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QRFP-26 enhances insulin's effects on glucose uptake in rat skeletal muscle cells

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Abstract

QRFP is expressed in central and peripheral regions important for nutrient intake and metabolism. Central administration of QRFP-26 and QRFP-43 induces a macronutrient specific increase in the intake of high fat diet in male and female rats. Recently, cell culture models have indicated that QRFP-26 and QRFP-43 are involved in glucose and fatty acid uptake in pancreatic islets and adipocytes. Since skeletal muscle is a major consumer of circulating glucose and a primary contributor to whole body metabolism, the current study examined the effects of QRFP-26 and QRFP-43 on insulin-stimulated uptake of glucose in skeletal muscle using L6 myotubes. The current experiments were designed to test the hypothesis that QRFP and its receptors, GPR103a and GPR103b are expressed in L6 myotubes and that QRFP-26 and QRFP-43 affect insulin-stimulated uptake of glucose in L6 myotubes. The results indicate that prepro-QRFP mRNA and GPR103a mRNA are expressed in L6 cells, though GPR103b mRNA was not detected. Using complementary assays, co-incubation with QRFP-26, increased insulin's ability to induce glycogen synthesis and 2-deoxyglucose uptake in L6 cells. These data suggest that QRFP-26, but not QRFP-43, is involved in the metabolic effects of skeletal muscle and may enhance insulin's effects on glucose uptake in skeletal muscle. These data support a role for QRFP as a modulator of nutrient intake in skeletal muscle.

Keywords

QRFP-26; QRFP-43; GPR103a; Glucose uptake; L6 cells

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1.1 Introduction

Members of the RFamide-related peptide family exert a large array of biological activities which include cardiovascular functioning, analgesia, food intake, blood pressure, locomotor activity and pituitary hormone regulation [3, 5, 11]. Recently, a 26-amino acid peptide exhibiting the Arg-Phe-NH₂ signature was isolated from frog brain, pyroglutamylated arginine-phenylalanineamide peptide (QRFP-26, also referred to as 26RFa) and the cDNA encoding QRFP-26 was characterized in rat, mouse, human and a number of other species [1, 2, 14]. The QRFP-26 precursor has been shown to generate an N-terminal extended form of 43-amino acids (QRFP-43, also referred to as 43RFa). Both QRFP-26 and QRFP-43 are potent ligands for GPR103a and GPR103b, G protein-coupled receptors.

Multiple studies have investigated the central effects of QRFP [10–12] on nutrient intake and recent studies have examined the effects of QRFP in regulating peripheral metabolic effects [4, 6, 9]. Prepro-QRFP mRNA is expressed in multiple peripheral tissues involved in metabolism, including white adipose tissue, pancreatic islets, and skeletal muscle [6, 8, 9]. Both prepro-QRFP mRNA and GPR103b mRNA are expressed in 3T3-L1 adipocyte cells and QRFP-26 and QRFP-43 administration increases triglyceride accumulation and fatty acid uptake in these cells [9]. In the isolated rat pancreas, QRFP-26 reduces glucose-induced insulin release, likely through an inhibition of adenylyl cyclase [4]. In human pancreatic islets, both QRFP and GPR103 are expressed and incubation with QRFP-43 increases glucose-stimulated insulin release in pancreatic islets, while incubation with QRFP-26 inhibits glucose-stimulated insulin release in these cells. Furthermore, QRFP-43, but not QRFP-26, increases 2-deoxyglucose (2-DG) uptake in pancreatic islets [6]. In skeletal muscle, dietary fat modulates prepro-QRFP mRNA and GPR103a mRNA expression in several rodent models (unpublished observations). These data suggest that QRFP plays an important role in both the central regulation of nutrient intake and peripheral factors important in metabolism [4, 6, 9].

Skeletal muscle is a main contributor to whole-body metabolism, is the largest insulin-sensitive tissue in the body and is an important site for insulin-stimulated glucose utilization [13]. The goal of the current study was to investigate the role of QRFP-26 and QRFP43 on insulin-stimulated glucose uptake in skeletal muscle, using L6 myotubes. In the first experiment, a time course analysis of the expression of prepro-QRFP mRNA and GPR103a and GPR103b mRNA in L6 cells was performed, which indicated that both prepro-QRFP and GPR103a were expressed in L6 cells. The second experiment tested the hypothesis that incubation of QRFP-26 and/or QRFP-43 would enhance insulin-stimulated glucose uptake. Two complementary assays, the glycogen synthesis assay and the 2-DG uptake assay, were used to test this hypothesis.

1.2 Material and Methods

1.2.1 Cell Culture

L6 myoblasts were obtained from American Type Culture Collection and maintained at 37°C, 95% O₂ and 5% CO₂ in low glucose DMEM supplemented with 10% CBS serum and antibiotics. Myoblasts were subcultured onto 6 well plates, grown to 80–90% confluence in

Experiment 1 and 50–60% confluence in Experiment 2, and then differentiated into myotubes by altering media to contain 2% equine serum.

1.2.2 Time Course Analysis of Gene Expression

The time course of gene expression of prepro-QRFP mRNA, GPR103a mRNA and GPR103b mRNA was determined by Real Time PCR. Pre-differentiated L6 cells were harvested upon 80–90% confluence and upon differentiation, cells were collected daily for 7 days. RNA was isolated using Tri-Reagent (Molecular Research Ctr, Cincinnati, OH USA) and RNeasy Minikit procedures (Qiagen, Valencia, CA USA) and based on previous experiments by Primeaux et al [11]. Reverse transcription (RT) was conducted using the High-Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, Foster City, CA, USA). Taqman Gene Expression Assays (Applied Biosystems) were used to assess levels of prepro-QRFP, GPR103a, GPR103b and the housekeeping gene, GAPDH. The quantity of prepro-QRFP, GPR103a, and GPR 103b mRNA expression was based on a standard curve and normalized to GAPDH levels (ABI Prism 7900 Sequence Detection System, Applied Biosystems).

1.2.3 Insulin Stimulated Glucose Uptake

For the glycogen synthesis assay, prior to the experiment, L6 cells were serum starved overnight. Glycogen synthesis was determined under basal and insulin stimulated states based on previous methods [7]. Myotubes were stimulated in PBS containing 0.2% BSA and glucose (30mM) with or without QRFP-26 (100nM) or QRFP-43 (100nM) and insulin (200 nM) for 2h. After removing media and washing with cold PBS (3x), 200µl of sodium acetate buffer (0.2 mol/L, pH 4.8) was added to each well. A glucose assay kit (Sigma Aldrich, St. Louis, MO) was used to measure glucose from the cell lysate. After the addition of glucose oxidase/oxidase reagent, cells were incubated at 37°C for 30 min. The reaction was stopped by adding 12N H₂SO₄ and measured by spectrophotometer (BioRad, Hercules, CA) at 540nm. 2-DG uptake was determined using the glucose uptake colorimetric assay kit (Sigma Aldrich). L6 myotubes were serum starved overnight then washed with PBS (3x) and incubated with Krebs-Ringer buffer with or without QRFP-26 (100nM) or QRFP-43 (100nM) for 40 min. Insulin (100nmol/L) was added to the culture medium for 20 min, followed by the addition of 2-DG (10mM) and incubated for 20 min. Following incubation, cells were washed with cold PBS (3x) and lysed with extraction buffer. Cell lysate was neutralized by addition of neutralization buffer and centrifuged. The remaining lysate was then diluted with assay buffer. 2-DG uptake was determined by an enzymatic reaction to oxidize 2-DG6P to generate NADPH, which is then amplified and utilized by glutathione reductase to produce glutathione. Glutathione produces TNB by reacting with the substrate DNTB added to the reaction. TNB was then detected at 412 nm by a spectrophotometer (BioRad). Values in both assays were normalized to protein levels, which were determined using a BCA protein assay (Life Technologies, Carlsbad, CA).

Statistical Analysis—For Experiment 1, a between subjects t-test was used to compare gene expression for each day post-differentiation to Day 0. For Experiment 2, a 2X3 ANOVA was used to compare glycogen synthesis and 2-DG uptake in the L6 myotubes.

Bonferroni post-hoc tests comparisons were used as needed. A significance level of $p < .05$ was used for all tests.

1.3 Results

1.3.1 Experiment 1

A time course analysis of prepro-QRFP mRNA expression in L6 myotubes revealed that, compared to pre-differentiation levels, prepro-QRFP mRNA expression was decreased at D1 ($p < .05$) and increased at D7 post-differentiation ($p < .05$; Fig. 1A). mRNA expression of QRFP's receptor, GPR103a, was increased at each time point post-differentiation ($p < .05$; Fig. 1B). GPR103b mRNA was not detectable in the L6 myotubes.

1.3.2 Experiment 2

In the glycogen synthesis assay, a significant interaction between insulin and QRFP was detected ($F = 3.39$, $p < .05$). Insulin increased glycogen synthesis in all conditions. Co-incubation with QRFP-26 enhanced insulin's effects on glycogen synthesis, while co-incubation with QRFP-43 did not alter insulin's effect on glycogen synthesis. (Fig. 2A). In the 2-DG assay, a significant interaction between insulin and QRFP was also detected ($F = 4.99$, $p < .05$). Insulin increased 2-DG levels in all conditions and co-incubation with QRFP-26 led to enhancement of 2-DG compared to insulin alone. QRFP-43 co-incubation did not enhance insulin's effects of 2-DG levels (Fig. 2B).

1.4 Discussion

QRFP and its receptors, GPR103a and GPR103b are expressed centrally, in various species, and peripherally in humans, rats and mice [1, 2, 6, 8–10, 12, 14]. Central administration of QRFP-26 and QRFP-43 has an orexigenic effect and in rats, this effect is specific to the intake of dietary fat [10, 12]. Additionally, prepro-QRFP mRNA levels are increased in the hypothalamus of rats consuming a high fat diet, suggesting that QRFP, in the brain, is an important modulator of fat intake and may be involved in obesity [10–12]. Studies investigating the effects of QRFP in peripheral tissues have shown that, in adipocytes, QRFP-26 and QRFP-43 promote fat storage [9]. In pancreatic islet cells, QRFP-43, but not QRFP-26, increases glucose-stimulated insulin release and increases 2-DG uptake [6]. These data led us to hypothesize that QRFP-26 and QRFP-43 would alter insulin-stimulated glucose uptake in skeletal muscle, since skeletal muscle is major consumer of glucose and important for overall metabolism.

In the current series of experiments, Experiment 1 demonstrated that both prepro-QRFP and GPR103a were expressed in L6 myotubes. Experiment 2 examined the effects of co-incubation with either QRFP-26 or QRFP-43 and insulin on glycogen synthesis or 2-DG uptake in L6 myotubes. Complementary assays were used to confirm that QRFP-26, but not QRFP-43 enhanced insulin's effects on glycogen synthesis and 2-DG uptake. These data indicate that QRFP-26 plays a role in insulin sensitivity at the level of the skeletal muscle, by promoting the uptake of glucose into the muscle cells. Though there is still a dearth of information regarding the peripheral effects of QRFP on metabolism, the current data, as well as previously published data [6, 9] suggest that QRFP may be developed as a novel

therapeutic agent in individuals suffering from metabolic syndrome. Further research is needed to fully understand the role of QRFP-26 in skeletal muscle and assessment of the mature forms of QRFP-26 and QRFP-43 in muscle cells is imperative.

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Highlights

- Prepro-QRFP mRNA and GRP103a mRNA is expressed in differentiated L6 myotubes.
- QRFP-26 enhanced insulin-stimulated glycogen synthesis and 2-DG uptake.
- QRFP-43 did not affect insulin's action on glycogen synthesis and 2-DG uptake.

Figure 1.

A. A time course analysis of prepro-QRFP mRNA expression indicated that prepro-QRFP mRNA was significantly elevated 7 days following differentiation in L6 myotubes. **B.** GPR103a mRNA levels were elevated throughout the differentiation of L6 myotubes. Data are shown as mean $\pm$ SEM, * $p < .05$.

Figure 2.

A. A glycogen synthesis assay was used to test the effects of QRFP-26 and QRFP-43 on insulin-stimulated glucose uptake. Co-incubation with QRFP-26 enhanced insulin's effects on glucose uptake in L6 myotubes. **B.** A 2-DG uptake assay was also used to examine the effects of QRFP-26 and QRFP-43 on insulin-stimulated glucose uptake. Co-incubation with QRFP-26 enhanced insulin's effects on glucose uptake in L6 myotubes. Data are shown as mean $\pm$ SEM, * $p < .05$ vs. basal; + $p < .05$ vs. control + insulin.