RESEARCH PAPER

A non-erythropoietic peptide derivative of erythropoietin decreases susceptibility to diet-induced insulin resistance in mice

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BACKGROUND AND PURPOSE

The haematopoietic activity of erythropoietin (EPO) is mediated by the classic EPO receptor (EpoR) homodimer, whereas tissue-protective effects are mediated by a heterocomplex between EpoR and the β-common receptor (βcR). Here, we investigated the effects of a novel, selective ligand of this heterocomplex – pyroglutamate helix B surface peptide (pHBSP) – in mice fed a diet enriched in sugars and saturated fats.

EXPERIMENTAL APPROACH

Male C57BL/6J mice were fed a high-fat high-sucrose diet (HFHS) for 22 weeks. pHBSP (30 µg·kg^{-1} s.c.) was administered for the last 11 weeks. Biochemical assays, histopathological and immunohistochemical examinations and Western blotting were performed on serum and target organs (liver, kidney and skeletal muscle).

KEY RESULTS

Mice fed with HFHS diet exhibited insulin resistance, hyperlipidaemia, hepatic lipid accumulation and kidney dysfunction. In gastrocnemius muscle, HFHS impaired the insulin signalling pathway and reduced membrane translocation of glucose transporter type 4 and glycogen content. Treatment with pHBSP ameliorated renal function, reduced hepatic lipid deposition, and normalized serum glucose and lipid profiles. These effects were associated with an improvement in insulin sensitivity and glucose uptake in skeletal muscle. Diet-induced overproduction of the myokines IL-6 and fibroblast growth factor-21 were attenuated by pHBSP and, most importantly, pHBSP markedly enhanced mitochondrial biogenesis in skeletal muscle.

CONCLUSIONS AND IMPLICATIONS

Chronic treatment of mice with an EPO derivative, devoid of haematopoietic effects, improved metabolic abnormalities induced by a high-fat high-sucrose diet, by affecting several levels of the insulin signalling and inflammatory cascades within skeletal muscle, while enhancing mitochondrial biogenesis.

Abbreviations

βcR, β-common receptor; ACR, albumin-to-creatinine ratio; BUN, blood urine nitrogen; COI, cytochrome c oxidase 1; EPO, erythropoietin; EpoR, EPO receptor; FGF-21, fibroblast growth factor-21; GLUT-4, glucose transporter type 4; HFHS, high-fat high-sugar; mtTFA, mitochondrial transcription factor A; NRF1, nuclear respiratory factor 1; OGTT, oral glucose tolerance test; PGC1-α, peroxisome proliferator-activated receptor γ co-activator 1-α; pHBSP, pyroglutamate helix B surface peptide; TG, triglyceride

Tables of Links

TARGETS		LIGANDS
Catalytic receptors[a] **Enzymes**[c]		IL-6
β-common receptor, CD131 Akt (protein kinase B)		Insulin
EPO receptor GSK-3β		
Transporters[b]		
GLUT4, glucose transporter 4 (SLC2A4)		

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b,c]Alexander *et al.*, 2013a,b,c).

Introduction

Erythropoietin (EPO), a 31 kDa glycoprotein that stimulates proliferation, differentiation and survival of erythroid progenitor cells by activation of the EPO receptor (EpoR), has been widely used for the treatment of chronic anaemia (Drueke *et al.*, 2006). A large number of experimental studies demonstrate a tissue-protective effect of EPO in many organs including the brain, the kidney, the heart, the vasculature and the gastrointestinal tract (Patel *et al.*, 2011). Interestingly, EPO deficiency has been noted in patients with diabetes (Craig *et al.*, 2005; McGill and Bell, 2006; Thomas, 2006), and EPO therapy decreased insulin resistance in haemodialysis patients by improving glucose metabolism and reducing chronic inflammation (Mak, 1998; Spaia *et al.*, 2000; Rasic-Milutinovic *et al.*, 2008; Khedr *et al.*, 2009). Collectively, these data raised the possibility that EPO and the signalling cascades activated by this pleiotropic hormone may affect insulin sensitivity and, thus, may be relevant to the management of diabetes. Potentially serious adverse effects of EPO including an increase in haematocrit, blood pressure and thrombosis may limit any potential therapeutic translation of these beneficial effects of EPO in diabetic patients.

Although the EPO-mediated signalling pathways are well characterized for erythroid cell types, they are less well defined for non-erythroid tissues and, therefore, there is limited information about the mechanisms underlying the EPO-induced improvements in insulin resistance. The extra-haematopoietic effects of EPO may be mediated, at least in part, by a putative 'tissue-protective receptor', which has been recently proposed to be a heterocomplex between the EpoR and the β-common receptor (βcR, known also as CD131) (Brines *et al.*, 2004). βcR is a common subunit of other heteroreceptors, including those of IL-3, IL-5 and G-MCSF (Murphy and Young, 2006). Very recently, the use of βcR knockout mice has shown that the activation of βcR by EPO is essential for the EPO-induced reduction of both kidney and cardiac dysfunction associated with sepsis, as well as in producing long-term relief of neuropathic pain (Swartjes *et al.*, 2011; Coldewey *et al.*, 2013; Khan *et al.*, 2013). Moreover, the development of EPO derivatives, which only activate the EpoR–βcR complex and do not stimulate erythropoiesis, has led to a better characterization of the role of this pharmacological target in mediating EPO effects (Leist *et al.*, 2004).

The pyroglutamate helix B surface peptide (pHBSP), also known as ARA290, is one of the the newest generation of EPO derivatives. This peptide mimics the external, aqueous face of helix B of EPO (including amino acids that are exposed at the helix B surface as well as three residues from the loop between helices B and C), sufficiently to exert the tissue-protective activities seen with the EPO protein. Thus, pHBSP is a small synthetic peptide consisting of 11 amino acids, which binds to the EpoR–βcR complex, but not the classical erythropoietic EpoR homodimer *in vitro* (Brines and Cerami, 2008). Several preclinical studies have revealed that pHBSP exerts pronounced tissue-protective properties without stimulating haematopoiesis (Brines and Cerami, 2008; Brines *et al.*, 2008; McVicar *et al.*, 2011; Seeger *et al.*, 2011; Swartjes *et al.*, 2011; Pulman *et al.*, 2013; van Rijt *et al.*, 2013). However, none of the previous studies investigated the role of the EpoR–βcR complex activation in the management of metabolic abnormalities or any other chronic disease where administration of the drug for several weeks is warranted. Hence, the present study was undertaken to determine the effects of the non-erythropoietic EPO derivative pHBSP in mice chronically fed a diet enriched in sugars and saturated fats (HFHS), which is known to promote obesity and insulin resistance. To characterize the extra-haematopoietic effects of EPO, we

investigated, in mice, the potential effects of the activation of the EpoR–βcR complex by pHBSP on signalling pathways involved in the pathogenesis of insulin resistance, focusing on skeletal muscle, which accounts for ~70–80% of insulin-stimulated glucose uptake and, thus, exerts a key role in regulating whole-body glucose homeostasis.

Methods

Animal model

All animal care and experimental procedures complied with the EU Directive and were approved by the Turin University Ethics Committee. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 42 animals were used in the experiments described here.

Male C57BL/6J mice, aged 4 weeks, were purchased from Harlan Laboratories (Udine, Italy). The animals were maintained on a pellet diet (Piccioni n. 48, Gessate Milanese, Italy) for 1 week, then randomly divided into two groups: normal diet (control, $n = 18$) and the HFHS diet ($n = 24$) for 22 weeks. The HFHS diet contained 40% fat (2% from soybean, 38% from butter), 15% protein and 45% carbohydrate (15% from corn starch and 30% from sucrose). The animals were housed in a temperature-controlled environment with a 12 h light/dark cycle. Food and water consumption and body weight were measured weekly.

Treatments

After 11 weeks on diet, 6 mice on the control diet and 12 mice on the HFHS diet were treated with pHBSP for further 11 weeks (control + pHBSP and HFHS + pHBSP respectively). The EPO derivative pHBSP was supplied by Araim Pharmaceuticals (Ossining, NY, USA). Mice were treated with pHBSP (30 µg·kg⁻¹, in 200 µL PBS) by s.c. injection at 2 day intervals. Vehicle treatment consisted of 200 µL of PBS at pH 7.4. The pHBSP dose used in this study is based on previous studies on tissue-protective effects of chronic pHBSP administration in mice (Brines and Cerami, 2008; Brines *et al.*, 2008; Schmidt *et al.*, 2011; Swartjes *et al.*, 2011). This dose of pHBSP was previously found to induce no change in the haematocrit, haemoglobin concentration and platelet count over a 28 day period of twice daily administration (Brines and Cerami, 2008; Brines *et al.*, 2008). The interval of drug administration was based on published preclinical and clinical data (Swartjes *et al.*, 2011; Heij *et al.*, 2012; van Velzen *et al.*, 2014), which clearly demonstrated that the repetitive (2 day intervals) i.p. administration of pHBSP exerted significant beneficial effects, despite the short half-life of this peptide ($t_{1/2\ \text{ELIM}}$ ~2 min).

Oral glucose tolerance test (OGTT)

OGTT was performed on mice fasted for 6 h at the end of the experiment (week 22). Glucose (2 g·kg⁻¹) was given orally by gavage. Serial blood glucose levels were measured at 0, 30, 60, 90 and 120 min in samples from the saphenous vein using the Accu-Check glucometer (Roche Diagnostics, Mannheim, Germany).

Biochemical parameters

Urine was collected for analysis by placing animals in metabolic cages for 18 h. The concentration of urinary creatinine and albumin was assessed using commercial kits (Arbor Assays, Ann Arbor, MI, USA and Bethyl Laboratories Inc., Montgomery, TX, USA respectively). Albumin excretions were related to urine creatinine concentrations (albumin-to-creatinine ratio, ACR) in order to take into account differences in urinary flows. Blood urine nitrogen (BUN) levels as well as plasma concentrations of triglycerides (TGs), total cholesterol, high-density lipoprotein (HDL) and low-density lipoprotein (LDL) were determined using colorimetric assays from Sigma Chemical (St. Louis, MO, USA) and Hospitex Diagnostics (Sesto Fiorentino, FI, Italy) respectively. Commercially available ELISA kits were used to measure plasma insulin, leptin, adiponectin, IL-6 and FGF-21 levels.

Kidney histopathological examination

Coronal sections of both kidneys and fragments from the left lateral and medial lobes were fixed in 4% buffered formaldehyde solution overnight at 4°C. Dewaxed 5 µm sections were stained with haematoxylin-eosin and examined under an Olympus Bx4I microscope (40× magnification) with an AxioCamMR5 photographic attachment (Carl Zeiss, Oberkochen, Germany).

Liver Oil Red staining

Neutral lipids were assessed on sections of frozen liver embedded in OCT (10 µm in thickness) by Oil Red O staining using an Olympus Bx4I microscope (40× magnification) with an AxioCamMR5 photographic attachment (Carl Zeiss).

Protein extraction and Western blot

Gastrocnemius muscle and liver were homogenized and fractionated as previously described (Benetti *et al.*, 2013). Briefly, frozen tissues were homogenized in RIPA buffer containing protease inhibitors. The protein content was measured by a protein assay kit using bicinchoninic acid and albumin as standard (Pierce Biotechnology Inc., Rockford, IL, USA). Protein-matched samples were separated by electrophoresis and then transferred to a PVDF membrane, which was then incubated with primary antibodies to: glucose transporter type 4 (GLUT-4), cytochrome c oxidase 1 (COI), peroxisome proliferator-activated receptor γ co-activator 1-α (PGC-1α), mitochondrial transcription factor A (mtTFA), nuclear respiratory factor 1 (NRF-1), and the total and phosphorylated forms of insulin receptor substrate 1 (IRS-1), Akt, GSK-3β and the Akt substrate 160 (AS160); all from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). After incubation with primary antibodies, membranes were incubated with secondary antibodies linked to HRP. Protein bands were visualized using enhanced chemiluminescence. For control of protein loading, the blots were stripped and reacted with tubulin monoclonal antibody. Bands were quantified by an optical densitometry using Gel Pro®Analyzer 4.5, 2000 software (Media Cybernetics, Silver Spring, MD, USA).

Liver TG level

Lipids were extracted from liver tissue as previously described (Collino *et al.*, 2013), and TG contents were determined enzymically using a TG quantification kit from Abnova Corporation (Heidelberg, Germany).

Skeletal muscle glycogen content

The glycogen concentration of the gastrocnemius was determined using a glycogen assay kit, following the protocol provided by the manufacturer (glycogen assay kit, Abnova Corporation, Taiwan).

EpoR/βcR co-immunoprecipitation

Co-immunoprecipitation was performed in order to examine whether there is a physical interaction between EpoR and βcR. Protein extracts were immunoprecipitated from gastrocnemius lysates using EpoR antibody for 3 h, followed by incubation with protein A agarose beads overnight at 4°C. The mixture was centrifuged for 5 min and supernatants were removed. Bead-antibody-protein complexes were washed in RIPA buffer, and proteins were eluted from protein A agarose beads by boiling in loading buffer. After centrifuge antigen–antibody complexes were subjected to SDS-PAGE using 8% gel, followed by transfer to PVDF membranes. The membranes were incubated with the primary antibodies anti-EpoR or anti-βcR and the appropriate secondary antibodies.

Immunohistochemical staining

Representative 10 µm cryostat sections of gastrocnemius were fixed with 100% acetone for 10 min and then incubated with rabbit anti-GLUT-4 antibody overnight at 4°C. After washing, sections were incubated with anti-rabbit IgG-HRP for 1 h at room temperature. Colour development was achieved by incubation with 0.02% 3,3′-diaminobenzidine tetrahydrochloride containing 0.02% hydrogen peroxide for 10 min at room temperature and the specific staining visualized with a Olympus-Bx4I microscope connected to a photographic attachment (Carl Zeiss, Oberkochen, Germany).

Data analysis

All values are presented as mean ± SEM for n observations. We analysed data using the Prism software package (GraphPad Software, San Diego, CA, USA). Comparisons among groups were performed using one-way ANOVA with Bonferroni's multiple comparison *post hoc* test. Differences between groups were considered statistically significant at P-values below 0.05.

Results

Effects of diet exposure and pHBSP treatment on metabolic parameters

After 22 weeks of feeding, mice of the HFHS group had greater body and adipose tissue (epididymal fat) weights than control diet-fed animals, which were significantly reduced by pHBSP treatment (Table 1). Moreover, an increase in the caloric intake in the mice fed with the experimental diet was recorded and this increase was slightly reduced by drug treatment without reaching statistical significance. As reported in Table 2, HFHS increased serum levels of TGs, total cholesterol and LDL and reduced HDL level, in comparison with control mice. Most notably, the changes in lipid contents were normalized by pHBSP treatment. When compared with control, chronic exposure to HFHS resulted in more than twofold increase in insulin levels, which was associated with almost 30% increase in fasting serum glucose concentrations (Figure 1A and B). Consistently, the OGTT revealed that the HFHS mice were glucose intolerant (Figure 1C). In contrast, pHBSP administration exerted significant insulin- and glucose-lowering ability. Serum concentrations of adiponectin and leptin, both of which play important roles in lipid and glucose homeostasis, showed different patterns: while the level of adiponectin in HFHS mice was lower than in control mice, that of leptin increased versus controls (Table 2). When pHBSP was administered to HFHS mice, there was a significant increase in the adiponectin levels, associated with a significant decrease in leptin levels, while pHBSP administration to mice fed with the control diet did not modify any of these metabolic parameters.

Chronic pHBSP treatment reduces diabetic nephropathy in HFHS mice

Representative sections from each experimental group were taken from the transition between the deeper cortex and the outer stripe of the outer medulla zones (Figure 2A). Kidneys from mice fed with a standard laboratory chow showed a normal morphology and treatment of control mice with pHBSP did not produce any significant changes. In contrast,

Table 1

Effects of diet and pHBSP chronic administration on metabolic parameters

	Control (n = 12)	Control + pHBSP (n = 6)	HFHS (n = 12)	HFHS + pHBSP (n = 12)
Body weight (g)	29.05 ± 0.58[§]	28.7 ± 1.21[§]	38.00 ± 0.82*	34.7 ± 0.73*[§]
Body weight gain (g)	9.17 ± 0.75[§]	8.14 ± 1.67[§]	16.21 ± 1.51*	10.03 ± 1.90[§]
Food intake (g per day)	3.80 ± 0.40	3.20 ± 0.50	2.90 ± 0.40	2.70 ± 0.40
Caloric intake (kcal per day)	10.80 ± 0.92[§]	9.92 ± 1.55[§]	14.05 ± 0.80*	12.25 ± 0.80
Epididymal fat weight (%BW)	2.41 ± 0.09	2.11 ± 0.21	3.34 ± 0.25*	2.52 ± 0.25[§]
Kidney weight (%BW)	1.06 ± 0.05	1.08 ± 0.09	1.19 ± 0.08*	1.08 ± 0.05[§]
Liver weight (%BW)	4.93 ± 0.25	4.59 ± 0.45	5.23 ± 0.28	4.88 ± 0.31
Gastrocnemius weight (%BW)	1.04 ± 0.08	1.08 ± 0.11	1.22 ± 0.06	1.09 ± 0.06

Data are means ± SEM. *$P < 0.01$ versus control; [§]$P < 0.05$ versus HFHS. BW, body weight.

Table 2

Effects of diet and pHBSP chronic administration on mouse blood biochemistry

	Control (n = 12)	Control + pHBSP (n = 6)	HFHS (n = 12)	HFHS + pHBSP (n = 12)
Triglycerides, mmol·L⁻¹	0.52 ± 0.08§	0.54 ± 0.09§	1.02 ± 0.07*	0.63 ± 0.03§
Total cholesterol, mmol·L⁻¹	2.30 ± 0.09§	2.54 ± 0.11	2.66 ± 0.09*	2.16 ± 0.08§
LDL, mmol·L⁻¹	0.65 ± 0.17§	0.61 ± 0.09§	1.08 ± 0.18*	0.78 ± 0.09§
HDL, mmol·L⁻¹	1.83 ± 0.14	1.81 ± 0.25	1.19 ± 0.17*	2.04 ± 0.17§
Adiponectin, ng·mL⁻¹	12.3 ± 0.69§	11.9 ± 1.18§	7.51 ± 0.35*	10.8 ± 0.63§
Leptin, pg·mL⁻¹	33.6 ± 2.06§	34.5 ± 2.87§	58.4 ± 3.87*	38.8 ± 6.81§

Data are means ± SEM. *$P < 0.01$ versus control. §$P < 0.01$ versus HFHS.

the HFHS diet produced an intense vacuolar degeneration of the S1 and S2 portions of the proximal convoluted tubules, which may be the consequence of specific features of these segments, including prominent endocytic system and large secondary lysosomes (Maunsbach, 1966). In contrast, the normal appearance of the S3 segments (Supporting Information Fig. S1) could be the result of a lesser development of the endocytic vacuolar system of the 'pars recta'. Interestingly, the HFHS-induced alterations were significantly attenuated by pHBSP treatment. The HFHS-induced renal pathology correlated with decline in kidney function. The ACR ratio, a reliable marker of glomerular damage and progressive renal dysfunction associated with diabetes and obesity (Praga and Morales, 2010), was markedly elevated in HFHS-fed mice and significantly reduced after pHBSP administration for 11 weeks (Figure 2B). Similarly, BUN levels were increased in the HFHS group compared with the control group and reduced by pHBSP treatment (Figure 2C).

Chronic pHBSP treatment reduces liver steatosis

Livers from control mice showed a normal appearance, and chronic treatment with pHBSP had no effect on liver histology. In contrast, livers from mice that had received the HFHS diet showed moderate-to-severe mixed vacuolar steatosis (Figure 3A and Supporting Information Fig. S2). The observed steatosis was more prominent in the periportal zone, where hepatocytes exhibited some degree of hydropic degeneration. Chronic administration of pHBSP attenuated the neutral fat accumulation (measured after Oil Red O staining; Figure 3A). This beneficial effect of pHBSP on lipid accumulation was confirmed by measurement of TG content (Figure 3B). Liver homogenates from HFHS mice showed a threefold increase in TG levels when compared with control animals. Most notably, the TG levels of HFHS animals that had been treated with pHBSP were similar than those seen in control animals.

Insulin signal transduction was enhanced in gastrocnemius muscle of HFHS mice exposed to pHBSP treatment

In this skeletal muscle, the expression levels of total IRS-1, Akt and GSK-3β proteins were not affected by either dietary manipulation or drug treatment (Figure 4). In contrast,
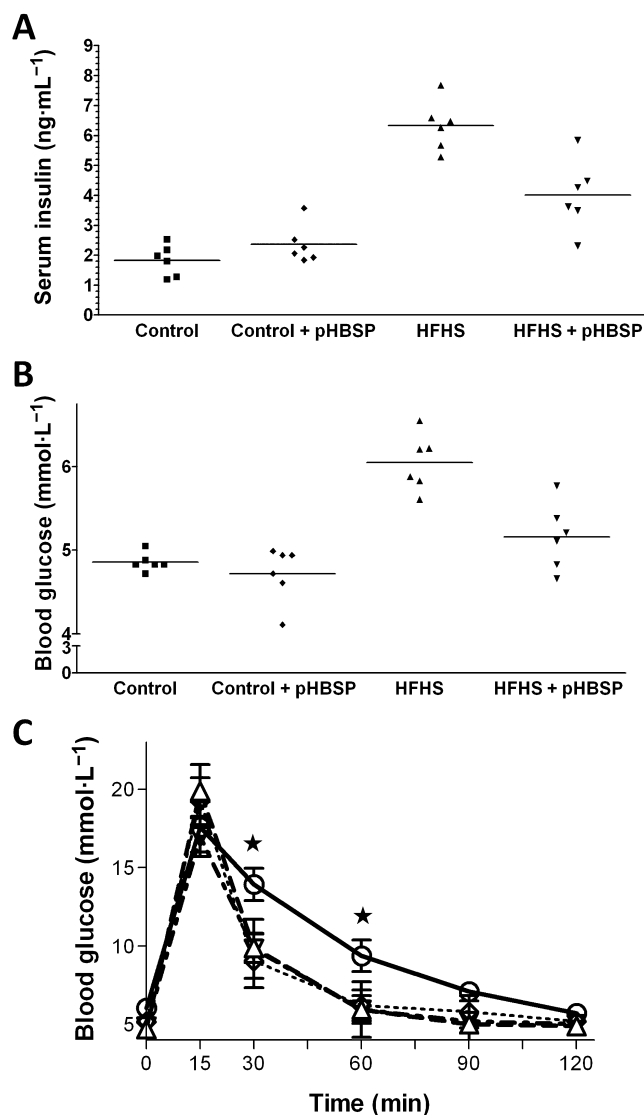
Figure 1

HFHS diet and chronic administration of pHBSP (30 μg·kg⁻¹, s.c.) on serum insulin (A), blood glucose (B) and oral glucose tolerance (C). Values are mean ± SEM of six animals per group. ★$P < 0.05$ versus control.
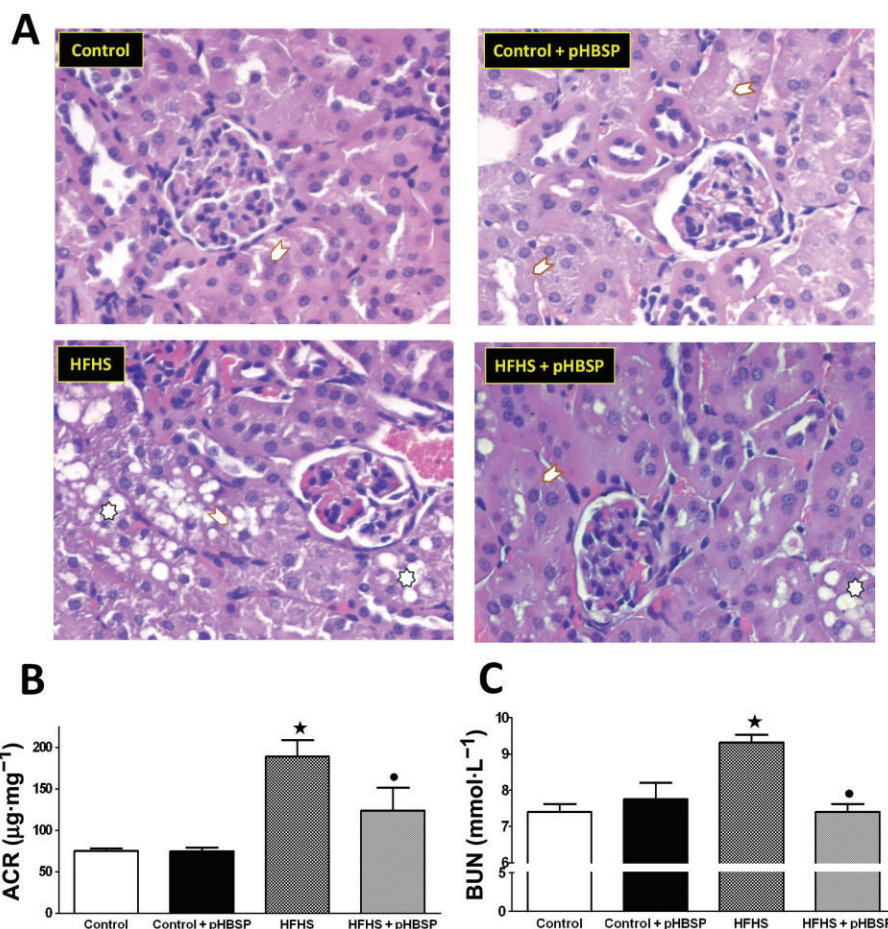
Figure 2

Effects of HFHS diet and pHBSP administration on kidney structure and function. Representative sections of kidneys (A) from mice maintained on the control diet or the HFHS diet and treated with pHBSP (30 μg·kg^{-1}, s.c.). Kidneys from mice fed with a standard laboratory chow showed a normal appearance in the presence or absence of drug treatment. The HFHS diet produced an intense vacuolar degeneration of the S1 and S2 portions of the proximal convoluted tubules. These alterations were significantly attenuated by pHBSP administration. (Arrows point to S1 and S2 portions of the proximal convoluted tubules; asterisks indicate tubular cells with vacuolar degeneration.) Urinary ACR (B) and BUN (C) levels were measured in mice exposed to the control diet or the HFHS diet in the absence or presence of pHBSP (30 μg·kg^{-1}, s.c.). Values are mean ± SEM of six animals per group. ★$P < 0.05$ versus control; •$P < 0.05$ versus HFHS.

22 weeks of HFHS diet caused a marked increase in Ser^{307} phosphorylation of IRS-1 in parallel with reduced Ser^{473} phosphorylation of Akt and Ser^9 phosphorylation of GSK-3β, a downstream target of Akt, indicating an impaired insulin signalling pathway. Chronic pHBSP administration significantly reversed all the effects of HFHS diet on IRS-1, Akt and GSK-3β phosphorylation.

GLUT-4 and glycogen levels in the mouse skeletal muscle

As shown in Figure 5B, GLUT-4 expression was slightly (although not significantly) reduced in gastrocnemius of HFHS mice compared with control animals, and increased following pHBSP administration. Interestingly, pHBSP administration not only increased transporter expression levels, but also induced a significant membrane translocation, as shown by a different pattern of GLUT-4 immunostaining between muscle sections of mice exposed to dietary manipulation in the absence or presence of pHBSP (Figure 5A). GLUT-4 translocation to the plasma membrane was confirmed by Western blot analysis of the Akt substrate 160 (AS160), a protein that regulates insulin-stimulated GLUT-4 trafficking in skeletal muscle (Kramer *et al.*, 2006). AS160 phosphorylation is required for GLUT-4 translocation to the cell surface. Here we showed that the total protein abundance of AS160 in the mouse gastrocnemius was not affected by either dietary manipulation or drug treatment. However, Thr^{642} phosphorylation of AS160 was significantly reduced by HFHS diet and this effect was prevented by pHBSP administration (Figure 5C). The HFHS-induced modulation of GLUT-4 membrane translocation was accompanied by a robust decrease in muscle glycogen content, which was blunted by pHBSP treatment (Figure 5D).
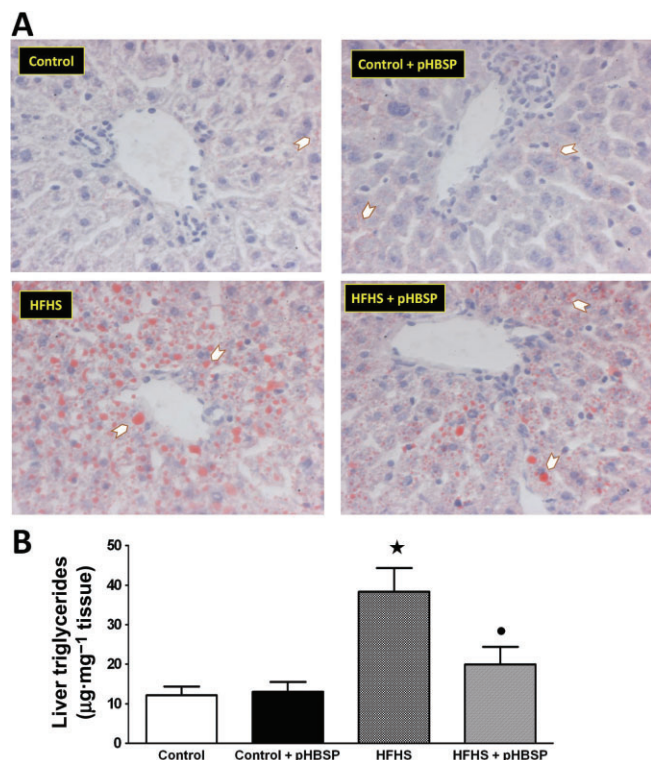
Figure 3

Effects of HFHS diet and pHBSP administration on liver lipid accumulation. (A) Representative photomicrographs (×40 magnification) of Oil Red O staining on liver sections from mice maintained on the control diet or the HFHS diet and treated with pHBSP (30 µg·kg^{-1}, s.c.). Arrows point hepatocytes with lipid droplets. (B) TG content in mouse liver. Data are mean ± SEM of six animals per group. ★$P <$ 0.05 versus control; •$P <$ 0.05 versus HFHS.

Administration of pHBSP prevented local and systemic myokine overproduction evoked by chronic consumption of HFHS

HFHS significantly elevated skeletal muscle production of two well-known myokines, IL-6 and fibroblast growth factor-21 (FGF-21), while pHBSP treatment resulted in a marked decrease in IL-6 and a slight reduction in FGF-21 levels (Figure 6A and B respectively). These changes in the muscle myokine production were paralleled by the changes in circulating levels of the myokines. In fact, circulating concentrations of IL-6 and FGF-21 increased after HFHS feeding and these increases were almost completely abolished in mice treated with pHBSP. In contrast, their levels were not affected by pHBSP treatment in mice fed with the control diet (Figure 6C and D).

Effect of chronic pHBSP treatment on markers of muscle mitochondrial production/biogenesis

To gain further insight into the effects of pHBSP, selective markers of muscle mitochondrial production/biogenesis were evaluated. Protein expression of the mitochondrial component of the electron transfer chain, COI, used as a marker of
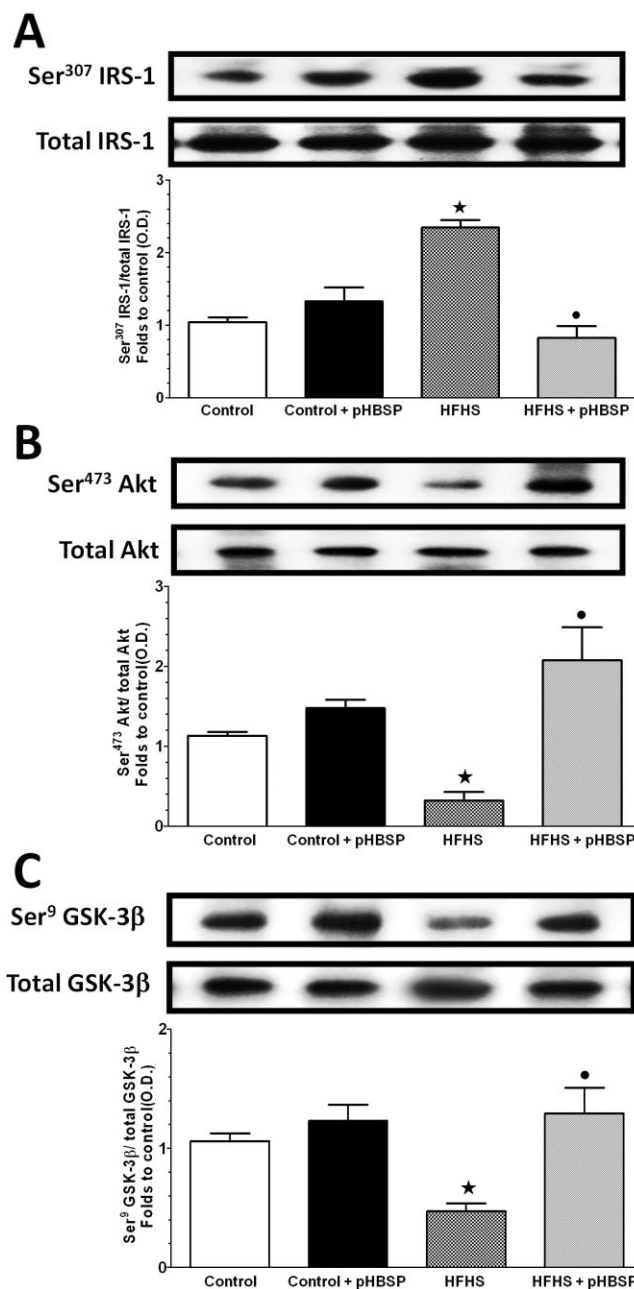

Figure 4

Alterations in insulin signalling pathway in the skeletal muscle of mice maintained on HFHS diet in the absence or presence of pHBSP. Total protein expression of IRS-1 (A), Akt (B) and GSK-3β (C) as well as their related phosphorylated forms were analysed by Western blot on the gastrocnemius homogenates obtained from mice exposed to the control diet or the HFHS diet and treated with pHBSP (30 µg·kg^{-1}, s.c.). Protein expression is measured as relative optical density (O.D.), corrected for the corresponding tubulin contents and normalized to the control band. The data are means ± SEM of three separate experiments. ★$P <$ 0.05 versus control; •$P <$ 0.05 versus HFHS.

mitochondrial content, and PGC1-α, a transcriptional coactivator mediating mitochondrial biogenesis, increased in the gastrocnemius muscle of animals fed with the HFHS diet (Figure 7A and B respectively). Interestingly, a further
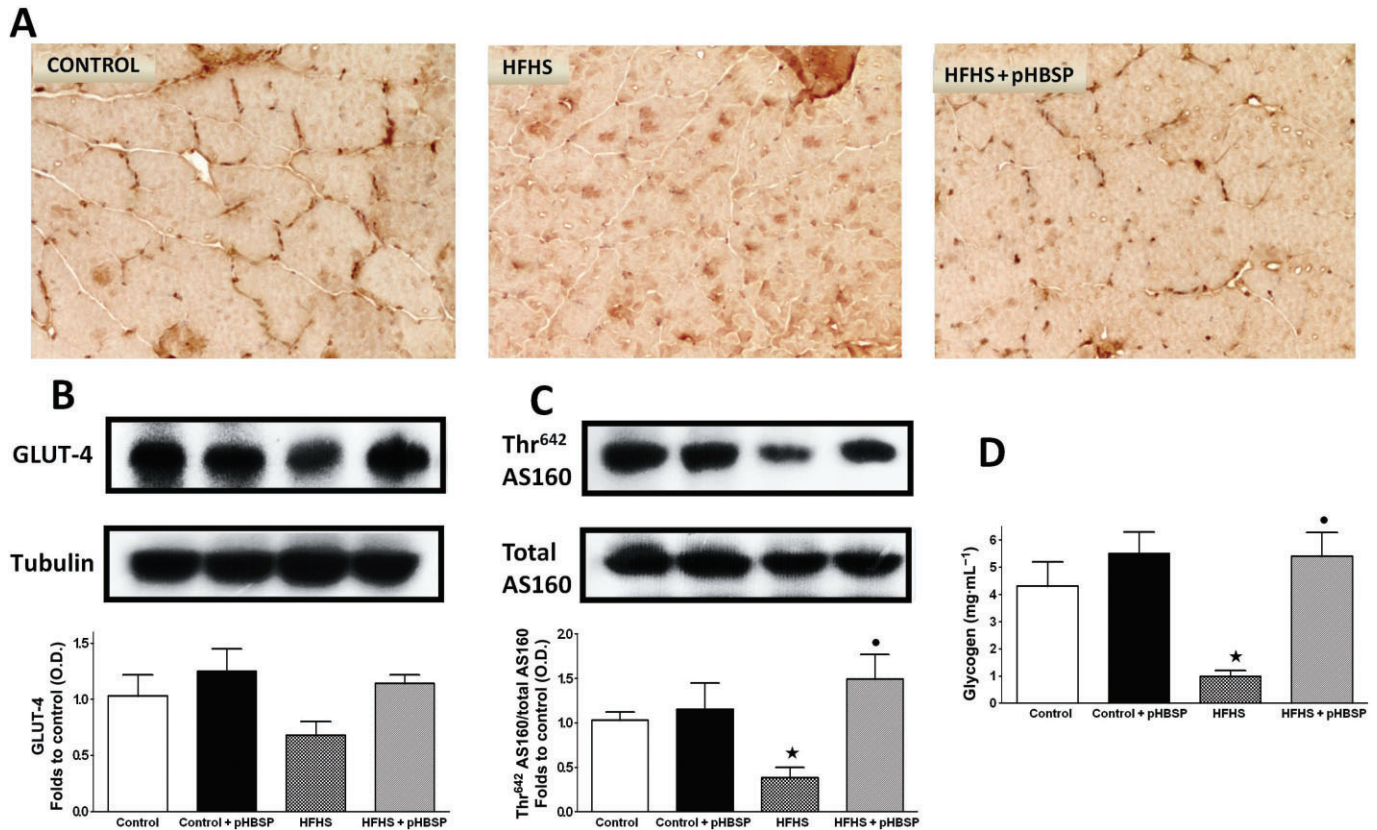
Figure 5

Effects of dietary manipulation and pHBSP treatment on GLUT-4 localization (A, original magnification: 400×) and expression (B), AS160 total protein expression and Thr^{642} phosphorylation (C), and glycogen content (D) in the skeletal muscle from mice exposed to the control diet or the HFHS diet and treated with pHBSP (30 µg·kg^{-1}, s.c.). Protein expression is measured as relative optical density (O.D.), corrected for the corresponding tubulin and normalized using the related control band. Data are means ± SEM of three separate experiments for Western blot analysis and six to eight animals per group for skeletal muscle glycogen content. ★$P < 0.05$ versus control; •$P < 0.05$ versus HFHS.

increase in PGC1-α expression was observed in the muscle of HFHS mice treated with pHBSP. Moreover, expression of the transcription factors, mtTFA and NRF1, which act in concert to increase mitochondrial oxidative phosphorylation and mitochondrial biogenesis, were not affected by HFHS diet, whereas they were markedly up-regulated by pHBSP (Figure 7C and D respectively).

Evidence of a heteromeric complex consisting of EpoR and βcR in the mouse skeletal muscle

Immunoprecipitation assays showed that EpoR and βcR were physically associated in the gastrocnemius muscle of control animals and this interaction was not altered in response to dietary modification and/or pHBSP treatment (Figure 8).

Discussion

The current study demonstrates for the first time that chronic administration of the EPO derivative pHBSP protects against the metabolic abnormalities caused by exposure to a typical unhealthy diet containing high concentrations of both fat and sugar. Mice fed with the HFHS diet for 22 weeks developed a significant increase in body weight associated with dyslipidaemia, hyperinsulinaemia and changes in insulin sensitivity and, most notably, treatment with pHBSP-normalized serum glucose and lipid profiles and evoked a slight, but still significant, decrease in body weight, with reduced amounts of epydidimal fat tissue. This decrease was not due to reduced food intake, as shown by our measurements of this variable. In addition, we observed several pathophysiological alterations in the kidneys and livers of mice that had been maintained on the HFHS diet, which were significantly attenuated by drug treatment. In HFHS animals, pHBSP reduced the diet-induced diabetic nephropathy (confirmed by histology, rise in BUN levels and albuminuria). Similarly, pHBSP reduced the HFHS-induced liver steatosis (confirmed by histological analysis and determination of tissue TG levels).

Overall, our data were compatible with recent studies showing that EPO may ameliorate organ dysfunction evoked by metabolic derangements and regulate glucose metabolism and insulin sensitivity. Specifically, treatment of diabetic mice with EPO attenuated both albuminuria and podocyte
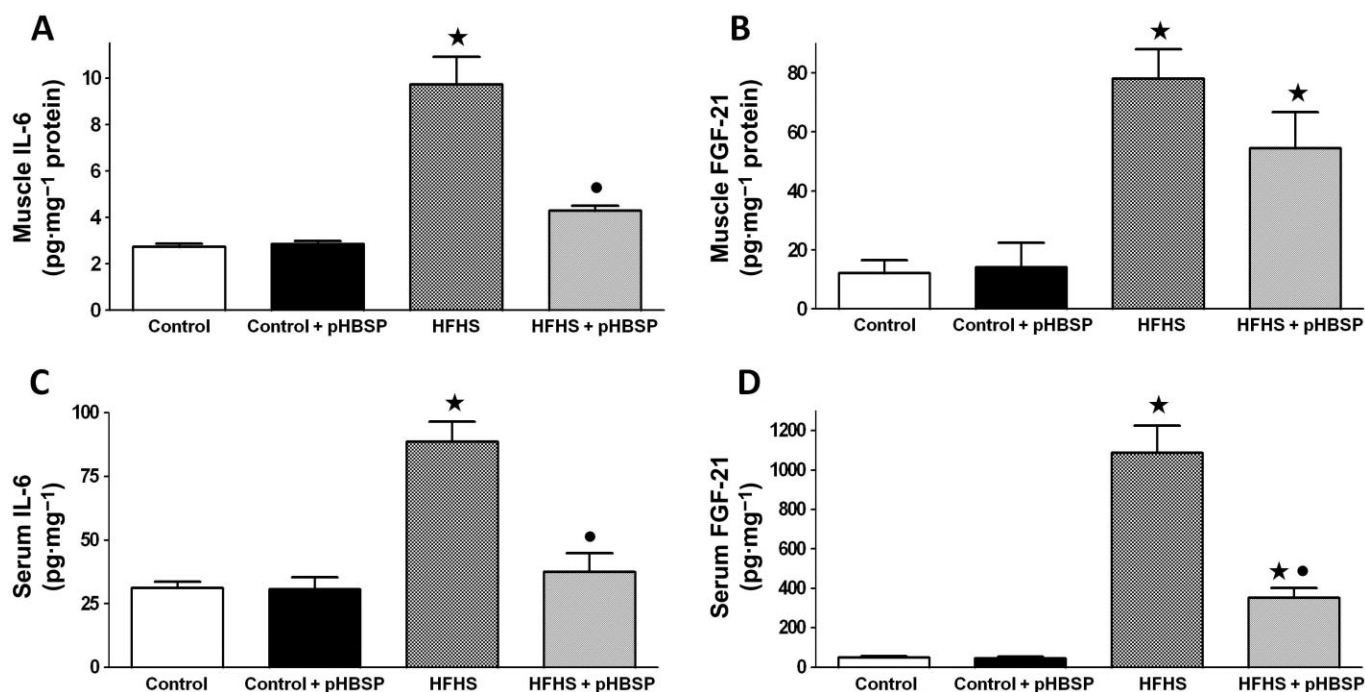
Figure 6

Effects of pHBSP on local and systemic levels of IL-6 and FGF-21 in mice fed with HFHS diet. IL-6 and FGF-21 concentrations were analysed by ELISA in gastrocnemius homogenates (A and B, respectively) and serum (C and D, respectively) of mice maintained on the control or the HFHS diet and treated with pHBSP (30 μg·kg^{-1}, s.c.). Data are means ± SEM of six to eight animals per group. ★$P < 0.05$ versus control; •$P < 0.05$ versus HFHS.

loss (Loeffler *et al.*, 2013) and improved mitochondrial oxidative phosphorylation and lipid metabolism in the liver (Meng *et al.*, 2013). Interestingly, transgenic mice constitutively overexpressing human EPO are resistant to high-fat diet-induced glucose intolerance and insulin resistance (Hojman *et al.*, 2009; Katz *et al.*, 2010). These data are supported by clinical evidence documenting beneficial effects of EPO on both glucose metabolism and insulin sensitivity in both diabetic and non-diabetic patients undergoing haemodialysis (Allegra *et al.*, 1996; Tuzcu *et al.*, 2004; Brown *et al.*, 2009; Khedr *et al.*, 2009). However, thus far any potential therapeutic applications of human-recombinant EPO in patients with metabolic diseases are limited by rises in haematocrit and platelet count (haematopoietic effect), resulting in an increase in thrombotic risk.

The EPO mimetic pHBSP, which was evaluated in our study, binds selectively to the heteromeric receptor composed of EpoR and βcR, which is responsible for the tissue-protective effects of EPO, but pHBSP does not interact with the erythropoietic EpoR homodimer, activation of which triggers a rise in haematocrit (Brines and Cerami, 2008). Therefore, our findings confirm the protective effects of EPO against diet-induced metabolic derangements and, most notably, demonstrate that these beneficial metabolic effects can also be obtained by selective activation of the EpoR–βcR complex, without stimulating haematopoiesis and, hence, potentially causing the severe side effects associated with chronic EPO administration.

The skeletal muscle, which is a major site of postprandial glucose metabolism and one of the insulin-sensitive tissues

most likely to manifest early signs of insulin resistance, is also a major target of the protective effects of EPO. EPO is produced by primary myoblasts isolated from gastrocnemius muscle and it contributes to the increased myoblast proliferation and survival, and to skeletal muscle repair and regeneration, by activating the Akt signalling pathway (Jia *et al.*, 2012). EPO overexpression in skeletal muscle protects mice against diet-induced obesity and associated metabolic abnormalities (Hojman *et al.*, 2009). Davenport *et al.* (1993) also demonstrated that 14 weeks of EPO administration in patients with chronic kidney disease on haemodialysis increased muscle glycogen content while, in rats, EPO induced a shift in muscle fibre composition from fast glycolytic fibres to slow oxidative fibres (Cayla *et al.*, 2008). Both EpoR and βcR are expressed in human skeletal muscle and are co-localized in the sarcolemma (Joshi *et al.*, 2014). Here we describe for the first time the physical interaction between EpoR and βcR in mouse skeletal muscle by immunoprecipitation assays, showing that the pharmacological target of pHBSP, the EpoR/βcR complex, was expressed in the skeletal muscle of both control and HFHS-fed mice. pHBSP is known to bind to the EpoR/βcR complex (Brines and Cerami, 2008; 2012; Bohr *et al.*, 2013) and no effect of pHBSP in mice lacking the βcR has been reported (Swartjes *et al.*, 2011; 2013). However, further studies on the effects of pHBSP in βcR knockout mice given a HFHS diet are necessary to confirm that the observed beneficial effects of this peptide are indeed due to activation of the EpoR/βcR complex.

In our study, pHBSP attenuated the impairment in insulin signalling, caused by HFHS diet in skeletal muscle, which
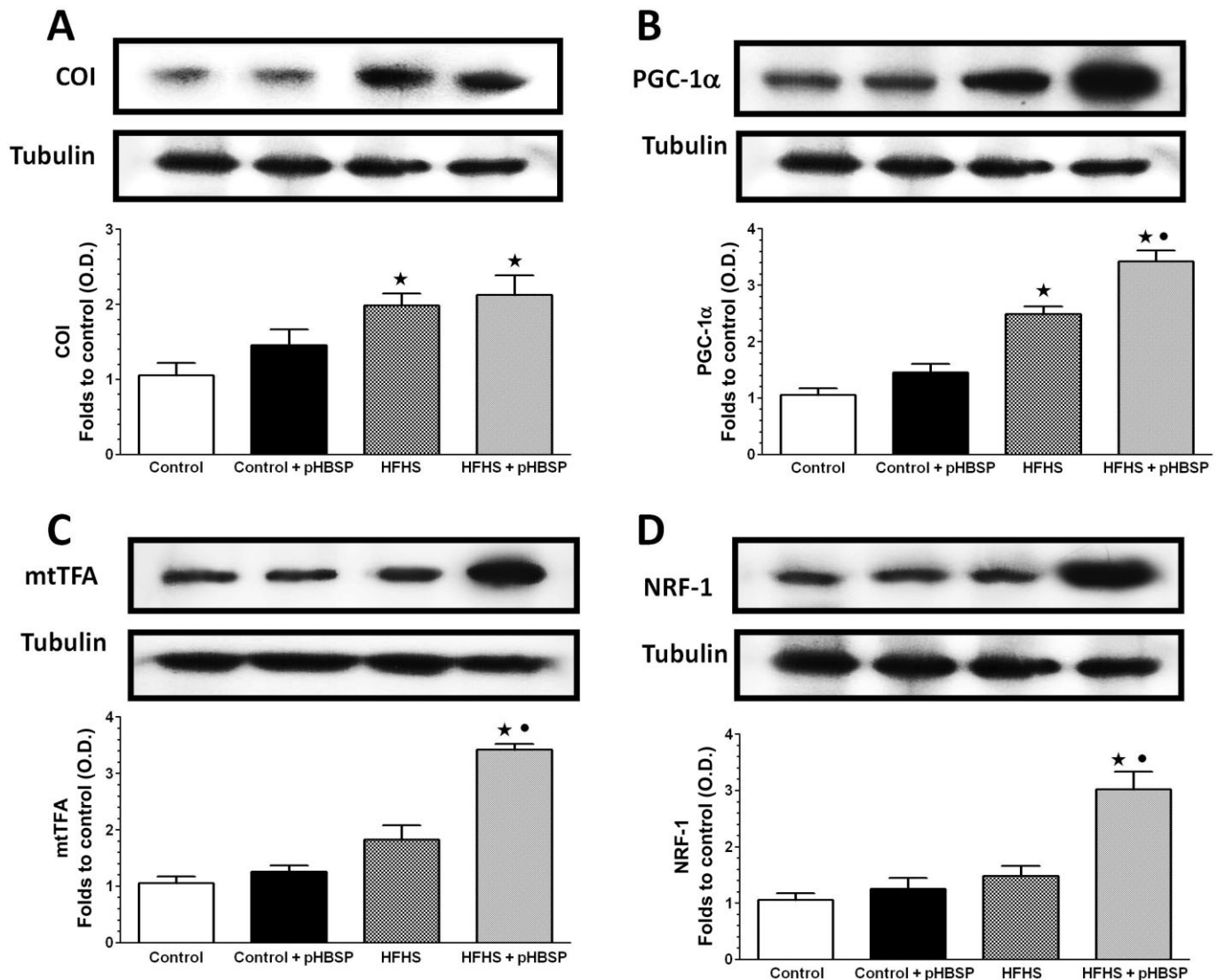
Figure 7
Effects of pHBSP treatment on markers of mitochondrial production/biogenesis in the gastrocnemius of mice fed with a HFHS diet. COI (A), PGC1-α (B), mtTFA (C) and NRF-1 (D) protein expression was analysed by Western blot on the gastrocnemius homogenates obtained from mice maintained on the control or the HFHS diet and treated with pHBSP (30 μg·kg^{-1}, s.c.). Protein expression is measured as relative optical density (O.D.), corrected for the corresponding tubulin contents and normalized using the related control band. The data are means ± SEM of three separate experiments. ★$P < 0.05$ versus control; •$P < 0.05$ versus HFHS.

may, at least in part, account for the changes in systemic insulin sensitivity. Specifically, pHBSP counteracted the HFHS-induced alterations in the phosphorylation of IRS-1 protein in skeletal muscle, as well as the activities of the downstream key insulin signalling molecules, Akt and GSK-3β, an Akt substrate. These effects were accompanied by a significant improvement in membrane translocation of GLUT-4, the most abundant glucose transporter isoform in skeletal muscle (Bouzakri et al., 2006), and by a robust increase in muscle glycogen content. As the inhibition of GSK-3β evokes an increase in the glycogen synthesis (Cross et al., 1995), the increased muscle glycogen storage evoked by pHBSP administration may be secondary to selective inactivation of GSK-3β. Similarly, the pHBSP-induced membrane

translocation of GLUT-4 could be secondary to the modulation of the insulin signalling pathway by activation of the EpoR/βcR complex. Indeed, both Akt and IRS-1 may regulate the translocation, targeting and fusion of GLUT-4-containing vesicles in mice skeletal myocytes (Gonzalez and McGraw, 2006; Leto and Saltiel, 2012; Zeng et al., 2012). Akt also regulates AS160 phosphorylation, which is a crucial step in GLUT-4 translocation in the skeletal muscle (Sano et al., 2003; Kramer et al., 2006), and here we showed a significant increase in the phosphorylation of AS160 in the presence of pHBSP, which may result in GLUT-4 exocytosis and reduced GLUT-4 endocytosis. We propose that pHBSP stimulates glucose transport in skeletal muscle by increasing AS160 phosphorylation throughout Akt modulation, thus implying
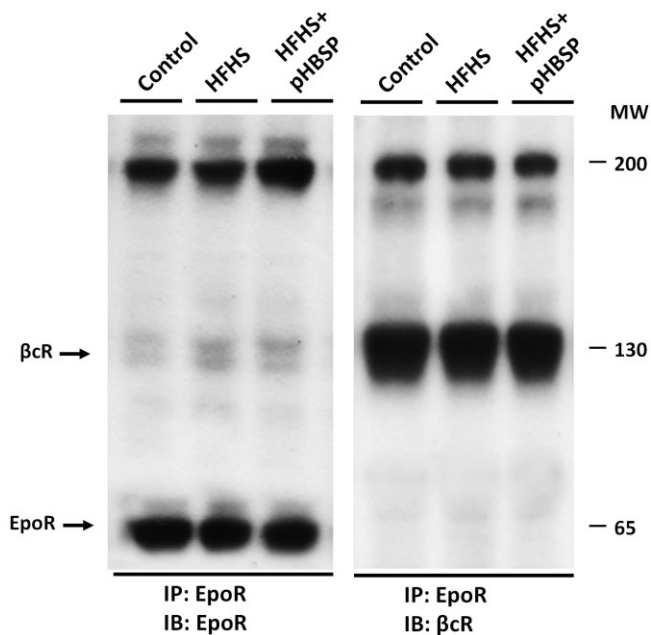
Figure 8

Heterodimerization between EpoR and βcR in the mouse skeletal muscle. Co-immunoprecipitation analysis illustrating the heteromeric complex consisting of EpoR and βcR in gastrocnemius homogenates obtained from mice maintained on the control or the HFHS diet and treated with pHBSP (30 μg·kg⁻¹, s.c.). Lysate from skeletal muscles was subjected to immunoprecipitation (IP) with anti-EpoR antibody and then exposed to immunoblot (IB) analysis with either anti-EpoR (left panel) or anti-βcR antibodies (right panel). The results, representative of two independent experiments, show interaction between the two receptors both under control conditions and after dietary manipulation and/or pHBSP treatment.

that the EpoR/βcR complex is a common target of convergence to counteract both diet-induced impaired insulin signal transduction and glucose transport.

Evidence that the skeletal muscle is an endocrine organ, producing and releasing a number of biologically active substances, such as IL-6, IL-8, IL-15 and FGF-21, collectively known as myokines, has grown over the last few years (Pedersen and Febbraio, 2008). These myokines participate in cell-to-cell and organ-to-organ crosstalk and play important roles in the development of diseases associated with low-grade inflammation, including insulin resistance (Pedersen, 2011). Here we found that HFHS diet evoked up-regulation of IL-6 and FGF-21 in skeletal muscle and that this effect was also associated with a strong increase in the plasma levels of IL-6 and FGF-21. Interestingly, administration of pHBSP led to dramatic reduction in the plasma levels of IL-6 and FGF-21. When measured in the gastrocnemius homogenates, however, the reduction in myokine levels by pHBSP was maximal for IL-6 and modest for FGF-21. This observation is intriguing, as the role of myokines in the aetiology of insulin resistance has been a matter of controversy. Although chronic exposure to IL-6 induced insulin resistance in skeletal muscle both *in vitro* and *in vivo* (Nieto-Vazquez *et al.*, 2008), FGF-21 has emerged as an important regulator of glucose metabolism, increasing glucose uptake and abolishing insulin resist-

ance in skeletal muscle cells (Ni *et al.*, 2012). In humans, serum FGF-21 levels are paradoxically increased in metabolic diseases (Chen *et al.*, 2008; Galman *et al.*, 2008; Han *et al.*, 2010). The mechanisms responsible for this paradoxical elevation of FGF-21 are not fully understood, although it might be partly explained by a compensatory overexpression of FGF-21 and/or FGF-21 resistance in peripheral tissues with insulin resistance. We may, thus, speculate that the HFHS-induced rise in IL-6 recorded in our study contributes to the loss of local insulin sensitivity, while the rise in FGF-21 is a defensive mechanism to counteract the deleterious effects of glucotoxicity. Accordingly, the reduction in IL-6 levels by pHBSP might be a consequence of the EpoR/βcR complex activation, which exerts beneficial effects on insulin sensitivity. In contrast, FGF-21 levels were not directly modulated by pHBSP. Thus, the FGF-21 reduction afforded by pHBSP may well be secondary to a reduced glucotoxicity following pHBSP, but this hypothesis warrants further investigation. It is noteworthy that our results do not argue against the role of EPO as potential regulator of adipose tissue inflammation in diet-induced obesity and insulin resistance, as recently suggested by Alnaeeli *et al.* (2014). Instead, our results imply that modulation of local inflammation in skeletal muscle may be an additional mechanism by which the EPO derivative can affect the actions of insulin in skeletal muscle.

Finally, we evaluated the effects of pHBSP on skeletal muscle mitochondrial function. Although mitochondrial dysfunction has been indicated as a cardinal feature of insulin resistance, most recent evidence suggest that a high caloric intake may lead to insulin resistance, even in the presence of increased mitochondrial production in muscle (Garcia-Roves *et al.*, 2007; Hancock *et al.*, 2008). Our results show that HFHS feeding resulted in increases in skeletal muscle expression of COI and PGC-1α, although no effects were observed on the expression of NRF1 (a transcription factor involved in controlling the expression of nuclear gene encoding for mitochondrial proteins) and mtTFA (a transcription factor involved in controlling the expression of mitochondrial genes) (Scarpulla, 2008; Hock and Kralli, 2009). This slight increase in selective markers of mitochondrial number and/or function detected in the skeletal muscle of HFHS mice may be suggestive of an early and weak compensatory mechanism to the deleterious consequences of dietary manipulation, which was dramatically improved in response to the activation of the EpoR/βcR complex, as shown by a further induction in PGC-1α expression as well as a robust up-regulation of NRF1 and mtTFA in the presence of chronic pHBSP administration.

In conclusion, chronic treatment with pHBSP, an EPO derivative devoid of any haematopoietic effects, triggers an amelioration of the metabolic abnormalities evoked by exposure to a diet containing high concentrations of both fat and sugar. The present study also increases our understanding of the mechanism of action of pHBSP and highlights a pivotal role for the EpoR/βcR complex in skeletal muscle as a key interface between impaired insulin pathway and local inflammatory response. The demonstration that an EPO derivative that is devoid of any EPO activity (that are, hence, potentially translatable to man) may act on diet-induced metabolic derangements points to a new pathway for the development of novel drugs for patients with diabetes and obesity, two

closely linked healthcare challenges of modern societies that continue to rise in prevalence, with important health and economic implications.

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Author contributions

M. C. designed the study, performed the experiments, analysed and researched data, and wrote the manuscript. E. B., M. R., F. C. and D. N. researched data. J. C. C. performed the kidney and liver histopathological examination. R. M. analysed and researched data, contributed to the study design, to analysis and interpretation of data. M. A., R. F. and M. A. M. contributed to discussion and reviewed the manuscript. C. T. designed the study, contributed to drafting and revising the manuscript, and edited the final version. M. C. is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. All the authors approved the final version.

Conflict of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

http://dx.doi.org/10.1111/bph.12888

Figure S1 Effects of dietary manipulation and pHBSP treatment on renal S3 morphological structure. As shown in the representative pictures, sections from S3 segments of the PCT exhibited a well-preserved morphological aspect normally compatible in all the experimental groups evaluated here.

Figure S2 Effects of dietary manipulation and pHBSP treatment on liver structure. Representative pictures of sections from fragments around 1–3 cm from the left lateral and medial lobes stained with haematoxylin-eosin and examined under an Olympus Bx4I microscope (40× magnification) with an AxioCamMR5 photographic attachment (Zeiss, Gottingen, Germany). Livers from mice fed with a standard (Control) diet showed a morphological normal appearance. The administration of pHSBP to control mice did not produce any significant alterations. When animals received HFHS diet, their livers developed steatosis. Marked fatty change together with some degree of hydropic degeneration predominantly involved the periportal hepatocytes. Concomitant administration of pHSBP exerted a protective action, as the degree of the degenerations mentioned above was significantly attenuated.