as glycogen, convert glucose to lactate via glycolysis and fully oxidise glucose to CO_2. Muscle has a limited capacity for gluconeogenesis but can convert lactate and alanine to glycogen. Gluconeogenesis requires FBP2, a muscle-specific form of fructose bisphosphatase that converts fructose-1,6-bisphosphate (F-1,6-bisP) to fructose-6-phosphate (F-6-P) opposing the activity of the ATP-consuming enzyme phosphofructokinase (PFK). In mammalian muscle, the activity of PFK is normally 100 times higher than FBP2 and therefore energy wasting cycling between PFK and FBP2 is low. In an attempt to increase substrate cycling between F-6-P and F-1,6-bisP and alter glucose metabolism, we overexpressed FBP2 using a muscle-specific adeno-associated virus (AAV-tMCK-*FBP2*). AAV was injected into the right tibialis muscle of rats, while the control contralateral left tibialis received a saline injection. Rats were fed a chow or 45% fat diet (HFD) for 5 weeks after which, hyperinsulinaemic-euglycaemic clamps were performed. Infection of the right tibialis with AAV-tMCK-*FBP2* increased FBP2 activity 10 fold on average in chow and HFD rats ($P<0.0001$). Overexpression of FBP2 significantly increased insulin-stimulated glucose uptake in tibialis of chow animals (control 14.3 ± 1.7; FBP2 17.6 ± 1.6 µmol/min/100 g) and HFD animals (control 9.6 ± 1.1; FBP2 11.2 ± 1.1µmol/min/100 g). The results suggest that increasing the capacity for cycling between F-1,6-bisP and F-6-P can increase the metabolism of glucose by introducing a futile cycle in muscle, but this increase is not sufficient to overcome muscle insulin resistance.

Key Words

- insulin resistance
- skeletal muscle
- fructose-1,6-bisphosphatase
- glucose metabolism

Journal of Endocrinology (2018) **237**, 101–111

Introduction

Reduced levels of glycolysis and glycogen synthesis are a well characterised feature of skeletal muscle in type 2 diabetes (Bouche *et al.* 2004, Abdul-Ghani & DeFronzo 2010). In general, the flux through glycolysis is determined by the opposing enzymes phosphofructokinase (PFK) in the glycolytic pathway, and fructose-1,2-bisphosphatase (FBPase) in the gluconeogenic pathway as well as the activity of glycogen synthase in the glycogenic pathway. Muscle performs glycolysis to provide energy for contraction at rates that vastly exceed the synthesis of new glucose and glycogen from lactate or other 3 carbon precursors via gluconeogenesis. This predominance of glycolytic over gluconeogenic pathways in muscle is largely governed by the low total activities of gluconeogenic enzymes in muscle (Newsholme & Leech 1984).

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https://doi.org/10.1530/JOE-17-0555

Newsholme and Challiss in 1980s hypothesised that the low level of FBPase activity in the muscle was a way of reducing energy wastage due to futile cycling between muscle FBPase (FBP2) and PFK-M (the muscle isoform of PFK) (Newsholme 1978, Newsholme *et al.* 1983). Futile cycling between these two enzymes has been shown to increase under physiological conditions such as exercise, hyperthyroidism or insulin stimulation in rodents and humans (Challis *et al.* 1985, Shulman *et al.* 1985). Furthermore, Newsholme proposed that futile cycling between PFK-M and FBP2 in the muscle increases metabolic sensitivity in adaptation to exercise training (Newsholme & Leech 1984). Exercise has been shown to increase the fructose-6-phosphate (F-6-P) to fructose-1,6-bisphosphate (F-1,6-bisP) ratio in frogs and humans, indicating a possible increase in cycling between PFK-M/FBP2 (Cheetham *et al.* 1986, Krause & Wegener 1996). Additionally, PFK-M and FBP2 transcription was induced with Nur77 overexpression, a transcription factor known to mediate the effects of exercise (Chao *et al.* 2007).

Therefore, increased levels of FBP2 activity may increase futile cycling and metabolic sensitivity to increase energy demand and consequently increase substrate oxidation. It has previously been shown that futile cycling in other pathways may be effective in creating an energy demand due to ATP wastage and increasing substrate usage (Elia *et al.* 1987).

Therefore, we hypothesised that AAV-mediated FBP2 overexpression in the skeletal muscle may increase gluconeogenic flux to glycogen and futile cycling, which could subsequently improve glucose uptake in the muscle.

Materials and methods

Vector construction and AAV propagation

The human *FBP2* was synthesised by Genscript, USA in the pcDNA3.1+ vector and then further subcloned into the pAMCBA vector backbone under the muscle-specific promoter tMCK. The plasmid was then used to generate a titre of 4.79×10^{13}/mL genome copies of AAV9-tMCK-*FBP2* by University of Pennsylvania Vector Core Facility (Philadelphia, PA, USA).

Animal maintenance

A titre of $2–3 \times 10^{11}$ genome copies of AAV9-tMCK-*FBP2* was introduced into the tibialis/EDL muscle bundle of the right (test) leg of young wistar rats (60 g) using 6×50 µL injections along the muscle. Saline (6×50 µL) was injected in the left (control) leg. Pilot studies using AAV-GFP determined that this methodology resulted in transfection of 60–70% of all fibres in the tibialis/EDL muscle bundle (data not shown). The rats were either fed a standard chow diet (8% calories from fat, 21% calories from protein, 71% calories from carbohydrate) from Gordon's Specialty Stock Feeds, NSW, Australia or a high-fat diet (HFD; 45% calories from fat (lard), 20% calories from protein, 35% calories from carbohydrates, based on Rodent Diet #D12451 Research Diets, Inc., New Brunswick, NJ, USA). The animals were maintained on a 12:12-h light–darkness cycle at 22 ± 0.5°C, with free access to food and water for four weeks, after which, the animals were used for either *ex vivo* or *in vivo* experiments. All experimental procedures were approved by the Garvan Institute / St. Vincent's Hospital Animal Experimentation Ethics Committee and were in accordance with the National Health and Medical Research Council of Australia Guidelines on Animal Experimentation.

Ex vivo muscle strip isolation

Four weeks after injection with the AAV, the animals on chow or HFD were euthanised and the EDL from each leg was stripped from tendon to tendon with a 27-gauge needle as described in Bruce *et al.* (2009). The EDL muscle comprises type I, type IIa, type IIx fibres to form the proximal red EDL muscle bundle and distal type IIb fibres, which appear white (Lexell *et al.* 1994, Delp & Duan 1996). These red and white EDL bundles were stripped longitudinally into two red and two white muscle strips, such that each muscle strip weighed 20–30 mg. The strips were incubated in pre-warmed, pre-gassed (95% O_2 and 5% CO_2) KHB (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 15 mM $NaHCO_3$, with 2% fatty acid free BSA, 5 mM glucose and 0.5 mM oleic acid) for 30 min with agitation at 30°C. Thereafter, the strips were transferred into vials containing the same buffer as well as 2 µCi/mL [^{3}H]-2-deoxy-glucose and 1 µCi/mL [^{14}C]-glucose. Muscle strips were incubated for 1.5 h to estimate glucose uptake (conversion of [^{3}H]-2-deoxy-glucose to [^{3}H]-2-deoxy-glucose phosphate), rate of glucose oxidation (conversion of [^{14}C]-glucose to $^{14}CO_2$), rate of glycolysis (conversion of [^{14}C]-glucose to [^{14}C]-lactate) and rate of glycogen synthesis (conversion of [^{14}C]-glucose to [^{14}C]-glycogen) as described below. The $^{14}CO_2$ gas released by the muscle reacted with NaOH in the centre well to form $NaH^{14}CO_3$, which was trapped in the centre

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well, while the lactate was released in the media. At the end of the incubation period, muscle strips were rinsed in saline, blotted dry and snap frozen in liquid nitrogen to determine glucose uptake and rate of glycogen synthesis.

The NaOH from the centre well was mixed with scintillation fluid to measure $^{14}CO_2$ by liquid scintillation counting (Beckman LS6000, Beckman Instruments, Fullerton, CA, USA), to give a measure of total [^{14}C]-glucose converted to $^{14}CO_2$.

Lactate in the media

The lactate in the media was measured by a modified ion exchange method (Hammerstedt 1980). The acidified media was neutralised with 1 M KOH and centrifuged at 16,200 ***g*** for 10 min to remove the salt precipitate. The supernatant was then run through an ion exchange acetate form of AG 1-X8 resin column, which was washed three times with 3 mL of water and the flow through was discarded. The lactate was eluted with three washes with 3 mL 0.5 M formic acid. The flow through collected was used to count radiolabelled lactate by liquid scintillation counting (Beckman LS6000, Beckman Instruments).

In vivo hyperinsulinaemic-euglycaemic clamp

Under isoflurane anaesthesia, and aseptic conditions, cannula were inserted into the right jugular vein and left carotid artery of animals fed chow or HFD. After the animals had recovered from the surgery (~1 week), they were fasted for 5 h and a hyperinsulinaemic-euglycaemic clamp was performed as described by James *et al.* (1985). Briefly, the cannulae from the concious rats were connected to an infusion line or sampling line. A constant insulin (0.3 U/kg/h) infusion was then commenced and 30% glucose was infused at a variable rate to achieve a stable blood glucose level (5 mM), which was determined using blood taken from the sampling line. After a steady state was established, a bolus injection of 50 µCi [^{3}H]-2-deoxy-D-[2,6]-glucose (GE Healthcare Life Sciences) and 22.5 µCi [^{14}C]-glucose was administered, and blood samples were taken at 2, 5, 10, 15, 20, 30 and 45 min. At the conclusion of the tracer period, rats were euthanised with intravenous administration of 0.1 mL of 60 mg/kg of pentobarbital sodium (Nembutal; Abbott Laboratories). The tibialis, red and white EDL muscles were rapidly dissected and freeze clamped and stored at −80°C for later analysis.

Glucose uptake

Both tibialis and EDL muscle were used to determine glucose uptake. Tibialis muscle was pulverised using a mortar and pestle cooled in liquid nitrogen and powdered muscle (~50 mg) was homogenised in water for the glucose uptake assay. Each EDL muscle strip was homogenised in 19 volumes of homogenising buffer (50 mM Tris HCl pH 7.5, 1 mM EDTA, 5 mM $MgCl_2$ and 1 mM DTT) to obtain a homogenate with a 1:20 (w/v) dilution. The homogenate was used to measure glucose uptake, FBPase activity, glucose incorporation into glycogen and glycogen content.

To determine the glucose uptake, the homogenate was spun at 16,200 ***g*** for 10 min and the supernatant was applied to an ion exchange column (2 cm of AG 1-X8 resin, Bio-Rad). The column was washed with 3 mL of water three times to remove any glucose and then washed twice with 3 mL of 1 M HCl. A 2 mL sample of the flow through obtained from the acid wash was mixed with liquid scintillation fluid and the [^{3}H]-deoxy-glucose-6-phosphate counted to enable calculation of glucose uptake.

Total glycogen and glucose incorporation into glycogen

The homogenate from the EDL strips or ~50 mg of powdered tibialis tissue was digested with 200 µL of 1 M KOH at 70°C and glycogen was precipitated, isolated and then digested to produce glucose as described previously (Thompson *et al.* 2000). The digested glucose was then used to estimate the total glycogen with a colorimetric assay and glycogen synthesis calculated by counting the [^{14}C]-glucose incorporated into glycogen using liquid scintillation counting.

FBP2 activity

Rat tibialis or EDL muscle was homogenised (1:20 weight of muscle/volume of buffer) in homogenising buffer (50 mM Tris HCl pH 7.5, 1 mM EDTA, 5 mM $MgCl_2$ and 1 mM DTT) and the homogenate was centrifuged for 16,200 ***g*** for 5 min. The supernatant (50 µL) was assayed for FBPase activity at 30°C in 0.5 mL of assay buffer (44 mM NADP, 0.1 U glucose-6-phosphodehydrogenase (G6PDH), 0.2 U phosphoglucoisomerase (PGI), 80 mM Bis–Tris propane pH 9.5, 1.6 mM EDTA, 8 mM $MgCl_2$ and 1.6 mM DTT). The reaction was started with 3.5 mM F-1,6-bisP and the increase in absorbance was followed for 20 min at 340 nm.

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PFK activity

The supernatant (20 µL) obtained from centrifuging the muscle homogenate was assayed for PFK activity at 30°C in 1 mL of assay buffer (0.2 mM NADH, 1 mM ATP, 2 mM AMP, 0.9 U aldolase, 100 mM Tris HCl pH 8.2, 10 mM $MgCl_2$, 400 mM KCl, 2 mM DTT). The reaction was initiated with 2.5 mM F-6-P. The decrease in absorbance was followed for 10 min at 340 nm.

Hexokinase activity

The supernatant (100 µL) obtained from centrifuging the muscle homogenate was assayed for hexokinase activity at 30°C in 1 mL of assay buffer (4 mM NADP, 25 mM ATP, 0.4 U glucose-6-phosphate dehydrogenase (G6PDH), 100 mM Tris HCl pH 7.5, 2 mM EDTA, 15 mM $MgCl_2$, 3 mM KCl). The reaction was initiated with 2 mM glucose. The increase in absorbance was followed for 10 min at 340 nm.

Western blotting

Tissue lysates were run on a 7.5% polyacrylamide gel and transferred on to a nitrocellulose membrane, which was then incubated for 2 h with 1:1000 dilution of FBP primary antibody (Novus Biologicals NBP1-56453). The membrane was washed and incubated in secondary antibody for 1 h. Any excess secondary antibody was washed away and FBP2 bands were visualised using chemiluminescence (Bradford 1976).

Metabolite extraction and LC/MS

Powdered tibialis muscle (~30 mg) was homogenised in 500 µL 50% (v/v) methanol:water mixture (at −30°C) with internal standards, deuterated thymine and D-camphor-10-sulfonic acid at 2.5 µM. Chloroform (500 µL) was added to create a phase separation. The aqueous phase was collected and spun in speed-vac for 1 h at room temperature to reduce any methanol. Particulate matter was removed by centrifugation and 5 µL of the extract was injected into a Synergi 2.5 mm 100 Å Hydro-RP column (2.0 mm I.D., 100 mm length; Phenomenax, Lane Cove West, Australia). LC–MS analysis was performed using an Agilent Infinity 1260 LC coupled to an AB Sciex QTRAP 6500 MS. LC separation was achieved with buffer A (97:3 (v/v) water:acetonitrile containing 10 mM tributylamine and 15 mM acetic acid (pH 4.95)) and buffer B (100% acetonitrile). MS ion source temperature voltage was set at 400°C and −4500 V respectively. Scheduled multiple reaction monitoring acquisition was performed. Metabolomic work was facilitated by SydneyMS and the cost subsidised by University of Sydney. Calibration standards were prepared using the same extraction procedure. Raw data were extracted into text files using ProteoWizard. Peak alignment and integration were performed using in-house MATLAB (The MathWorks) scripts.

Statistical analysis

All results are presented as mean±S.E.M. Since the overexpression and control muscle were contralateral legs of the same animal, repeated measures within the 2-way ANOVA was used to study the effects of overexpression and diet, followed by Sidak's *post hoc* test to compare between treated and control groups of the same diet. Where appropriate, paired *t* tests were used. Statistical analysis was performed in GraphPad Prism software (Prism 6, version 6.04, for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com). A *P* value of less than 0.05 was considered significant.

Results

There are two different isoforms of fructose-1,6-bisphosphatase (FBPase), liver FBP1 and muscle FBP2. These two enzymes are encoded by different genes and have only a 71% amino acid sequence homology in rats and even lower homology in humans (Tillmann *et al.* 2002). Liver FBP1 is the slightly bigger isoenzyme of the two (39.6 kDa) and is also expressed in the kidney, but not expressed in the other tissues as shown in Fig. 1. The smaller (36.8 kDa) FBP2 muscle-specific enzyme is expressed in relatively low levels in muscle compared to FBP1 expression in liver based on the signal from the immunoblot and FBPase activity measurements in muscle (0.12±0.02 µmol/min/g fresh weight) and in liver (9.8±0.5 µmol/min/g fresh weight), as well as other previous studies (Krebs & Woodford 1965, Newsholme *et al.* 1979).

Muscle-specific *FBP2* was cloned under a muscle-specific promoter and incorporated into adeno-associated virus, serotype 9 (AAV9). The AAV9-tMCK-*FBP2* was injected into the right tibialis/EDL muscle bundle and the left leg was used as the contralateral control. Both FBP2 protein and FBPase activity were significantly increased (13-fold on average) in the test leg (Fig. 2), compared to

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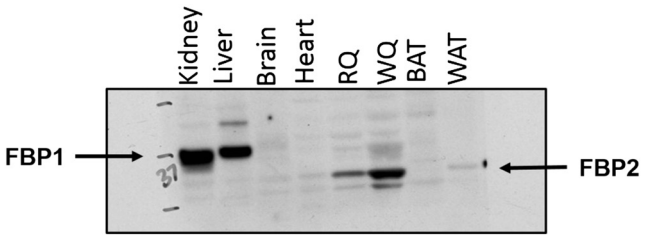

Figure 1
FBP enzyme tissue distribution. The tissue expression profile for FBP was studied by Western blot. The FBP antibody (NBP1-56453) also bound to the larger FBP1 protein in the kidney and the liver, $n=1$. BAT, brown adipose tissue; RQ, red quadricep; WAT, white adipose tissue; WQ, white quadricep.

the control leg in both chow and HFD-fed rats. The effect of increased FBPase activity on glycolysis was examined by measuring glycolytic intermediates in control and FBP2-overexpressing tibialis muscle of both chow and HFD animals. HFD reduced levels of dihydroxyacetone phosphate (DHAP) in the tibialis muscle. FBP2-expressing muscle had significantly increased F-6-P levels (the product of FBP2 activity) (Fig. 2C). FBP2 overexpression did not alter any other glycolytic intermediates in chow or HFD tibialis muscle.

FBP2 was also overexpressed in the right EDL muscle in animals fed a normal chow diet. EDL from the test leg and control leg of these rats was stripped into white and red and were then incubated with radiolabelled glucose and 2-deoxy glucose to study glucose metabolism under basal conditions. Control leg EDL muscle strips had an average FBPase activity of 0.21 µmol/min/g of tissue. FBPase activity in EDL muscle strips from the AAV transfected leg were variable and only strips with FBPase activity of 0.9 µmol/min/g of tissue or higher (Fig. 3A and B) were considered to have significant overexpression of FBP2 and used for further analysis. Of the 6 animals injected with AAV-tMCK-*FBP2* in right EDL muscle, 5 distal white and 3 proximal red EDL muscle strips had an FBPase activity above 0.9 µmol/min/g of tissue. The PFK activity in the white EDL on average was 12.2 µmol/min/g of tissue and in the red EDL was 9.5 µmol/min/g of tissue. This resulted in a decrease in PFK/FBPase activity ratio from 60–1 to 11–1 in white EDL, and 80–1 to 8.5–1 in red EDL. The glucose uptake from these strips was significantly higher in both white and red EDL muscle strips overexpressing FBP2 compared to the control EDL muscles (Fig. 3C and D).

White EDL strips overexpressing FBP2 had increased glucose uptake (14% higher) (Fig. 3C and D) and increased glycolytic rate, as measured by [^{14}C]-lactate released into the media (Fig. 4C and D). FBP2 overexpression in white EDL appeared to reduce glucose oxidation (Fig. 4A and B). Overexpression of FBP2 in the test red EDL increased glucose uptake by 30% (Fig. 3C and D) and was reflected in increased glycolysis, as well as glucose oxidation (Fig. 4). Glycogen synthesis was not altered in either white or red EDL muscle strips overexpressing FBP2 (Fig. 4E and F).

To further examine the effect of FBP2 overexpression in muscle, *in vivo* studies were undertaken to examine how increased FBPase activity might alter muscle glucose metabolism in chow and insulin-resistant, high-fat-fed rats. The differences between the chow and HFD-fed animals due to high-fat feeding are displayed in Table 1. The high-fat fed animals on average had 46% larger fat pads and a 10% increase in body weight. The basal plasma insulin levels were higher in the HFD group compared to the chow fed group. Therefore, the HFD animals exhibited evidence of lipid-induced insulin resistance. During the hyperinsulinaemic-euglycaemic clamp, the insulin levels were increased such that both chow and HFD animals had similar levels of plasma insulin. The higher insulin levels

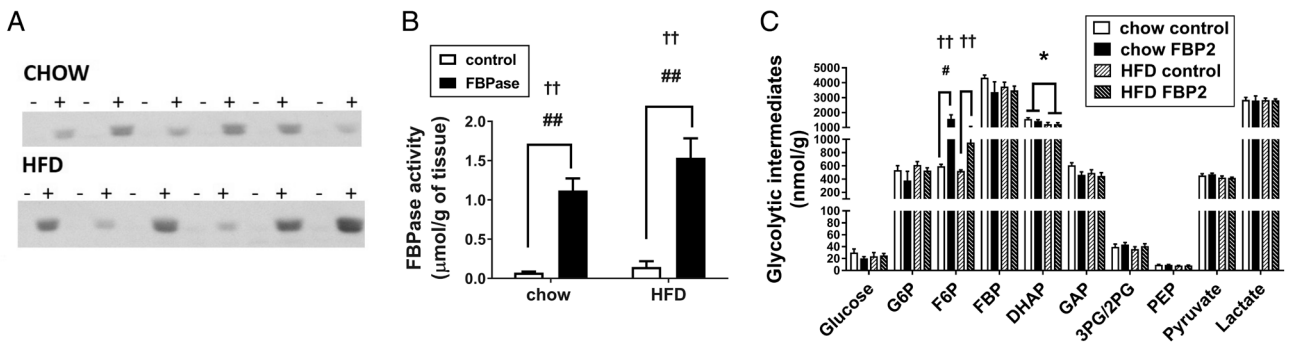

Figure 2
Overexpression of FBP2 in rat tibialis muscle after 4 weeks of AAV9-tMCK-*FBP2* administration. FBP2 protein levels (A) FBPase activity (B) glycolytic intermediates (C) in control and test tibialis muscle. $N=6$–8 rats for A and B and $n=3$–4 rats for C. $^{*}P<0.05$, 2 way ANOVA, effect of diet; $^{\dagger\dagger}P<0.01$, 2 way ANOVA, effect of FBP2; $^{\#}P<0.05$, $^{\#\#}P<0.01$ *post hoc* Sidak test, effect of FBP2.

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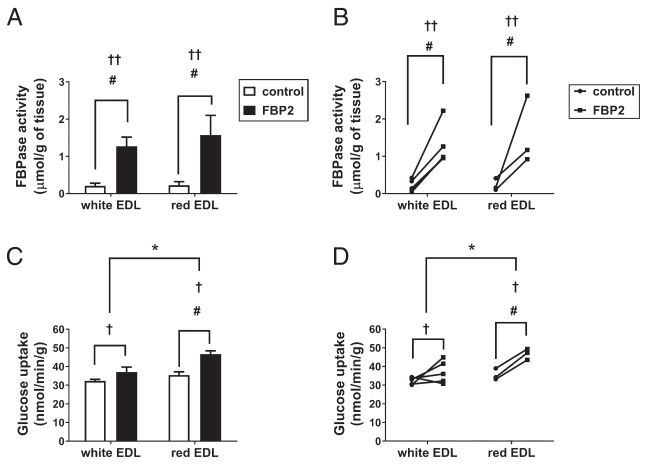

Figure 3
Ex vivo effect of FBP2 overexpression in EDL muscle on glucose uptake under basal conditions. FBPase activity (A and B), glucose uptake (C and D) in the white and red EDL muscle. $N=3$–5 rats. $^{*}P<0.05$, 2 way ANOVA, effect of muscle; $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$, 2 way ANOVA, effect of FBP2; $^{\#}P<0.05$, $^{\#}P<0.01$, *post hoc* Sidak test, effect of FBP2.

during the clamp reduced the plasma triglyceride and free fatty acid levels in both groups of animals.

As would be expected, the HFD animals showed signs of whole body insulin resistance and muscle insulin

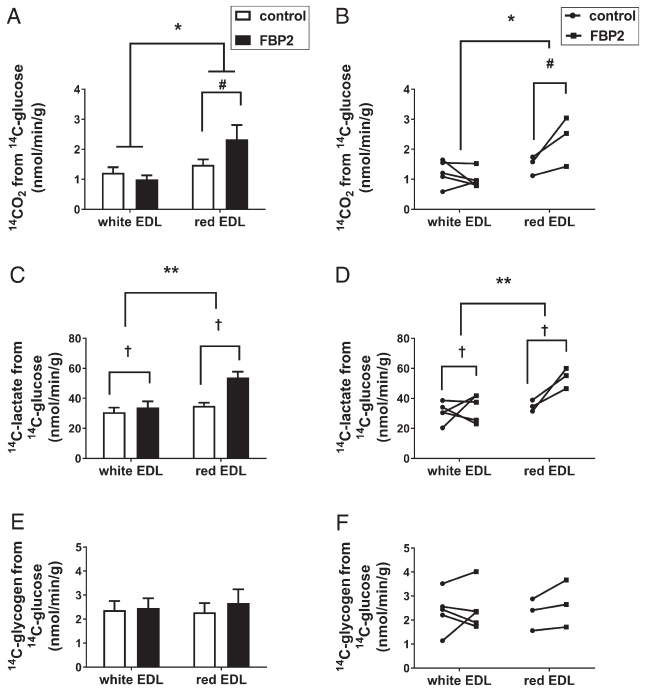

Figure 4
Ex vivo effect of FBP2 overexpression on glucose metabolism under basal conditions. [^{14}C]-glucose was converted to $^{14}CO_2$ (A and B), [^{14}C]-lactate (C and D), [^{14}C]-glycogen (E and F). $N=3$–5. $^{*}P<0.05$, $^{**}P<0.01$, 2 way ANOVA, effect of muscle type; $^{\dagger}P<0.05$, 2 way ANOVA, effect of FBP2; $^{\#}P<0.05$ *post hoc* Sidak test, effect of FBP2.

resistance. The HFD animals required a lower level of glucose infusion (Table 1) to maintain euglycaemia indicating a lower rate of glucose disposal in HFD rats compared to the chow-fed animals (Table 1). Additionally, the glucose uptake in tibialis muscle from HFD rats was reduced to, on average, 67% of control tibialis muscle of chow rats (Fig. 5).

There was on average a 13-fold increase in FBPase activity in right tibialis muscle transfected with AAV-tMCK-*FBP2* from chow- and HFD-fed rats (Fig. 2). These changes were associated with a significant increase in insulin-stimulated glucose uptake in the right tibialis muscle compared to the left, untransfected tibialis muscle in both chow and HFD groups (Fig. 5). It should be noted that although the total increase was small, every right tibialis muscle overexpressing FBP2 had a higher glucose uptake than the contralateral control muscle (Fig. 5B). The effect of FBP2 overexpression was greater in tibialis from chow-fed rats (23% increase in glucose uptake) compared to muscle from HFD-fed tibialis (16% increase). In a pilot study, glucose uptake in tibialis muscle transduced with the control vector AAV9-GFP from two chow-fed rats was 8.5 and 11.6 µmol/min/100 g. In a HFD tibialis transfected with AAV9-GFP glucose uptake was 10.4 µmol/min/100 g. These estimates of insulin-stimulated glucose uptake were similar to the range of values observed in saline-treated muscle in chow animals and in HFD animals in Fig. 5 indicating that transfection with the AAV *per se* is unlikely to have any significant effect on insulin-stimulated glucose uptake.

Tibialis from both chow and HFD-fed animals overexpressing FBP2 had a 15% and 17% increase in hexokinase activity, respectively (Fig. 6A). PFK activity in control HFD muscle appeared to be reduced and this was restored to control chow fed muscle levels in FBP2 overexpressing HFD muscle (Fig. 6B).

HFD reduced insulin-stimulated glycogen synthesis irrespective of FBP2 overexpression, but there was no effect on total glycogen content (Fig. 6C and D).

Discussion

The flux through glycolysis or gluconeogenesis is largely dependent on the activity of the key enzymes PFK and FBPase, both of which play a crucial role in different tissues to regulate blood sugar levels. Overexpression of FBPase has been studied in pancreas and liver and found to reduce pancreatic sensitivity to increased circulating glucose (Kebede *et al.* 2008) and increase hepatic

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Table 1 Parameters for hyperinsulinaemic-euglycaemic clamp performed on rats fed chow or HFD and overexpressing AAV9-tMCK-*FBP2* for 4 weeks.

	CHOW	HFD	*t*-Test	2-way ANOVA
Number of animals	7	8		
Body weight (g)	289±6	318±8	*	
Epididymal fat pad (g)	1.6±0.3	2.6±0.5	**	
Subcutaneous fat pad (g)	2.9±0.2	3.7±0.23	*	
Clamped plasma glucose (mM)	7.3±0.3	7.7±0.4		
Glucose infusion rate (mg/kg/min)	37.1±2.8	21.2±2.3	**	
Glucose disposal rate (mg/kg/min)	33.8±1.5	24.4±1.7	**	
Basal insulin (U/L)	46.7±8.7	95.1±16.3	*	**
Clamp insulin (U/L)	290.8±40.3	298±27.3		
Basal NEFA (mM)	0.6±0.1	0.8±0.2		**
Clamp NEFA (mM)	0.1±0.02	0.2±0.1		
Basal TAG (mM)	0.6±0.1	1.4±0.4		
Clamp TAG (mM)	0.3±0.1	0.9±0.4		

Data are mean±S.E.M. All animals received a AAV9-tMCK-*FBP2* in the right leg and a saline in the left contralateral leg.
*$P<0.05$, **$P<0.01$, unpaired *t*-test and 2-way ANOVA effect of clamp between chow and HFD animals.
NEFA, non-esterified fatty acid; TAG, triglyceride.

glucose production (Visinoni *et al.* 2008), respectively. Additionally, compounds that inhibit hepatic FBPase and subsequently gluconeogenesis have been shown to reduce hepatic glucose output, and these are being clinically developed for diabetes treatment, as reviewed in van Poelje *et al.* (2011).

The FBPase enzyme is encoded by a different gene in muscle, compared to the liver and pancreas. The rat muscle isoenzyme amino acid sequences are 97% similar to human muscle FBPase but only 72% and 52% similar to the rat and human liver isoenzyme, respectively (Tillmann *et al.* 2002). The major difference between the liver and muscle enzymes is the regulation of the enzyme activity because of the different roles the enzymes play in different tissues. Muscle has low levels of gluconeogenic enzymes and does not require an intricate regulation of the opposing reactions of F-6-P phosphorylation and de-phosphorylation. Therefore, the small amount of bifunctional PFKFBP2 (the enzyme that regulates fructose-2,6-bisphosphate levels to control PFK and FBPase activity in liver) lacks any known regulatory phosphorylation sites in muscle, in contrast to the liver isoform (Kurland & Pilkis 1995). Instead, FBP2 in muscle is regulated by AMP levels (Rakus *et al.* 2005, Shi *et al.* 2013).

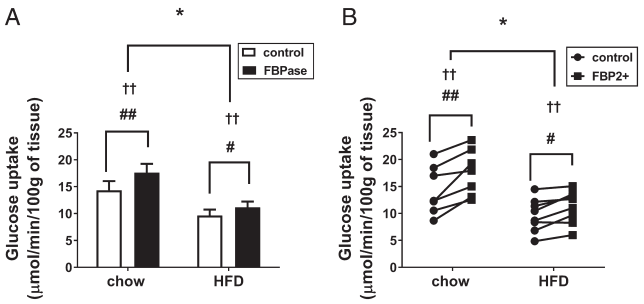

Figure 5
In vivo effect of FBP2 overexpression on insulin-stimulated glucose uptake in the tibialis muscle. Insulin-stimulated [^{14}C]-glucose uptake in the tibialis muscle (A and B). $N=7$–8 rats. *$P<0.05$, 2 way ANOVA, effect of diet; $^{\dagger\dagger}P<0.01$, 2 way ANOVA, effect of FBP2, $^{\#}P<0.05$, $^{\#\#}P<0.01$ *post hoc* Sidak test, effect of FBP2.

The high activity of glycolytic enzymes and very low levels of gluconeogenic enzymes ensures that glycolytic flux is favoured in muscle. Nevertheless, muscle does have glycogen stores and about 13% of which may be replenished after exercise from lactate through gluconeogenesis (Bangsbo *et al.* 1991). Since, muscle has negligible levels of glucose-6-phophatase activity, any newly synthesised glucose-6-phosphate through gluconeogenesis is converted to glycogen, instead of being released into circulation. Overexpressing FBP2 in the muscle had little effect on glycogen synthesis or total glycogen levels in both chow and HFD animals under both *ex vivo* and *in vivo* conditions. Additionally, contrary to other tissues, increasing FBP2 activity increased glycolytic flux in EDL muscle. This effect of concomitant increase in substrate cycling in muscle between F-6-P and F-1,6-P and glycolytic flux was also observed with insulin and adrenaline stimulation (Challis *et al.* 1985). This effect was more pronounced in the red EDL muscle, than the white EDL muscle from chow animals. In chow animals, the increased rate of glycolysis in EDL muscle *ex vivo* with FBP2 overexpression was paralleled by an increased hexokinase activity and glucose uptake in the tibialis muscle *in vivo*. Furthermore, overexpression of FBP2 in red EDL led to an increase in glucose oxidation.

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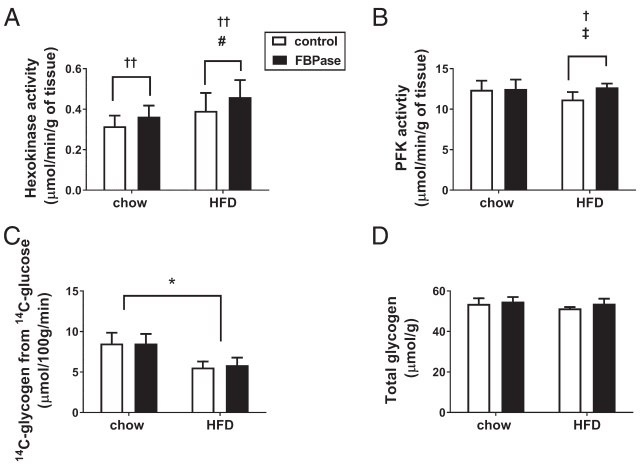

Figure 6

Effect of FBP2 overexpression on glucose metabolism in the tibialis muscle. Hexokinase activity (A), phosphofructokinase (PFK) activity (B), [^{14}C]-glucose incorporated into glycogen (C), total glycogen (D). $N=7$–8 rats. *$P<0.05$, 2 way ANOVA, effect of diet; $^{\dagger\dagger}P<0.01$, 2 way ANOVA, effect of FBP2, $^{\#}P<0.05$, *post hoc* Sidak test, effect of FBP2; $^{\ddagger}P<0.05$, *post hoc* test Sidak test, effect of diet.

Conversely, overexpression of FBP2 in white EDL led to a decrease in glucose oxidation, which may reflect the metabolic differences between white (predominantly comprises of type IIb or fast glycolytic fibres) and red EDL (predominantly comprises of type IIa fibres also known as fast oxidative glycolytic fibres) that could contribute to differences observed in insulin-stimulated glucose uptake (Lexell *et al.* 1994, MacKrell *et al.* 2012).

The presence of low levels of gluconeogenic enzymes in muscle have been hypothesised to ensure limited futile cycling between glycolytic and gluconeogenic pathways to prevent thermogenesis or energy wastage (Newsholme & Crabtree 1970, Newsholme 1978, Newsholme & Parry-Billings 1992). Mammals use different methods other than futile cycling between PFK-M and FBP2, to maintain body temperature. Therefore, to avoid energy wastage, low level FBP2 activity may be inhibited by AMP (Newsholme & Crabtree 1970, Rakus *et al.* 2005, Shi *et al.* 2013). However, futile cycling could occur under physiological situations such as exercise, hyperthyroidism or cold exposure (Challis *et al.* 1985, Shulman *et al.* 1985) or under adrenergic stimulation such as exposure to catecholamines and also insulin stimulation (Challiss *et al.* 1984, Challis *et al.* 1985), to increase metabolic sensitivity. Therefore, any increased FBPase activity may lead to some energy wastage, which can be calculated based on the assumptions that each futile cycle is limited to maximal FBP2 enzyme activity and that each cycle consumes one ATP. The maximal FBPase activity in muscle of control tibialis muscle is 0.05 µmol/g/min at 30°C and in the tibialis that was overexpressing FBP2, this activity increased to 1–2 µmol/g/min. Using the Arrhenius equation to adjust this activity to 37°C would give maximal activities for FBPase of approximately 0.1 µmol/g/min in the control muscle and 1.7–2.8 µmol/g/min in the overexpressing tibialis muscle. In human muscle at rest, the ATP turnover has been measured at 8–12 µmol/g/min (Szendroedi *et al.* 2008, Lim *et al.* 2011). Based on these estimates, it is possible to speculate that in normal muscle, cycling between F-6-P and F-1,6-bisP could contribute at most 1% of total ATP turnover, which may increase up to 10–20% of total ATP turnover in muscle overexpressing FBP2, given that cycling was operating at the absolute maximal activity of FBP2. At rest, human muscle contributes approximately 20% of total energy expenditure (Müller *et al.* 2002). Therefore, if whole body musculature had an increase in FBP2 similar to that observed in the rat tibialis and futile cycling was maximally active, the contribution of this cycling to whole body energy expenditure would be approximately 1%. This small increase in basal energy expenditure may have a significant impact on energy expenditure over many years, but these calculations are based on maximal rates of cycling, at a temperature of 37°C, and there being no compensatory changes in other parameters such as food intake or reduced physical activity. Muscle temperature at rest is probably closer to 35°C and can increase in proportion to intensity of exercise (Castle *et al.* 2006, Costello *et al.* 2012).

In HFD-fed animals, a transient reduction in PFK-M activity (Nemeth *et al.* 1992), and a concomitant 30% increase in FBP2 activity (Newsholme *et al.* 1979, Nemeth *et al.* 1992) has been reported to reduce muscle glycolysis while simultaneously increasing glycogen synthesis, as a way of coping with metabolic impairment before insulin resistance develops (Kim *et al.* 1996). Diabetic rats have also been shown to have increased FBPase activity in muscle and liver (Prince & Kamalakkannan 2006). In humans, some studies have reported type 2 diabetic subjects with either normal (Mandarino *et al.* 1987, Vestergaard *et al.* 1993) or decreased (Falholt *et al.* 1988) or increased (Hansen *et al.* 2015) levels of PFK-M. Results presented in this study support the idea that PFK-M activity levels may be reduced with HFD feeding. This reduction in PFK-M activity was restored in HFD muscle overexpressing FBP2. Hexokinase activity was also increased in HFD muscle overexpressing FBP2 which slightly, but significantly, increased glucose uptake in HFD muscle.

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Various compounds such as berberine, rutin, rosiglitazone and BM15.2054 that can increase glycolytic rate, have been demonstrated to increase skeletal muscle glucose uptake (Furnsinn *et al.* 1999, Jucker *et al.* 2002, Kim *et al.* 2003, Prince & Kamalakkannan 2006, Yin *et al.* 2008). Furthermore, genetic manipulations in animal models with improvements in glycolysis or glucose oxidation, with or without increase in fatty acid oxidation, have been shown to increase muscle glucose uptake (Choi *et al.* 2007*a*,*b*, Kanzleiter *et al.* 2010). Exercise and nitric oxide can also increase glucose transport in conjunction with an increase in hexokinase activity and glucose oxidation to facilitate an increase skeletal muscle glucose uptake (Wahren *et al.* 1971, Balon & Nadler 1997, Bergman *et al.* 1999, Fueger *et al.* 2004, Paik *et al.* 2005). Therefore, a concomitant increase in hexokinase activity, glycolysis and glucose oxidation in response to FBP2 overexpression could be responsible for the observed increase in glucose uptake for both chow and HFD animals without any changes in muscle size or fibre type. However, effect of FBP2 overexpression on muscle function was not studied.

It should be noted that an increase in FBP2 activity in muscle appears beneficial in contrast to the potential hyperglycaemic effects of increased activity of FBP1 in liver. Due to structural similarities between FBP1 and FBP2 proteins, FBP1 inhibitors under development to reduce FBP1 activity may also inhibit FBP2 (Erion *et al.* 2005). Therefore, studies aimed at developing FBP1 inhibitors for diabetes therapy should also include studies on muscle FBP2 activity, muscle glucose utilisation and the consequences for whole body glucose homeostasis.

In conclusion, there was a clear increase in insulin-stimulated glucose uptake in muscle overexpressing FBP2 in the hyperinsulinaemic-euglycaemic clamp experiments. This increase in glucose uptake was probably due to changes in metabolic flux through PFK-M and FBP2, rather than energy wastage and subsequent increase in substrate usage. Although, the increase in glucose uptake was significant in skeletal muscle from HFD fed animals, the effect was small. Therefore, increasing FBP2 activity in the muscle to improve glucose uptake in the skeletal muscle may not be a viable treatment strategy for lipid-induced insulin resistance.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

I B was the recipient of an International student scholarship from UNSW. G C was supported by a Fellowship from the NHMRC (1003313) and N T was supported by a Future Fellowship from the ARC. The work was supported by a Program grant from the NHMRC (535921) and DART (Y17G-BRAA).

Author contribution statement

I B, N T and G C devised the study and the majority of the experimental work was carried out by I B with the help of E S and G C. The hyperinsulinaemic-euglycaemic clamp was performed by A B, I B, E S, and L S. L Q performed the metabolomics study. The analysis of data and drafting of the manuscript was carried out by I B, N T and G C edited the manuscript.

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Received in final form 25 January 2018
Accepted 5 March 2018
Accepted Preprint published online 5 March 2018